EFFICACY OF A LIQUID AMNION ALLOGRAFT ON EQUINE HEALING

By

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EFFICACY OF A LIQUID AMNION ALLOGRAFT

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Abstract: Amnion is an excellent source of growth factors and cytokines important in healing, and thus considered a regenerative therapy. We hypothesized that distal limb wounds on horses treated with an equine-derived liquid amnion allograft (LAA) would heal faster than saline-treated controls. A randomized, blinded, controlled study was performed. Full-thickness skin wounds were surgically created on both metacarpi of eight horses. The margin of each wound was injected with either LAA or saline (control) nine days later. The wounds on each horse were randomly assigned to both groups. Bandage changes were performed at regular intervals until wounds had healed. At each bandage change, wound size was measured. The mixed model found no significant difference in wound size over time between the treatment and control groups using $P = 0.99$ for the interaction term. Using a Kaplan-Meier survival curve with survival defined as a wound not reaching 95% healed, the log-rank test found no significant difference in survival between the groups using $P = 0.2$. It was concluded that LAA did not accelerate wound healing. We also hypothesized that treatment of equine tendon and ligament injuries with local injection of LAA would result in an ability to return to work comparable to published reports using alternative regenerative therapies. A prospective, multi-center, non-blinded clinical trial was conducted. Criterion for inclusion was a horse presenting with lameness attributed to tendonitis or desmitis using diagnostic anesthesia and imaging, and subsequently treated by local injection of the lesion with LAA. Standardized questionnaires describing each horse's signalment, discipline, and ability to return to work were completed by attending veterinarians after at least six months of follow-up. Questionnaires for 100 horses with 128 tendonitis or desmitis lesions met the inclusion criterion. Of these, 72 horses with 94 lesions returned to or exceeded their original level of work, 10 horses with 13 lesions returned to work but could not perform to previous standards, and 18 horses with 20 lesions did not return to work as a result of the injury. These results were comparable to published reports using platelet-rich plasma, autologous conditioned serum and mesenchymal stem cells.

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CHAPTER I

INTRODUCTION

This paper investigates the effectiveness of an equine-derived liquid amnion allograft (LAA), as a regenerative medicine therapy, on healing in horses. It incorporates an experimental component that investigates the outcome of using LAA to treat experimentally-created distal limb wounds in horses, and a clinical component that investigates the outcome of using LAA to treat naturally-occurring tendon and ligament injuries on the limbs of horses.

The paper begins by describing the significance and character of traumatic distal limb wounds and musculoskeletal injuries involving tendons and ligaments in horses followed by a brief introduction to the concept of regenerative medicine. This is followed by a relatively extensive review of the literature pertaining to the use of amnion, the innermost fetal membrane, to treat wounds and musculoskeletal injuries in both human and veterinary medicine. Amnion and amniotic membrane are used synonymously. The historical progression of amnion use is considered imperative to understand how it has become valued for alleged healing powers. The methodology used for both the experimental and clinical components of the study are then presented. This is followed by the findings of both components. Finally, conclusions are presented.

CHAPTER II

REVIEW OF LITERATURE

Traumatic Distal Limb Wounds

Horses commonly incur traumatic wounds to their distal limbs, more than 50% of total wounds treated according to one large report. (Wilmink et al., 2002) Wounds are often left to heal by second intention due to massive tissue loss, excessive contamination, excessive skin tension, or an unacceptable delay since the injury occurred. (Theoret, 2004) Even with primary closure, partial or total dehiscence often occurs, mainly as a result of infection but also due to tension on the wound margins, motion, and natural debridement. (Wilmink et al., 2002)

Healing by second intention is generally a lengthy process. It involves the formation of granulation tissue to fill the defect, the migration of epithelium over the granulation bed, contraction of the wound edges to reduce wound size, and remodeling of connective tissue to improve tissue strength. (Theoret, 2004) Growth factors and cytokines play an important role in the complex interactions that take place during wound repair by coordinating cells such as platelets, macrophages, keratinocytes, fibroblasts, and endothelial cells. (Theoret, 2004; Barrientos et al., 2008) Growth factors and cytokines are often used interchangeably in the literature, although this is strictly incorrect. The most important growth factors and cytokines include epidermal growth factor (EGF), transforming growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth

factor (PDGF), connective tissue growth factor (CTGF), interleukin-1 (IL-1), interleukin-6, and tumor necrosis factor-α (TNF-α). (Barrientos et al., 2008)

Anatomic and physiologic particularities of the distal limb present unique challenges to wound healing. These include skin with relatively poor blood supply, bony prominences, lack of supporting deep musculature, highly mobile joints, and a relatively high degree of contamination. (Hendrickson & Virgin, 2005) Distal limb wounds in horses are prone to complications such as chronic inflammation and excessive fibroplasia, which subsequently retards normal epithelialization and contraction. (Theoret, 2004) Further perpetuating excessive fibroplasia, granulation tissue continues to proliferate without epithelial cover. (Schwartz et al., 2002) The chronic nature of the inflammatory response in horses is attributed to persistence of neutrophils and macrophages within the wound while excessive fibroplasia is attributed to fibroblasts not undergoing timely apoptosis or not differentiating into contractile myofibroblasts. (Lepault et al., 2005) In comparison to ponies, for example, horses have a weaker and protracted initial inflammatory response to injury which may inhibit the proliferative and remodeling phases of wound healing from proceeding normally. (Wilmink, Stolk, et al., 1999; Wilmink, van Weeren, et al., 1999; Wilmink et al., 2003; Theoret, 2004; Wilmink & van Weeren, 2005) Distal limb wounds are also subject to expansion in the first week or two due to tension on the wound margins from adjacent tissues. (Jacobs et al., 1984; Bertone et al., 1985; Bigbie et al., 1991)

Tendon and Ligament Injuries

Tendon and ligament injuries are another significant cause of morbidity in horses, particularly the performance horse, where they can compromise a return to previous level of activity, perhaps even forcing early retirement. (Denoix, 1994; Dowling et al., 2000; Smith & Webbon, 2005; Dahlgren, 2007; O'Sullivan, 2007; Nixon et al., 2008; Ely et al., 2009; Thorpe et al., 2010) The initial response in the healing process is the acute inflammatory phase characterized by pain,

intra-lesional hemorrhage, and edema. Circulating inflammatory cells infiltrate the injury and release growth factors and cytokines. These factors subsequently orchestrate biological processes involved in healing. (Dahlgren, 2007; Durgam & Stewart, 2017) The acute inflammatory response is followed by the proliferative phase where fibroblastic cells produce scar tissue comprised of far more type III collagen than the original tendon. (Watkins et al., 1985; Dowling et al., 2000; Dahlgren, 2007; Richardson et al., 2007) The remodeling phase follows, resulting in the gradual transformation to more type I collagen though not to the same proportion as the original pre-injury tissue. (Dowling et al., 2000; Dahlgren, 2007; Richardson et al., 2007) The end result is a tendon that lacks pre-injury biomechanical properties. (Dowling et al., 2000; Dahlgren, 2007; Richardson et al., 2007; Durgam & Stewart, 2017)

Regenerative Medicine

Regenerative medicine seeks to restore structure and function to injured tissue. (Fortier & Smith, 2008; Lopez & Jarazo, 2015; Dahlgren, 2018; Ortved, 2018) Extracellular matrix scaffolds, growth factors, and stem cells are the main components of regenerative therapies. (Fortier & Smith, 2008; Lopez & Jarazo, 2015; Dahlgren, 2018; Ortved, 2018) Scaffolds provide a framework for cell adhesion and migration across a defect, growth factors recruit and stimulate cells necessary for healing, while stem cells provide tropic mediators as well as the possibility of differentiation into mature cell types that contribute to healing. (Dahlgren, 2018)

Current regenerative therapies for tendinitis and desmitis in horses include local injection of mesenchymal stem cells (MSCs), (Smith & Webbon, 2005; Richardson et al., 2007; Fortier & Smith, 2008; Renzi et al., 2013; Van Loon et al., 2014; Lopez & Jarazo, 2015; Ortved, 2018; Schnabel et al., 2019) platelet rich plasma (PRP), (Fortier & Smith, 2008; Waselau et al., 2008; Castelijns et al., 2011; Zuffova et al., 2013; Scala et al., 2014; Romagnoli et al., 2015; Geburek et al., 2016; Ortved, 2018; Schnabel et al., 2019) and autologous conditioned serum (ACS). (Fortier

& Smith, 2008; Easter & Watts, 2014; Geburek et al., 2015; Ortved, 2018; Schnabel et al., 2019) A practical disadvantage associated with these autologous therapies is the time delay between sample collection and administration due to the required processing, which, at best, is generally overnight, but up to three or four weeks in the case of MSCs that undergo culture and expansion.

Amnion is one of two membranes that, together with chorion, are referred to as the fetal membranes. (Mamede et al., 2012) Chorion is the outer fetal membrane, in contact with the maternal cells, while amnion is the inner membrane in contact with the amniotic fluid that surrounds the fetus. (Mamede et al., 2012) Histologically, the amnion is made up three distinct layers. (Borazjani et al., 2011; Mamede et al., 2012; McCoy, Smith, et al., 2019) The most inner layer, in contact with the amniotic fluid, is a single row of cuboidal epithelial cells characterized by apical microvilli. (Mamede et al., 2012) The adjacent layer is a basement membrane to which the epithelial cells are firmly attached. (Mamede et al., 2012) The basement membrane is acellular and made up of collagen types I, II, and III as well as proteoglycans. The basement membrane impedes the movement of large molecules from the epithelial cells or amniotic fluid. The third layer that makes up amnion is a mesenchymal matrix rich in type III collagen but low in cell numbers. (Mamede et al., 2012) In humans, this layer is in contact with the chorion (Borazjani et al., 2011); however, in horses, it faces and is in loose contact with the allantois. (Coli et al., 2011) The elastic nature of amnion is attributed to the type III collagen-rich mesenchymal layer. Fresh amnion contains significant amounts of growth factors and cytokines such as EGF, TGF-α, TGF-β, bFGF, KGF, and VEGF that are important in tissue regeneration and healing. (Litwiniuk & Grzela, 2014; Bomfim Pereira et al, 2016) The epithelial cells are responsible for producing the cytokines and factors. (Litwiniuk & Grzela, 2014)

Amnion and Healing

Origins of Amnion Use in Medicine

The use of amnion to treat wounds such as ulcers, burns, and traumatic denudations in human patients was reported over a century ago. (Davis, 1910; Stern, 1913; Davis, 1919) Intended as a substitute for skin grafts to provide permanent coverage, the amnion failed due to immunological rejection. (Gruss & Jirsch, 1978) The original justification for considering amnion a suitable transplant tissue was based on it being derived from ectoderm and composed of embryonic skin elements. (Stern 1913, Davis 1919) Down the years, this characteristic was, in one way or other, repeatedly mentioned in studies. (Douglas 1952, Pigeon 1960, Robson and Krizek 1973) Douglas (1952) reported that amnion was believed to be have been continuous with the actual skin of the fetus at one time, Pigeon (1960) claimed it was formed by the ectoderm of the fetus and an extension of its skin, while Robson and Krizek (1973) reported it to be derived from the epiblast and continuous with the ectoderm.

