

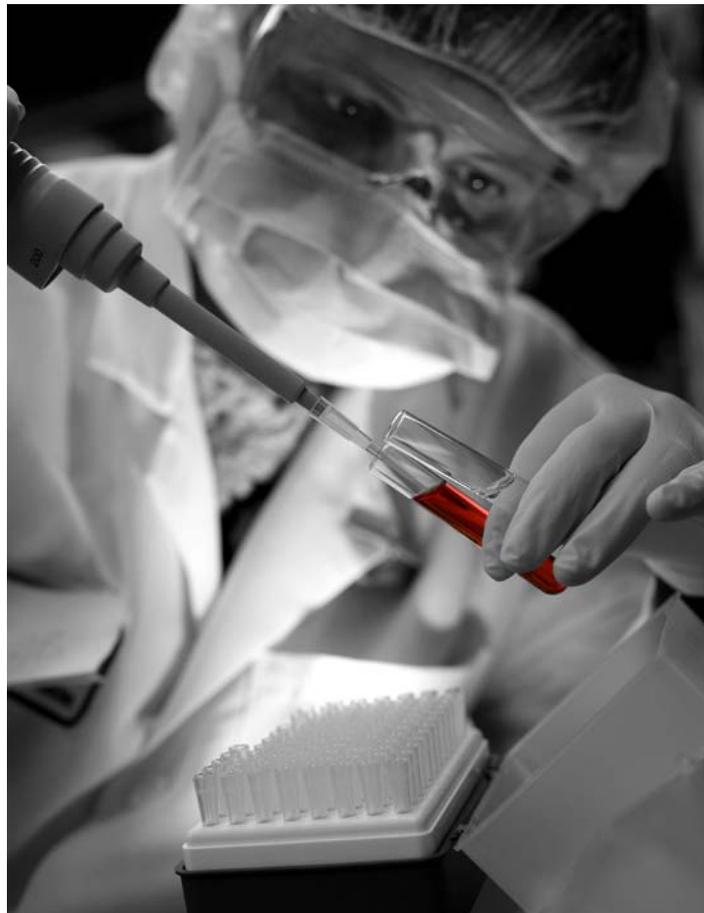
# Advances in Regenerative Medicine: High-density Platelet-rich Plasma and Stem Cell Prolotherapy For Musculoskeletal Pain

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**P**rolotherapy is a method of regenerative injection treatment designed to stimulate healing.<sup>1</sup> Prolotherapy is used for the treatment of chronic musculoskeletal pain, including ligament, tendon and joint injuries, as well as osteoarthritis. The term *prolotherapy* is short for *proliferation therapy*, as it stimulates the proliferation and repair of injured tissue.

