WONDER WHY?

Stem Cell Prolotherapy in Regenerative Medicine

Background, Theory and Protocols

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ABSTRACT

Prolotherapy is a proven technique for resolving musculoskeletal pain, but can have limitations if tissue damage is too severe. Platelet Rich Plasma (PRP) Prolotherapy offers a physiologic tool in some of those cases, but this too may fail. One explanation for deficient repair is when undifferentiated adult stem repair cells are inadequate in number or cannot be stimulated within the damaged tissue site. With improved understanding of tissue healing and regeneration, stem cell Prolotherapy is gaining significant clinical importance and potential. Using Prolotherapy technique, with ultrasound guidance, placement of a living bioscaffold of autologous adipose (fat) tissue and its mesenchymal stem/stromal cell population, mixed with critically important high-density PRP (defined as a ***minimum*** *concentration of >4 times circulating baseline platelet levels), provides enhanced musculoskeletal healing, shifting the clinical paradigm. The protocol described within this paper for stem cell Prolotherapy can be done in the physician's office, at the point-of-care, within the same procedure on the same day, and without violation of current FDA regulations. This paper also discusses the theories and background leading up to the protocol, and presents representative clinical examples of its use in the treatment of musculoskeletal injuries, with documented high-definition ultrasonic evidence of healing.*

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INTRODUCTION

Prolotherapists have known since the 1930's that a solution as innocent as dextrose, used as an irritant and properly placed, stimulates injured musculoskeletal connective tissue to heal, often dramatically. In the 1990's platelet rich plasma (PRP) gained acceptance in many surgical circles, and in the 2000's, Prolotherapists and other physicians in the orthopedic and sports medicine field, began using high-density PRP concentrates (HD-PRP), defined as a *minimum* of 4 times patient baseline platelet levels, to stimulate musculoskeletal connective tissue repair.[1, 2] Recently, Prolotherapists have begun to utilize the potential of autologous adipose (fat)-derived stem/stromal cells (AD-SC) within non-manipulated fat graft scaffolding, combined with high-density PRP concentrates (HD-PRP) to provide a potent biological therapeutic combination. With high levels of platelet-derived growth factors and cytokines, this combination provides both a living bioscaffold and a multipotent cell replenishment source useful for enhanced musculoskeletal healing.[3] In veterinary medicine, AD-SC's have been utilized effectively for over ten years in the treatment of osteoarthritic joints[4, 5] and connective tissue injuries, showing an over 80% success rate in blinded placebo controlled canine clinical trials.[6] Cosmetic-plastic surgeons have studied autologous fat grafting for structural augmentation via transplantation of lipoaspirants for many years. In the past decade, better understanding of the cellular mechanisms responsible for successful soft tissue augmentation has been reported, focusing on the plentiful undifferentiated stromal elements rather than the survival of mature adipocytes.

Recognition of the vast number of undifferentiated cells associated with the stromal vascular fraction has resulted in extensive research demonstrating the heterogeneity of such cells, and their ability to participate in production of all mesodermal-derived tissues.[7] There has been some variation and question regarding the correct terminology for this population of stromal adipose cells. At first, the mesenchymal stem cell was thought to be the primary component of this undifferentiated cell type, however it is now evident that within the adipose extracellular matrix are also adipocytic precursors (known as progenitor cells) adherent to adipocytes, and in close approximation to a variety of additional undifferentiated multipotent and pluripotent cells, including pericytes and endothelial

cells, all thought to play important roles in mesenchymal-stromal derived tissue regeneration. Therefore we have chosen to use the term "adipose-derived stem/stromal cells" (AD-SC's), rather than simply "mesenchymal stem cells."

Multiple investigations have clearly demonstrated the *in Vitro* ability of AD-SC's to differentiate into, and repair, musculoskeletal connective tissues including ligament,[8] tendon,[9-12] cartilage,[13-15] disc,[16] muscle,[17-19] nerve tissue,[20-22] bone,[23-25] hematopoietic-supporting stroma,[26-28] and to actively participate in tissue homeostasis, regeneration, and wound healing.[29-31] AD-SC's have demonstrated pluripotent capabilities where they were shown to have the ability to differentiate into a variety of non-mesodermally derived tissues including: hepatic,[32] pancreatic, and keratocytic tissue and to be effective in skin anti-aging and tissue regeneration,[33-35] cardiovascular muscle and vascular tissue repair,[36] rheumatoid arthritis,[37] diabetes,[38] and other diseases.[39-41] Historically, mesenchymal stem cells (MSC's) have been studied from bone marrow aspiration. However, bone marrow possesses very few true MSC's, and is gradually being replaced with AD-SC's as a primary tissue source. Fat is a complex tissue that is not only easier to harvest, but offers markedly higher nucleated, undifferentiated stem cell counts[42] than bone marrow. Research has shown as much as 500 to 1000 times as many mesenchymal and stromal vascular stem-like cells exist in adipose as compared to bone marrow.[43-45] This additional quantity of adipose-derived cells helps to obviate the need for FDA prohibited cell expansion often required for successful use of bone marrow.[46] Further, harvesting and retrieval of autologous adipose tissue via modern lipoaspiration methods is less invasive, procedurally easier, available in abundant amounts, and has lower morbidity than bone marrow harvest.[47] A simple means for harvesting adipose tissue is available utilizing the Tulip™ Medical microcannula system.[48] Lipoaspiration and concentration of platelets are procedures easily carried out at the point-of-care (POC) and delivered to targeted treatment sites via guided injection.

The purpose of our article is to detail these advances for application in musculoskeletal regenerative medicine, review current regulatory issues, and present a workable in-office protocol for the Prolotherapist or other physician engaged in the treatment of musculoskeletal connective tissue injury, degeneration and pain. We also report representative clinical human case examples with this novel regenerative protocol using ultrasound diagnosis and injection guidance, and present objective ultrasound evidence demonstrating tissue repair. While no formal statistical analysis was done, high definition ultrasonic analysis and tracking of patient subjective outcomes was carefully followed. In all cases to date, every participant has reported improvement of clinical symptoms and/or function.

REVIEW OF DEXTROSE PROLOTHERAPY (THE ORIGINAL "REGENERATIVE MEDICINE")

Prolotherapy is a method of injection treatment designed to stimulate healing.[49] This treatment works by locally raising growth factor levels to promote tissue repair and regeneration.[50-52] Prolotherapy began in the 1930's, when an osteopathic general surgeon, Earl Gedney, treated his severely sprained thumb after it was mangled in malfunctioning operating room doors.[53] Dr. Gedney developed the technique by extrapolating from the practice of "herniologists" who would inject irritating solutions into hernia fibrous rings to stimulate connective tissue repair. After the dramatic success on his thumb, Gedney spent the rest of life researching and forwarding Prolotherapy for use in musculoskeletal pain, publishing case reports and protocols, along with others, throughout the 1940's, 50's and 60's.[54, 55] Solutions used in dextrose Prolotherapy vary depending on the preference and experience of the practitioner, but are usually dextrose or saline based, and may contain Sarapin, morrhuate or other natural ingredients, combined with a local anesthetic.

Prolotherapy is based on the premise that chronic musculoskeletal pain is due to inadequate repair of fibrous connective tissue, resulting in ligament or tendon weakness and relaxation (laxity),[56] also known as connective tissue insufficiency.[57] When connective tissue is weak, there is insufficient tensile strength or tightness,[58] resulting in excessive "loading" of the tissues which stimulates pain mechanoreceptors.[59] As long as connective tissue remains functionally insufficient or ineffective, these pain mechanoreceptors continue to fire with use, causing significant pain and limitation of function.[60] If the laxity or tensile strength deficit is not corrected sufficiently to stop pain mechanoreceptor stimulation, chronic sprain/strain and pain result.[61] Prolotherapy works by stimulating a temporary, low grade inflammation at the site of ligament or tendon weakness (fibro-osseous junction),

"tricking" the body into initializing a new healing cascade cycle. Inflammation (characterized by increased blood flow) activates fibroblasts and native growth factors which stimulate the microenvironment to produce collagen, resulting in reinforcement of local connective tissue.[62-67] Inflammation amplifies local undifferentiated cellular and chemical responses commonly associated with secondary growth factor and cytokine elevation.[68] It has been well documented that direct exposure of fibroblasts to growth factors causes new cell proliferation and collagen deposition. This inflammatory stimulus effectively raises the level of these various elements to resume or initiate a new connective tissue repair sequence to complete one which was prematurely aborted, or never started.[69]