Research in 1940s and 1950s

While amnion was originally used for skin wounds, other applications were later investigated. Chao et al. (1940) reported the successful experimental use of amnion in cats to prevent meningocerebral adhesions while allowing regrowth of lepomeninges (i.e. arachnoid mater and pia mater) over the brain after cerebral laceration. They referred to the processed form of amnion they used surgically as amnioplastin to distinguish it from fresh fetal membranes. Processing included sterilization by either autoclaving or boiling. Rogers (1941) reported the use of amnioplastin in 12 human cases to treat peripheral nerve lesions resulting from involvement of nerve trunks in scar tissue. After freeing the nerve trunk from scar tissue, the site of the lesion was wrapped with amnioplastin. While acknowledging it was too early to draw ultimate results, he concluded that the results to date were encouraging. Pinkerton and Edin (1942) reported the

use of amnioplastin in four human patients to mobilize tendons fixed by adhesions following sheath infection. After debriding scar tissue, the tendons were wrapped with amnioplastin. Satisfactory results were reported up to three months after surgery.

Law and Philip (1941) reported satisfactory healing using amnioplastin as a conjunctival graft in a clinical human case involving a lower eyelid injury. Compared to using a mucous membrane graft, it did not require an additional wound at the graft source. De Rotth (1940) had previously reported unsatisfactory results using the complete fetal membranes as a conjunctival graft.

Troensegaard-Hansen (1950) reported the clinical use of amnion as a graft to treat chronic leg ulcers in six human patients and considered the treatment remarkably successful. His patients noted that pain was no longer evident after two or three days, which was not the case with other dressings. He remarked how, in principle, the amnion kept the wound closed similar to an approach to wound care developed by Winnett Orr and Trueta using a plaster-of-Paris cast. As an aside, Dr. H. Winnett Orr and Dr. J. Trueta were both credited with establishing the use of plaster-of-Paris casts to treat wounds and open fractures as an alternative to the frequent change of dressing they believed to delay healing.

In a later study, Troensegaard-Hansen (1956) reported the clinical use of amnion to treat intermittent claudication (i.e. pain caused by inadequate blood supply to muscles, usually in the leg) in 40 cases of peripheral vascular disease. The procedure involved implanting amnion deep to fat on the affected thigh and was adopted after observing that amnioplasty performed for the treatment of leg ulcers had a beneficial effect on concurrent intermittent claudication. He concluded that one possible explanation was a reaction to the amnion implants giving rise locally to a vigorous outgrowth of capillaries.

In a review of 100 cases, Troensegaard-Hansen (1960) suggested that the epithelium of implanted amnion appeared to influence outcome since results were unsatisfactory in control cases where the amnion had been treated to remove epithelium.

Douglas (1952) reported the experimental and clinical use of fetal membranes for treating wounds. Placentas were collected from recent deliveries in human patients. The amnion and chorion were either separated or left attached to each other. Membranes were washed with normal saline and, if not used immediately, stored in a refrigerator one or two degrees above freezing for up to 4 days and kept moist with saline. The intent was to provide a "living covering" that retained its vitality and would last for a prolonged, not necessarily permanent, period. In the experimental portion of the study, wounds were created on dogs and dressed with either amnion or chorion. Membranes remained viable for as long as 29 days. The clinical treatment of human wounds included just three cases. The study concluded that the widespread use of fetal membranes on extensive wounds required more research before their use was advocated.

It appears that the potential benefits of amnion to promote healing were recognized and investigated by several authors with positive impressions generally. As will be discussed later, amnion is still used in human ophthalmology as a conjunctival graft. Another interesting insight was that of Troensegaard-Hansen recognizing how amnion might close wounds just as plaster-of-Paris was claimed to do decades earlier.

Cold War Impetus for Burn Research

The possibility of having to treat large numbers of burn victims at short notice in an atomic war motivated research into the treatment of burns with amnion. (Pigeon, 1960) Since the harvesting of skin grafts from elsewhere on a victim's body would be undesirable, a great deal of attention was being paid to the use of allographs. Pigeon (1960) reported the clinical use of amnion to treat second-degree burns in nine human patients. These burns involved loss of the epidermis, which

could result in excessive fluid loss and shock. Pigeon (1960) asserted that, since it was formed by the ectoderm of the fetus, amnion was similar to epidermis, and concluded that it was the most effective and practical treatment in his experience. The fetal membranes were collected in a sterile towel at birth, the amnion and chorion separated, the amnion cleaned with an antiseptic solution, then stored in an antiseptic solution in a refrigerated sterile container. When applied to burns, the amnion dressings were left in place for 10 to 15 days. Pain relief was reported generally as soon as the wound was dressed with amnion. Amnion was not used for third-degree burns or older burns where infection was already present.

Dino et al. (1965) reported the clinical use of amnion and chorion, either alone or in combination, to treat 23 human patients with burns covering between 15% and 60% of body surface areas. The fetal membranes served as an allograft to temporarily replace lost epidermis and protect against fluid loss and infection. It was acknowledged that autographs would be ideal but, since that creates additional raw surfaces, it was hardly considered particularly in the case of extensive wounds. Placentas were acquired from the obstetrics department. In some cases, the amnion and chorion were kept together after separation from the placenta while, in other cases, the amnion was stripped from the chorion. The membranes were then washed in running water, rinsed in sterile normal salt solution, immersed in normal salt solution with penicillin and streptomycin, and refrigerated until used.

Of the 23 cases, five had old burns that had already developed granulation tissue while the remaining 18 cases had recent burns with no granulation. Amnion alone was used in 19 cases, chorion alone in two cases, and a combination of amnion and chorion in the remaining two cases. The grafts were removed between nine and 20 days of application. Initially, the older wounds remained infected and the allograft was rejected as early as two days after application; therefore, grafting of old burns was discontinued in the later part of the study. In contrast, recent burns receiving the graft displayed no gross infection, and immediate pain relief was noted in all cases

when fetal membranes were applied. It was proposed that the analgesic effect might have been due to the soothing effect of the membrane's soft mucoid surface, protecting nerve endings from irritants, even surrounding air. No allergic reactions were observed in any of the cases. Similar to Pigeon's report, (Pigeon, 1960) pain relief was reported when amnion was used to dress burns.

Antibacterial Properties Investigated

Galash and Synder (1970) investigated antimicrobial factors in amniotic fluid. They obtained samples of amniotic fluid by abdominal amniocentesis from three healthy human patients in their third trimester of pregnancy. Samples were subsequently refrigerated for use the same day, or frozen at -20ºC and used within a week. Prior to analysis, samples were centrifuged to remove particulate matter and then sterilized by filtration through a Millipore filter. Lytic activities of the samples were compared to purified lysozyme, and provided evidence for the presence of lysozyme in the amniotic fluid. Immunoelectrophoresis of the samples demonstrated the presence of certain immunoglobulins characteristic of IgG. The samples were found to have significant antibody titers against influenza A2 and parainfluenza 3. It was mentioned that progesterone was known to be present in amniotic fluid and had previously been shown to be bacteriostatic for certain gram-positive organisms. The study concluded that the presence of antimicrobial factors in amniotic fluid was demonstrated.

Robson and Krizek (1973) reported the effect of human fetal membranes and human skin grafts on the bacterial population of experimentally infected burn wounds in rats as well as a limited number of clinical cases in human burn patients. The purpose of these studies was the re-evaluation of fetal membranes as a temporary biological dressing. Placentas were acquired at delivery from healthy mothers and the fetal membranes removed aseptically. No attempt was made to separate amnion from chorion. The combined membranes were passed through five rinses of sterile isotonic saline and, those not being used immediately, were passed through one

rinse of 0.025% sodium hypochlorite followed by an additional five rinses of saline before storage at 4°C. All membranes underwent bacterial culture at regular intervals up to six weeks.

In the first part of the report, full-thickness scald burn wounds were created in rats and inoculated with *Pseudomonas aeruginosa*. Five days later, the eschar was removed. Each wound was subdivided into three areas, and a bacterial count performed for each area from tissue biopsies. Each of the three areas was subsequently treated by suturing human skin or membranes into the defect, or leaving the defect untreated as a control. Every 48 hours, the skin grafts and membranes were replaced and bacterial count repeated. After two changes of biological dressings, virtually all of the wounds treated with skin grafts and membranes had a decreased bacterial count compared to only 40% of control wounds. Wounds treated with membranes had a geometric mean bacterial count of 10^3 organisms per gram compared to 10^6 organisms per gram for wounds treated with skin grafts. This raised the question of whether the membranes contained an antibacterial substance, with allantoin and lysozyme both mentioned as possibilities. However, an in vitro comparison of bacterial growth in membranes and split-thickness skin was performed using inoculums of *P. aeruginosa* and *Escherichia coli* but no antibacterial effect was observed. It was proposed that the membranes resulted in a biologically closed wound, thus allowing the host immune system to act against the bacterial population. This hypothesis was also applicable to the decrease in bacterial count observed using skin grafts.

