Original Article

Ultrasound-guided lipoaspiration for mesenchymal stromal cell harvest in the horse

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Summary
Mesenchymal stromal cells (MSC) are being used to treat a variety of conditions in the horse. Traditional lipectomy and bone marrow aspiration for MSC harvest have disadvantages including cosmetic issues with lipectomy and extensive time for cell expansion after bone marrow harvests. This article describes a new technique of adipose harvest in the horse, utilising ultrasound and liposuction that can be safely and effectively performed in an ambulatory environment. Ultrasound-guided lipoaspiration offers a minimally invasive technique using small portals and a diffuse area of collection significantly improving aesthetic outcome and increasing the likelihood of treating within a matter of days vs. a matter of weeks.

Introduction
Regenerative medicine is a growing field of equine ambulatory and referral practice. Adult MSC therapy has widely gained acceptance in the treatment of a variety of musculoskeletal conditions. The commonality and recurrence of equine tendinopathies (Rooney and Genovese 1981; Dowling et al. 2000; Williams et al. 2001) has motivated MSC research in horses. Recent publications have supported MSC use demonstrating improved healing quality and reduced reinjury in horses with tendonitis (Del Bue et al. 2008; Nixon et al. 2008; Smith 2008; Carvalho et al. 2009; Williams 2005; Dahlgren et al. 2012; Lim 2014). The mechanism by which MSCs improve healing outcomes is likely a combination of anti-inflammatory, immunomodulatory and growth factor effects (Puissant et al. 2005; Dahlgren et al. 2006; Wolbank et al. 2007; Nixon et al. 2008). Additionally, recent work investigating anti-apoptotic effects of MSCs in man and mice (Kim et al. 2012; Lim et al. 2013) offer further support of MSC use as apoptosis appears to significantly influence degeneration in inflamed equine tendons (Hosaka et al. 2004).

Currently, the two most common sources of adult MSCs are bone marrow and adipose tissue. The choice of adipose tissue (AT-MSC) or bone marrow (BM-MSC) derived mesenchymal stromal cells is of current debate and clinician preference. Historically, wound care, hair deformation and cicatricial inherent of incisional lipectomy have deterred use of AT-MSC in horses. With the introduction of lipoaspirate as a source of MSCs (Zuk et al. 2001) in man and horses (Bruno et al. 2014) these disadvantages become inconsequential due to minimally invasive skin portals and diffuse collection site distant from vital organs.

Lipoaspiration is combined with tumescence, a technique that utilises saline, local anaesthetic and epinephrine to provide regional anaesthesia and haemostasis limiting the need for heavy sedation or general anaesthesia. The tumescent lipoaspiration technique in the horse originated from human plastic surgery principles shown to be a safe and effective method of adipose harvesting (Habbema 2009; Boeni 2011). However, lipoaspiration requires surgical techniques not routinely covered in veterinary didactic training or clinical practice; thus the authors advocate utilising diagnostic ultrasound during the procedure. In the authors’ experience, ultrasonographic guidance during tumescent infusion and lipoaspiration improves cosmetic outcome and reduces risk of iatrogenic trauma and contamination of lipoaspirate with other tissues. This paper will provide a detailed review of ultrasound-guided tumescent lipoaspiration in horses. In the author’s experience, knowledge of lipoaspiration of adipose tissue has limited recognition amongst equine practitioners and their clientele and the use of ultrasound guidance throughout the technique has not been published elsewhere in the horse.

Materials and methods
The procedure was performed on 21 clinical cases (14 geldings, six mares, one stallion) from 2011 to 2014 for AT-MSC application in a variety of musculoskeletal injuries. Age ranged from 1 to 18 years and a diversity of breeds represented including Quarter Horse, Warmblood, Thoroughbred, Andalusian, Arabian and pony.

