

TITLE PAGE

**Autologous Fat Grafts As Mesenchymal Stromal Stem
Cell Source For Use In Prolotherapy: A Simple
Technique To Acquire Lipoaspirants**

Submitted by:

Robert W. Alexander. M.D., D.M.D., F.I.C.S.

Summary Page

Robert W. Alexander, M.D., D.M.D., F.I.C.S.

Associate Professor, University of Washington School of Medicine and Dentistry
Seattle, WA 98195

Private Practice of Aesthetic & Reconstructive Surgery

715 Main Street, Suite B

Stevensville, MT. 59870

Phone: (406) 777-4477

FAX: (866) 766-5458

Email: rwamd@cybernet1.com

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Attached: Author photograph and Curriculum Vitae

Abstract

Objectives: Background for use of autologous adipose tissue as a source of adult progenitor (stem) cells for use in prolotherapy. Present a means of lipoaspiration to harvest adipose-derived mesenchymal stromal cells (AD-MSC) and the stromal vascular fraction (SVF) for use in prolotherapy and regenerative medical applications by non-plastic surgeons.

Design: Explain the patented super luer-lok and microcannulas for use with the Tulip™ Medical closed syringe system. A sequential explanation and equipment selection for minimally traumatic lipoaspiration in small volumes is presented.

Results: Thousands of autologous fat grafts (AFG) have proven safe and efficacious for lipoaspiration techniques for large and small structural fat grafting procedures. Addition of platelet-rich plasma (PRP) to AFG has been used in several thousand cases of HD ultrasonic-guided injection prolotherapy for musculoskeletal purposes in the past 4 years with excellent clinical outcomes.

Conclusions: Use of Tulip™ closed syringe lipoaspiration system with microcannulas offers a safe and effective means of harvesting small volumes of non-manipulated adipose tissues and its accompanying progenitor cells within the SVF. It offers a simple and effective means to gather undifferentiated cells for use in prolotherapy and regenerative medical applications. Syringe and microcannulas offer a compact system and practical protocol for non-plastic surgical practitioners.

Keywords: Lipoaspiration; Lipoharvest; Adipose-derived stem cells; Adipose-derived stromal cells; Adipose-derived progenitor cells; Adipose-derived adult mesenchymal stem cell, Platelet-rich plasma; PRP; Prolotherapy; Bioscaffolds; Stromal vascular fraction; SVF

Introduction & Background

For many years, cosmetic-plastic surgeons have recognized the value of low pressure lipoaspiration for successful transplantation of adipose tissue for structural augmentation. In the introductory years (1980-1990) of liposuction techniques, autologous fat grafting (AFG) was considered unpredictable. Once bioengineers discovered the mechanisms by which lipoaspiration worked, the closed syringe system for gentle harvesting and transplantation was developed and patented. Early belief that effective lipoaspiration was directly related to force of vacuum was replaced by understanding, that, introduction of fluid into the fat layers permitted the fat cells and stroma to enter into a suspension. This suspension was easily extracted through use of closed syringes, and provided adipose tissues with reduced damage and improved grafting results.¹

As the importance of tumescent fluid distribution was appreciated, more value was placed in extensive pre-tunneling (moving cannula without applying vacuum). This better distributed local solution and enhanced the ability to mobilize the adipose tissues into a suspension, which yielded more successful and predictable AFG. During the late 1990s, surgeons began to include utilization of platelet-rich plasma (PRP) to further enhance the successes and acceptance of the graft tissues, in both large and small volume transfers.²

In the early 2000s, appreciation of the potentials of adipose tissue and its related stromal elements, led to examination of the adipose-derived adult mesenchymal stem cell content (AD-MSC). Evidence has clearly shown the key importance of the progenitor cells (SVF) and extracellular matrix (ECM) as integral contributors to the tissue maintenance and healing processes.³ Studies of adipocyte replenishment (following normal senescence and cellular death) showed that these attached progenitor cells were activated to form adipocytes and thereby maintain adipose tissue integrity over time.⁴ Since there is easy accessibility and greater availability of pluripotential progenitor cells for all mesogenic lines within adipose tissues, utilization of AD-MSC/SVF has become a central focus in clinical regenerative medicine.⁵⁻¹⁰