In the second part of the report, 10 human patients with deep partial-thickness or full-thickness burns were also studied. (Robson & Krizek, 1973) Full-thickness wounds were first treated with topical antibacterial agents until eschar separation. Partial-thickness wounds were also treated with topical antibacterial agents in the acute stage. At that point, topical treatment was discontinued, bacterial counts were performed from tissue biopsies, and the wounds were dressed with either membranes or split-thickness skin allographs. These dressings were replaced every 48 hours and bacterial counts performed from tissue biopsies. Biological dressings continued for

full-thickness burns until replaced with autographs or, in the case of partial-thickness burns, until the wound had re-epithelialized. Bacterial count was well controlled by the topical antibacterial with no count greater than 10^6 organisms per gram prior to commencing biological dressings. Dressing the burns with either amnion or skin grafts caused a further decrease in bacterial counts with no apparent difference between the two treatments. In these clinical cases, results were considered difficult to interpret given the non-uniformity of the burns. In addition, the sample size was too small for statistical analysis. Notwithstanding these factors, the membrane dressings appeared to decrease bacterial count similar to the skin grafts.

Inge et al. (1991) investigated the presence of antibacterial factors in amnion using a disc-diffusion susceptibility test. Three categories of discs were tested: amnion alone, amnion unseparated from the chorion, and a synthetic membrane that served as a control. Susceptibility of five species of bacteria were tested: coagulase-positive staphylococcus, *E. coli*, *Klebsiella pneumonia*, *P. aerginosa*, and *Proteus mirabilis*. While the presence of a halo representing bacterial susceptibility was not observed around any of the disks, bacterial growth was completely inhibited immediately beneath all the discs. The study proposed that the close adherence of amnion to wound beds may be the primary contributing factor where amnion is found to have an antibacterial effect on wounds.

1970s Surge in Interest

An upsurge of research interest into amnion appeared to take place in the 1970s. Robson and Krizek (1974), building on their previous research (Robson & Krizek, 1973), reported on the clinical use of fetal membranes as a temporary biological dressing to treat wounds in 150 patients. As in their prior report, the amnion and chorion were not separated. When applied to full-thickness wounds, the combined membranes were placed with the chorion surface against the wound and changed every 48 hours. In contrast, when applied to partial-thickness wounds, the

amnion surface was placed against the wound and left undisturbed while epithelialization progressed underneath. These membranes provided results at least as good as skin allografts or xenografts used as biological dressings. All three types of dressings decreased pain and controlled heat and fluid loss from the wound. Compared to xenografts, fetal membranes adhered better and had a superior ability to decrease bacterial counts. Compared to allografts, they were more readily available and less expensive.

Colocho et al. (1974) reported their clinical experience with amnion used to dress burns and split-thickness skin graft donor sites. In 20 of 65 human patients, only a portion of the donor site was dressed with amnion while the remaining portion was covered with petrolatum-impregnated fine gauze. All 20 patients noted less pain associated with the amnion-covered portions of donor sites. The amnion was applied just once and allowed to desiccate spontaneously and separate from the wound bed. No vascularization of the amnion was observed.

Trelford et al. (1975) reported on the experimental use of amnion to treat surgically created wounds in sheep with subsequent histological evaluation of healing. Two 5x5cm square wounds were created on the back of nine lambs delivered by cesarean section at six hours of age. The wounds were then covered with a double layer of amnion and the orientation of the epithelial cell layer noted. The amnion was sutured in place and covered with a furacin and vaseline gauze. Excisional biopsies were performed daily for 12 days. All wounds healed within 10 days. Histology showed the surgically excised skin incorporated epidermis, dermis, and underlying muscle fascia. It also found little difference in whether the epithelial cell or the mesenchymal surface was oriented towards the wound bed. Healing appeared to progress with epithelium undermining the amnion as well as by wound contraction. The amnion did not persist as a permanent structure and it appeared capable of preventing bacterial invasion. No acute immunological rejection was noted.

Gruss and Jirsch (1978) reported the use of fetal membranes as a temporary biological dressing for wounds in 120 human patients. The amnion and chorion were not separated. Full-thickness wounds were treated by dressing the wound with the combined membranes, the chorion surface against the wound. Placing the chorion surface against the wound was driven by the supposed findings of Douglas (1952), that initial neovascularization occurred on experimental wounds with that surface orientation; however, review of the report by Douglas (1952) does not clearly support that assertion. Dressings were changed every 48 hours or more frequently if heavy contamination or infection occurred. Once healthy granulation tissue had formed, the wounds were treated with either permanent skin autografts or delayed closure. Partial-thickness wounds were treated by placing the amnion surface against the wound allowing epithelialization to take place underneath. Placing the amnion surface against the wound was driven by the findings of Colocho et al. (1974) that no vascularization occurred with that membrane orientation. Gruss and Jirsch (1978) concluded that membranes were successful as a temporary biological dressing in their patients.

Bose (1979) reported on the use of fetal membranes as a physiological dressing in 15 burn patients. No attempt was made to separate the amnion from chorion. The combined membranes were applied with the amnion surface against the wound. The first wound inspection was performed after 24 hours with any non-adherent portion being removed and replaced; thereafter, the entire dressing was replaced every 48 hours. The removal of membrane adhering to the wound bed helped to debride the wound in preparation for grafting. After four to six days, it was usually straightforward to identify the areas that required skin grafting. After harvesting grafts, donor sites were dressed with amnion. Bose reported that the outcome of 14 of 15 cases was satisfactory. In all cases, analgesia after application of membrane was considered impressive. Bose (1979) considered that membrane adherence to the wound was a significant factor in the positive outcomes, describing membrane as a fibrin-elastin biological bond that protected the wound from the environment, possibly explaining some of its anti-bacterial properties.

Quinby et al. (1982) reported a clinical trial using fetal membranes to treat several categories of burn and donor site wounds in human patients. The amnion and chorion were not separated. The combined membranes were applied with the chorion surface against the wound. Donor sites in 40 patients were treated and achieved a rate of healing equivalent to the standard dressing of 5% scarlet red ointment gauze, and analgesia was marked. Membranes were applied to clean shallow burns in 11 patients with healing similar to topical silver agents and allografts. Improved analgesia was again noted when membranes were used. When membrane was temporarily applied to 12 freshly excised burn wounds in 10 patients in preparation for eventual skin grafting, the success of subsequent autografts was equivalent to that of skin allografts typically used for the same purpose. Membrane was applied to contaminated open burn wounds in 10 patients with suppression of bacterial growth similar to skin allografts. Quinby et al. (1982) considered it probable that control of bacterial growth in contaminated wounds treated with membranes resulted from intimate conformity of the membrane to the wound. Analgesia appeared to result from decreased inflammation and, possibly, hydration of the wound bed. The study concluded that the beneficial effects of amnion, similar to skin allografts, was the preservation of healthy cells in the wound and protection from environmental contamination.

Treatment of Ulcers

Bennett et al. (1980) used amnion as a dressing in preparation for eventually using autografts on chronic leg ulcers in 15 human patients. Fetal membranes were collected from patients that underwent elective cesarean section and maintained in a tissue culture until used. Prior to use, the amnion was separated from chorion. Despite conflicting information between text and tables in the study, it appears that dressing a wound for approximately five days with amnion generally resulted in wounds filled with granulation tissue. The study concluded that the best clinical responses to subsequent autografts were noted in patients with healthy granulation tissue after amnion treatment, and an angiogenic effect of the amnion was proposed.

In a related report, Faulk et al. (1980) reported on biopsies obtained from leg ulcers on the same 15 patients (Bennett et al., 1980) prior to and following application of amnion. Histology of pre-treatment samples found isolated groupings of thick-walled vessels within dense connective tissue. After treatment, specimens displayed an increased number of more evenly dispersed thin-walled vessels in more delicate connective tissue. Immunohistology found increased factor VIII granules within endothelial cells in post-treatment specimens as well as increase in the number of vessels. These findings suggested the presence of angiogenic growth factors in amnion that might explain the success of amnion in clinical practice.

Egan et al. (1983) reported on the use of human amnion to treat chronic lower limb ulcers prior to skin grafting. This was a prospective study involving 24 ulcerated limbs in 18 patients where limbs were randomly assigned to a treatment group (amnion) or a control group (conventional treatment). Conventional treatment involved frequent application of eusol, an antiseptic, in paraffin followed by saline soaks in the period immediately prior to grafting. For the treatment group, placentas were sourced from elective cesarean sections, transported in saline, and the amnion applied to wounds within four hours. Tissue biopsies were taken from each wound before amnion treatment and again before grafting. Wounds treated with amnion were considered ready for skin grafting after a mean of 10.7 days compared to 24.9 days for the conventional group. Analgesia was reported to be noticeably better in the amnion group. While histology revealed that pre-treatment wounds tended to have sparsely distributed, thick-walled vessels in dense connective tissue, wounds in both post-treatment groups displayed thin-walled vessels with wider lumens. This change was more pronounced in the amnion group.

Ward and Bennett (1984) reported on the long-term results of using human amnion to treat leg ulcers prior to skin grafting. This was a retrospective study and included 38 ulcers in 28 patients with at least 18 months of follow-up after grafting. After collection from elective cesarean sections, fetal membranes were maintained in a tissue culture. The amnion and chorion were

separated prior to use and just the amnion used. Half of the wounds showed no sign of ulcer recurrence within the grafted area one year after grafting while some recurrence was noted in the remaining wounds. The long-term recurrence rates were considered similar to alternative surgical treatments. Direct application of angiogenic growth factors sourced from amnion to enhance wound healing was proposed.

Growth Factors Investigated

The previously mentioned publication by Faulk et al. (1980) appears to be one of the earliest to propose the benefits of growth factors provided by amnion, specifically angiogenic factors. Robson (1991) acknowledged growth factors as messengers that orchestrated the various processes involved in wound healing. Rather than considering wound healing a general term, Robson (1991) described it as the summation of processes such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasia, epithelialization, contraction, and remodeling. The involvement of each process depended on the nature and stage of healing of a wound. For example, a partial-thickness skin wound heals almost entirely by epithelialization while a deep, chronic ulcer depends far more on angiogenesis, fibroplasia, and contraction.