Procedure preparation
Prior to the procedure, ultrasonographic examination with a linear 12.5 MHz transducer (SonoSite) is performed to confirm appropriate adipose layer for harvest. A variety of other transducers such as a rectal probe also provide adequate imaging. Optimal adipose thickness is 3–4 cm. If adipose depth is less than 1.5 cm owners are cautioned that cell expansion by culture may be necessary. The depth (4.9 cm is typical for these authors) and gain should be adjusted appropriately in accordance with practitioner preference. A large fat depot is located approximately 8–15 cm abaxial to the insertion of the tail. A trough formed by the junction of the semitendinosus and biceps femoris muscles is typically the thickest portion of the adipose field (Fig 1). The authors place each portal entry site just caudal to the deepest portion of the adipose trough. The trough is followed abaxially, axially and cranially to ensure at least 1–2 cm of adipose tissue are

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present for harvest and the boundaries are then marked with Wite-Out® (Fig 2).

After wrapping the tail with cohesive bandage (Vetrap)3 the marked area is clipped with a No. 40 blade and aseptically prepped. The patient is mildly sedated intravenously using detomidine hydrochloride (Dormosedan)4 (0.01–0.02 mg/kg bwt) and butorphanol tartrate (Torbugesic)5 (0.01–0.02 mg/kg bwt). Additional sedation is used as it is necessary to ensure the patient stands still.

**Ultrasound-guided tumescent infusion**

After applying a twitch or other appropriate restraint the skin portal site is desensitised with 2–3 ml (40–60 mg) of 2% lidocaine6 using a 25 gauge x 16 mm needle ensuring a visible bleb is left behind (Fig 2). After additional scrub is applied and the site rinsed with alcohol, an adhesive sterile surgical drape (Loban)3 is applied to the ventral one-half of the preparation area and secured distally to prevent iatrogenic contamination with unclipped hair of the caudal hindlimb. The dorsal one-half is left undraped to allow adequate access for the ultrasound probe to guide the cannulas.

After appropriate delay for local anaesthesia the twitch is reapplied and a portal hole for the cannula made with a percutaneous punch (Pocar)7 (Fig 3) or scalpel blade within the desensitised bleb. The portal entry site should be sufficiently sized to allow cannula penetration with minimal tissue drag but small enough to prevent loss of suction and entry of air. A 60 ml Toomey® syringe is then loaded with tumescent fluid and infusion cannula (CellFriendly infiltrator)7 connected and primed to displace air from the cannula lumen. The infusion cannula was chosen in accordance with manufacturer’s recommendations based on the fibrous nature of equine adipose tissue and intended use of the lipoaspirate. Alternatively, a spinal needle may be used, although the infusion distribution will likely vary. The tumescent fluid consists of 230 ml sterile NaCl 9%, 20 ml (400 mg) 2% lidocaine and 0.2 ml (0.2 mg) 1/1000 epinephrine9. A small amount of alcohol for acoustic coupling is sprayed above the draped field and the ultrasound probe applied by a technician. Both transverse (authors’ preference) and sagittal probe orientations can be used to visualise advancement of cannula through the adipose layer. The probe is redirected through practitioner-technician communication, which is quickly mastered even with minimal knowledge of ultrasonography by the technician (Fig 4).

The infusion cannula should be advanced in a systematic manner, with the authors preferring to begin by aiming the cannula to the most axial and cranial aspect as determined with ultrasound. The tumescent solution is then slowly infused as the cannula is retracted but not withdrawn from the portal and then re-advanced slightly deeper than the previous stroke staying within the adipose layer as confirmed with ultrasonography. Once empty, the 60 ml syringe is then
reloaded with tumescent fluid and infusion repeated aiming in a radial pattern from axial to abaxial. This is repeated with 180–250 ml until the relatively hypoechoic adipose tissue becomes evenly expanded and relatively hyperechoic ultrasonographically, indicating optimal infusion of tumescent infiltrate (Fig 5). During infusion, meticulous avoidance of the muscle layer is advocated. If muscle disruption occurs, the area of haemorrhage is readily visible and can be avoided ultrasonographically during harvesting. Once the infusion is complete a timer is set for 15–45 min to allow distribution of tumescent fluid into the tissue. In the authors’ experience, a minimum of 20 min allows for optimal anaesthesia, haemostasis (via vasoconstriction) and tumescence (i.e. becomes firm).