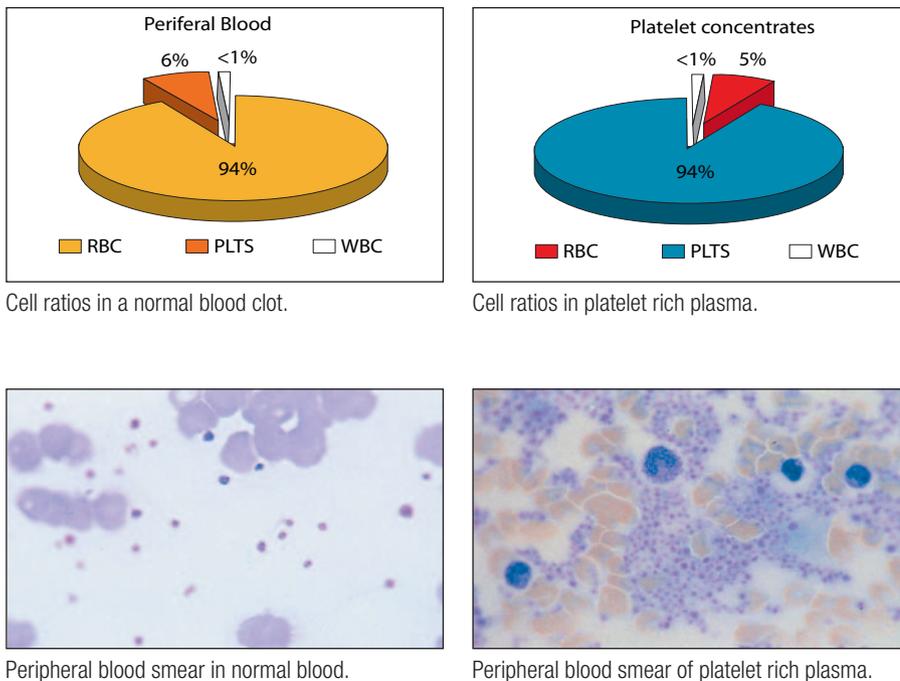
Traditional dextrose prolotherapy originated in the 1930s and continues to be used successfully. In the 2000s, in-office platelet-rich plasma (PRP) prolotherapy was introduced. This method uses a patient's own blood, centrifuged to concentrate growth factor-rich platelets as the proliferation formula. In the past few years, physicians have begun using adult stem cells, harvested from an individual's fat tissue or bone marrow during an in-office procedure, then combined with the individual's PRP as the proliferation formula for injection into injured musculoskeletal tissue.

This newest form of prolotherapy, known as *stem cell prolotherapy*, is used in difficult cases or where accelerated musculoskeletal healing is desired. Popular media reports have been emerging that cite the use of stem cell prolotherapy in professional athletes such as Bartolo Colon, starting pitcher for the New York Yankees, who had the procedure successfully done for a rotator cuff injury earlier this year.<sup>2</sup> Our article will review the history, science, methodology, and evidence for these types of prolotherapy, and offer a treatment algorithm.

## Prolotherapy: The Original Regenerative Medicine

Prolotherapy was "discovered" in the 1930s by Dr. Earl Gedney, an osteopathic surgeon, before the term *regenerative medicine* existed. However, prolotherapy is a true regenerative medicine, working by locally raising growth factor levels to promote tissue repair and regeneration.<sup>3-5</sup>

Multiple studies confirm the effectiveness of prolotherapy in the resolution of musculoskeletal pain, including low back pain,<sup>6,7</sup> neck pain, and whiplash injuries<sup>8</sup>; chronic sprains and/or strains; tennis and golfer's elbow<sup>9</sup>; plantar



**Figure 1.** Difference between whole blood and platelet-rich prolotherapy. Top left, cell ratios in a normal blood clot. Top right, cell ratios in platelet-rich plasma. Bottom left, peripheral blood smear in normal blood. Bottom right, peripheral blood smear of platelet-rich plasma. PLTS, platelets; RBC, red blood cells; WBC, white blood cells

fasciitis<sup>10</sup>; knee,<sup>11</sup> ankle, shoulder pain, coccydynia<sup>12</sup>; chronic tendonitis/tendonosis,<sup>13</sup> including Achilles tendonitis/tendonosis<sup>14</sup>; and other joint pain or musculoskeletal pain related to osteoarthritis.<sup>4</sup>

**How Prolotherapy Works**

Prolotherapy is based on the premise that chronic musculoskeletal pain is caused by an inadequate repair of fibrous connective tissue, resulting in ligament or tendon weakness and relaxation (laxity),<sup>1</sup> also known as connective tissue insufficiency.<sup>15</sup> Weak connective tissue results in insufficient tensile strength or tightness,<sup>16</sup> causing excessive “loading” of the tissues that stimulates pain mechanoreceptors.<sup>15</sup> 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.<sup>17</sup> If the laxity or tensile strength deficit is not corrected sufficiently to stop pain mechanoreceptor

stimulation, chronic sprain/strain, and pain results.<sup>3</sup>

Prolotherapy works by stimulating a temporary, low-grade inflammation at the site of ligament or tendon weakness, “tricking” the body into initiating a new healing cycle cascade.<sup>3</sup> A common formula used in classic prolotherapy is dextrose, however, the choice of solution varies depending on practitioner preference and may contain sarapin, morruate, zinc, or other natural ingredients, combined with a local anesthetic.

