Platelet Rich Fibrin Matrix with Facial Collagen Genesis and Epidermal Regeneration

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Abstract

Objective: To determine if Platelet-rich fibrin matrices can induce an improvement in facial volume and mollify epidermal age-related negative remodeling.

Introduction

Dr. Anthony Stefani has demonstrated that PRP is effective for restoring facial volume in such regions as the nasolabial folds. Currently, there has not been any published study which shows that multiple injections of PRP throughout the face could induce collagen genesis or thickening of the dermis as well as overall epidermal rejuvenation in the older phenotype. It is our intent to determine in this pilot study if platelet-rich fibrin matrices are an appropriate aesthetic protocol for facial rejuvenation.

Methods

Twelve subjects between the ages of 44 and 56 who are healthy with a normal BMI as well as non-smokers were selected to participate in this study. We injected these individuals with their own autologous PRP throughout the mid and lower face. These injections were within the dermis and subdermis. The injection sites were three across the face and there were four rows from the zymatic arch to the jaw line. Thus, we injected twelve distinct areas on each side of the face. The amount of PRP plasma was approximately fifteen milligrams, which was calibrated as a function of the mild elevation of the epidermis. This aesthetic strategy was executed twice a month, or nine times during a four month period. Furthermore, facial volume loss was treated with a more robust prophylaxis, as several cc’s of PRP was injected into those facial regions.

Results and Discussion

In our view, autologous PRP prophylaxis engendered an improved collagen genesis which translated to enhanced facial volume as well as significant epidermal texture rejuvenation. It is likely in certain phenotypes that PRP cosmetic treatments may be as effective as current laser protocols and fat grafting. We observed no patient down time, and as a function of our questionnaire, subject's satisfaction was overwhelmingly substantial. PRP prophylaxis appears to be a viable strategy with multiple injections to treat a face between the ages of 35 and 55 which exhibits age and sun related facial aberrancies in terms of unattractive and negative remodeling.

Introduction

There has been a myriad of studies demonstrating the efficacy of PRP for orthopedic and cosmetic purposes.1,2,3,4 and 5 Dr. David Crane and Peter Everts, 2008, have stated that "PRP matrix grafts, along with other biological graft techniques are becoming more prevalent in the treatment paradigms of musculoskeletal medicine. These PRP matrix grafts provide effective, safe, and amelioratively low cost treatment options to patients who have time and wherewithal to allow collagen synthesis and maturation at the graft site. PRP matrix grafts appear to restore tissue homeostasis and the biotensegrity of collagen".6 David Karli and B. Robinson, 2010, have likewise stated that the body's own biological modulations can be efficacious in terms of enhancing the wound healing process. The literature is extensive, demonstrating that an increase in growth factor concentrations can be propitious in the wound healing process. Moreover, Karli and Robinson, 2010, purport that "this case report demonstrates sustained, subjective and functional improvement with near complete repair on MRI with a single application of platelet rich plasma in a severe tendon injury".7 J Menetrey and Etal show that higher concentrations of growth factors improved muscle healing.8 Conversely, a recent article in the British Journal of Sports Medicine, 2010, revealed that the IOC consensus paper on the use of platelet-rich plasma in sports medicine was far more conservative in its investigation of the use PRP in the treatment of sports related musculoskeletal perturbations.9 Clinical researchers like Stephen Barret have argued that higher concentrations of platelet rich growth factor provides excellent wound healing profile.10

Molecular medicine in the twenty-first century comprehends that cytokine activation of cellular dynamics is preferable to exogenous non-specific pharmacological agents as well as highly invasive surgical

protocols. Kilroy et al., 2007, purported that “the cytokine expression profile has a direct relevance to adipose tissue function and healing disease”.11 The literature is expanding with studies which indicate that higher concentrations of growth factors can have edifying effects on transcriptional and translational activities on a diverse array of human cell types. Elizaveta Kon et al. 2008, have reported that PRP intervention for the utilization of the higher concentration of growth factors engenders a rejuvenation of tissue. This group stated that “this report outlines the first in vivo investigation of the use of autologous growth factors to treat jumper’s knee by means of PRP injection, and demonstrate that this is a method to improve tendon healing and promising results”.12

The intent of this investigational study is not on the heuristic merit of PRP therapies for wound healing for sports injuries; but rather whether this biological modulatory protocol can actually have significant cosmetic efficacy. Anthony Sclafari’s initial 2009 study indicates that PRP therapies can have significant cosmetic benefits.13 This plastic surgeon employed higher concentration of platelets with their growth factors into a patient’s nasolabial folds to mitigate deficiency in volume in this particular facial region. His results were promising and cosmetically effective.