While waiting for the first infusion to diffuse, tumescent solution is applied to the contralateral side in the same manner. The twitch is then removed and is usually no longer required. Prior to sterile glove removal, manual massage of the adipose field is advocated to enhance distribution of tumescent fluid. During the resting phase the fat pad becomes palpably swollen and firm as tumescent fluid causes the adipose to swell, become turgid and ready for harvest (Dhami 2008; Venkataram 2008).

**Ultrasound-guided lipoaspiration**

Using a sterile technique, the aspiration harvest cannula (Cobra Bibevel) is connected to a new, dry Toomey syringe ensuring no liquid is present to weaken the seal. A small amount of sterile saline is used to fill the cannula lumen to again prevent air from obscuring ultrasound image quality. Additional alcohol is applied for acoustic coupling and the cannula inserted through the portal hole. Once in place within the adipose layer, the syringe plunger is pulled back to the fullest extent and locked in place with a syringe plunger lock (Johnny Lok) to create an even, gentle vacuum pressure within the syringe and adipose field (Fig 6).

With scrupulous ultrasound visualisation the cannula is then sharply directed forward into the tumescent adipose tissue. As the cannula is advanced, the wrist is rotated (e.g. from pronated to supinated and back again). This is repeated 25-40 times to yield 45 ml of lipoaspirate in the same radial pattern used to inflate the tumescent fluid with ultrasound guidance to avoid muscle tissue. Ultrasonographically, gradual depletion of the hyperechoic tumescent-adipose field occurs as the tissue is aspirated, indicating a need to redirect the cannula to a different location within the fat pad. After completion the lipoaspirate is then carefully deposited into an empty sterile transport container without disconnecting the Toomey syringe and harvesting cannula (to maintain dry, adequate seal for the other side). The cannula manufacturer advises caution when handling to prevent scratching of the specialised cell-friendly coating designed to preserve cell viability. The same steps for aspiration are then repeated on the contralateral side, yielding a total harvest of 90 ml of lipoaspirate. Generally, after between 30 and 45 ml the surgeon will notice a significant reduction in volume extraction per stroke and an increase in serosanguinous character of the lipoaspirate. When this occurs the authors generally discontinue lipoaspiration on that side.
Post operatively, the portal holes are left open to heal by second intention to allow drainage of any tumescent fluid left behind after aspiration post operatively (Dhami 2008; Venkataram 2008). Mild serosanguinous drainage is normal and expected for 2–3 days. Ports may be sutured closed; however, performing this procedure in an ambulatory environment assumes some level of contamination in the authors’ opinion. With this in mind, the authors’ surmise primary closure may actually lead to more complications such as prolonged swelling, pain, seroma or infection (Dhami 2008). Antimicrobial and nonsteroidal anti-inflammatory administration is based on veterinary discretion.

The harvested lipoaspirate is typically shipped overnight on ice to a processing laboratory. Samples are then processed aseptically to separate the adipocytes from other nucleated cells as reported in the literature (Bunnel et al. 2008; Carvalho et al. 2009; Taylor and Clegg 2011). The resulting heterogenous cell population, termed the stromal vascular fraction (SVF), can either be injected as is or subjected to cell expansion to produce a relatively pure population of MSCs. The cell processing laboratory10 used by this practice monitors all samples for contamination. To date, all ultrasound-guided samples we have performed have been free of contamination. Samples were tested for aerobic bacteria, anaerobic bacteria and fungi via USP 71 (http://www.pharmacopeia.cn/v29240/usp29nf24s0_c71.html) as well as tested for mycoplasma. Additionally, this laboratory has unpublished internal data validating lipectomy and lipoaspirate samples in the horse. The cells produced meet the minimal criteria for MSC defined by the International Society for Cellular Therapy (ISCT) (Dominici et al. 2006) and International Federation of Adipose Therapeutics and Science (IFATS) (Bourin et al. 2013) based on plastic adherence, CD markers and differentiation assays. Current literature also supports lipoaspirate meeting ISCT minimum criteria for MSC in dolphins (Johnson et al. 2012).