Pre-clinical and clinical applications have been reported in many scientific studies in the biological, bioengineering, and clinical medical literature.¹¹ Cosmetic-plastic surgeons initially focused on understanding the mechanisms to achieve safe and effective AFG. It was believed that intact cellular (mature lipocytes) transplantation was the most important goal. However, it has become crystal clear that the actual adult adipocytes transplanted may be the *least* important feature producing the success, even in structural fat graft applications. Current beliefs are that success in long-term AFG is actually due to *activation* of adherent progenitor cells (attached to mature adipocytes, ECM and SVF), and *proliferation* of those progenitor cells to *differentiate* into the target cell for replacement.¹²⁻¹⁴ As example, placement of lipoaspirants into existing adipose tissue favors differentiation into adipose cell types. As understanding of the maintenance and replenishment of cell cycles *in Vivo* increases, extensive research has been devoted to the study of microenvironment (niche), cell-to-cell/cell-to-matrix factors, and

autocrine/paracrine signaling system functions. Since AD-MSC/SVF are capable of differentiation for all mesogenic lines including: (1). Chondrogenic; (2). Fibro-muscular (including tendon, ligament, skeletal and cardiac muscle); (3). Osteogenic; (4). and, Adipogenic cell lines, uses in clinical applications have increased.¹⁵⁻¹⁸ Addition of PRP concentrate-derived of growth factors and signal proteins (cytokines) to the progenitor cells, have clinically improved abilities to promote healing within an injury site.²

Rapidly accumulating early clinical data on the safety and efficacy of AD-MSC/SVF *in Vivo* provides clear evidence that adipose tissue grafts possess extensive potentials, well beyond the structural augmentation in plastic surgical uses. Understanding these mechanisms resulted in important application potentials for prolotherapy and regenerative medicine. Reporting of pre-clinical, early clinical, and controlled studies in animal and human models, shows worldwide recognition of the potentials uses of these cells in diverse areas of medicine and surgery.¹⁹⁻²⁰

For more than 5 decades, most research and clinical application reports have used bone marrow-derived stem cells (BM-MSCs).²¹ It is now recognized that AD-MSCs have essentially the same capabilities while offering significant advantages over BM-MSCs utilization. These include: (1). Very similar progenitor differentiation capabilities; (2). Found in much higher concentrations compared to bone marrow aspirants (>100X); (3). Less expensive to harvest; (4). Less invasive (more safe); (5). Readily available tissue; and, (6). Less technically demanding than bone marrow penetration and cellular harvest. These have led to increased uses in many areas of medicine and surgery.²²⁻²⁷

Prolotherapy has clearly demonstrated how mechanical and chemical irritants can effectively stimulate damaged musculoskeletal (MSK) and connective tissues and “restart” the healing process. In the early 1990s, the value of adding complex elements which are naturally derived via platelet degranulation proved effective in further promotion of healing mechanisms. Marx²⁰⁰⁴; Since that time, prolotherapy has recognized the value of PRP concentrate placement into local musculoskeletal and joint areas in more complex cases.²⁸⁻³⁵

The natural next step is application of AD-MSC/SVF + PRP placement by guided high-definition (HD) ultrasonography in complex and difficult cases. This has proven to be of significant value in healing outcomes, particularly in area of complex damage or surgically failed sites.³⁶ It is now believed that improved efficacy is a result of direct stimulation of the microenvironment (niche) and simultaneous provision of needed undifferentiated (progenitor) cell types to damaged tissue sites *in Vivo*.³⁷⁻³⁹