Multiple studies confirm the effectiveness of Prolotherapy in the resolution of musculoskeletal pain, such as low back,[70-73] neck pain and whiplash injuries,[74] chronic sprains and/or strains, tennis and golfer's elbow,[75] plantar fasciitis,[76] knee,[77] ankle, shoulder, coccydynia[78] and chronic tendonitis/tendinosis[79] including Achilles tendonitis/tendinosis,[80] and other joint pain or musculoskeletal pain related to osteoarthritis.[81]

REVIEW OF PLATELET RICH PLASMA PROLOTHERAPY

Platelet Rich Plasma (PRP) Prolotherapy is based on the same theory and methodology as dextrose Prolotherapy, however, the solution used is a high-density concentration of the patient's circulating platelet levels isolated and concentrated by bidirectional centrifugation. Enhanced healing capability is possible when platelet concentrations are increased within injured or damaged tissue.[82] For many years, the importance of platelets was thought to be formation of "plugs," useful in reduction of bleeding in the tissues. It is now recognized that this may represent the least important function served by platelets. Platelets contain a significant number of key signal proteins, growth factors, chemokines, cytokines and other proinflammatory bioactive factors that initiate and regulate basic aspects of the inflammatory cascade resulting in natural wound healing.[83] Elevated platelet concentrations are also known to stimulate the proliferation, differentiation and migration of needed mesenchymal and stromal repair cells to an injury site.[84] Similar to dextrose Prolotherapy, addition of high-density PRP concentrates result in an inflammatory and proliferative response that enhances healing and promotes tissue regeneration.[85]

High-density platelet rich plasma (HD-PRP) is defined as autologous blood with concentrations of platelets at equal to or greater than four (4) times circulating baseline levels[86] and which increases the important bioactive protein load (growth factors) in a direct correlative fashion.[87] Cell ratios in average circulating whole blood contain only 6% platelets. In true high-density PRP preparations, the concentration achieved is 94%.[88] The average patient platelet count is 250,000 platelets/dl. Four times this is 1 million platelets/dl, which is considered the desired benchmark for "therapeutic PRP."[89] The use of clinically proven devices to obtain this degree of concentration is considered essential to ensure platelet numbers and their important contents achieve therapeutic effects. Circulating platelets, when activated, begin a degranulation process which secretes a variety of important growth factors and cytokines/chemokines, such as platelet-derived growth factor (PDGF: stimulates cell replication, angiogenesis), transforming growth factor beta-1 (TGF-B1: angiogenesis), vascular endothelial growth factor (VEGF: angiogenesis), fibroblast growth factor (FGF: proliferation of myoblasts and angiogenesis), and insulin-like growth factor-1 (IGF-1: mediates growth and repair of skeletal muscle), among others.[90] Activated platelets also secrete stromal cell derived factor 1 alpha (SDF-1a) which supports primary adhesion and migration of mesenchymal stem/stromal cells.[91] (*See Figure 1.*)

Figure 1. Common growth factors found in PRP.

Platelet-Derived Growth Factor aa,bb,ab	PDGF
Transforming Growth Factor B1, B2	TGF-B1, TGF-B2
Platelet-Derived Epidermal Growth Factor	PDEGF
Platelet-Derived Angiogenesis Factor	PDAF
Platelet Factor 4	PF-4
P-Selectin	GMP-140
Interleukin 1	IL-1
Fibroblast Growth Factor	FGF
Interferons: Alpha, Gamma	IFN-α, IFN-γ
Insulin-like Growth Factor	IGF

Various portable commercial centrifugation units exist which process blood samples, resulting in platelet rich plasma concentrates. There are two commercially available systems which are capable of consistently concentrating platelets to the therapeutic levels. The one used for our investigation was the patented Harvest Technologies Smart PReP2 centrifugation system which

has been cleared by the FDA. This system uses a sterilized blood collection kit which allows in-office phlebotomy and processing in a tabletop bidirectional centrifugation unit. The Harvest system is capable of consistently concentrating four to five times, or more, patient's circulating level of platelets,[92] therefore, achieving the needed therapeutic HD-PRP considered of most value.

THE ADULT STEM CELL

Some researchers believe there are really only two kinds of stem cells, the embryonic (prenatal) stem cell and the adult (postnatal) stem cell.[93] Although most lay people recognize the term "embryonic stem cells", attention to the important potentials of "adult" stem cells has been discussed in the medical literature since 1963 when Becker et al. reported on the regenerative nature of bone marrow.[94] Embryonic stem cells are, in theory, able to transform into any type of tissue; they are "totipotent" or "omnipotent" when an egg is fertilized, then after several divisions are "pluripotent" and able to differentiate into any of the three germ layers.[95] However there are religious, political and ethical issues which inhibit their use. Postnatal "adult" stem cells are those cells present which remain in an individual after birth, in an undifferentiated state, and available to maintain tissue homeostasis and regeneration in a tissue or organ system. These stem cells can be activated to proliferate and differentiate to yield some or all of the major specialized cell types of their tissue type when required for maintenance or repair.[96] Because they typically differentiate into a variety of cellular phenotypes from one germ layer, they are recognized as "multipotent," with some cells demonstrating transdifferentiation capabilities in tissue culture. Multipotent stem cells facilitate tissue maintenance, regeneration, growth and wound healing throughout life.[97] Adult stem cells can be found in all tissues in the body[98] in various quantities, with major reservoirs in adipose[99] (fat) and, to a lesser extent bone marrow.[100] Like bone marrow, adipose tissue is derived from embryonic mesodermal tissues and contains a well described microvascular network including extracellular matrix and extensive perivascular stroma, which suggested promise for use in regenerative medicine.[101] In 2001 and 2002, Zuk et al. confirmed that adipose stroma contains relatively large numbers of undifferentiated cells capable of producting cartilage, ligament, tendon, muscle and bone as well as adipose tissues.[102, 103] Multiple other studies have since confirmed the potential for these cells to differentiate to skeletal muscle,[104] smooth muscle and even cardiac muscle.[105] Further investigations have shown that AD-SC cells also have the potential to differentiate into tissue derived from ectodermal and endodermal origins, such as organ tissue,[106] nerves[107-111] and skin,[112, 113] suggesting them to be pluripotent, rather than exhibiting only multipotent, capabilities.[114, 115]

Recent studies have determined the safety and efficacy of implanted/administered AD-SC's in various animal models, as well as human clinical trials in some medical subspecialties. AD-SC also meet the criteria suggested by Gimble et al. that an ideal stem-stromal cell for regenerative medicinal applications should meet certain criteria, such as: 1. Found in abundant quanitites; 2. Harvested with a minimally invasive procedure; 3. Can be differentiated along multiple cell lineage pathways in a regulatable and reproducible manner; and 4. Can be safely and effectively transplanted.[116, 117] Adipose tissue meets these criteria, and has become an important resource for research and patient care applications.

MESENCHYMAL STEM CELLS STIMULATE CONNECTIVE TISSUE REPAIR

In the early 1990s, existence of adult mesenchymal stem cells, described as "non-committed progenitor cells of musculoskeletal tissues," were discovered to have an active role in connective tissue repair.[118] These cells were first labeled by Caplan (1991) as "mesenchymal" stem cells ("MSC")[119] because of the ability to differentiate to lineages of mesenchymal tissue, and were recognized to be an essential component of the tissue repair process.[120] An interesting observation made about MSC's is the ability to "hone in" and help repair areas of tissue injury.[121] While bone marrow has historically been used as a source of MSC's, adipose-derived MSC's have been shown to have nearly identical fibroblast-like morphology and colonization (CFU-F), immune phenotype, successful rate of isolation, and differentiation capabilities.[122-124] The healing potentials of adipose-derived MSC's were demonstrated in early clinical use for cranial defect and chronic fistula repair, without side effects.[125, 126]