Results
At the time of writing this article none of the 21 patients demonstrated complications during adipose collection or portal wound healing. Subtle, diffuse depressions over the aspiration fields are noted transiently (Fig 7), which fills in over time. Owner satisfaction with the procedure has been excellent with no aesthetic complaints up to 2 years post operatively. The average adipose-derived nucleated cells collected in this lipoaspirate cohort was 383,460 cells/g. Greater than 85% (18/21) of lipoaspirates resulted in treatment within 48 h of harvest, while the remainder (3/21) required cell expansion prior to therapy.

Discussion
Tumescent lipoaspiration is widely used in human plastic and reconstructive surgery and considered extremely safe (Alexander 2011). Early in the developmental phase of human lipoaspiration surgeons attributed increased yields to increased vacuum force. It is now understood the addition of a tumescent solution allows the adipose matrix (a mixture of adipose cells and stroma) to become suspended within the fluid, allowing for reduced suction force with an apparatus as simple and cost effective as a syringe and plunger. The use of gentle, constant suction with a closed syringe system reduces cell damage and significantly improves fat grafting results in man (Alexander 1992). Modification of human lipoaspiration for use in horses has proven to be a safe and effective means of AT-MSC harvest in this practice. The use of ultrasound provides improved assurance of adipose tissue collection and intentional cannula avoidance of surrounding tissues and blood vessels limiting complications. This is especially advantageous for veterinarians learning a new technique. None of the 21 patients experienced infection, excessive drainage or soreness whereas dehiscence, infection and prolonged wound healing were occasionally experienced with lipectomy in this practice (R. Thaler, unpublished data 2011). In addition to reduced complications, owner compliance and satisfaction significantly improved with lipoaspiration due to reduced cicatrix and tissue deformation when compared to lipectomy. The improved aesthetics are secondary to minimally invasive portal incisions and a more diffuse, bilateral
harvest area that result in relatively unnoticeable alterations in gluteal contour. This practice has experienced a significant increase in client acceptance of AT-MSC therapy since adopting a minimally invasive technique, possibly due to the loss of marketability and poor owner perception inherent of an animal with a scar. Lipoaspiration additionally offers the advantage of expedited time to treatment compared to techniques that require cell expansion.

Disadvantages over lipectomy include lower cell counts [average 28% fewer nucleated cells per gram (C. Orava, unpublished data 2014)], increased procedure time and cost. Arguably, the 90 ml harvest is a mixture of adipose tissue and tumescent fluid which is the primary cause for lower cell counts when compared to incisional en bloc lipectomy harvests. If allowed to settle gravitationally, the cell layer will separate from the tumescent fluid and it has been proposed to decant the top layer. However, literature supports MSC presence in both the fluid and fatty layers of the lipoaspirate and decantation prior to shipment is not currently advocated (Toshimura et al. 2006). Other probable factors include cell trauma from repeated cannula advances. The infusion and harvest cannulas were chosen based on manufacturer recommendation due to the fibrous nature of equine adipose tissue. There are a variety of cannula options and the optimal choice is an area for future research. Additionally, tumescent fluid itself may impose negative effects on cell homeostasis. Recent in vitro work with human adipose lipoaspirates did not find deleterious effects of epinephrine but determined a dose and time dependent cytotoxicity with lidocaine (Girard et al. 2013). Mepivacaine has also been shown to significantly reduce equine chondrotoxicity when compared to lidocaine and bupivacaine (Park et al. 2011). This practice has shown success with lipospiration in the horse despite using lidocaine as a local anaesthetic. However, cellular results may be optimised with alternatives and should be explored in the future. Lastly, human tumescent lipospiration procedures advocate ‘pretunneling’ where the infusion cannula is repeatedly advanced within the adipose layer to prevent pooling of tumescent fluid, improve adipose suspension and ease harvest collection (Alexander 2011). Although, the authors have good success using the technique described here and currently do not advocate pretunneling, these data suggest future research regarding instrumentation, local anaesthetics and manipulation techniques are warranted to optimise equine MSC yield and viability.