Robson et al. (1998) reported mixed results from clinical trials spanning the previous 10 years treating chronic wounds with topical exogenous recombinant growth factors. Trials included use of PDGF, bFGF, EGF, GM-CSF, TGF-β, insulin-like growth factor I (IGF-I), and human growth hormone (HGH). The use of specific growth factors to influence processes currently active within a particular wound was considered important. It was believed that the sequential administration of growth factors, or sequential therapy, might be the solution.

In addition, bacterial infection and ischemia were both considered detrimental to the effectiveness of growth factors and had to be controlled before growth factor therapy. (Robson et al., 1998) Proteases produced by bacteria and leukocytes degraded growth factors, while the impact of

growth factors on aerobic processes in ischemic tissue was impaired. The means of delivering exogenous growth factors was also a consideration with liquids, gels, and collagen sponges being used in the past. The possible role of gene transfer in the future was mentioned.

Robson et al. (1998) also raised the question whether additional growth factors were actually needed in chronic wounds. Cooper et al. (1994) had previously reported a method of determining levels of endogenous cytokines in wounds. Twenty chronic pressure ulcers were included in the study, which discovered that endogenous cytokine levels were much lower in chronic wounds compared to acute wounds. There was also a significant variation in cytokine levels which might have explained the conflicting results between clinical trials investigating exogenous cytokines.

PDGF was the first recombinant growth factor approved by the United States Food and Drug Administration for topical application, specifically for diabetic leg ulcers. (Robson et al., 1998) Rees et al. (1999) reported the results of topically treating pressure ulcers with becaplermin, a recombinant human PDGF. This was a double-blinded, randomized, placebo-controlled study that assigned 124 patients to four treatment groups: a) becaplermin gel 100 µg/g (i.e. becaplermin per gram of sodium carboxymethylcellulose vehicle gel) once daily; b) becaplermin gel 300 µg/g once daily; c) becaplermin gel 100 µg/g twice daily; and d) placebo twice daily. The primary endpoint for assessing the effectiveness of treatment was incidence of complete healing. The study found that the incidence of complete healing was significantly greater with either concentration of becaplermin gel applied once daily compared to the placebo, although there appeared little difference between the two concentrations themselves with no clear explanation. There was no significant difference between the placebo and becaplermin gel 100 µg/g applied twice daily, again with no clear explanation. The study concluded that becaplermin gel 100 µg/g applied once daily increased incidence of complete healing.

Robson et al. (2000) subsequently reported the results of sequential growth factor therapy in the treatment of chronic ulcers. This was a double-blinded, randomized, placebo-controlled study that divided clinical cases of pressure ulcers into four groups for daily topical treatment: a) 10 days of GM-CSF followed by 25 days of bFGF; b) 35 days of GM-CSF alone; c) 35 days of bFGF alone; and d) 35 days of placebo. Sixty-one patients completed the 35-day acute phase of the study. The primary outcome of interest was decrease in ulcer volume. Previous work by Robson suggested that GM-CSF acted early in healing to cause contraction, hence its inclusion to determine if it potentiated bFGF. Ulcer size was measured prior to initial treatment and again weekly for five weeks. In addition, endogenous levels of GM-CSF, bFGF, PDGF, TGF, and IL-1B, as well as mRNA levels of GM-CSF and bFGF were measured prior to treatment and again at 10 and 36 days following start of treatment. The study concluded that treatment with bFGF resulted in greater healing than treatment without bFGF. The study also reported that bFGF alone was most superior followed by the sequential GM-CSF/bFGF. It acknowledged that the sequential therapy did not result in the best outcome, and that delaying treatment with bFGF decreased its response.

Marvin et al. (2002) reported on the expression of growth and development related genes in human amnion and chorion. Fetal membranes were collected at delivery. Human cytokine expression arrays were probed using complementary DNA prepared from RNA extracted from the amnion and chorion. Amnion and chorion were analyzed separately. A range of angiogenic and neurotropic factors were represented on the complementary DNA arrays. A number of growth factors were found to be expressed, the strongest of which were three angiogenic factors, including vascular endothelial growth factor.

Contemporary Use of Amnion in Medicine

Pesteil et al. (2009) was reported that amnion was no longer being widely used to treat wounds in developed countries due to the risk of transmitting bacterial or viral diseases but was being used

to treat ocular surface disorders. Although its use to treat ocular burns was reported in the 1940s, it was not until the 1990s that it became more widely used in North America. (Dua et al., 2004) Prabhasawat et al. (1997) reported that amnion grafts were an acceptable treatment for primary pterygium (i.e. abnormal growth of conjunctiva that spreads across onto adjacent cornea) based on a recurrence rate significantly less than primary closure although the recurrence rate was significantly higher compared to conjunctival autografts. Azuara-Blanco et al. (1999) reported mixed results using amnion as a transplant to treat corneal disorders. Amnion promoted rapid healing in four of five patients with superficial defects; however, amnion was unsuccessful in the treatment of severe corneal ulcers in four patients. Dua et al. (2004) published an extensive review of amnion used for ophthalmic conditions involving the ocular surface. They concluded that, while its use as a viable alternative had been demonstrated in some situations, a similar outcome could have been achieved without amnion and that it was "not the panacea" that some reports may have suggested.

A recent retrospective study reported 771 amnion transplants performed at a hospital in Germany between 2001 to 2016. (Rock et al., 2018) The mean number of transplants in the second 8-year period was twice that of the first 8-year period, believed likely driven by new applications and increased awareness of amnion's beneficial growth factors.

Although human amnion-derived products are commercially available, there is very limited clinical data to support their use in human sports injuries. (Riboh et al., 2015; Huddleston et al., 2020) Studies investigating treatment of orthopedic conditions involving cartilage, tendons and ligaments, and osteoarthritis appear to be largely in vitro or animal models. (Riboh et al., 2015; Huddleston et al., 2020) Product formulations available include sheets, liquids, and powders. (Riboh et al., 2015; Huddleston et al., 2020) However, based on the effects of different processing methods on mechanical, biological, or cellular characteristics, equivalency of different formulations or products cannot be assumed. (Riboh et al., 2015; McCoy, Arrington, et al., 2019)

Amnion Use in Equine Medicine

Compared to human medicine where amnion has been widely used, there is lack of published reports using amnion in equine clinical cases and may reflect a difference in product availability. (McCoy, Smith, et al., 2019)

Three early studies stand out investigating the use of amnion to treat distal limb wounds in horses and ponies (Bigbie et al., 1991; Howard et al., 1993; Goodrich et al., 2000) All three studies involved collecting placentas at foaling, separating the amnion from chorion, processing the amnion in a series of steps involving antimicrobials, and storing in an antimicrobial solution at either -20ºC (Bigbie et al., 1991; Howard et al., 1993) or 4ºC. (Goodrich et al., 2000) Prior to use, frozen amnion was thawed in a refrigerator. (Bigbie et al., 1991; Howard et al., 1993)

Bigbie et al. (1991) published the results of using amnion to treat horses with experimentally created distal limb wounds. The number of days to complete healing of wounds was significantly less when wounds were dressed with amnion beneath a non-adherent dressing compared to control wounds with just the non-adherent dressing. Wounds treated with amnion, unlike control wounds, did not experience expansion in the first few days following surgery. This lack of expansion was attributed to amnion adhering to the wound and shrinking, conceivably acting as a splint at the wound edges and preventing spread. If this were indeed the case, it seems that the amnion provided a biomechanical rather than biological advantage.

Howard et al. (1993) also published the results of using amnion to treat experimentally created distal limb wounds in horses. Four different types of dressings were compared: non-adherent, semi-occlusive, equine amnion (described as biologic semi-occlusive), and fully occlusive. There was no significant difference in number of days to complete healing between the amnion and non-adherent dressing groups, or between the amnion and synthetic semi-occlusive dressing

groups. However, the number of days to complete healing was significantly longer for wounds dressed with the fully occlusive dressing compared to any of the other three types of dressing.

Goodrich et al. (2000) published a comparison between the use of amnion and a non-adherent dressing after pinch grafts were performed on experimentally created distal limb wounds in ponies. They found that wounds dressed with amnion had a significantly lower median healing time. The possible role of growth factors originating from the amnion was not mentioned in any of these three equine studies. (Bigbie et al., 1991; Howard et al., 1993; Goodrich et al., 2000)

In 2013, the clinical treatment of tendinitis and desmitis in horses using mesenchymal stromal cells from either amnion or bone marrow was investigated. (Lange-Consiglio, Rossi, et al., 2013) Ninety-two client-owned sports horses were randomly assigned to receive allogenic frozen-thawed amnion-derived mesenchymal stromal cells (51 horses) and fresh autologous bone marrow-derived mesenchymal stromal cells (44 horses). Horses were treated with intra-lesional injections of mesenchymal stromal cells and followed for two years after return to training. It was found that, while recovery time was similar, the difference in re-injury rate was significantly lower in horses treated with cells derived from amnion (4.00%) compared to those treated with cells derived from bone marrow (23.08%). The duration between injury and treatment ranged from 6 to 15 days and 16 to 35 days respectively for both groups, reflecting the delay incurred to culture and expand autologous cells compared to the immediate availability of allogenic cells. This delay in treatment was one proposed explanation for the significant difference in re-injury rates. The main contribution of mesenchymal stromal cells to healing remained uncertain with possibilities including differentiation into tenocytes, the supply of growth factors, or both.

In a follow-up study, tendon and ligament injuries in 13 client-owned horses were treated using an acellular conditioned medium derived from the culture of amnion-derived mesenchymal stromal cells. (Lange-Consiglio, Tassan, et al., 2013) The conditioned medium was prepared by

collecting supernatant from plates in which mesenchymal stromal cells had been cultured, centrifuging the supernatant to remove cellular debris, and lyophilizing the final supernatant. Prior to use, the lyophilized supernatant was dissolved in sterile water. Horses were again followed for two years after return to training and a re-injury rate of 15.38% of reported. It was concluded that the positive effect of the product on healing was achieved by factors released by the mesenchymal stromal cells.