MSC's, along with other cells within the adipose stroma, react to cellular and chemical signals, and have been shown *in Vitro* to differentiate and assist in healing for a wide variety of cellular types. This includes cartilage repair,[127] angiogenesis in osteoarthritis,[128-130] tendon defects,[131-133]

ligament tissue,[134] intervertebral disc repair,[135, 136] ischemic heart tissue,[137, 138] graft-vs-host disease[139] and osteogenesis imperfecta.[140] (*See Figure 2.*) Of particular interest in musculoskeletal medicine is the observation in degenerative diseases, such as osteoarthritis, an individual's adult stem cell frequency and potency may be depleted, with reduced proliferative capacity and ability to differentiate.[141, 142] It has been suggested that addition of these missing stem cell elements might help these conditions. Studies have demonstrated such improvement with adult stem cell therapy by the successful regeneration of osteoarthritic damage and articular cartilage defects.[143, 144] In 2003, Murphy et al. reported significant improvement in medial meniscus and cartilage regeneration with autologous stem cell therapy in an animal model. Not only was there evidence of marked regeneration of meniscal tissue, but the usual progressive destruction of articular cartilage, osteophytic remodeling and subchondral sclerosis commonly seen in osteoarthritic disease was reduced in MSC-treated joints compared with controls.[145] In 2008, Centeno et al. reported significant knee cartilage growth and symptom improvement in a human case report using culture expanded autologous MSC's from bone marrow.[146]

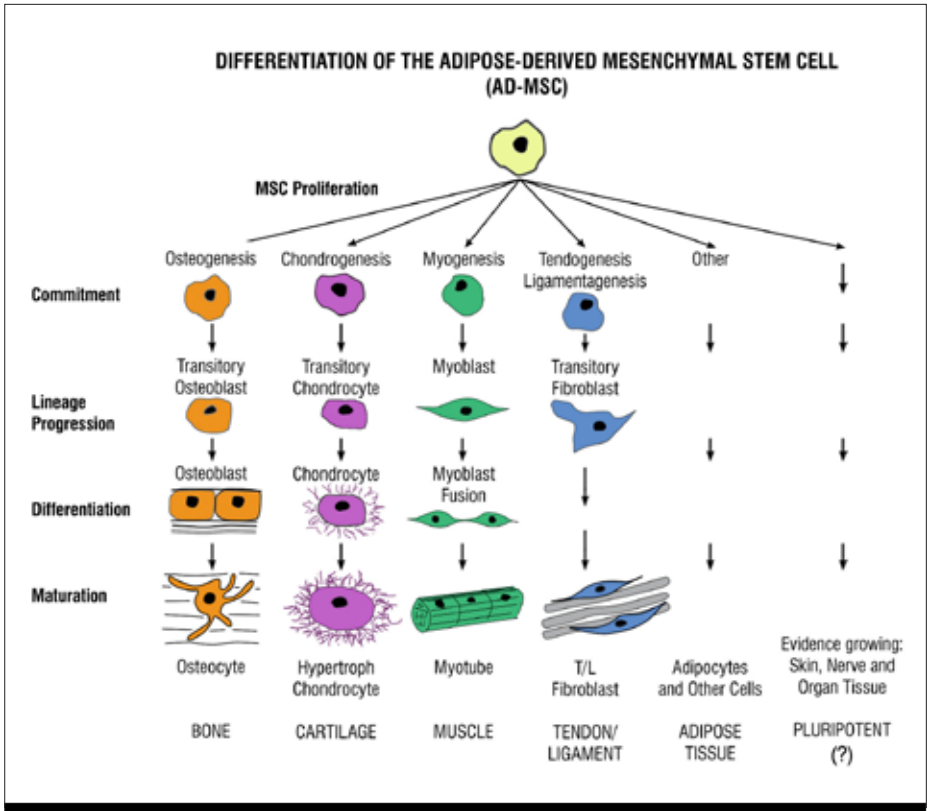

Figure 2. Flow chart elucidating possible commitment, lineage progression and maturation of adipose-derived mesenchymal stem cells.

AUTOLOGOUS ADIPOSE FAT GRAFTS IN COSMETIC-PLASTIC SURGERY

Adipose tissues have long been a proven safe and efficacious structural tissue amenable to successful transplantation.[147] For more than 50 years, cosmetic-plastic surgeons have attempted such transfers with variable success. It is clear that control of cellular fate and extracellular environment is critical in tissue regeneration and cell-based therapies.[148] It was not until the advent of a patented, closed syringe system was introduced in 1990 (Tulip Medical™) that predictability of structural augmentation was fully appreciated.[149] For many years cosmetic-plastic surgeons believed the key to a successful structural autologous fat graft (AFG) was transplantation of intact cellular elements (mature adipocytes) into environments that had existing adipose tissues. However it was recognized that mature adipocytes did not undergo mitosis, therefore further understanding of how adipose tissue maintained its structural integrity and volumes became an important undertaking. The past decade in cosmetic-plastic surgery has been spent increasing understanding of the importance of adipose-derived stem-stromal elements to the replenishment and restoration of adipocytes *in Vivo*. As adult adipocytes enter senescence stages, adherent (cell-to-cell) adipose progenitor cells directly differentiate into adipocytes to replace the aging cells. These progenitor cells are capable of undergoing mitosis, however do so in an asymmetric manner, producing another, now adipose-lineage committed (unipotent or terminally commited), progenitor cell and a less differentiated progenitor cell, in order to maintain precursor numbers for future differentiation and restore stem-like progenitor availability. Further understanding of the importance of the autocrine and paracrine functions of such cells within their niche has demonstrated the complex microenvironmental factors involved in tissue maintenance and regeneration.

It is now understood that the adult adipocyte life cycle is between 2-10 years for complete turnover.[150] The undifferentiated cells, including mesenchymal stem cells and those in the stromal vascular fraction (SVF) such as the progenitors (pre-adipose cells), as well as the pericytes and endothelial cells, (characterized as those cells which adhere on and inside the walls of blood vessels), are felt to serve the functions of replacement and maintenance in adipose and many other tissues. Examination of this undifferentiated population of mesenchymal-like stem cells showed between 4-8 stem cells (true progenitor cells) to be attached to mature adipocytes, with the vast majority of nucleated undifferentiated cells adherent to the extracellular matrix and SVF structures.[151] (*See Figure 3.*)

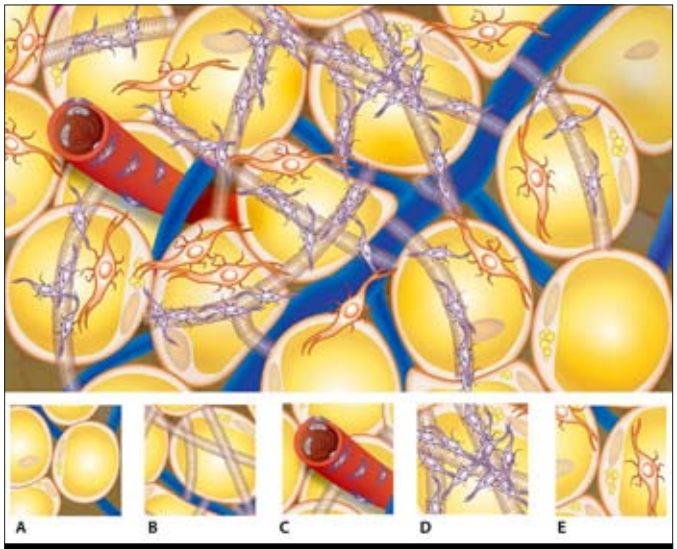

Figure 3. Adipose tissue with undifferentiated elements in stroma. A) Adipose Cells; B) Extracellular Matrix (most stems there); C) Pericytes (surround vessels, important in angiogenesis); D) Mesenchymal Stem Cells (the little guys); E) Pre-adipocyte (progenitor cell).

During the 1990s, further understanding and enhancements to improve the "take" of fat grafts led to the effective addition of HD-PRP concentrates to further enhance the success of these autologous fat grafts (AFG).[152] Several publications within the cosmetic-plastic surgical literature have reported significant contributions to successful adipose tissue transplantation (including their AD-MC/SVF fraction) when these autologous grafts were blended with highly concentrated platelet elements (PRP).[153-155] Recognition of the significant clinical contribution to structural fat grafting when transplanted with the multitude of platelet-derived growth factors, cytokines and chemokines, became a valuable aid in retaining improved structural augmentation. It is believed that these effects are largely a result of PRP's ability to improve active angiogenesis, stimulate and promote undifferentiated cell adherence, proliferation, and differentiation activities of precursor cells in the grafts, reflecting the niche in which they are received. (*See Figure 4.*) Thousands of these successful autologous fat grafts (AFG) with HD-PRP have been reported and performed within the aesthetic and plastic surgical literature for more than ten years proving safety and efficacy.[156]

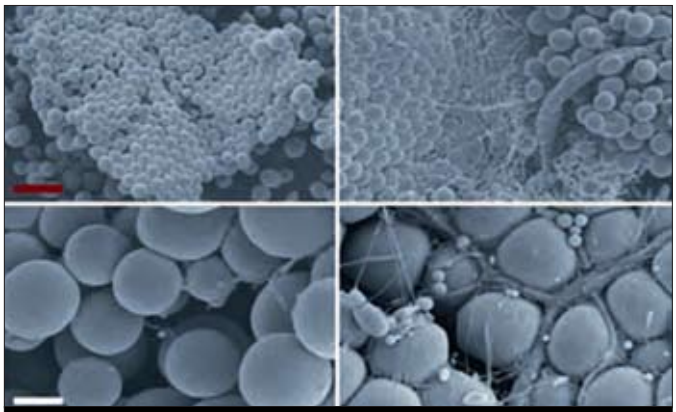

Figure 4. Photomicrograph of adipose derived mesenchymal stem cells treated with PRP in vitro.