Bob Jackson’s 2003 article stated that PRP therapy actually significantly reduced senoma formation during the abdominoplasty procedure.14 This cosmetic surgeon stated that “the application of platelet rich plasma as a natural fibrin matrix delivers growth factors to the wound and seems to promote more rapid healing”.15 Ferdinand Becker in an unpublished pilot study showed that PRP therapies provided patients with superior aesthetic results after undergoing a cosmetic procedure. He writes that “the use of platelet concentrate has demonstrated excellent results by enhancing and accelerating wound healing. Patient’s own platelet concentrate has included experiencing significantly less swelling, bruising, and overall morbidity”. 16 Dr. Clevens work in Melbourne, Florida, likewise has shown an enhanced healing time when utilizing PRP in conjunction with a midface surgical procedure.17 Moreover, many others such as Thomas Tzikas have stated that the use of PRP for cosmetic purposes during a surgical procedure improves healing and reduces bleeding.18 Dr. Patrick Abuzeuni and Robert Alexander found that PRP with fat grafting enhances fat transplantation. They discovered that “this technique is intended to promote or accelerate the healing face after grafting, enhanced the

intended augmentation retention value of volume, potentially reduced secondary calcification, and microcystic formation, and maximized the transplant unit volume by reducing extracellular fluids transferred with grafts".19

Walter Tom wrote the following about the use of PRP for the use of aesthetic rejuvenation. "The new paradigm for natural facial rejuvenation is based on revolumizing the aging deflation of the face…an answer may be our own plasma with a concentrated fraction of platelets. Platelet rich plasma [PRP] has the potential not only to fill deflated volume, but may indeed trigger cell migration and differentiation. If this is born out, then we have a relatively inexpensive filler that is autologous with long term benefits and a minimum of side effects". 20 Recently, Katherine St. Louis discussed in a New York Times article that the benefits of the use of PRP for facial rejuvenation.21 The dermatological phenomena of what she referred on the "vampire face lift", which was licensed by Dr. Charles Reynolds; and represents a new frontier in biological medicine. However, the antidotal commentaries which were cited in this article seem puerile, and more hypothetical than a sophisticated clinical procedure.8 It is our purpose in executing this pilot study to utilize a complex methodology to determine if it is possible to reduce and alleviate volume deflation; and aesthetically improve the impoverished epidermal texture in the older phenotype in a manner which is consistent with what is attained with an average SMAS face lift in conjunction with a fractionated laser resurfacing. In summary, it is our intention to determine if many facial injections of PRP can induce dramatic dermatological remodeling of an older cosmetically compromised face. The literature seems to support that autologous platelet growth factors are able to remodel cellular tissue in a manner which is consistent with facial fat grafting.

These are some the essential aesthetic issues which are the etiological basis of this dermatological study. In general, we are interested in understanding whether we can reeducate the transcriptional machinery of senescent cells to behave in a more robust, youthful manner with respect to overall protein translation and synthesis. This study involves nine treatment sessions over a five month period where both the mid and lower face are treated. The second phase of this study will examine how long the aesthetic benefits of multiple PRP sessions are sustained over a six month time period. It appears that growth factors, in most instances, have more ameliorative merit than stem cells and are very important in terms of cellular remodeling.

Methodology

Study Design: Ten subjects were selected who met our criteria. We excluded subjects who were overweight (40 lbs over normal BMI), smokers, and those taking several or more prescribed medications, especially for depression and anxiety. The age range was from 44 to 57. Gender was not a delimiting factor. Some of the subjects previously had minimum cosmetically invasive procedures. One patient underwent a midface lift five years ago with limited cosmetic benefit. Several of the subjects have been treated with fillers, botox, and laser protocols. Their skin types ranged between two and four on the Fitzpatrick rating scale.

All the subjects exhibited excessive epidermal sun damage and aging. Some of them had letiginious lesions and dyschromia. Every subject had considerable volume loss in the nasolabial fold region as well as adjacent facial quadrants. None of the subjects were pregnant, nursing, or with any kind of skin lesion which was inflamed or infected. These individuals did not have a herpes simplex outbreak within the preceding five years. Furthermore, the subjects were not using any kind of dermatological prescription topical agent like retinoid acid. All subjects never reported an increased sensitivity to light or any kind of skin-related debilitating perturbation.