**Platelet-rich Plasma Prolotherapy**

In the 1990s, the use of PRP to accelerate healing gained acceptance in surgical circles. However, the machines were large, expensive, and only used in hospital operating rooms. By the 2000s, the machines were smaller and available for use in an office setting.

Prolotherapists, and other physicians in the orthopedic and sports medicine fields, began using PRP

injections to stimulate musculoskeletal connective tissue repair.<sup>18-20</sup> PRP prolotherapy is based on the same theory as traditional dextrose prolotherapy, however, the formula 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.<sup>21</sup>

High-density PRP (HD-PRP) is defined as autologous blood with concentrations of platelets at no less than four times the circulating baseline levels,<sup>22</sup> and which increases the important bioactive protein load (growth factors) in a direct correlative fashion.<sup>23</sup> Cell ratios in average circulating whole blood contain only 6% platelets. In true HD-PRP preparations, the concentration achieved is 94%.<sup>22</sup> An 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 (Figure 1).<sup>24</sup>

Circulating platelets, when activated, begin a degranulation process that 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- $\beta$ 1; angiogenesis), vascular endothelial growth factor (VEGF; angiogenesis), fibroblast growth factor (FGF; proliferation of myoblasts and angiogenesis), and insulin-like growth factor-1 (ILGF-1; mediates growth and repair of skeletal muscle), among others.<sup>25</sup> Activated platelets also secrete stromal cell-derived factor 1- $\alpha$  (SDF-1 $\alpha$ ), which supports primary adhesion and migration of mesenchymal stem/stromal cells (Table, page 58).<sup>22</sup>

Platelets contain a significant number of key signal proteins, growth

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**Table. Common Growth Factors and Abbreviations Found in Platelet-rich Plasma**

Platelet-derived growth factor aa,bb,ab	PDGF
Transforming growth factor- $\beta$ 1, $\beta$ 2	TGF- $\beta$ 1, TGF- $\beta$ 2
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: $\alpha$ , $\gamma$	I- $\alpha$ , I, $\gamma$
Insulin-like growth factor	ILGF

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factors, chemokines, cytokines, and other proinflammatory bioactive factors that initiate and regulate basic aspects of the inflammatory cascade resulting in natural wound healing.<sup>26</sup> Elevated platelet concentrations are known to stimulate the proliferation, differentiation, and migration of needed mesenchymal and stromal repair cells to an injury site.<sup>27</sup>

Similar to dextrose prolotherapy, the addition of HD-PRP concentrates results in an inflammatory and proliferative response that enhances healing and promotes tissue regeneration.<sup>28</sup> The use of clinically proven devices to obtain this degree of concentration is considered essential. Various portable commercial centrifugation units exist that can process blood samples, resulting in PRP concentrates, however only a few have been shown to concentrate platelets to therapeutic levels as does the FDA cleared Harvest Technologies' SmartPrep2 system.

### Stem Cell Types

It is believed that there are really only two kinds of stem cells: the embryonic (prenatal) stem cell and the adult (postnatal) stem cell.<sup>29</sup> Embryonic stem cells are, in theory, able to transform into any type of tissue; they are totipotent or omnipotent when an egg is fertilized. After several divisions, the stem cell is considered pluripotent, and able to differentiate into any of the three germ layers.<sup>30</sup>

Postnatal stem cells are those cells present that remain in an individual after birth, in an undifferentiated state, and available to maintain tissue homeostasis and regeneration in a tissue or organ system. 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.<sup>31</sup> These adult 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.<sup>32</sup> 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.<sup>33</sup> Adult stem cells can be found in all tissues in the body in various quantities.<sup>34</sup>