Extrapolation of extensive human uses with proven safety and success in aesthetic and reconstructive surgical structural fat grafting/PRP has led prolotherapists to adapt the techniques for uses in musculoskeletal (MSK) tissues. Technology is available to effectively isolate/ concentrate these progenitor cells, but such manipulation involving chemical digestion is not permitted for clinical use within the United States. Therefore, utilization of non-manipulated AFG/PRP concentrates remains the only option at this time.⁴⁰ This paper is intended to provide

prolatherapists and sports medicine practitioners a simple and safe means of gathering lipoaspirates to be utilized in advanced cases. The patented Tulip™ closed syringe system with its array of microcannulas is a well recognized lipoaspiration system, and suitable for non-plastic surgeons to harvest small volume lipoaspirates for use in guided musculoskeletal injection therapies. Explanation and discussion of a reasonable and safe protocol for adipose harvest for non-plastic surgeons will be provided in this paper.

Materials & Methods

Selection of Lipoaspiration Sites

The lower abdomen and flank areas of both males and females are considered ideal sites due to distribution of human adipose tissues and relatively large deposits. Choice of aspiration sites in the medial and lateral thighs-buttocks areas are sometimes used in female patients due to genetic distribution within the gynoid body type. In very low percentage body fat patients, use of the ultrasound is helpful to determine the thickness and depth of adipose deposits.

Preparation of Lipoaspiration Sites

The patients may be placed in either supine or lateral decubitus position to facilitate the preparation and sterile isolation of the proposed donor area (s). It is considered important to follow a standard sterile protocol for both the harvesting and placement sites.

Instrumentation

The patented Tulip Medical closed syringe system for lipoaspiration features very smooth cannulas and a “super” luer-lok connection for use with standard luer-lok syringes. (Figure 1) This hub connection is an important component of the microcannula system, in that it provides an excellent seal for maintaining even vacuum forces during lipoaspiration. In addition, it provides a very stable, rigid base when using very small cannulas and their associated flexibility within the tissues.

Two standard options for microcannula selection are offered within the Tulip system. They are:

- (1). *Cell-Friendly Microcannula option* (autoclavable) (Figure 2):

These cannulas are internally polished by a proprietary extrusion process to maximize internal smoothness and reduce adipose tissue damage to the adipocytes and their accompanying matrix. External cannula anodizing processes provide a smoother surface for ease of passage within the subdermal adipose plane. This is the most popular design in use within the plastic-cosmetic autologous fat grafting procedures;

- (2). *Gel Coated Disposable option* (Figure 3):

Use of microcannulas in small diameters (range 0.9-2.1 mm OD) presents a challenge in proper cleaning and sterilization, making a disposable option attractive. These are packaged and labeled in a sterile wrap, and can be opened directly onto the sterile field or back table. Featuring the super luer-lok base, the stainless steel cannulas are coated with a hydrophilic gel which provides slick coating and pass through adipose tissues with minimal resistance.

Selection of Microcannula Length and Diameters

For prolotherapy applications, it is recommended to use a small, multiport *Infiltrator Cannula* for even and thorough distribution of local throughout the adipose donor layer. Openings near the tip are multiple and oriented such that 360 degree distribution of local while moving through the subdermal fat layers is possible. It is recommended that practitioners use this cannula in a diameter of 2.1 mm OD and a length of 10 cm. (Figure 4 a,b)

Harvesting Cannulas are designed to actually remove the adipose tissues from the subdermal plane, following the same pattern and location of local anesthesia distribution. It is recommended that prolotherapists use one of two diameters, smaller for thinner and lower percent body fat patients, and larger for the majority of patients. The openings are offset, meaning in a non-linear pattern near the tip of the cannula. These are in diameters of 1.67 mm and 2.1 mm (OD), and a length of 8 cm. Use of a slightly shorter harvesting cannula versus infiltrator, makes it easier to remain within the local anesthesia distribution areas.