The autologous platelet concentration was prepared from a 60 mL or 20 mL kit with anti-coagulant. The blood was extracted and prepared using the Smart PREP system of Harvest Technology, Plymoth, Mass. This process provides a 9cc or 3.5cc of platelet concentrate with higher levels of growth factors. In general, a 60 or 20cc syringe is prefilled with 5cc of a citrate based on an anticoagulant (ACD-A) which is part of Harvest's disposable kit. Approximately 55cc or 20cc of patient blood is withdrawn from a venous puncture in the upper arm into either a 60cc or 20cc syringe.

The anti-coagulated blood is then placed into a blood chamber of a processing vessel which is disposable. This disposable unit is then set into the centrifuge locator cup of the Smart PREP system. The counter balance weight is placed in the opposite rotator cup, and in most instances, we had a second disposable kit to balance the unit. The lid of the system is closed and the processing of the blood is commenced. The process is automatic and takes twelve minutes. The centrifugation separates red blood cells from the plasma. This process enables platelets to create a pellet at the bottom of the disposable kit's plasmid

chamber.

The disposable kit's plasmid chamber contains red blood cells and in a second part harbors platelet concentrate. The platelet poor plasma (PPP) is removed. We primarily utilize the platelet concentrate. However, there was some degree of platelet poor plasma which was infused to create the concentrated platelet rich plasma or PRP solution. We did not use an activator for the platelet rich plasma; and it was placed by our technician into 31 gauge 1cc syringes. A 20cc kit produced 3.5cc of viable PRP concentrate, whereas a 60cc system yielded 9cc or more of PRP concentrate.

All patients were not treated with lidocaine. For most subjects, the pain of multiple injections was tolerated. One patient got her own ice packs to cope with the perceived pain. Moreover, we did not use any kind of topical lidocaine. A medical assistance cleaned the facial surface of all patients. A plastic surgeon marked which facial regions were to be treated with PRP injections. In general, a large kit of 60cc was used for subjects with excessive volume loss. Most patients had at least two regions on each side of their face which needed to be treated for excessive volume deflation.

The injections involved three points across, and a total of four rows for each side of the face from the zyomatic arch to the lower jaw line. Each injection into a particular mark represents a titration into the dermal and subdermal region; and in most instances the 31 gauge needle only punctured 50% into the facial surface. In most instances, a 1cc 31 gauge syringe treated most of the twelve marks on each side of the face. Each injection was approximately 5 milligrams. The clinical practitioner utilized a direct lateral approach into the surface of the face. Conversely, when treated the nasolabial fold the clinician employed a direct vertical angle, where the needle was placed fully into a specific subdermal vector plane. These individuals also received twelve injections on each side of their face involving 1cc of PRP concentrate. In general, there was some degree of variability in terms of treatment strategies for a particular phenotype as a function of their level of volume deflation. Furthermore, all patients did receive twelve standardized injections on each side of their face, but there was sufficient variability in terms of treatment protocols for facial volume deflation.

The injection sessions were fourteen days apart and took between ten and fifteen minutes. For most patients, the pain was well tolerated.

There were nine separate injection sessions over a five month period. We did not observe any severe complications in any of the ten phenotypes who participated in the study. Most patients did not experience pain after each treatment session. There was never a need for the use of prescription medication for any subject who was involved in the study. Some of the subjects did observe in areas of deeper injection, such as in the nasolabial fold region, bruising which was resolved in a few days.

We did not see any extensive erythema or any edema; and limited facial swelling resolved within twelve hours. Thus, these injections were well tolerated by subjects with limited complications. However, one patient seemed to have a heightened pain sensitivity after each session. This woman reported that she was suffering from severe general anxiety syndrome which was not currently being treated. All subjects were given fourteen questions to answer at the final session which was concerned with their satisfaction with these treatment protocols.

Patient Questionnaire for PRP Study

Please circle numbers on the scale of 1 to 10 (1 being lowest 10 the highest) to score how strongly you agree with the following statements.

Question 1: I've received significant facial rejuvenation:

1 2 3 4 5 6 7 8 9 10

Question 2: The epidermal texture of my face has improved:

1 2 3 4 5 6 7 8 9 10

Question 3: These injections made me look younger:

1 2 3 4 5 6 7 8 9 10

Question 4: My face looks like I had a facelift:

1 2 3 4 5 6 7 8 9 10

Question 5: This procedure added volume to my face:

1 2 3 4 5 6 7 8 9 10

Question 6: This cosmetic strategy enabled me to become more attractive:

1 2 3 4 5 6 7 8 9 10

Question 7: I would undergo these treatments again:

1 2 3 4 5 6 7 8 9 10

Question 8: I will recommend PRP facial treatments to my friends:

1 2 3 4 5 6 7 8 9 10

Question 9: This cosmetic strategy improved my quality of life:

1 2 3 4 5 6 7 8 9 10

Question 10: I prefer this treatment over a facelift:

1 2 3 4 5 6 7 8 9 10

Question 11: The aesthetics of my face are still improving with multiple treatments:

1 2 3 4 5 6 7 8 9 10

Question 12: I think this medical protocol is appropriate for the general public in terms of enhancing facial beauty:

1 2 3 4 5 6 7 8 9 10

Question 13: I think this aesthetic therapy is a superb strategy for reducing the effects of facial aging:

1 2 3 4 5 6 7 8 9 10

Question 14: I am very satisfied with the cosmetic results I achieved from participating in this study:

1 2 3 4 5 6 7 8 9 10

Results

This initial pilot study involving twelve subjects was to determine if platelet-rich plasma actually had sufficient cosmetic efficacy in terms of observable and viable aesthetic changes. It is essential in a larger cohort study to begin doing genetic analysis of the cellular system protein expression changes in the dermis; and even 3-D imaging in order to understand the degree of dermal thickening as well as other molecular ameliorative modifications. Moreover, histological analysis and 3-D imaging was not utilized in this study, but would be an essential facet of any other future investigation. In general, we relied on a patient satisfaction rating scale and their overall perceptions to verify the effectiveness of this innovative cosmetic protocol.

All patients who participated in this study perceived that they received facial rejuvenation; and they all rated their results at the highest level. These subjects evaluated the first seven questions of this study with the highest rating possible. In general, they were extremely satisfied with the improvement of their epidermal texture; and they felt that these injections enabled them to look younger in appearance. They all believed that this procedure induced their face to appear as though they underwent some type of invasive surgical procedure.

All subjects regarded that this cosmetic strategy significantly added volume to their face; and therefore they felt more attractive. All eight subjects who completed this investigative study would unanimously undergo these treatments again. Thus, the eight patients of this study answered the questions concerned with their satisfaction with the highest rating possible in terms of improvement of facial appearance. They all perceived their epidermal texture as improving, looking younger with greater volume, and would undergo these treatments again in order to become more attractive.

Two of the eight subjects answered seven and eight whether they would recommend this procedure to their friends. These two women did not enjoy having these injections without lidocaine; and they believed that their friends would not necessarily be able to cope with the discomfort associated with this protocol. Six of these patients stated that this procedure absolutely improved their quality of life, and one responded with a nine rating, one with an eight, and another with a seven. All seven subjects stated that they preferred this strategy over that of a face lift, and one woman responded with a score of an eight. It

seems that all the subjects who completed this study preferred multiple treatments of PRP to that of having a highly invasive surgical procedure. Six of the eight subjects rated this question a ten to the fact that their face is still improving, and one person responded with a seven and another with a score of eight. All seven subjects expressed a rating of ten in terms of the appropriateness of this procedure for the general public. These subjects evaluated the use of PRP treatments as an effective strategy for reducing facial aging. Furthermore, the subjects who participated in this study were 100% satisfied with their cosmetic results; However, one woman rated her satisfaction with a score of nine.

Clinical Observations of Medical Practitioners who Participated in this Investigational Study

We all concurred that four to six injections of PRP in the older phenotypes who participated in this study was highly efficacious in terms of cosmetic benefits. In certain subjects, multiple injections seemed to be as effective as a robust fractionated CO2 treatment with respect to facial rejuvenation of the epidermal texture and mitigating skin laxity. However, the results may not necessarily be consistent with a larger cohort population. We believe that there is a certain degree of variability in the subject population in terms of the degree of skin tightening, which is affected by age and overall physical health status. Furthermore, we all observed results in the majority of subjects consistent with one pass of a fractionated CO2 treatment.

The two clinicians who participated in this study have performed many facial fat grafting procedures. They observed that after multiple treatments of a PRP protocol, that volume restoration for several patients is as judicious as employing facial fat grafting. PRP injections provides a patient with a very naturalistic symmetry as well as ameliorating facial volume loss compared to the unpredictability associated with facial fat grafting. There appears to be sufficient evidence from this initial pilot study that multiple PRP injections in a particular facial quadrant may be preferable in some instances to facial fat grafting.