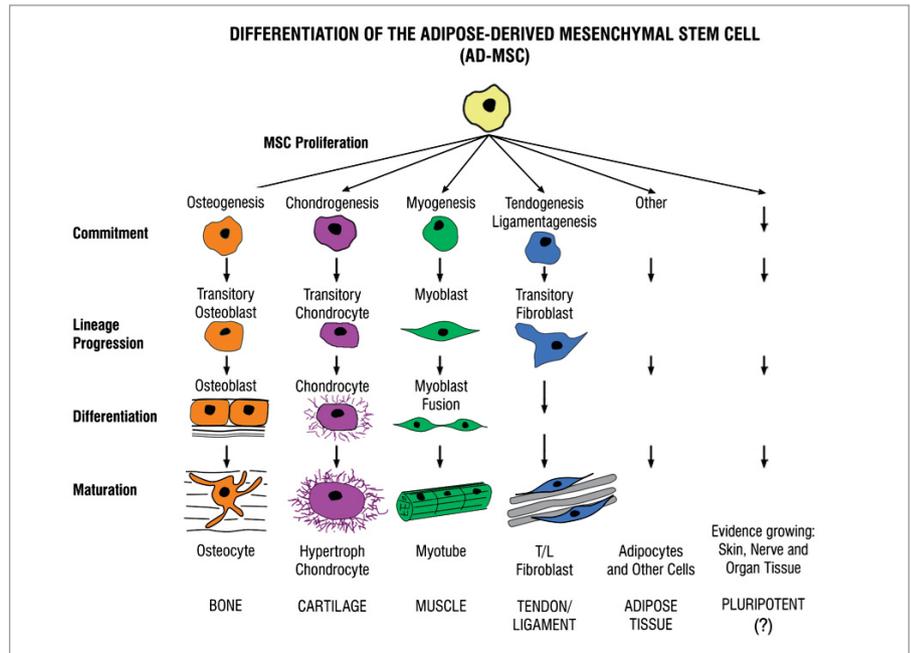
### Adult Mesenchymal Stem Cells

In the early 1990s, existence of adult mesenchymal stem cells (MSCs), described as "non-committed progenitor cells of musculoskeletal tissues," were discovered to have an active role in connective tissue repair.<sup>35</sup> These cells were first labeled by Caplan as mesenchymal stem cells<sup>36</sup> because of their ability to differentiate to lineages

of mesenchymal tissue, and were recognized to be an essential component of the tissue repair process.<sup>27</sup> An interesting observation made about MSCs is their ability to “home in” and help repair areas of tissue injury.<sup>35</sup>

Although bone marrow historically has been used as a source of MSCs, adipose-derived MSCs have been shown to have nearly identical fibroblast-like morphology and colonization (CFU-F), immune phenotype, successful rate of isolation, and differentiation capabilities.<sup>37</sup> The healing potential of adipose-derived MSCs was demonstrated in early clinical use for cranial defect and chronic fistula repair, without side effects.<sup>38</sup> MSCs, 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,<sup>39</sup> angiogenesis in osteoarthritis,<sup>40,41</sup> tendon defects,<sup>42-44</sup> ligament tissue,<sup>45</sup> intervertebral disc repair,<sup>46,47</sup> muscle,<sup>48</sup> nerve tissue,<sup>49</sup> bone,<sup>50</sup> and hematopoietic-supporting stroma.<sup>51</sup> MSCs also actively participate in tissue homeostasis, regeneration, and wound healing<sup>52</sup>; ischemic heart tissue<sup>53,54</sup>; graft-vs-host disease<sup>55</sup>; and osteogenesis imperfecta (Figure 2).<sup>56</sup>

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.<sup>57,58</sup> It has been suggested that addition of these missing MSC elements might help these conditions. A number of studies have demonstrated such improvement with adult stem cell therapy by the successful regeneration of osteoarthritic damage and articular cartilage defects.<sup>59,60</sup> In 2003, Murphy et al reported significant improvement in medial meniscus and