Recent contributions utilizing isolation and concentration of AD-SC/SVF elements have increased the effectiveness of adipose transplantation.[157] In 2007, Yoshimura et al. reported on cell-assisted lipotransfer, concentrating a portion of the lipoaspirate to AD-SC elements then adding back to the AFG, resulting in more successful natural breast fat transfer augmentation.[158] In his studies, lipoaspirants were documented to have a little more than one-half the number of stem-stromal cells, and that by addition back of these cells the native adipose stroma concentrations were effectively restored. However, at this time, no chemical manipulation of the adipose-derived tissues for isolation and concentration is permitted in the United States by the FDA. This is discussed in the next portion of this article. It is the authors' opinion that the ability to pelletize and concentrate the stem cell elements, and add them back to an adipose graft carrier (AFG) for delivery with guided ultrasound placement will prove clinically advantageous. Outside the United States, these procedures are proving safe and efficacious,[159] including use in musculoskeletal applications.[160]

FDA CONSIDERATIONS

Controversy surrounding use of fetal stem cells can be avoided with the use of autologous adult stem cells, but regulation still exists in terms of how these cells may be altered. Autologous adult stem cells are considered "Human Cells, Tissues and Cellular-Based Products (HCT/P's)" and thus regulated by the FDA.[161] However, exemption from regulation exists if the physician "*removes HCT/P's from an individual and implants such HCT/P's into the same individual during the same surgical procedure.*"[162]

To be considered as occurring "during the same surgical procedure" the cells must be "autologous," "minimally manipulated," and "used within a short period time."[163] "Minimally manipulated" is defined as "processing that does not alter the relevant biological characteristics of cells or tissues"[164] while "short period of time" is not exactly defined, but per the "FDA Guidance for Industry" is considered to be "a matter of hours (or less), without the need for shipping."[165] "More than minimal" manipulation involves: "the use of drugs, biologics, and/or additional devices that warrants regulation of the manufacturing process and the resulting cells as biological products." This is where the use of enzymes such as collagenase or culture expansion of cells comes into question.[166] Therefore, chemical isolation, concentration, and culture expansion of stem cells, while delivering higher yields, remains problematic in terms of existing FDA requirements. It is clear that harvesting native autologous adipose stem-stromal cells does not currently pose any problem as far as FDA regulation is concerned as long as exemption criteria are met.

CLINICAL TRIALS

While use of culture expansion or chemical digestion to isolate undifferentiated stem/stromal cells is considered "more than minimally manipulated," and has not yet been approved by the FDA in the United States, many ongoing controlled clinical trials using these methods are being reported or in progress at the time of this writing. In fact, there are more than 43 ongoing U.S. IRB controlled clinical trials now with approximately half of them still recruiting participants.[167] Studies include the use of AD-SC's for degenerative arthritis. In that trial, AD-SC's will be culture expanded, then administered into a cartilage tissue lesion via orthopedic surgery.[168] Another trial pending (Scarpone and Alexander, sponsored by Trinity Health Systems) is: "Autologous Tissue Grafting Using Platelet-Rich Plasma And Fat (Expanded and Non Expanded), A Randomized Trial For Treatment Of Knee Osteoarthritis." There are four legs planned: 1) PRP only, 2) AD-SC's only, 3) PRP and AD-SC's, and 4) a control.[169] Other ongoing studies include AD-SC's for the treatment of diabetes, recto-vaginal and perianal fistulas, peripheral vascular disease, ischemic heart disease, coronary arteriosclerosis, hemifacial atrophy, liver cirrhosis, breast reconstruction after breast cancer, anti-aging, polycystic ovary syndrome, metabolic syndrome X, fecal incontinence, graft vs. host disease, chronic critical limb ischemia in diabetic patients, lipodystrophy, Crohn's Disease, spinal cord injury, Buerger's disease, and neurologic diseases such as ALS.[170]

THEORY OF STEM CELL PROLOTHERAPY

The ability of AD-SC's to support and serve as a cell reservoir for connective tissue and joint repair is the basic theory of stem cell Prolotherapy. With stem cell Prolotherapy, a stem cell niche (microenvironment which favors healing) is moved from one tissue in which these niches are abundant (adipose) into one where they are scarce (a non-repairing connective tissue).[171] AD-SC's have been shown, in multiple studies, to improve wound healing and stimulate fibroblast proliferation, migration and collagen secretion, thereby increasing connective tissue tensile strength and healing.[172] As discussed earlier, AD-SC's have differentiation potential to become cartilage, tendon, ligament, bone and skeletal or smooth muscle, and are also capable of expressing multiple growth factors that influence, control and manage damaged neighboring cells.[173] AD-SC's have also been reported helpful in intervertebral disc regeneration,[174] tendon and ligament regeneration,[175] and in accelerating tendon repair and strength.[176] It is reasonable then that when traditional dextrose Prolotherapy and/or platelet rich plasma Prolotherapy, or other stronger proliferants, have not resulted in complete resolution of a musculoskeletal problem, stem cell Prolotherapy would be the logical next step. Our reported technique uses well established Prolotherapy injection protocols with autologous AD-SC, harvested via microcannula lipoaspiration, together with high-density PRP concentrates. Lipoaspirates are obtained via the patented closed syringe Tulip™ microcannula system, a technique commonly employed by cosmetic-plastic surgery in structural fat grafting. The harvested autologous fat graft complex can then be decanted by gravity, or low g-centrifugation (less than 1000 g for 3 minutes), and combined with highly concentrated

platelet-rich plasma obtained via Harvest Technologies' SmartPrep2 system.[177]

The combination of PRP and AD-SC in a fat graft matrix is then accurately injected into injured musculoskeletal and connective tissue via ultrasound guided injection. In our early technique, and in the clinical cases reported in this paper, gravity decanting was used, which has been shown to provide a large number of viable AD-SC's.[178] We are now beginning to use low g-centrifugation to effectively compress the adipose tissues and separate the lipid oil fraction. This is believed to provide a more cellular graft and stroma to enhance clinical effectiveness.

HIGH-DENSITY PRP CREATES A FAVORABLE GROWTH FACTOR ENVIRONMENT

A concentrated growth factor environment, coupled with a living bioscaffolding, has been found to be important for AD-SC used in orthopedic applications.[179] High-density PRP (HD-PRP) has shown the ability to enhance musculoskeletal healing and stimulate local microenvironmental regenerative capabilities,[180] especially during the early phase of tendon healing.[181] Proliferation of AD-SC's and their differentiation is also believed to be directly related to platelet concentration.[182] HD-PRP releases large quantities of Platelet Derived Growth Factor (PDGF), Transforming Growth Factor-Beta 1 (TGF-B1), and many others which, when activated, significantly enhance stem-stromal cell proliferation and angiogenesis,[183, 184] as well as enhancing the survival of the fat scaffolding.[185] The fat tissue complex used in this protocol provides a cell source and matrix (bioscaffolding) serving to provide improved adherence capabilities for proliferative stromal cell activity, which is then amplified with addition of HD-PRP.