Tulip Syringe Locks are designed for use on BD or Monoject 10/12 and 20 cc luer-lok syringes to hold the syringe plunger in a fully drawn position during the application of vacuum. (Figure 5) When in the locked position, and all air removed from the system, this permits the physician to apply even and gentle vacuum pressures while moving the cannula through the adipose layer.

Tulip Anaerobic Transfers are available to facilitate loading of treatment syringes for both combining PRP + adipose grafts into one syringe. They are also useful for transferring the treatment mix into syringe sizes of physician's preference for injection. (Figure 6 a,b)

Tulip Injector Gun is a device for placement of controlled 0.5 cc aliquots of treatment mix. When AFG is added to the PRP, the density of the injection material is increased. This may result in the prolotherapist requiring more force to inject into the tissue site. Single trigger pull provides exact volumes of solution to be placed with less pressure required by the provider. (Figure 7 a,b)

Sequential Technique For Lipoaspiration

It is recommended that the area of donor fat be outlined with a skin marking pencil. This will become the area prepped-draped to expose the thickest deposit of palpable fat tissues and serve as a distribution pattern for local anesthetic infiltration. (See Diagram 1)

After marking, preparation, and isolation of the donor area, an 18-20 g needle is utilized to create a small opening, extending through the epidermis and dermis, into the subdermal fat plane below Scarpa's fascia. This opening is made larger by selectively cutting the dermal layer (under the skin surface) with edge of the needle. This allows the introduction of the multiport infiltration cannula through the skin and the superficial fascia (Scarpa's in the abdomen). (See Diagram 2) Following entry, passage of the infiltrator is approximately in a horizontal fashion, above the muscular layer, in a "spokes-of-a-wheel" pattern. Pinching the skin-fat tissues may help in passing the cannula. During movement of the infiltrating cannula, slow injection of the tumescent local anesthesia fluid is provided on both the entry and withdrawal strokes, evenly, and in layers. The importance of avoiding "pooling" of local is that evenly distributing liquids improve the efficiency of harvest and well as providing excellent patient comfort. Typical volumes of local solution range from 20-30 cc during the infiltration process. Most practitioners select 50 cc multi-dose vials of local anesthetic (e.g. 0.5 to 1.0 % Xylocaine with, or without, 1:100,000 epinephrine) to supply tumescent fluid for lipoaspiration. The remaining local is available to provide local anesthesia for the skin opening for lipoharvest, and for entry injection therapy sites for guided treatment.

Upon completion of distribution of local anesthesia within the donor area, it is recommended that re-passage of the infiltrating cannula throughout the donor area (termed "pre-tunneling") multiple times is helpful. This more thoroughly distributes local fluids, and begins the mobilization of adipose tissue prior to use of any vacuum. This is an important step which will improve comfort during harvest, plus make extraction more efficient and effective.

Most practitioners use a 20 cc BD luer syringe attached to the harvesting microcannula with a mounted locking device. A very small volume (1-2 cc) of sterile 0.9% saline is drawn into the cannula to displace air from the system prior to insertion into the harvest (donor site). Once the harvesting cannula is in the local infiltrated adipose layer, the syringe plunger is drawn to a full extension, and twisted to provide a "lock", permitting the physician to be free to move the harvesting cannula in a forward and back series of passages. It is important that these passages are within the same plane and same pattern as was used in placement of local solution. Adipose return, at first, will be slower, as the tissue must be in suspension to be able to be extracted. Continuing these movements with vacuum applied will yield yellow adipose tissues which float on the small fluid volume within the syringe system. When the desired volume is attained, lipoaspiration is finishing, and the small opening covered with a triple antibiotic ointment and a Coverlet bandage (adhesive on all sides).