A commercially available, decellularized, dehydrated equine amniotic membrane scaffold in combination with a decellularized, liquid morselized equine amniotic membrane product was recently investigated treating experimentally created distal limb wounds. (Fowler et al., 2019) Collectively, both treatments are referred to as equine amniotic membrane allograft (eAM). The liquid component used was found to contain detectable concentrations of TGF-β1 and VEGF but neither EGF nor PDGF-BB were detected. Wounds were assigned to four different groups: a) eAM; b) eAM control (untreated wound but within diffusion distance of liquid component used in eAM group; c) occlusive silicone gel dressings, and d) non-adherent dressing (negative control). No significant difference between the four groups was found in either healing time or histology of healed wounds. It was noted, however, that wounds in the first group (i.e. treated with eAM) showed greater production of granulation tissue early in the healing process.

Despite decades of research, however, the exact mechanism through which amnion influences wound healing is not yet fully understood. (Dahlgren, 2018)

Objectives of Study

An alternative equine-origin acellular LAA, comprised of amniotic membrane and fluid, has recently become commercially available. However, scientific data relating to the efficacy of this product is lacking.

The objective of the experimental component of this study was to report the outcome of treating experimentally created distal limb wounds in horses with locally administered LAA. It was hypothesized that wounds treated with LAA would heal faster than saline-treated controls.

The objective of the clinical component of this study was to evaluate the outcome (i.e. ability of horse to return to work) following local injection of LAA for the treatment of tendinitis and desmitis lesions. Additionally, these outcomes were to be compared to those reported in published clinical studies using regenerative modalities such as MSCs, PRP, ACS, and gene therapies for the treatment of tendonitis and desmitis. Our hypothesis was that treatment of specific equine tendonitis and desmitis lesions with local injection of LAA would result in outcomes comparable to horses with similar lesions treated with other regenerative modalities.

CHAPTER III

METHODOLOGY

Treatment of Experimentally-Created Wounds

All procedures in this experimental component of the current study were approved by and performed in accordance with the Oklahoma State University Institutional Animal Care and Use Committee (Protocol #VM-18-36).

Horses

Eight university-owned adult horses were included in a randomized, blinded, placebo-controlled study. Mares (n = 4) and geldings (n = 4) were evenly represented. Breeds included Quarter Horse (n = 4), Thoroughbred (n = 2), Oldenburg (n = 1), and Paint (n = 1). Median age was s years (range, 3 to 10 years). All horses were free of any obvious sign of previous trauma to the middle third of the metacarpi. All horses were judged healthy prior to the study based on physical examination and bloodwork (complete blood count, serum chemistry). Throughout the study, horses were housed indoors in individual 12 x 12 foot stalls with access to ad lib grass hay and water, and hand-walked for 15 minutes twice daily.

Surgical Procedure

After pre-medication with xylazine (1 mg/kg IV), anesthesia was induced with midazolam (0.05 mg/kg IV) and ketamine (2.5 mg/kg IV). Once intubated, horses were positioned in dorsal recumbency. General anesthesia was maintained with triple-drip (500 ml 0.9% NaCl, 500 mg

xylazine, 50 mg midazolam, and 2000 mg ketamine) at a rate of 1 ml/kg/hr. Both forelimbs were extended from the ceiling. Each metacarpus was circumferentially clipped and prepped with chlorhexidine scrub and isopropyl alcohol. A surgical staple was placed as a marker in the middle one-third of each metacarpus on the dorsomedial aspect. With the preplaced staple at its center, a sterile pliable template was used to outline a 2.5 x 2.5cm square area with a sterile marker, and a full thickness skin incision was made along the margins with a 15 scalpel blade. The square section of skin was resected, taking care to leave the subcutaneous tissue in situ. Hemostasis was achieved by applying pressure to the wound bed with sterile 4x4 inch gauze sponge. With a sterile metric ruler held adjacent to the wound for reference, a photograph of each wound was taken with a digital camera (Canon) allowing wound area to be measured later.

A calcium alginate dressing (Maxorb Extra Alginate Wound Dressing, Medline Industries, Mundelein, IL) was applied directly to the wound and secured with a stretch gauze bandage (Stretch Gauze Bandage Roll - 4", Non-Sterile, Dynarex Corp., Orangeburg, NY). A standard distal limb bandage consisting of 14-inch cotton combine secured with brown cling gauze and cohesive tape was applied over the initial bandage. Horses were administered one dose of flunixin meglumine (1.1mg/kg IV) post-operatively for analgesia. Horses recovered unassisted in a padded recovery stall. The day that wounds were created was designated as day 0 and used as a timeline reference for the remainder of the study. On day 3 and 6, the bandages were removed and the wound margins gently cleaned of any exudate with dry, non-sterile gauze sponges. The wounds were photographed and bandaged as previously described.

Treatment versus Control

On day 9, one investigator (MW) randomly assigned a wound from each horse (left or right) to the treatment group (n = 8) while the remaining wound was subsequently assigned to the control group (n = 8) using a random number generator (www.random.org). All other investigators were

blinded to these assignments. The bandages were removed and each wound was gently cleaned with saline-soaked sterile gauze sponges and photographed as before. After sedation with detomidine (0.01 mg/kg IV), a subcutaneous line block was performed on the dorsal aspect of each proximal metacarpus with 10 to 15 mL of 2% mepivacaine. The margins of each wound in the treatment group were injected with LAA (RenoVo, Equus Innovations, Phoenix, AZ) while those in the control group were injected with sterile 0.9% NaCl solution. The non-blinded investigator (MW) pre-loaded 1mL syringes with either the treatment or the saline control. These unidentified syringes were given to one blinded investigator (HD) with instructions on which wound (L or R) to inject. Injections were performed at the corners and midway between each corner for a total of eight injection sites per wound. A separate syringe and 25G x 5/8" needle was used for each injection. Each needle was inserted to its hub into the wound bed approximately 1cm from the wound margin and directed into the subcutaneous tissue so that the tip rested approximately 5mm beyond the wound margin. For the first two horses, 0.18 mL of LAA (treatment) or saline (control) was administered at each injection site. For the remaining six horses, the total volume administered at each injection site was doubled to 0.36 mL by adding an additional 0.18 mL of saline; however, the total volume of LAA injected remained the same (1.5 mL). After injection, the wounds were bandaged in a similar manner to day 0 except that the calcium alginate dressing was discontinued and replaced with a hydrophilic foam dressing (Kendall Hydrophilic Foam Dressing 4" x 4", Covidien, Mansfield, MA). Thereafter, bandages were removed and reapplied in a similar manner every three days to day 42, and then weekly until day 91, up to the time both wounds were judged healed. Bandages were maintained on both wounds until both were judged healed regardless if one wound healed sooner.

Wound Assessment

At each bandage change following injection, the wound margins were gently cleaned of any exudate with dry, non-sterile gauze sponges and photographed as before. The granulation tissue

bed was scored as previously described (Bigbie et al., 1991). If exuberant (i.e. grade 4), granulation tissue was resected with a 20 scalpel blade to the level of the surrounding epithelium. Wounds were considered healed when completely covered by epithelium. On completion of the study, all of the wound photographs were examined in random order (www.random.org) regardless of horse or day. For each photograph, a single blinded investigator (MS) traced the wound margin, allowing calculation of wound area (cm^2) using an imaging software (ImageJ).

Statistical Analysis

A mixed effects model with random intercept for each animal and fixed effects of time, treatment, and "time × treatment" interaction was used to determine if there was a significant difference between the treatment and control groups over time. Kaplan-Meier survival analysis was performed to compare treatment and control groups using time-to-heal as the variable, and a log-rank test (using wound size on day 9 as the initial size of each wound) was used to test whether there was a significant difference in survival (i.e. time not to reach a certain percentage of healing) between the groups. Statistical analysis was performed using R (version 4.0.3, The R Foundation). $P < 0.05$ was considered significant.

Treatment of Naturally-Occurring Tendonitis/Desmitis

The clinical component of this study was a prospective, multi-center, non-blinded clinical trial. Experienced equine veterinarians at 14 hospitals (sites) were selected for participation in the study. The LAA was shipped frozen by the manufacturer to the sites with instructions to keep the product frozen at -80˚C until use to preserve the beneficial proteins. Each site was provided with a calibrated -80˚C portable freezer (Shuttle C Model ULT-25NE, Stirling Ultracold) to store the LAA when received. The LAA was provided in 1.5 mL and 3.0 mL volumes aseptically packaged in individual vials. Instructions for use directed the veterinarian to thaw the LAA at room temperature and administer it locally in or around the lesion using strict aseptic technique

including aseptic preparation of the administration site and the use of sterile gloves and administration paraphernalia. The volume of LAA used to treat an individual lesion was left to the attending veterinarian's discretion.

Client-owned horses were selected for enrollment and subsequent treatment with LAA at the discretion of the attending veterinarian following examination, diagnosis and client education. Written informed client consent for treatment was obtained by the attending veterinarian prior to treatment. Criteria for inclusion in the study was defined as a horse displaying fore or hind limb lameness attributed to a specific diagnosis (lesion) of tendonitis or desmitis proven or supported by diagnostic anesthesia and imaging (ultrasound or magnetic resonance imaging) of the regional anatomy. Additionally, the identified lesion(s) must have been treated by local injection of LAA with follow-up information available for a period of six months or more. All horses remained in the care of the owners throughout the study period.

For each horse enrolled, a standardized questionnaire was completed by the attending veterinarian which included the date of LAA administration, LAA product code and serial number, horse identification and signalment, diagnosis(es), duration of injury/lameness, initial and re-evaluation lameness grade, ability to return to work, adverse events, and product satisfaction (Appendix 1). The studied outcome measure was the ability of the horse to return to work following LAA treatment. This was categorized as either returning to or exceeding the horse's previous level of work, returning to work but not able to perform to the horse's previous standards, or inability to return to work as a result of the injury and showing no improvement.

Following review of the completed questionnaires, information from those meeting the above criteria was compiled and those deemed not to have met the study criteria or those missing pertinent information (diagnosis, initial lameness grade, or ability to return to work) were

excluded. If a horse was administered LAA for treatment of two or more separate lesions concurrently, each was considered a separate lesion.