In the eight phenotypes who participated in this study, only two subject had aesthetic results which would be applicable to a SMAS mid facelift. These two women are in their late forties, and demonstrated at

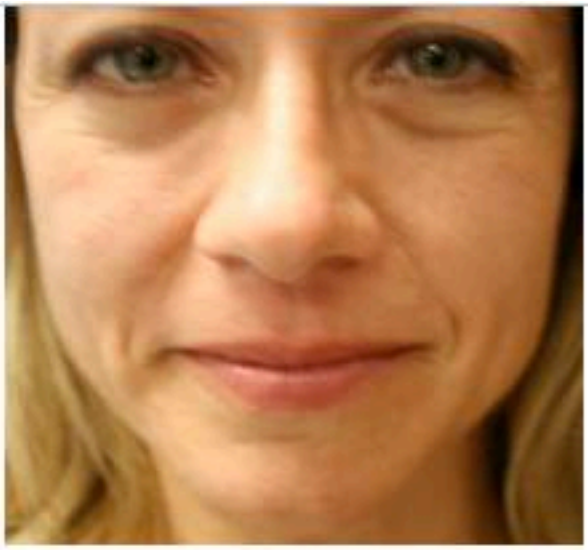
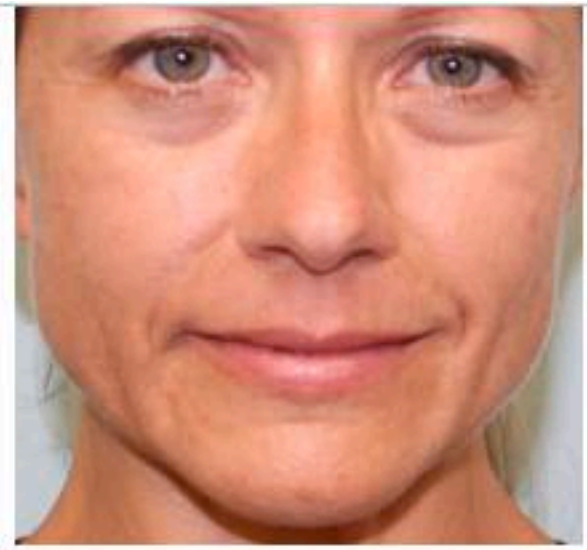
the initial phase of the study limited facial laxity and had only moderate volume deficiency. We observed after multiple PRP treatments that there was a mollification of facial laxity and restoration of facial volume loss as well as robust epidermal remodeling. In our view, eight injections in phenotypes over fifty would not be comparable to a mid-facelift in most phenotypes. However, in some patients in their late forties with a healthy lifestyle, they could possibly achieve the aesthetic benefits consistent with a mid-facelift. This particular cohort population now have the option to undergo multiple treatments of PRP in lieu of a highly invasive surgical procedure.

Three subjects were eliminated from this investigation for missing two consecutive treatment sessions. One woman, who was 57, with excessive skin laxity dropped out, as her results were more minimum than the other cohorts who completed this research study. This female was advised as a function of her aberrancies in soft tissue remodeling that she should pursue an invasive mid-facelift procedure as it would be the only cosmetic protocol to mitigate her inordinate facial aging. Thus, in our opinion, multiple PRP treatments are not appropriate for anyone who is over sixty with excessive skin laxity or in general with pejorative soft tissue remodeling. This biological cosmetic protocol is most appropriate for a phenotype with sun damage in their late thirties to early fifties without excessive soft tissue aberrant remodeling.

We have discerned that younger subjects who are physically active in their forties will have the most dramatic aesthetic results with multiple treatment sessions. Moreover, two younger females in their late twenties with non-age related volume deficiencies were treated twice with PRP injections; and their aesthetic results were dramatic in terms of improving their overall appearance. Thus, we believe that younger women could have significant aesthetic enhancement with two treatment sessions of PRP therapy. This innovative cosmetic protocol is definitely efficacious with respect to improving facial beauty in the younger phenotype.

One male patient who was treated twice with PRP injections in the scalp has demonstrated a greater hair density in areas of his scalp which were formerly very thin. In our view, it is necessary for other investigational studies to be conducted in a larger male cohort population, if PRP injections can actually thicken hair density. In summary, patients receiving the greatest benefit of PRP injections had to be treated at least five times. We did view smaller cosmetic benefits