In the event of loss of vacuum (evidenced by a hissing sound), the cannula is simply removed from the adipose tissues, fluid advanced into the harvesting cannula to eliminate air from the

system, cannula reinserted and plunger pulled open and locked to restart the aspiration. During the displacement of air, it is recommended that 4X4 sterile gauze be held over the harvesting tip openings to avoid spraying contents. Occasionally, in cases where there is a larger volume of infranatant fluid (the layer immediately below the fat tissues), simply express the liquid portion, and re-insert the harvesting cannula to the harvest site, lock the plunger, and gather more graft tissue. One common cause of increased infranatant volume is inadequate distribution of local fluid, creating a “pooling” effect, which reduces efficiency of adipose harvest.

Upon completion of aspiration of approximately 12-20 cc, the harvester cannula is removed, and the syringe end capped and placed in a vertical position in a standard test tube rack and allowed to decant. Some advanced techniques include a centrifugation (700 g for 3 minutes) to help further separate fluid from harvested graft. If ultrasound guidance is employed, preparation of the treatment sites can be completed during the decanting period (3-5 minutes). There is often a small liquid layer (infranant) noted below the fat grafts. This may be expressed through an anaerobic transfer connection prior to loading of treatment syringes. [Note: It is advised that the most superficial layer on the top of the autologous fat graft not be included in the treatment syringes (represents residual free oils)]. Some practitioners perform 1-2 rinses with sterile saline to help reduce any residual local anesthetic solution and red blood cells.³⁸

Patients undergoing AFG will also be prepared for addition of previously harvested platelet-rich plasma (PRP) concentrates. (Harvest Tech, SmartPrep II™) The desired amount of PRP can be loaded to the treatment syringes using the anaerobic transfer. Most cases utilize the AFG:PRP ratio of 50% (i.e. added at a 50:50 ratio graft::PRP volume). This preparation is then ready for placement into treatment sites with practitioners using 18-20 gauge needles of lengths needed to deliver the combination to the specific treatment site. Although these needles are larger than in common prolotherapy use, they are necessary due to the increased viscosity of the AFG-PRP combination.

Discussion

When the mechanisms involved in liposuction technologies were recognized in the late 1980s, the ability to provide small and large volume liposuction via the Tulip closed syringe system was proven. Due to even, low vacuum pressures with syringe use, enhanced abilities to provide autologous fat grafting with safety, efficacy, and predictability within aesthetic surgical applications was noted. Structural fat grafting, utilizing the exact techniques herein described has been completed many thousands of times. In the mid-1990's this author began the addition of PRP to provide more effective graft acceptance. Recognizing the lipospirant concentration of AD-MSC/SVF played an important role in such autologous grafting and maintenance, we participated in examination for use of these progenitor cells for treatment of chronic musculoskeletal and joint disorders. With the ability of these adipose-derived stromal cells to physically differentiate into cartilaginous, skeletal, teno-ligamentous, skeletal muscle tissues *in Vitro*, early clinical prolotherapy treatments utilizing these stem cells began in 2006.

Protherapists have extensive experience in using irritants to “trick the body” into returning to a previously incompletely or unhealed tissue state, stimulating the natural inflammation and encourage musculoskeletal connective tissue and joints to effectively heal. The utilization of a patient’s own tissues (autologous) to enhance a prolotherapy treatment and promote the healing abilities in complex sites has become a practical clinical option. By providing many of the body’s own healing growth factors and cytokines (derived from degranulating platelets), plus undifferentiated progenitor cells into damaged connective tissues, remarkable successes have been accomplished. The safety and efficacy of prolotherapy with platelet-rich plasma concentrates is now well recognized by many practitioners providing sports injury care.

Appreciation of regenerative capabilities within bone marrow-derived mesenchymal stromal cells (BM-MSC) and AD-MSC have led protherapists to clinical applications for use of non-manipulated AFG and autologous PRP to injury sites. Simultaneously, adipocytes and the associate stromal elements serve an important role, serving as a living bio-scaffold (attachment to adipocytes and SVF) which is critical to encourage available stem cells to activate. Both the AFG and PRP effects are further enhanced in the damaged tissues through established “signaling” mechanisms of autocrine and paracrine pathways *in vivo*.^{2,36} Accurate introduction of AD-MSC/SVF, adipocytes, and platelet-derived factors is important. When placed in a targeted injury site, it is believed that the interaction within the local microenvironment (niche) actually “guides” the activation and proliferation toward site-specific cell types. In doing so, it leads to improved inflammatory and healing capabilities.³⁹⁻⁴³ Enhanced by the inclusion of the PRP concentrate, effectiveness of therapy has improved, even in the most complex cases.