CHAPTER IV

FINDINGS

Treatment of Experimentally-Created Wounds

Horses remained in the study no longer than day 91, at which time the study ended regardless of whether the wounds were judged healed or not. Of the 16 wounds, only one wound was not judged healed by day 91. Median (range) days to heal from day 0 were 77 (33 to 91) and 66.5 (33 to >91) for treatment and control wounds, respectively (Table 1).

Table 1. Number of days for wounds to heal

	Days to Heal	
Horse	***Treatment***	***Control***
1	33	33
2	33	36
3	91	>91
4	70	56
5	84	33
6	42	39
7	36	56
8	77	91

Comparing wound size to that measured on day 9 (day of injections), the mixed model found no significant difference in wound size over time between the groups using P = 0.99 for the interaction term (Figure 1). The main effect of treatment was also not significant.

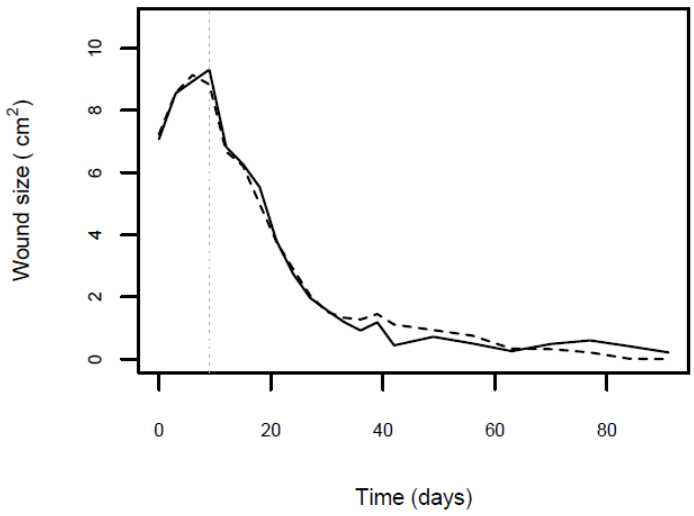

Figure 1. Mean wound size over time (treatment vs. control). Day 0 represents when wounds were *created*. The dashed line represents the treatment group while the solid line represents the control group.

Defining survival as a wound not reaching 95% healed, the log-rank test found no significant difference in survival between the treatment and control groups using P = 0.2 (Figure 2).

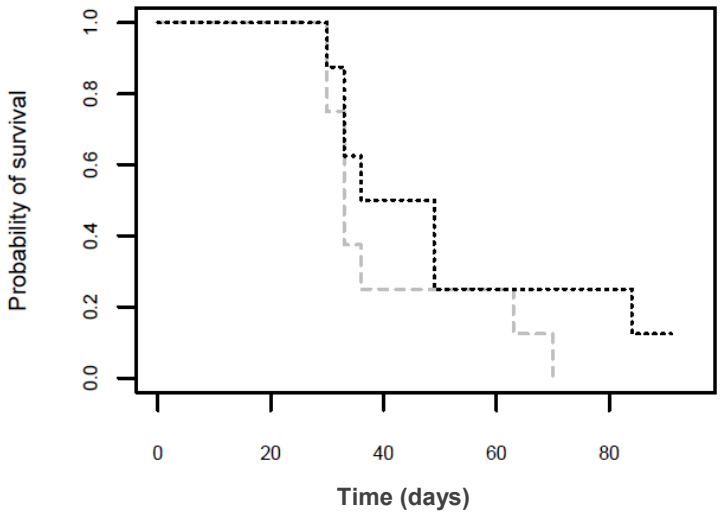

Figure 2. Kaplan-Meier survival curve (treatment vs. control). Survival is defined as a wound not reaching 95% healed. Day 0 represents when wounds were *treated*. The dashed gray represents the treatment group while the dotted black represents the control group.

Exuberant granulation tissue was identified and resected only twice throughout the study. Both instances involved wounds in the treatment group, horse 3 on day 57 and horse 5 on day 48.

Treatment of Naturally-Occurring Tendonitis/Desmitis

Nineteen veterinarians submitted 170 questionnaires describing their experiences with LAA in horses. Following review, 100 questionnaires describing outcomes for 100 horses were determined to be complete and meet the study's inclusion criteria; only these were included in the assessment (Appendix 2). A total of 128 tendonitis or desmitis lesions were described in these 100 questionnaires: 78/100 questionnaires described local injection of a single lesion, 18/100 described local injection of two lesions, 2/100 described local injection of three lesions, and 2/100 questionnaires described local injection of four lesions. Two of the 100 horses were treated twice for the same lesions, one horse with a single lesion and the other with two lesions.

Overall, 72/100 horses (72%) were reported to have either returned to or exceeded their previous level of work following local injection with the LAA: 18/72 horses at less than three months, 40/72 horses at between three and six months, and 14/72 horses at greater than six months. Ten out of the 100 horses (10%) were reported to have returned to work but not perform to previous standards: 2/10 horses at less than three months, 6/10 horses at between three and six months, and 2/10 horses at greater than six months. Eighteen of the 100 horses (18%) were reported to have not returned to work as a result of the injury and showed no improvement.

Duration of injury was reported on the questionnaire as < 6 months for 51/100 horses, 6 months to 1 year for 19/100 horses, 1 to 2 years for 10/100 horses, and > 2 years for 11/100 horses, while the duration of injury was not reported for 9/100 horses. The individual lesions were categorized based on their anatomic location and ability of the horse to return to work (Appendix 3). Horses associated with 94 of the 128 lesions were reported to have either returned to their previous level of work or exceeded their previous level of work: horses associated with 20/94 lesions at less than

three months, 53/94 lesions at between three and six months, and 21/94 lesions at greater than six months. Horses associated with 13 of the 128 lesions were reported to have returned to work but not perform to previous standards: horses associated with 2/13 lesions at less than three months, 6/13 lesions at between three and six months, and 5/13 lesions at greater than six months. Horses associated with 21 of the 128 lesions were reported to have not returned to work as a result of the injury and showed no improvement.

On 92 of 100 questionnaires, the attending veterinarians reported they were satisfied with both the LAA and the response to therapy. Seventy-two of these 92 questionnaires, reported the horse to have returned to or exceeded their previous level of work, 10/92 reported the horse to have returned to work but not perform to previous standards, and 10/92 reported the horse to have not returned to work as a result of the injury and showed no improvement. Six of 100 questionnaires indicated that the attending veterinarian was not satisfied and reported the horse to have not returned to work as a result of the injury and showed no improvement. A satisfaction response was not documented on 2/100 questionnaires.

Questionnaires indicated that one or more adverse reactions occurred following 14/131 local LAA injections in 13/100 horses. The most reported adverse events were swelling (11) and/or redness (7) at the administration site. Four horses were reported to have an increase in lameness post-LAA injection, two of those being non-weight bearing. In three of the four cases, the increased lameness was noted within 24 hours of LAA treatment and resolved within 72 hours. In the fourth case, an increase in lameness was not noted until 14 days after injection and resolved within 48 hours

CHAPTER V

CONCLUSION

The results of the experimental component of this study indicate that LAA, administered once by local injection, did not accelerate distal limb wound healing in horses. This was contrary to our hypothesis that wounds treated with LAA would heal faster than saline-injected controls. There was, in fact, no significant difference in healing time between the treatment and control groups.

This finding is similar to a recent study by Fowler et al. (2019) that incorporated a locally administered subcutaneous injection of an allogenic decellularized, liquid morselized amniotic membrane product on experimentally created distal limb wounds in horses. In addition to a subcutaneous injection along the wound margins, treated wounds were dressed with a decellularized, dehydrated equine amniotic membrane. No difference in time to heal was detected between treated and control wounds either grossly or histologically. While both Fowler et al. (2019) and the current study injected amnion at the wound margins, there was a fundamental difference related to timing of treatment. Fowler et al. (2019) performed injections three days after creation when the wounds were in the acute phase of healing. The current study performed injections nine days after creation so the wounds were more chronic when treated. Over time, wounds are reported to suffer growth factor depletion (Cooper et al., 1994), thus chronic wounds may benefit from therapies that replenish needed factors at the appropriate stage. However, a difference in time to heal between treated and untreated wounds was not observed in either

Fowler et al. (2019) or the current study. Perhaps the timing of treatment was inappropriate and further studies evaluating treatment of more chronic wounds with LLA may be indicated.

Bigbie et al. (1991) investigated the use of amnion to treat horses with experimentally created distal limb wounds. That investigation was preceded by multiple reports in human medicine that found amnion an effective biological dressing (Troensegaard-Hansen, 1950; Pigeon, 1960; Dino et al., 1965; Colocho et al., 1974; Bennett et al., 1980; Egan et al., 1983; Ward & Bennett, 1984). Bigbie et al. (2019) reported that the number of days to complete healing of wounds was significantly less when wounds were dressed with amniotic membrane beneath a non-adherent dressing compared to control wounds with just the non-adherent dressing. Bigbie et al. (2019) also reported that wounds treated with amnion, unlike control wounds, did not experience expansion in the first few days following surgery. This lack of expansion was attributed to amnion adhering to the wound and shrinking, possibly acting as a splint at the wound edges and preventing spread. If this were indeed the case, it seems that the amnion provided a biomechanical rather than biological advantage. The LLA used in the current study provided no mechanical structure or support.

Amnion is considered a regenerative therapy in equine wound management. (Dahlgren, 2018) The main components of regenerative therapies are stem cells, scaffold, and bioactive factors. (Dahlgren, 2018) Cell-based therapies provide mesenchymal stem cells to deliver trophic mediators or differentiate into mature cells such as fibroblasts; (Dahlgren, 2018) however, LAA is acellular and cannot contribute in this way. Scaffold-based therapies provide an extracellular matrix that gives structural support to a wound and allows cell adhesion and migration into the defect (Dahlgren, 2018) but LAA has no extracellular matrix to offer. Bioactive factor therapies provide growth factors and cytokines important to healing, (Dahlgren 2018); this is the category into which LAA falls. In contrast to LAA, amnion does provide an extracellular matrix. Amnion antiseptically processed in the same manner used by Bigbie et al. (1991) and Goodrich et al.