STEM CELL FATE DEPENDANT ON MICROENVIRONMENT

Stem cell fate is controlled by a complex set of physical and chemical signals dictated by the cellular and chemical microenvironment (niche).[186] ***It is important to understand that undifferentiated stromal cells must be adherent to other cells (cell-to-cell contact) or to ECM-perivascular tissues in order to proliferate effectively.*** Therefore, if AD-SC's are placed within and adherent to damaged connective tissue, uncommitted progenitor and stem-stromal elements within the AD-SC graft should be stimulated towards that specific connective tissue lineage for growth and repair. For example, if placed within osteoarthritic degenerated cartilage, chondrogenic differentiation is believed to be encouraged.[187-190] In the 1990's, Young et al. showed repair of an Achilles tendon tear when placed in a collagen matrix, then placed in a tendon defect.[191] Little et al. (2010) demonstrated the successful differentiation of human AD-SC's to ligament when adipose lipoaspirate was placed in a simulated ligament matrix composed of native ligamentous material combined with collagen fibrin gel. Cells placed in this manner showed changes in gene expression consistent with ligament growth and expression of a ligament phenotype.[192] Albano and Alexander successfully reported an autologous fat graft as a mesenchymal stem source and living bioscaffold ("Autologous Regenerative Matrix") to repair a persistent patellar tendon tear.[193] Growth factors and chemical elements, such as present in HD-PRP provide additional influence within the microenvironment to enhance adherence, proliferation, differentiation and migration of cells towards this end.[194]

USE OF ULTRASOUND FOR DIAGNOSIS AND INJECTION GUIDANCE

Musculoskeletal ultrasound has been gaining in popularity in the United States since the early 2000's. The first publication using musculoskeletal ultrasound was in 1958 by K.T. Dussik who measured the acoustic attenuation of connective tissues including skin, adipose tissue, muscle, tendon, articular capsule, articular cartilage and bone, laying the foundation of diagnostic musculoskeletal ultrasound.[195] Since that time, evolution of ultrasound technology has led to dramatic and ever increasing image quality in laptop sized machines, as well as lowering the price so the average practitioner can now afford such a modality. For example, a high resolution ultrasound machine with one probe can now be purchased for less than $25,000 dollars as compared to the average price for an ultrasound machine with one probe in 1999 which was $100,000 dollars with the images requiring dark room exposure.

In 1994, the European Society of Skeletal Radiology (ESSR) established technical guidelines and protocols for scanning the shoulders, wrists, hips, knees and ankles.[196] Since then, additional musculoskeletal texts have emerged for additional areas such as the spine.[197, 198] By following these guidelines, a systematic approach can be used to identify the tendons and ligaments and then to identify musculoskeletal pathology.

Musculoskeletal ultrasound has also been shown to be comparable in accuracy to MRI. Teefey et al. demonstrated that ultrasound can diagnose full-thickness rotator cuff tears with an accuracy of 96%.[199] This study evaluated 100 shoulders with arthroscopic surgery confirmation. Both ultrasound and MRI had similar accuracies of 92–97% for the identification and measurement of the size of rotator cuff tears.[200] While MRI has been shown to be less operator dependant, ultrasound has several advantages. Not only is ultrasound more convenient because assessments can be done in the doctor's office, but the physician can palpate the area of complaint, linking the imaging directly with symptomatology in a way not possible with other types of imaging. In addition, ultrasound allows scanning while moving the relevant anatomy thereby enabling the detection of abnormalities only visible with movement which might otherwise be missed.[201]

In an effort to evaluate, perform, and monitor the therapeutic outcomes of our clinical case examples, a Sonosite Turbo Max ultrasound machine was used to diagnose and identify pathogenic connective tissue before and after stem cell Prolotherapy. Ultrasound guidance was used to inject directly into the affected sites, and subsequently, to re-identify the treatment site after stem cell Prolotherapy treatments. (*See Figures 5a-5g.*) While some PRP studies have been shown to be effective without ultrasound guidance[202, 203] the authors believe ultrasound guidance has the clear advantage of visual confirmation of accurate placement and documentation of clinical changes. This is especially valuable in treatment of small tendon or ligament tears enabling stem cell Prolotherapy to be injected into both the tear and the sheath with improved accuracy. In addition, the enhanced density of the AFG-scaffolding provides a visual confirmation of the actual location and placement of the biologic products.

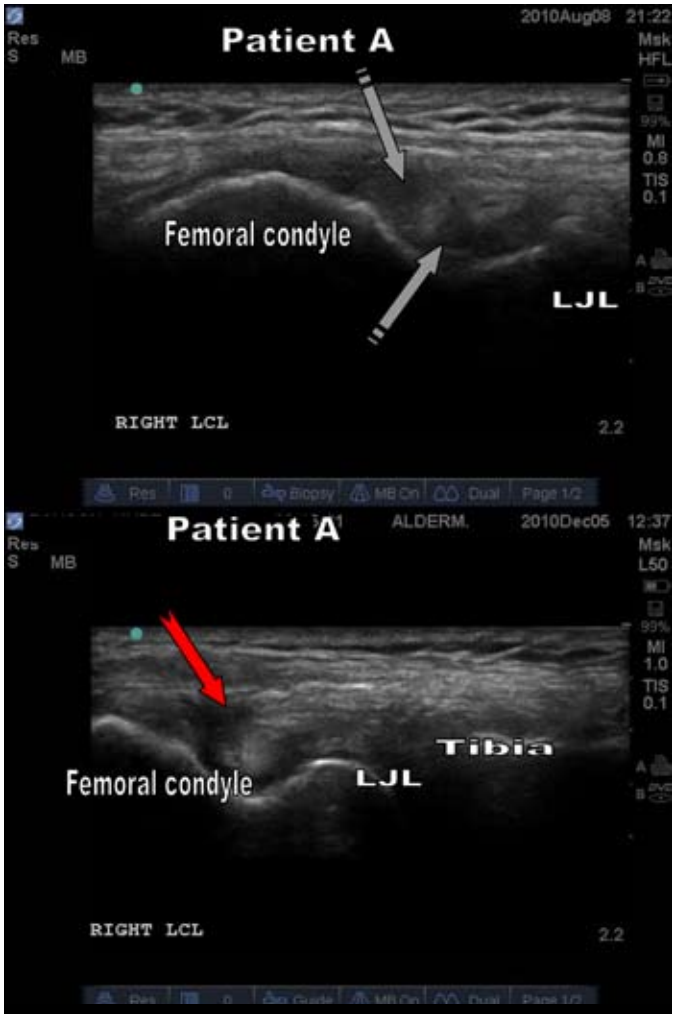

Figure 5a. Patient A ultrasounds "Before" and "After." Left side of screen is medial, anterior or proximal (MAP). Hypoechoic = darker than surrounding tissues; Hyperechoic = whiter/denser than surrounding tissues. AD-SC = Adipose-derived stem/stromal cells with high density PRP. **Top: Before AD-SC.** Lateral aspect of the knee. Segmented arrows showing hypoechoic lateral collateral ligament at its insertion. LJL = lateral joint line. **Bottom: After AD-SC.** Solid arrow showing hyperechoic new tissue in the lateral collateral ligament at its insertion four months after one stem/stromal cell treatment.

MATERIALS AND METHODS

***Materials*:** For details on materials used for infiltration and harvest, see Alexander article in this issue: "*Autologous Fat Grafts As Mesenchymal Stromal Stem Cell Source For Use In Prolotherapy: A Simple Technique To Acquire Lipoaspirants*". (Note: non-disposable cannulas were utilized, however disposable Tulip™ microcannula are currently in production and, when available, can be used). Additional materials for injections: 0.5% lidocaine without epinephrine, combined with calcium chloride (100 mg/ml) in a ratio of 1:10, i.e. 1 cc lidocaine/0.1 cc calcium chloride (for use prior to each AD-SC/HD-PRP injection); blunt tip needles to stir; 18 guage needles, various lengths, and Injector Gun. (*See Figure 6.*) Note that large gauge needles were needed because of viscosity of fat graft/PRP mixture, however injector gun was utilized when smaller gauges desired.

***Patient Selection*:** Patients used for our clinical investigation were consented volunteers with documented musculoskeletal pathology (by ultrasound), a history of pain greater than six weeks, and some level of disability measured by pain and decrease in work or sports activity.