Conclusion

This paper presents a simple and effective method of lipoaspiration to harvest adipocytes and their accompanying progenitor (stem) and stromal elements for use in prolotherapy applications. The safety of using the patented Tulip closed syringe microannula system has been proven for two decades. For the past 12 years, addition of PRP to the AFG protocol has provided great evidence of safety and influences on structural augmentation. As pre-clinical and human early clinical studies accumulate in MSK applications, many involved in prolotherapy and regenerative medicine are finding a valuable paradigm shift favoring use of autologous cells and platelet derivatives for therapeutic purposes. Controlled clinical trials are now in progress to further examine the mechanisms and *in vivo* tissue engineering.

[Note: The author has no financial interests in Tulip Medical, Harvest Technology or known conflicts of interest in presenting this material].

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Figures & Legends

Figure 1.



Tulip™ Medical Closed Syringe Microcannula System For Lipoaspiration & Injection.
Left to Right: Anaerobic Transfer (Super Luer Lok); Cell Friendly™ Microcannulas;
Mechanical Injector Gun; BD Syringes With Locks In Place

Figure 2.



Prolotherapy Microcannula Set (Cell-Friendly). Top: Tulip™ Multiport
Infiltrator; Middle: Tulip™ Carraway 3 Port Harvester 2.1 mm; Bottom:
Tulip™ Carraway 3 Port Harvester 1.67 mm

Figure 3.



Tulip™ Medical Disposable Microcannula System

Figure 4 a, b



a. Close up multiport infiltrator cannula



b. Close up 3 Port Carraway Harvester Cannula

Figure 5

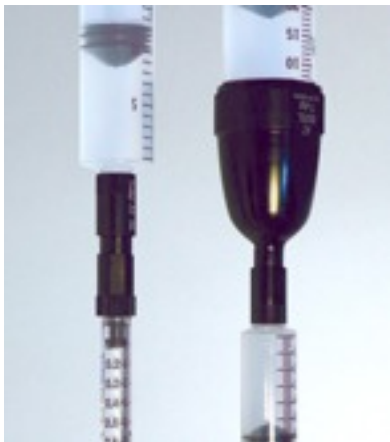


Close Up Of Jonnie Lock Attached To BD Syringe

Figure 6 a, b



- a. Anaerobic Transfers, Super Luer-Lok Attachment. Left: Cell-Friendly™ (Non-Disposable); Right: Disposable



- b. Anaerobic Transfers (Luer-to-Luer) For Loading
Syringes For Actual Guided Placement

Figure 7 a, b



- a. Mechanical Injector Gun (0.5 cc Per Trigger Pull)



- a. Mechanical Injector Gun (0.5 cc Per Trigger Pull) Prepared For

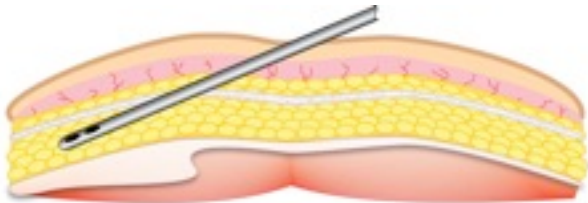
Injection Placement

Diagram 1



Marked Abdominal Skin For Infiltration and Harvest. X Marks Skin Opening Site(s)
Arrows Outline Pattern Of Infiltration Of Local and Harvest

Diagram 2



Lateral View Abdominal Wall, Cannula Placed Under Scarpa's Fascia