with several more injections. In the second phase of this study, we will comprehend how long these cosmetic benefits will be sustained after six months.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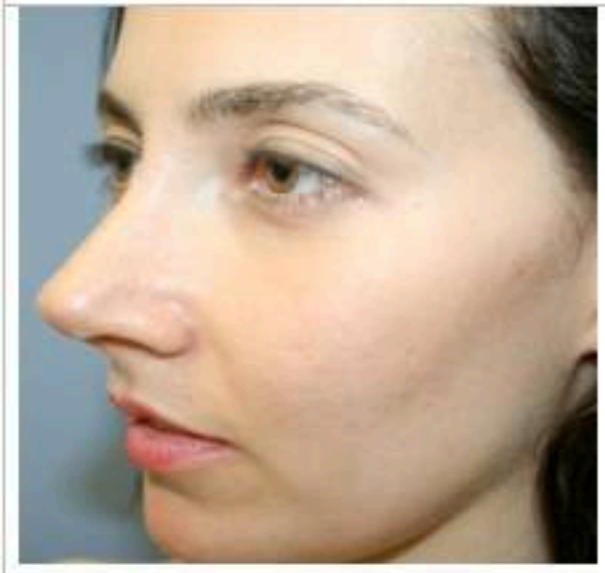	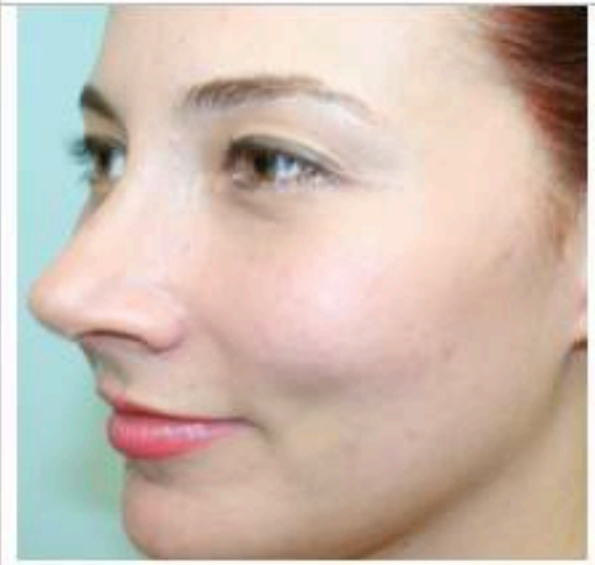
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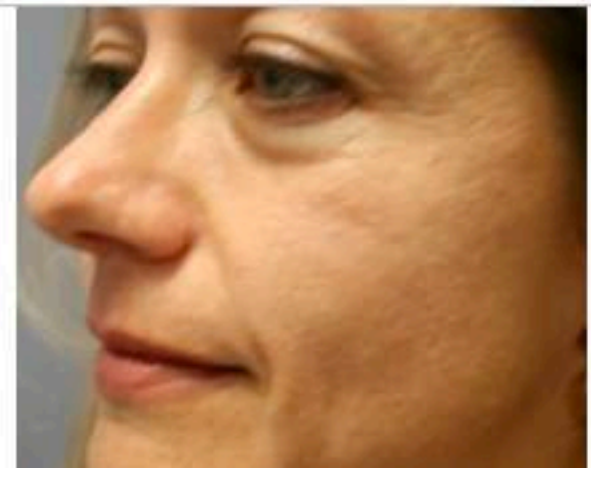	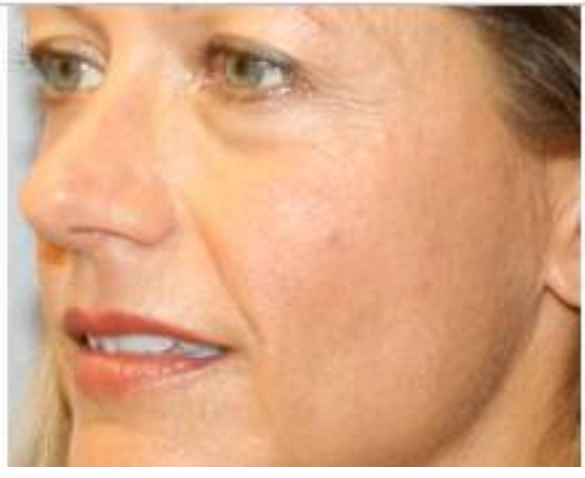

Discussion

Twenty-first century cosmetic medicine will be in the ensuing decades, much more reliant on the body's own endogenous molecular machinery to ameliorate protein expression and overall tissue remodeling. Cytokine matrices will be the molecular modulatory agents to govern cellular transcriptional processes in a manner which would be cosmetically beneficial to the patient. It is likely that highly invasive surgical aesthetic procedures will be replaced to a certain degree by cosmetic molecular science. This current pilot study showed that ten or more of the body's own endogenous growth factors expressed by platelets in higher concentrations can attenuate the aberrant physiological remodeling associated with excessive sun damage and molecular aging.