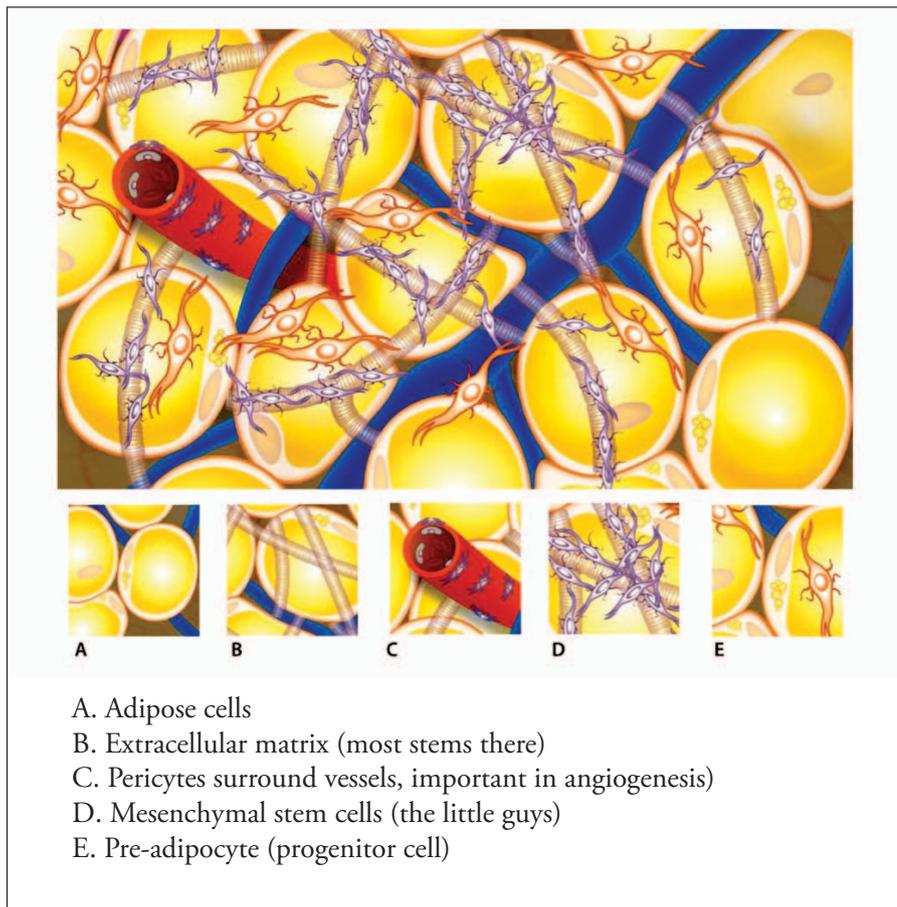
**Figure 2.** Flowchart elucidating possible commitment, lineage progression, and maturation of adipose-derived mesenchymal stem cells.

cartilage regeneration with autologous stem cell therapy in an animal model.<sup>61</sup> 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.<sup>61</sup> In 2008, Centeno et al reported significant knee cartilage growth and symptom improvement in a case report using culture expanded autologous MSCs from bone marrow.<sup>62</sup>

Multiple studies support the effectiveness of adipose-derived MSCs for use in connective tissue repair, among other potential clinical uses, with more than 40 institutional review board clinical trials ongoing at this time.<sup>63</sup> Current FDA restrictions prevent the manipulation or culture expansion of cells, however, they do allow removing cells from an individual and returning them to the same individual during the same procedure.