(2000) was found to maintain its structural integrity for up to 12 months before use. (McCoy, Smith, et al., 2019) However, while fresh amnion contains significant levels of growth factors and cytokines (Litwiniuk & Grzela, 2014), it was recently reported by McCoy, Arrington, et al. (2019) that a number of important factors including EGF, TGFα, KGF, bFGF, and VEGF were not detected in amnion that underwent long-term storage after processing in the same manner used by Bigbie et al. (1991) and Goodrich et al. (2000). This finding has far reaching implications since, if it were assumed that the amniotic membranes used by Bigbie et al. (1991) and Goodrich et al. (2000) were largely devoid of these growth factors, then the positive effect of the amniotic membranes on time to heal in their studies was likely due to some other property of the membranes. This would support the results of the current study as well as the Fowler et al. (2019) study where the administration of growth factors made no difference.

Exuberant granulation tissue did not appear to be a problem in the current study with just two instances. While both involved wounds in the treatment group, sample size was considered too small to interpret statistically. Development of exuberant granulation tissue was generally more common in previous studies (Bigbie et al., 1991; Howard et al., 1993). Bigbie et al. (1991) found that wounds treated with amnion developed exuberant granulation tissue on significantly less occasions than wounds dressed directly with a non-adherent pad, and concluded that amnion suppressed the formation of exuberant granulation tissue. There was, in fact, just one incident of exuberant granulation tissue out of 12 wounds treated with amnion. Howard et al. (1993) found no significant difference in the incidence of exuberant granulation tissue among wounds dressed with amnion, non-adherent, or synthetic semi-occlusive dressings, while the incidence of exuberant granulation tissue was significantly greater in wounds dressed with the fully occlusive dressing than any of the other three groups. Both of these previous studies (Bigbie et al., 1991; Howard et al., 1993) used the same classification system as the current study for defining exuberant granulation tissue although interpretation remains inherently subjective. While the

current study dressed all wounds from day 9 onwards with a hydrophilic foam dressing, the synthetic semi-occlusive dressing used by Howard et al. (1993) was also hydrophilic and this group developed exuberant granulation tissue with some frequency. The most plausible reason for the relative absence of exuberant granulation tissue in the current study may be the pressure bandage maintained on the distal fore limbs. This entailed cotton combine, brown cling gauze, and bandaging tape. In contrast, Bigbie et al. (1991) followed dressings with an absorbent layer and elastic tape while Howard et al. (1993) followed dressings with gauze and elastic adhesive tape. The effect of a distal limb pressure bandage on development of exuberant granulation tissue may, therefore, warrant further investigation.

One limitation of the current experimental study is that the wounds were created surgically rather than occurring naturally and dressed as soon as they were created with little opportunity for contamination. Naturally occurring wounds are generally traumatic in origin and often not discovered immediately. Both the inciting cause of the trauma as well as the lapse in time before treatment present opportunities for substantial contamination. A further limitation is the lack of histopathologic assessment of wound healing. Wounds in the current study were judged to be healed subjectively based on visual and digital examination, however, such an approach would be similar to wound assessment in the field.

In conclusion, a single treatment of LAA does not appear to reduce distal limb wound healing time. While the findings of the current study are consistent with another recent study that also injected wound margins with an amniotic membrane product (Fowler et al., 2019), they contrast to previous studies in horses (Bigbie et al., 1991) and ponies (Goodrich et al., 2000) where amnion was used as a biological dressing and significantly reduced total healing time. Due to the recent discovery that processed amnion appears to lose important growth factors while stored (McCoy, Arrington, et al., 2019), further studies may be warranted investigating amnion as a dressing. Two hypotheses, in particular, that have been proposed by investigators in the past

might be considered: 1) amnion adherence to the wound provides biological closure and protection from the environment (Troensegaard-Hansen, 1950; Robson & Krizek, 1974; Trelford et al., 1975; Bose, 1979, Quinby et al., 1982); and 2) amnion acts as a splint at the wound edges and prevents expansion of the wound. (Bigbie et al., 1991)

The clinical component of the current study represents the first study to evaluate local injection of LAA for treatment of equine tendonitis and desmitis, and reports that 72% of treated horses returned to or exceeded their previous level of work after at least six months following treatment. In a previous report describing the outcome of 99 horses with tendon and ligament injuries treated with local injection of a PRP preparation, 81% of the horses returned to their previous use, 12% had clinical improvement but could not perform at the previous level, and 7% were classified as failures. (Scala et al., 2014) Another controlled clinical study evaluating local injection of MSCs and PRP for the treatment of tendonitis and desmitis lesions in the cannon region of 23 horses reported 13 (57%) to returning to racing within 15 months of treatment. (Renzi et al., 2013) Differences in the type and location of lesions as well as the breed or discipline of horses could account for the discrepancy between these success rates.

The most common lesion in the current study was categorized as suspensory branch desmitis (SBD), representing 38 of the 128 (30%) lesions reported. A prior case series described 11 horses with 18 SBD lesions treated with a single intra-lesional injection of PRP reported that five horses (45%) returned to their previous level of work within three months. (Castelijns et al., 2011) In another study, 22 warmblood sport horses were treated for SBD using intra-lesional allogeneic umbilical cord-derived MSCs resulting in 15 (68%) had returning to at least the same level of work after six months or more. (Van Loon et al., 2014) In the current study, 25 of 38 (66%) SBD lesions were associated with horses that either returned to or exceeded their previous level of work following local injection of LAA, a similar or more favorable rate than these prior reports. (Van Loon et al, 2014; Castelijns et al., 2011)

In the current study, proximal suspensory desmitis (PSD) lesions accounted for 15 (12%) of the lesions, and 73% of those were associated with horses that either returned to or exceeded their previous level of work following local injection of LAA. In a previous study, 107 of 127 (84%) horses with forelimb PSD and 108 of 144 (75%) horses with hindlimb PSD were treated with a series of three peri-ligamentous injections of ACS, entered full training by 110 days from the initial injection, and had no recurrence of lameness after a month of training. (Easter & Watts, 2014) In the current study, five of six (83%) forelimb PSD lesions and six of nine (67%) hindlimb PSD lesions were associated with horses that either returned to or exceeded their previous level of work, similar rates for forelimbs and slightly less favorable rates for hindlimb PSD compared to the previous study. (Easter & Watts, 2014)

Superficial digital flexor (SDF) tendonitis accounted for 15 (12%) of the lesions in the current study. A case series of 22 racing Thoroughbreds with SDF tendonitis of varying severity reported that 64% of horses returned to racing following intra-lesional injection with PRP. (Zuffova et al., 2013) In a controlled clinical trial of 20 horses with forelimb SDF tendonitis, 8 of 10 (80%) horses treated with intra-lesional injection of PRP were performing at their previous level or higher 12 months after injury while only 6 of 10 (60%) control horses shared that success. (Geburek et al., 2016) A case series of 40 racing Thoroughbreds with SDF tendonitis treated with a series of four or five intra-lesional injections of insulin-like growth factor-type 1 reported that 21 of 34 (62%) horses for which race data were available raced at least once after treatment. (Witte et al., 2011) Additionally, 23 warmblood sport horses treated with intra-lesional injection of allogeneic umbilical cord-derived MSCs for SDF tendonitis resulted in 20 (87%) of these horses returning to at least the same level of work at least six months following treatment. (Van Loon et al., 2014) In the current study, 67% of the SDF tendonitis lesions treated with local injection of LAA were associated with horses that returned to or exceeded their previous level of work. The range of success rates reported in these previous reports (Van Loon et al., 2014;

Geburek et al., 2016; Witte et al., 2011) and the current study may be explained by differences in number, breed and discipline of the horses as well as the location (fore vs hind, proximal vs. distal) and severity of the lesions.

In the current study, deep digital flexor (DDF) tendonitis lesions accounted for 30 of the 128 (23%) lesions and were categorized anatomically as either cannon region or foot/pastern region. All eight DDF tendonitis lesions in the cannon region as well as 15 of 22 (68%) in the foot/pastern region were associated with horses returning to or exceeding their previous level of work. A study treating six warmblood sport horses with intra-lesional injection of allogeneic umbilical cord-derived MSCs for DDF tendonitis reported that four of these horses (67%) returned to their previous level of work six or more months after treatment, (Van Loon et al., 2014) a rate similar to the current study. A search of the current literature could find no reports evaluating the use of regenerative modalities in the treatment of DDF tendonitis of the foot/pastern region exclusively. A retrospective case study of 20 horses with 22 DDF tendonitis lesions treated with endoscopic debridement and/or navicular suspensory desmotomy reported that only nine (45%) returned to pre-treatment performance levels after six or more months. (Smith et al., 2007) Thus, it would appear that local injection of LAA may be beneficial in the treatment of these lesion types.

The current study also describes treatment of collateral (15), distal sesamoidean (11), SDF accessory (2), and patellar (2) desmitis lesions with local injection of LAA. A search of the current literature found very few reports describing long term outcomes of horses diagnosed with these desmopathies with none evaluating the use of regenerative modalities for treatment. A study of the long-term outcome of 20 horses diagnosed with collateral desmitis of the distal interphalangeal joint and treated with a variety of modalities reports 12 (60%) horses returning to and maintaining their previous level of exercise for at least three months while eight (40%) were described to have a poor outcome. (Gutierrez-Nibeyro et al., 2009) One horse in that report was

treated with intra-lesional injection of a urinary bladder matrix powder but the outcome of that specific horses was not described. (Gutierrez-Nibeyro et al., 2009) The current report includes 10 horses diagnosed with collateral desmitis of the distal interphalangeal joint with 9 of these (90%) returning to or exceeding their previous level of work. A previous study described 27 horses that were diagnosed with desmitis of the oblique (18), straight (3), or both (6) sesamoidean ligaments (10 forelimbs, 17 hindlimbs), and treated with various non-regenerative modalities in addition to rest and rehabilitation. (Sampson et al., 2007) Of the 21 available for follow-up, 16 (76%) were competing at the same or better level of performance. Another study reported nine cases of straight sesamoidean desmitis (5 forelimbs, 4 hindlimbs) with five (56%) able to return to their intended use. (Schneider et al., 2003) Four of the nine horses underwent ligament splitting of the straight sesamoidean ligament as well as injection of the tendon sheath with hyaluronic acid and methylprednisolone, three of these four horses being among the five able to return to their intended use. In the current report, six of seven (86%) horses diagnosed with straight sesamoidean desmitis and all four horses diagnosed with oblique sesamoidean desmitis returned to or exceeded their previous level of work following local injection of LAA, rates more favorable than previously reported. In a prior report, six of eight (75%) horses suffering from SDF accessory desmitis returned to their previous level of performance within six months following anti-inflammatory injection of the carpal sheath and rest. (Denoix et al., 1995) Both of the horses diagnosed with SDF accessory desmitis in the current study returned to or exceeded their previous level of work following local injection of with LAA. In a study of nine clinical cases of patellar desmitis, five were treated with rest and rehabilitation, three were treated surgically, and one was euthanized. (Dyson, 2002) Of the nine, just one was able to return to its former level of competition. Both cases of patellar desmitis in the current report returned to or exceeded their previous level of work following local injection of with LAA. It is the authors' opinion that the results presented in the current study support the use of local injection of LAA for these desmopathies.