Cytokines or growth factors expressed by platelets are normally involved with body's wound healing process. PRP rich plasma has at least five times greater concentration of growth factors than would normally occur in circulation. PRP rich concentrate has been shown to impact cellular and molecular processes of soft tissue. For example, Timothy Foster et al. 2009 writes the following concerning the positive effects that growth factors have on gene expression. "Several recent studies have clearly shown that PRP positively affects gene expression and matrix synthesis in tendon and tendon cells. Cell proliferation and total collagen production is increased in human tenocytes…explants cultured in PRP showed enhanced gene expression of type I collagen, types III collagen, and cartilage oligometric matrix protein… Several cytokines contained in PRP had a positive effect on muscle healing. For example, FGF [BFGF] and IGF-1 improved muscle healing in a gastrocnemius muscle laceration model in mice."22 The following growth factors are the prevalent cytokines which are released by higher concentrations of platelets as a result of PRP injections; TGF-B promotes matrix synthesis, TDGF is related to cell proliferation; IGF-1 and IGF-2 induces cellular synthesis as well as anabolic signaling; FGF causes angiogenesis and fibroblast proliferation; EGF is associated with cell proliferation; VEGF is involved with angiogenesis: and ECGF induces cell activation and angiogenesis; and fibronectin engenders cell growth. These are some of the essential growth factors which are secreted by platelets. In general, these cytokines impact many other cellular transcriptional activities, and most importantly, the nature of post-translational protein synthesis. It is likely that higher concentrations of these growth factors likewise impact

how seemingly unrelated proteins are also being translated both intra and extra-cellular. The matrix complexity of all these interactions is far too abstruse to establish a paradigm which explicates these biological processes.

Patrick Abuzei and Robert Alexander in an earlier article on PRP also showed the ameliorative molecular dynamics of platelet-rich plasma as a way to engender positive soft tissue remodeling. These medical researchers have written the following. “It appears that PDGF and TGF-B1 are among the most important growth factors in wound healing…PDGF appears to have a direct mitogenic influence on the target cells by binding to cell surface receptors and by indirectly enhancing the proliferative response of cells lacking detectable TDGF receptors…TGF-B1 is a chemotactic for macrophages and fibroblasts and is well established to be a potent stimulator of granulation tissue formation.”23

Katherine St. Louis’ article in the New York Times, published March 22, 2011, substantiated that there is now tremendous interest in the public and the cosmetic medical community in the use of PRP for facial rejuvenation. However, there are not any published studies aside from inflated antidotal commentary on the supposed efficacy of PRP injections into the face. Anthony Sclafari stills remains the only published investigative study employing PRP as facial filler. This particular research study involved one injection of PRP into the nasolabial fold. Conversely, we have concluded that one injection of PRP into the nasolabial fold would not be very effective in patients over 30 years of age. Furthermore, there are not any viable studies which involve a comprehensive cosmetic prophylaxis of mid and lower facial regions in the older phenotype. This study does indicate that multiple treatment sessions of PRP can have provocative aesthetic remodeling in a cosmetic manner similar to facial fillers, fat grafting strategies, and even fractionated CO2 laser protocols. We have not determined how long these results can be sustained. It is possible that there could be greater improvement over time or after stopping these treatment protocols, as the tissue structures will continue to remodel in a catabolic fashion since they are not being titrated with high concentrations of growth factors to modulate cellular transcriptional processes.

We have found that all patients with multiple PRP treatment session exhibit a more robust and renewed epidermal texture consistent with a younger phenotype. Most subjects showed a pronounced regeneration in facial volume concordant with fat grafting or the use of

fillers. All patients displayed a certain degree of skin tightening. However, we did not observe this dermatological phenomenon when the subject had inordinate skin laxity. We have concluded that multiple PRP injections is most propitious in patients between ages 33-55 who do not exhibit the effects of excessive sun damage or skin laxity.

In our view, our PRP facial prophylaxis or treatment protocols established in this study is an efficacious strategy for inducing overall skin tightening, alleviation of rhytides, the enhancement of facial volume, as well as improving the epidermal texture. We believe for certain phenotypes this cosmetic procedure could be utilized in lieu of a more invasive surgical protocol. We have not determined if PRP cosmetic intervention can be sustained over a year. It has been discussed in the introduction of this study that most of these studies concerning PRP therapy's effectiveness is related to musculoskeletal perturbation. The literature does indicate that these medicinal benefits, in terms of soft tissue healing of tendon or ligament, are long-lasting.

In conclusion, biological modulation of soft tissue will be replacing other more highly invasive cosmetic therapies in the ensuing decades. There needs to be future studies to demonstrate that PRP injections can actually induce hair density in the scalp. It is not known which molecular pathways are activated in terms of collagen genesis from the exogenous PRP injections. In addition, there needs to be 3-D imaging to substantiate the nature of soft tissue remodeling associated with this cosmetic procedure. In our view, there is considerable benefit from multiple treatment sessions of PRP as a facial aesthetic therapy.