Historically, MSCs have been studied from bone marrow aspiration. However, bone marrow possesses very few true MSCs, and is gradually being replaced with adipose(fat)-derived stem/stromal cells (AD-SCs) as a primary tissue source.<sup>64</sup> Like bone marrow, adipose (fat) tissue is derived from embryonic mesodermal tissue. Fat is a complex tissue that is not only easier to harvest, but offers markedly higher nucleated, undifferentiated stem cell counts<sup>65</sup> than bone marrow. Research has shown as much as 500 to 1,000 times as many mesenchymal and stromal vascular stem-like cells exist in adipose as compared with bone marrow (Figure 3, page 60).<sup>66-68</sup>

In 2001 and 2002, Zuk et al confirmed that adipose stroma contains relatively large numbers of undifferentiated cells capable of producing cartilage, ligament, tendon, muscle, and bone.<sup>64,69</sup> AD-SCs also appear to have an increased angiogenic capability versus bone marrow,<sup>70</sup> and have been shown to promote neovascularization



**Figure 3.** Adipose tissue with progenitors and mesenchymal stem cells in stroma.

in skin flaps,<sup>71</sup> as well as safely treat depressed scars.<sup>72</sup>

AD-SCs meet the criteria suggested by Gimble et al that an ideal stem/stromal cell for regenerative medicinal applications should:

- Be found in abundant quantities;
- Be harvested with a minimally invasive procedure;
- Be differentiated along multiple cell lineage pathways in a regulatable and reproducible manner;
- Be safely and effectively transplanted.<sup>73,74</sup>

#### Addition of HD-PRP to AD-SC

During the 1990s, further understanding and enhancements to improve the success of fat grafts in cosmetic plastic

surgery led to the effective addition of HD-PRP concentrates to these autologous fat grafts (AFG).<sup>75-77</sup> 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. Studies have determined the safety and efficacy of implanted/administered AD-SCs and suggest that AD-SC in combination with HD-PRP also can regenerate articular cartilage<sup>78</sup> and reverse hip osteonecrosis.<sup>79</sup> With high levels of PDGF and cytokines, this combination provides both a living bioscaffold and a multipotent cell replenishment source useful for enhanced musculoskeletal healing.<sup>80</sup>