In the current study, the attending veterinarians reported local adverse reactions in 13% of horses following injection of LAA. Although most of the events were transient swelling and redness, there were four reports of increase in lameness. In all four cases, the increase in lameness was reported to have resolved within 72 hours with non-steroidal anti-inflammatory treatment. In a prior report, a local injection site reaction rate of 4.35% was reported for local injection of allogenic MSCs in 164 horses with 230 lesions, which included 208 tendon or ligament lesions. (Ursini et al., 2019) The disparity in local adverse reaction rate between this report (Ursini et al., 2019) and the current study could relate to the difference in study design or the regenerative product used. Additionally, transient local adverse reaction rates as high as 12% are reported following injection of sodium hyaluronate. (Hyvisc package insert)

The attending veterinarian reported satisfaction with the LAA product in 92 of 100 horses, indicating an overall positive experience. Interestingly, the attending veterinarian reported satisfaction in 10 horses that failed to show improvement following local injection of LAA. Reasons for this may be that improvement was not expected due to the severity of the lesion or satisfaction referred to the LAA product's availability and ease of administration.

Limitations of the current study include the non-blinded design and the lack of a control group. The longest duration of follow-up was described as 6 months or more, thus some horses many have been evaluated at 6 months, while others may have been evaluated several weeks to months later. Having more precise follow-up periods would have allowed for more uniform reporting of outcomes. In addition, a longer duration of follow-up, perhaps 12 months or more, may have provided relevant information. The number of attending veterinarians likely introduced variability in patient selection and exact treatment methodologies (volume of LAA injected per lesion, LAA administration technique, etc.). Additionally, some of the specific lesion categories were low in numbers and may not represent an appropriate sample size, thus making interpretation difficult.

Results of this clinical trial indicate that local injection of LAA achieves comparable rates of return to previous level of work as other regenerative modalities such as MSCs, PRP and ACS in horses diagnosed with tendonitis or desmitis. However, blinded, controlled studies focusing on specific lesion types are indicated to more accurately evaluate the usefulness of local injection of LAA for the treatment of equine tendonitis or desmitis.

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APPENDICES

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Appendix 1 Standardized questionnaire completed for each horse in clinical trial

QUESTIONNAIRE

Thank you for completing this questionnaire to allow us to investigate and report outcome of horses with soft tissue injuries using the liquid amnion allograph product RenoVō™.

Your Full Name _____

Facility _____

Date of Product Administration: _____

Product Serial Number _____

Product Code _____

Horse Information

1. Horse Identification _____
2. Weight:
   - ☐ < 500 kg
   - ☐ 500 – 800 kg
   - ☐ > 800 kg
3. Age:
   - ☐ ≤ 3 years
   - ☐ 4 – 10 years
   - ☐ ≥ 11 years
4. Breed:
   - ☐ AQHA/APHA
   - ☐ Thoroughbred
   - ☐ Other (Please specify _____)
5. Sex:
   - ☐ Stallion
   - ☐ Gelding
   - ☐ Mare
6. Discipline:
   - ☐ Western (☐ reining ☐ cutting ☐ working cow)
   - ☐ Show
   - ☐ Sport (☐ dressage ☐ eventing ☐ hunter/jumper ☐ racing)
   - ☐ Other (Please specify (_____)
7. Type of Injury: _____

8. Duration of Injury:
   - ☐ < 6 months
   - ☐ 6 months – 1 year
   - ☐ 1 – 2 years
   - ☐ > 2 years

9. Lameness Grade (AAEP grading scale):
   - a. Pre-Treatment ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   - b. ____days Post-Treatment ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   - c. ____days Post-Treatment ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   - d. ____days Post-Treatment ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5
   - e. ____days Post-Treatment ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

10. Did the horse experience any of the following reactions upon administration of the product and if so, when did those reactions **resolve**?

Symptoms	1-3 hrs	Within 24 hrs	Within 48 hrs	Other (Please Specify)	Did Not Experience
Non-weight bearing	☐	☐	☐	☐ _____	☐
Swelling	☐	☐	☐	☐ _____	☐
Fever	☐	☐	☐	☐ _____	☐
Redness or Warmth	☐	☐	☐	☐ _____	☐
Other: _____	☐	☐	☐	☐ _____	☐

11. Work activity level post product administration?
   - ☐ Returned to or exceeded original level of work
     - ☐ < 3 months ☐ 3 – 6 months ☐ > 6 months
   - ☐ Returned to work but could not perform to previous standards or required more maintenance
     - ☐ < 3 months ☐ 3 – 6 months ☐ > 6 months
   - ☐ No return to work as a result of the injury and no improvement

12. Were you satisfied with the product and response to therapy?
   - ☐ Yes
   - ☐ No

Appendix 2 Outcome by signalment of 100 horses with tendonitis/desmitis treated with a liquid amnion allograft.

	Returned to or exceeded previous level of work	Returned to work but could not perform to previous standards	No return to work as a result of the injury and no improvement
Age			
≤ 3 years	4	0	3
4 to 10 years	40	6	9
≥ 11 years	26	4	5
ND	2	0	1
Weight			
< 500 kg	8	3	2
500 – 800 kg	59	7	15
> 800 kg	3	0	0
ND	2	0	1
Breed			
AQHA/APHA	38	2	17
Arabian/½ Arabian	17	3	0
Warmblood	9	1	0
Thoroughbred	2	2	1
Other	4	2	0
ND	2	0	0
Sex			
Stallion	4	0	0
Mare	31	4	6
Gelding	36	6	12
ND	1	0	0
Discipline			
Western Performance			
Barrel racing	14	1	4
Roping	5	1	4
Cutting	4	0	3
Reining	3	0	4
Not specified	2	0	0
Show	22	4	1

	Returned to or exceeded previous level of work	Returned to work but could not perform to previous standards	No return to work as a result of the injury and no improvement
Sport			
Racing - TB	2	2	1
Racing - Other	4	0	0
Hunter/jumper	6	2	0
Dressage	2	0	0
Not specified	1	0	0
Other	2	0	1
ND	5	0	0
Location of Injury			
Fore	47	6	13
Hind	25	4	5
Pre-Treatment Lameness Grade			
1	7	0	3
2	27	1	2
3	33	6	11
4	5	3	2
Duration of Injury			
< 6 months	37	4	10
6 Months - 1 Year	13	3	3
1 - 2 years	6	1	3
> 2 years	10	1	0
ND	6	1	2

ND – Not documented. TB – Thoroughbred

Appendix 3 Outcome of 128 tendonitis/desmitis lesions in 100 horses treated with a liquid amnion allograft.

	Returned to or exceeded previous level of work	Returned to work but could not perform to previous standards	No return to work as a result of the injury and no improvement
Suspensory branch desmitis			
All	25 (66%)	4 (10%)	9 (24%)
Fore	16 (73%)	0 (0%)	6 (27%)
Hind	9 (56%)	4 (25%)	3 (19%)
Proximal suspensory desmitis			
All	11 (73%)	1 (7%)	3 (20%)
Fore	5 (83%)	1 (17%)	0 (0%)
Hind	6 (67%)	0 (0%)	3 (33%)
DDF tendonitis – foot/pastern region			
All	15 (68%)	3 (14%)	4 (18%)
Fore	12 (67%)	3 (17%)	3 (17%)
Hind	3 (75%)	0 (0%)	1 (25%)
DDF tendonitis – cannon region			
All	8 (100%)	0 (0%)	0 (0%)
Fore	5 (100%)	0 (0%)	0 (0%)
Hind	3 (100%)	0 (0%)	0 (0%)
SDF tendonitis			
All	10 (67%)	2 (13%)	3 (20%)
Fore	8 (62%)	2 (15%)	3 (23%)
Hind	2 (100%)	0 (0%)	0 (0%)
SDF-AL desmitis			
Fore	1 (50%)	1 (50%)	0 (0%)
Collateral desmitis – coffin joint			
Fore	9 (90%)	1 (10%)	0 (0%)
Collateral desmitis - Other			
All	3 (60%)	1 (20%)	1 (20%)
Fore	2 (67%)	0 (0%)	1 (33%)
Hind	1 (50%)	1 (50%)	0 (0%)

	Returned to or exceeded previous level of work	Returned to work but could not perform to previous standards	No return to work as a result of the injury and no improvement
Straight sesamoidean desmitis			
All	6 (86%)	0 (0%)	1 (14%)
Fore	4 (80%)	0 (0%)	1 (20%)
Hind	2 (100%)	0 (0%)	0 (0%)
Oblique sesamoidean desmitis			
All	4 (100%)	0 (0%)	0 (0%)
Fore	2 (100%)	0 (0%)	0 (0%)
Hind	2 (100%)	0 (0%)	0 (0%)
Patellar desmitis	2 (100%)	0 (0%)	0 (0%)

DDF – Deep digital flexor, SDF – Superficial digital flexor, AL – Accessory (check) ligament

VITA

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