The lipoaspirate is enzymatically digested to produce the SVF. Based on lipectomies in the horse, approximately 43% of the cells in the SVF are MSCs (Vidal et al. 2007). The percentage of MSCs from lipoaspirate has not been published in the horse; however, a comparable average of 35% was reported from lipoaspirate in man (Varma et al. 2007). Lipoaspiration may still occasionally require cell expansion by culture and 3/21 (15%) of our lipoaspirate cohort required cell expansion. Expansion was expected in one horse with 0.7 cm adipose depot thickness. The second case was an 18-year-old mare with a metabolic condition while the third horse was a middle aged, healthy, but lean gelding with a very fibrous feel during aspiration harvest. Retrospectively, the first two cases may have been better candidates for an alternative source of MSC but would have likely required cell expansion regardless. The third case was unexpected and may be explained by the inherent individuality of adipose tissue. For example, even lipectomy occasionally requires cell expansion as a select few horses have low resident MSC within adipose but this is relatively uncommon (R. Thaler, personal observations 2014). The majority (85%) of lipoaspirates yielded an average of two fresh doses, allowing rapid initial treatment 48 h after sample harvest with ample time for cell expansion prior to the next scheduled treatment should this serve as an issue. The dose used in this practice was adopted from processing laboratory recommendations based on internal data. Standardisation of dosage regimens is needed to provide comparison reliability amongst future treatment protocols and success outcomes and is beyond the scope of this technique paper. Patient age and body condition score may also affect adipose MSC harvest as research on BM-MSCs and AT-MSCs has shown an inverse relationship with age and stem cell regenerative potential in equine, murine and human models (Zhou et al. 2008; Chen et al. 2012; Choudhery et al. 2012; Carter-Arnold et al. 2014).

The authors currently allot 60–75 min for actual procedure time in an ambulatory setting. Procedure duration depends highly on veterinarian competence with the technique and will improve with increasing familiarity. This is prolonged when compared to unilateral lipectomy (15–30 min). The cost of infusion and harvest cannulas also add expense when compared to a scalpel and suture material required for lipectomy. The harvest cannula can be resterilised and used approximately 10 times prior to replacement. Beyond 10 uses, the manufacturer cites a reduction in cell viability may be noted due to deterioration of the cell-friendly coating. The infusion cannula can be reused indiscriminately. It is of strong author opinion that these shortcomings are worth the extra financial investment and time commitment in exchange for a minimally invasive technique that conserves aesthetic appeal, reduces patient discomfort and improves client satisfaction while preserving an expedited time to treatment.

Conclusion

This paper outlines a novel procedure combining ultrasound and minimally invasive techniques adapted from human plastic surgeons for use in equine adipose harvests. Consistent with human tumescent liposuction, equine ultrasound-guided tumescent liposuction is a safe and effective technique for AT-MSC harvest and should be considered when aesthetic outcome or imminent treatment of the injury is a primary concern. Further investigation to determine effects of liposuction instrumentation, technique and tumescent interaction on equine MSC health to optimise cell viability is warranted. Analysis of MSC handling post harvest and treatment dose regimens in horses are also needed to reliably compare therapy outcomes by investigators in the future.

Authors’ declaration of interests

No conflicts of interest have been declared.

Ethical animal research

All animals used for this study were client-owned. Consent was obtained from the owners of the animals.
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Authorship
J. Lawver contributed to study design, study execution, data analysis and interpretation, and preparation of the manuscript. R. Thaler contributed to study design and study execution. Both authors gave their final approval of the manuscript.

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6Spartkoh Laboratorios, Lenexa, Kansas, USA.
7Tulip Medical, San Diego, California, USA.
8Abbott Laboratories, North Chicago, Illinois, USA.
93M, St. Paul, Minnesota, USA.
10Hospira, Inc. Lake Forest, Illinois, USA.
11Veil-Stem Regenerative Veterinary Medicine, Poway, California, USA.

References


Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Supplementary Item 1: A video outlining the procedure is permanently available online at https://vimeo.com/87289133.