#### Theory of Stem Cell Prolotherapy

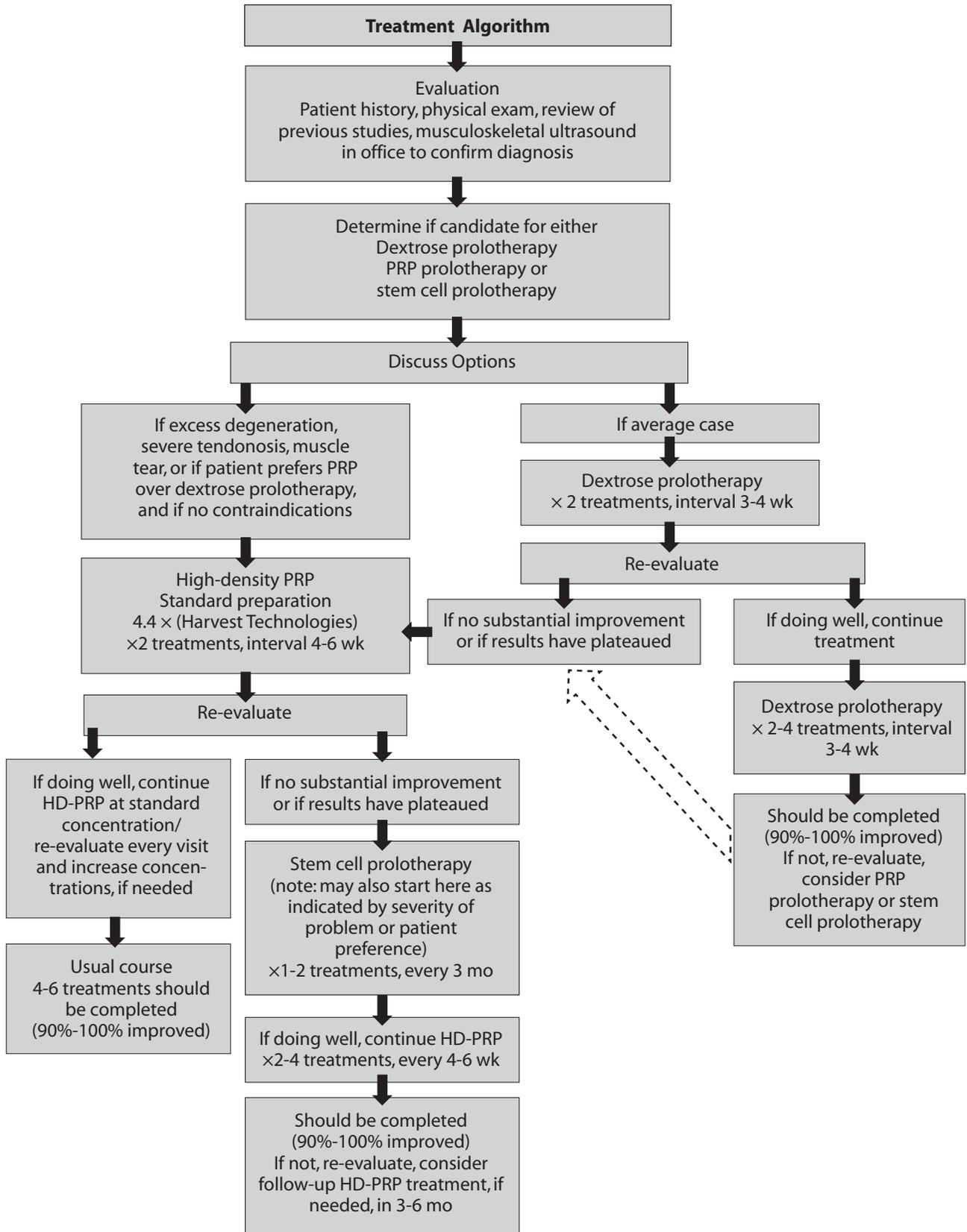
The ability of AD-SCs to support and serve as a cell reservoir for connective tissue and joint repair is the basic theory of stem cell prolotherapy. With stem cell prolotherapy, a stem cell niche (microenvironment that favors healing) is moved from one tissue in which these niches are abundant (adipose) into another where they are scarce (a nonrepairing connective tissue).<sup>81</sup> Multiple studies have shown that AD-SCs improve wound healing and stimulate fibroblast proliferation, migration, and collagen secretion—thereby increasing connective tissue tensile strength and healing.<sup>82</sup>

As discussed, AD-SCs have the potential to differentiate to become cartilage, tendon, ligament, bone, and skeletal or smooth muscle. They also are capable of expressing multiple growth factors that influence, control, and manage damaged neighboring cells.<sup>83</sup> Additionally, AD-SCs have been reported to be helpful in intervertebral disc regeneration,<sup>84</sup> tendon and ligament regeneration,<sup>85</sup> and in accelerating tendon repair and strength.<sup>86</sup> It is reasonable to hypothesize, therefore, that when traditional dextrose prolotherapy and/or PRP prolotherapy have not resulted in complete resolution of musculoskeletal pain and injury, stem cell prolotherapy would be the logical next step.

In veterinary medicine, AD-SCs have been used effectively for more than 10 years in the treatment of osteoarthritic joints<sup>87</sup> and connective tissue injuries in dogs. In fact, in double-blind placebo-controlled trials, AD-SC prolotherapy has been shown to be successful in more than 80% of cases.<sup>88</sup>

#### HD-PRP Creates Favorable Growth Factor Environment

A concentrated growth factor environment, coupled with a living bioscaffolding, has been found to be



**Figure 4.** Treatment algorithm for dextrose, PRP, and stem cell prolotherapy. PRP, plasma-rich prolotherapy

important for AD-SC use in orthopedic applications.<sup>89</sup> HD-PRP has shown the ability to enhance musculoskeletal healing and stimulate local microenvironmental regenerative capabilities,<sup>80</sup> especially during the early phase of tendon healing.<sup>90</sup> Proliferation of AD-SCs and their differentiation also is believed to be directly related to platelet concentration.<sup>27</sup> HD-PRP releases large quantities of PDGF, TGF- $\beta$ 1, and many other growth factors that, when activated, significantly enhance stem/stromal cell proliferation and angiogenesis,<sup>91-92</sup> as well as enhancing the survival of the fat scaffolding.<sup>93</sup>