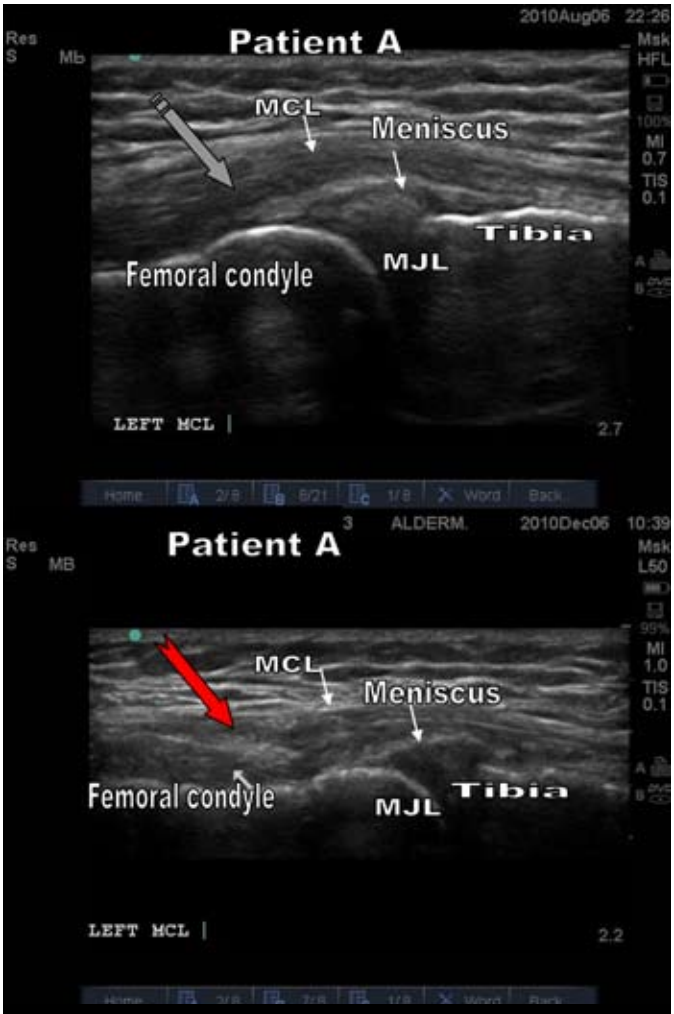

Figure 5b. Patient A ultrasounds "Before" and "After." Top: Before AD-SC. Medial aspect of the knee. Segmented arrows showing hypoechoic medial collateral ligament at its insertion. Proximal part of the tendon is tendonotic. MJL = medial joint line. **Bottom: After AD-SC.** Solid arrow showing hyperechoic new tissue in the medial collateral ligament at its insertion four months after one stem/stromal cell treatment.

Some of these patients had received previous Prolotherapy and/or standard PRP Prolotherapy treatments, and all were educated on the theory and methodology of the proposed treatment. Each patient received at least one AD-SC/HD-PRP treatment, with documented ultrasound follow up at different intervals of between one to six months.

***Procedure*:** Upon arrival to the clinic the patient scanned by ultrasound and pathology noted. Blood was then withdrawn, and placed for bidirectional centrifugation within Harvest Smart PRep-2 system. Sterile protocol

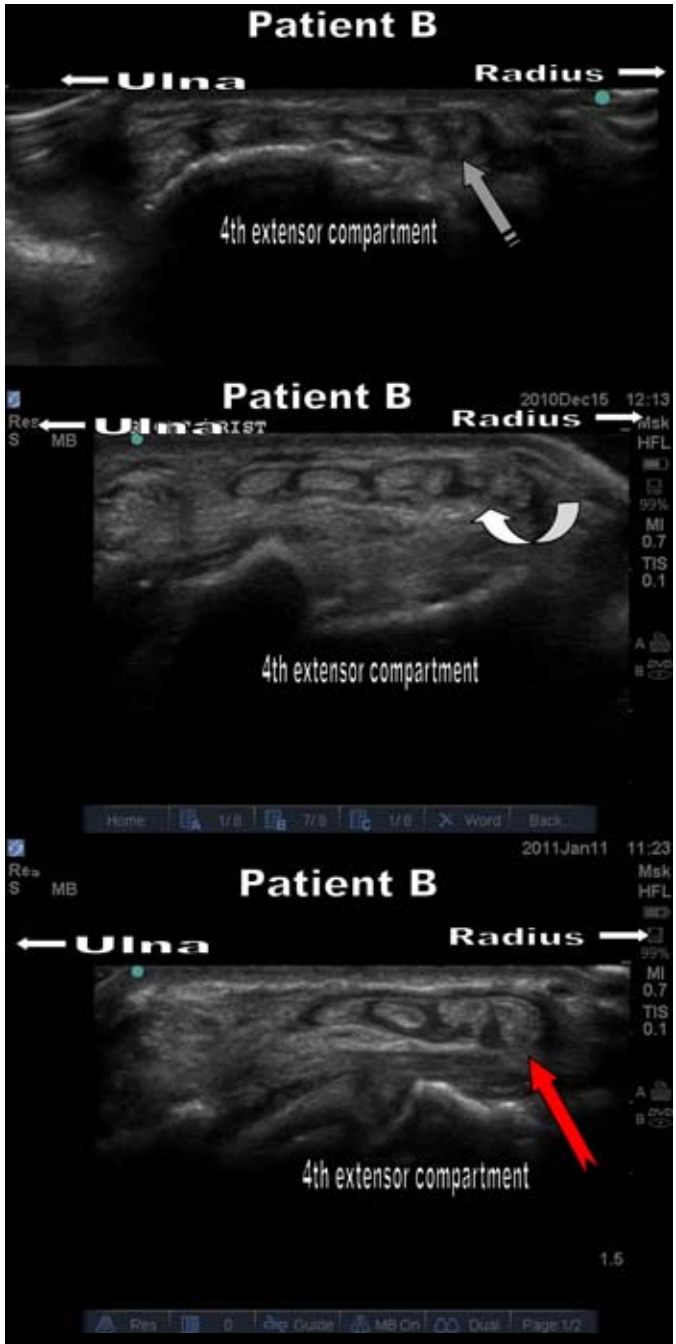

Figure 5c. Patient B ultrasounds "Before" and "After." Top: After Prolotherapy / Before PRP. Segmented arrow showing evidence of a tendonitis with a longitudinal tear through the radial aspect of the palmaris longus tendon. Patient had received several prior Prolotherapy treatments (ultrasound not available proir to Prolotherapy). **Middle: After PRP / Before AD-SC.** Curved arrow showing evidence of a healing but still torn palmaris longus tendon after five PRP treatments done over a 12 month period. **Bottom: After AD-SC.** Solid arrow showing hyperechoic "normal" appearing palmaris longus tendon without evidence of tearing one month after one stem/stromal cell treatment.

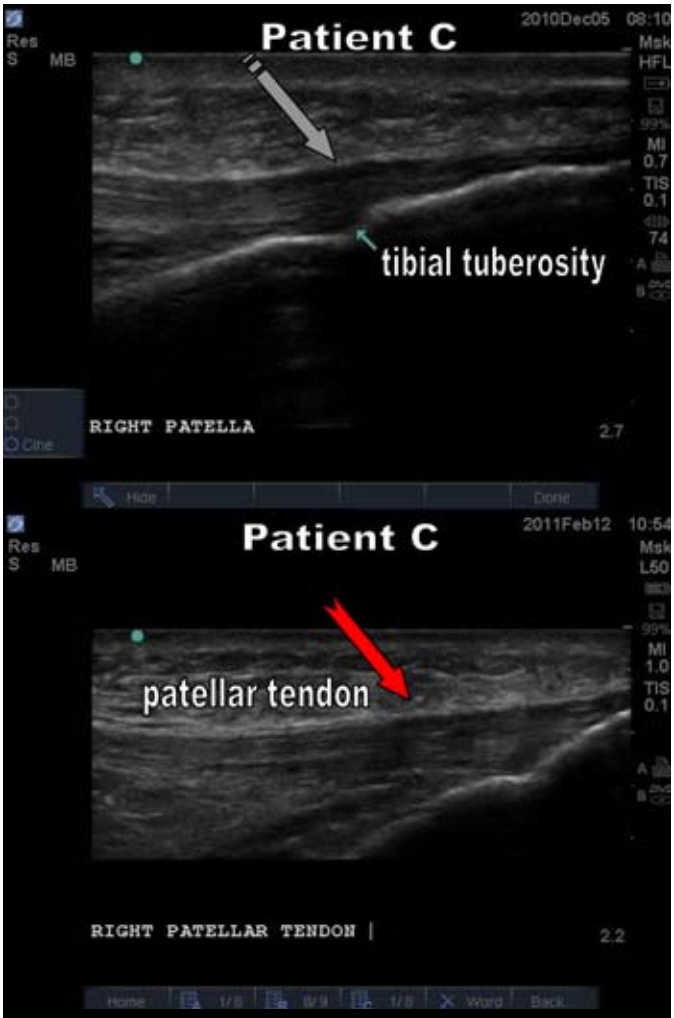

Figure 5d. Patient C ultrasounds "Before" and "After." Top: Before AD-SC. Long view. Segmented arrow showing a long image of a hypoechoic patellar tendonitis at the insertion. **Bottom: After AD-SC.** Long view. Solid arrow showing long view with marked improvement of previous hypoechoic area after one stem/stromal treatment.