### **Stem Cell Fate Dependent on Microenvironment**

It is clear that control of cellular fate and extracellular environment is critical in tissue regeneration and cell-based therapies.<sup>94</sup> Stem cell fate is controlled by a complex set of physical and chemical signals dictated by the cellular and chemical microenvironment (niche).<sup>95</sup> Therefore, if AD-SCs are placed within, and adherent to, damaged connective tissue, uncommitted progenitor and stem/stromal elements within the AD-SC graft should be stimulated toward that specific connective tissue lineage for growth and repair. For example, if placed within osteoarthritic degenerated cartilage, chondrogenic differentiation is believed to be encouraged.<sup>96-99</sup>

In the 1990s, Young et al showed repair of an Achilles tendon tear when AD-SC was placed in a collagen matrix, then placed in a tendon defect.<sup>100</sup> In 2010, Little et al demonstrated the successful differentiation of human AD-SCs 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.<sup>101</sup> Albano and Alexander successfully reported an autologous fat graft as a mesenchymal stem source and living bioscaffold (termed "Autologous Regenerative Matrix") to repair a persistent patellar tendon tear.<sup>102</sup>

### **Protocol for Stem Cell Prolotherapy**

A detailed protocol for stem cell prolotherapy was discussed in the August 2011 issue of the *Journal of Prolotherapy*.<sup>103</sup> A simple means for harvesting adipose tissue is detailed using the patented Tulip Medical™ microcannula system, which harvests cells and stroma in a safe and nontraumatic manner, preserving the mesenchymal stem/stromal cell elements.<sup>104</sup> Lipoaspirates are decanted by gravity, or low g-centrifugation (<1,000xg for 3 minutes), and combined with highly concentrated PRP obtained via Harvest Technologies' SmartPrep2 system. 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.<sup>103</sup>

In a trial series of patients, favorable outcomes were noted (reduced pain, improved function) with regenerative repair of ligament and tendon tears and defects in those patients, documented by musculoskeletal ultrasound. The determination of whether to start a patient with dextrose prolotherapy versus PRP versus stem cell prolotherapy is based on the severity of the physical findings in combination with patient preference. This is addressed in the treatment algorithm (Figure 4, page 61).

### **Conclusion**

Prolotherapy has come a long way since those early days in the 1930s when Dr. Gedney injected his own injured and painful thumb, searching

for a way to get his body to do what all bodies are programmed to do: heal and regenerate. Prolotherapy is, in fact, the original musculoskeletal "regenerative medicine." Traditional dextrose prolotherapy is still used with a high success rate in various musculoskeletal complaints. However, should dextrose prolotherapy fail or plateau, HD-PRP prolotherapy can be used to further enhance the healing process. Should HD-PRP prolotherapy fail or plateau, autologous AD-SCs combined with HD-PRP concentrates have proven very effective in the several thousand successful injections in preclinical use by physicians in the United States and elsewhere. HD-PRP prolotherapy and/or stem cell prolotherapy also can be used as a starting point treatment in more difficult cases.

Adipose tissue effectively delivers a living bioscaffold of adult mesenchymal-directed stem and stromal cells to devitalized tissue. The addition of HD-PRP concentrates to the adipose cells enhances healing capabilities and cellular repair. Although multiple articles have shown 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 prolotherapist, sports medicine, regenerative medicine, or orthopedic physician until recently. However, now these protocols are available and being used to obtain documented successful patient outcomes. Recent protocols can be completed at the point of care within the outpatient office setting and do not violate current FDA guidelines.

Outcomes and evidence so far is encouraging and positive, however, as this science continues to grow, more research needs to be done to refine these techniques and provide larger patient trials and longer term outcomes. ■

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