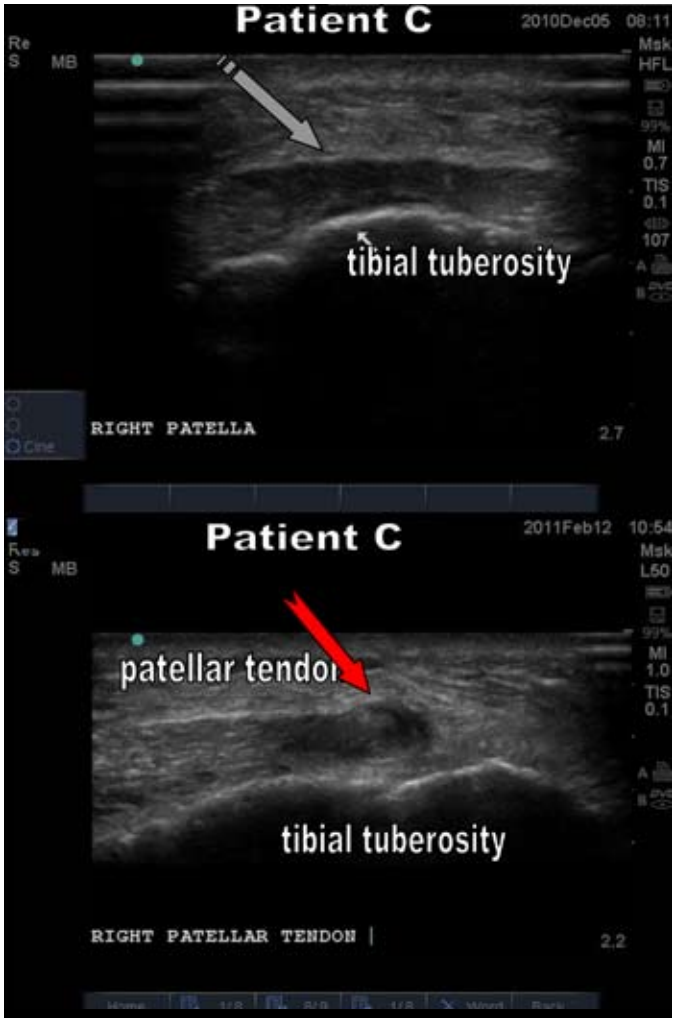

Figure 5e. Patient C ultrasounds "Before" and "After." Top: Before AD-SC. Transverse view. Segmented arrow showing an image of a hypoechoic patellar tendonitis at the insertion. **Bottom: After AD-SC.** Transverse view. Solid arrow showing marked improvement of previous hypoechoic area nine weeks after one stem/stromal treatment.

was strictly followed with tray set up to expedite treatment (*See Figure 7.*) Patient fat extraction site (typically abdomen or flank) was isolated and prepped with Technicare™ or Clorascrub™. For thinner patients where fat donor site was unclear, ultrasound was used to locate the thickest adipose stroma. While platelet concentrate was being processed, the closed syringe Tulip™ microcannula technique was utilized to harvest approximately 5 to 20 cc of lipoaspirate (volume depending on area being treated). Exact details on how the infiltration and harvesting are done is found within this issue of *Journal of Prolotherapy* (See Alexander article: "*Autologous Fat Grafts As Mesenchymal Stromal Stem Cell Source For Use In Prolotherapy: A Simple Technique To Acquire Lipoaspirants*"). Lipoaspirant obtained in this manner was gravity decanted for 3 to 4 minutes, infranatant expelled and adipose/stromal vascular fraction combined with approximately equal volume of HD-PRP. Patents were treated fully awake and without sedation. Targeted sites were located via ultrasound and with ultrasound guidance introduction of leur lock syringe and needle with lidocaine mixture made at site; a small amount of lidocaine injected (varied depending on site) then removal of syringe while needle still in place and syringe containing therapeutic preparation (fat graft/PRP) attached for delivery, then injected with continued needle visualization ultrasound to the targeted area.

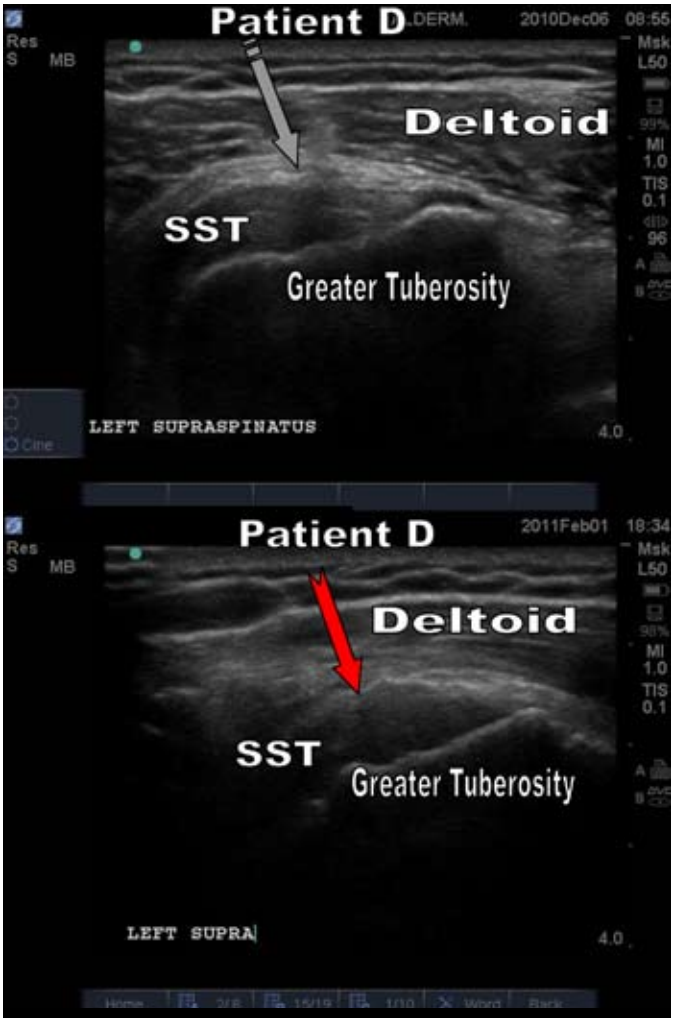

Figure 5f. Patient D ultrasounds "Before" and "After." Top: Before AD-SC (left). Segmented arrow showing a high grade tear of the supraspinatus at its insertion. Patient D had bilateral rotator cuff tears. **Bottom: After AD-SC (left).** Solid arrow showing the supraspinatus with evidence of new hypoechoic tissue within previous hypoechoic area two months after one stem/stromal cell treatment.

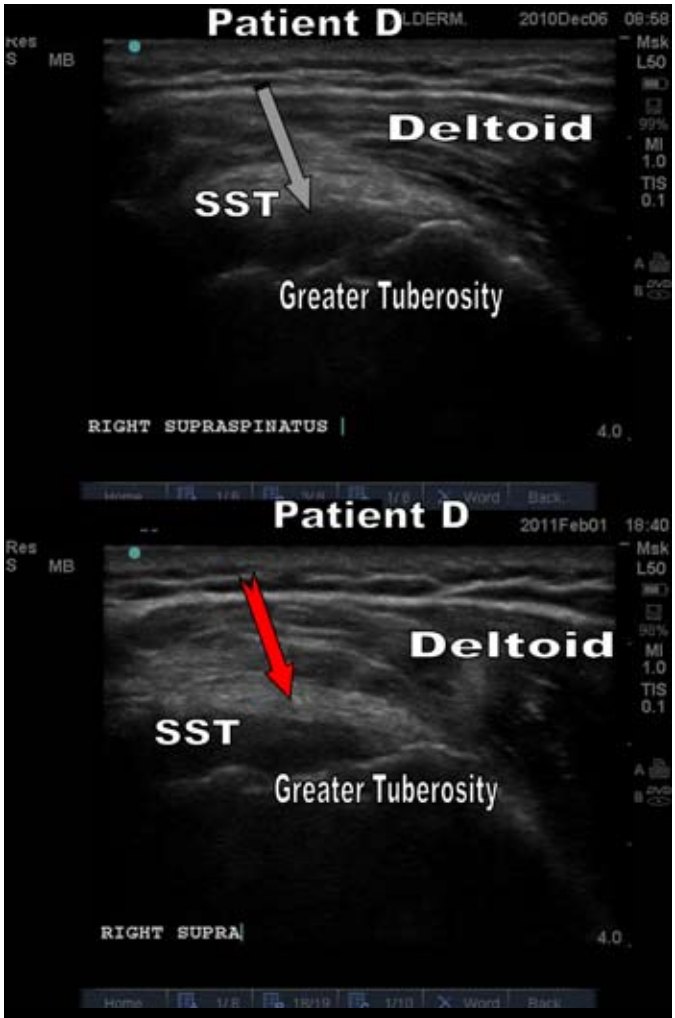

Figure 5g. Patient D ultrasounds "Before" and "After." Top: Before AD-SC (right). Segmented arrow showing an image of a hypoechoic partial tear of the supraspinatus tendon. **Bottom: After AD-SC (right).** Solid arrow showing the supraspinatus with evidence of new hypoechoic tissue within previous hypoechoic area two months after one stem/stromal cell treatment.

***Injection style*:** Injections were done "Prolo-style" with delivery of the AD-SC/PRP mixture accompanied by very mild needle irritation of the tendon, tendon sheath, or ligament injected. This could be compared to, but not nearly as aggressive, as percutaneous needle tenotomy, a dry needling procedure which has been found effective in several studies for tendonopathies.[204, 205] The needle irritation helps to activate the tissue to release thrombin and other mediators which help to activate the HD-PRP/AD-SC complex and attract additional growth factors and AD-SC's to the injury site. This becomes especially important in tendonosis, chronic degeneration without inflammation,[206] where tissue signaling is reduced or silent.

LIDOCAINE CONTROVERSY

Some controversy exists regarding use of lidocaine in adult stem cell procedures, although a thorough search by these authors did not locate any specific studies directly supporting the claim that lidocaine had any long term negative impact on stem-stromal cell survival at the low concentrations used in these procedures. The question of local anesthetic effect on human adipose survival has been discussed since the 1970's when Arner et al. studied the effect of prilocaine chloride on these cells and concluded that an inhibitory effect, while present at higher doses, could be regarded as minimal at low concentrations.[207]

Figure 6. Injector gun.

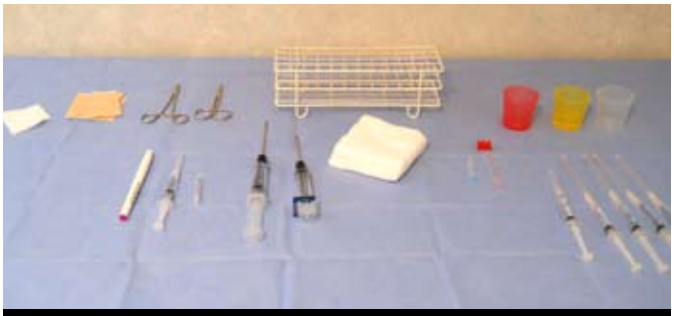

Figure 7. Simple tray set up.

Desai et al. confirmed that lidocaine does not appear to have any detrimental effect to fibroblast growth or wound healing when used at lower doses.[208] Kim et al. assessed urinary incontinance after transplantation of rat muscle-derived progenitor cells to a defect and concluded that lidocaine concentrations of less than 500 uM had no effect on muscle progenitor cells, even with continuous exposure, although at higher concentrations (1 to 5 mM) there was some cell impact. However, improvement in urinary incontinence occurred in all concentrations of lidocaine/stem cell treated groups vs. controls. The authors concluded that cytotoxicity due to lidocaine was minimal at physiologic concentrations and could be used without decreasing the efficacy of the therapy.[209] In cosmetic-plastic surgery studies have suggested that lidocaine may potentially inhibit glucose transport in adipocytes, putting them in "stasis," however this effect persisted only as long as lidocaine was present, and cells were able to fully regain their function whether exposure was as short as 30 minutes or as long as 10 days.[210] The lipophilic nature of lidocaine very quickly makes its way into adipocytes, but it does not appear to influence the mesenchymal/stromal/stem cell elements in the same way as it does to adipocytes, suggesting that studies relating to lidocaine toxicity may not be relevant. In a cosmetic-plastic surgical study, there is evidence that intracellular lidocaine in adipocytes is lost very slowly, and is not totally removed by multiple rinsings. The fact that retained intracellular lidocaine is present has not shown to have any clinically significant effects on autologous structural fat grafting effects.[211] Interestingly, procaine, an alternative local anesthetic, may have a preservation effect on pluripotent hemopoietic stem cells (HSC) in an animal study.[212] To more directly address this important question the authors are conducting an investigation to compare lidocaine exposed adipose samples vs. procaine exposed adipose samples vs. control with laboratory assessment of viable nucleated cell counts which will be published when complete.

DISCUSSION

Utilization of autologous adipose-derived stem/stromal cells, adipose scaffolding, and high-density platelet rich plasma concentrates have proven very effective in the several thousand of successful injections in pre-clinical use by physicians in the U.S. and elsewhere. The purpose of this paper is to provide: 1) A safe and effective protocol for stem cell/stromal Prolotherapy for physicians treating musculoskeletal injury; 2) A protocol that can be completed at the point-of-care within the outpatient office setting; and, 3) A protocol that does not violate current FDA guidelines. Stem cell Prolotherapy is an attractive option for connective tissue repair, especially when traditional Prolotherapy alone or high-density PRP Prolotherapy have not resulted in complete resolution of a connective tissue problem. Although multiple articles exist as to the benefit of mesenchymal and stromal stem cells in cosmetic-plastic surgery and orthopedic surgery, there has not been a standardized, effective protocol addressing an outpatient, bedside procedure for the Prololotherapist, sports medicine, regenerative medicine, or orthopedic physician. This protocol uses the Tulip™ patented micocannula system to harvest cells and stroma in a safe and non-traumatic manner, preserving the mesenchymal stem/stromal cell elements. Adipose tissue effectively delivers a living bioscaffold, felt to be very important in the repair and regenerative process. The use of HD-PRP concentrates in conjunction with the stromal and bioscaffold elements is believed to further enhance the healing capabilities and cellular repair.

Multiple studies support the effectiveness of adipose-derived mesenchymal stem cells for use in connective tissue repair, among other potential clinical uses, with over 40 IRB clinical trials ongoing at this time. Current FDA restrictions prevent the manipulation of cells, however do allow removing cells from an individual and returning them to the same individual during the same procedure. Methods employed in our experiences confirm this as a safe and efficacious means of providing significant patient successes in cases of chronic inflammatory, degenerative, and/or damaged musculoskeletal tissues. Ideally the ability to concentrate the cell elements and add them back to the adipose bioscaffolding will be permitted at some point in the future, potentially allowing an even more effective repair and regeneration within damaged or diseased sites. The authors believe that use of high definition ultrasonography can provide enhanced ability to diagnose tendon, ligament and joint defects accurately at the point-of-care, while insuring very accurate placement of the therapeutic combination of stem-stromal cells, adipose scaffolding, and HD-PRP concentrates. It also provides a metric to compare pre-treatment, time of treatment and follow-up documentation of tissue changes.

It is important to standardize terms and definitions when studies are reported in the literature. Various terms have been used to describe the mesenchymal stromal stem cell complex in adipose, however since the microenvironment of adipose has multiple active undifferentiated cell types, we believe the term "adipose-derived stem/stromal cell" (AD-SC) best describes this stem cell complex. At this time it is also believed to be of critical importance to clearly define platelet concentrates used, as there are a variety of systems reporting use of PRP, but no consistent definition documented. We propose that reports claiming PRP concentrate use be defined in terms of increases above baseline circulating levels. High-density, therapeutic, PRP should equal or exceed four (4) times individual patient baselines.

As controlled clinical trials are evolving which will provide statistical documentation of the safety and efficacy, early pre-clinical uses have proven very successful with extremely low morbidity. More studies need to be done, especially regarding the controversy of lidocaine use with stem-stromal cell viability.

CONCLUSION

Stem cell Prolotherapy offers a safe and clinically effective option in cases of musculoskeletal and connective tissue injury or joint degeneration which may be utilized by physicians to assist in their treatment of the patient with unresolved musculoskeletal pain. The efficacy of the treatment will need to be assessed by studies with larger patient numbers and under more controlled parameters. ■

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