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ONLINE FIRST

Induction of Dermal Collagenesis, Angiogenesis, and Adipogenesis in Human Skin by Injection of Platelet-Rich Fibrin Matrix

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Objective: To evaluate the histological changes induced in human skin by injection of autologous platelet-rich fibrin matrix (PRFM).

Methods: Four healthy adult volunteers were included in the study. Platelet-rich fibrin matrix was prepared from 9 mL of autologous blood using a proprietary system (Selphyl; Aesthetic Factors, Wayne, New Jersey) and injected into the deep dermis and immediate subdermis of the upper arms of subjects. Full-thickness skin biopsy specimens were taken from the treated areas over a 10-week period, and the specimens were processed for histological evaluation.

Results: Findings from histological examination supported the clinical observation of soft-tissue augmentation. As early as 7 days after treatment, activated fibroblasts and new collagen deposition were noted and continued to be evident throughout the course of the study. Development of new blood vessels was noted by 19 days; also at this time, intradermal collections of adipocytes and stimulation of subdermal adipocytes were noted. These findings became more pronounced over the duration of the study, although the fibroblastic response became much less pronounced. No abnormal mitotic figures were observed at any point, and a very mild chronic inflammatory response was noted only at the earliest time points of the study.

Conclusions: Injection of PRFM into the deep dermis and subdermis of the skin stimulates a number of cellular changes that can be harnessed for use. Coupled with prior in vitro and in vivo studies, we now have a much clearer picture of the cellular effects of PRFM and its potential uses in facial plastic surgery. Further work is planned to more clearly elucidate the potential role of PRFM in aesthetic and reconstructive surgery.

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SINCE THE TIME OF PARÉ, MODERN surgical care has relied on optimization of local tissue conditions to allow wounds to heal unimpeded. With an improved understanding of the effects of local growth factors, surgeons have begun to manipulate the wound environment to promote more rapid and effective healing. Isolated growth factors have been applied with some success topically (becaplermin for diabetic foot ulcers and palifermin for radiation induced mucositis), but platelet-rich plasma (PRP) has been promoted in the last decade as a more natural and more potent method of manipulating wound healing. However, the process can be time consuming and the results, equivocal.[1,2]

Platelet-rich fibrin matrix (PRFM) has been used successfully to promote healing of venous leg ulcers[3] and in orthopedic surgery[4,5] and has been used clinically in facial plastic surgery since 2009. Several reports[6-10] have described adding PRFM to autologous fat prior to transfer to enhance fat survival. In vitro studies have shown that PRFM can enhance endothelial cell and fibroblast proliferation. One of us (A.P.S.) has reported on the use of PRFM for treatment of deep nasolabial folds (NLFs),[11] as well as in other scenarios in a clinical facial plastic surgery practice.[12,13]

However, the cellular effects of dermal and subdermal injection of PRFM are unclear. In a study evaluating intradermal and subdermal PRFM injection for the treatment of deep NLFs, clinical improvement could be seen as early as 1 week and was statistically significant 2 weeks after treatment. The histological basis for such a rapid clinically apparent change was not immediately evident. This study was de-

signed to evaluate the dermal and subdermal histological changes induced by PRFM.

METHODS

Four healthy adult volunteers were included in the study. Nine-milliliter aliquots of peripheral blood from each subject were placed sterilely into 2 collection tubes (Selphyl; Aesthetic Factors, Wayne, New Jersey). Each tube was placed in a centrifuge for 6 minutes at 1100 rpm. The platelets were then resuspended in the supernatant plasma by gently inverting the tube 10 times, and the resulting mixture was transferred sterilely to a second tube containing a regulated amount of calcium chloride. This was mixed by gentle inversion, and then 0.5 mL of the mixture was injected into 4 distinct points (at least 15 mm apart from each other) intradermally and subdermally in the skin of each upper arm. At specified time points after treatment between 30 minutes and 10 weeks, 5-mm full-thickness skin biopsy specimens were taken from each injection site and the wounds closed with 3-0 chromic sutures.

Specimen were placed in formalin, sectioned, and stained with hematoxylin-eosin (HE) and trichrome stain (for examination of collagen deposition), periodic acid-Schiff stain (for mucopolysaccharide production) and adipophilin stain (for immunohistochemical evaluation of the presence of adipophilin).

This study was approved by the Institutional Review Board for Human Experimentation at The New York Eye and Ear Infirmary.

RESULTS

Clinically, there was little evidence of inflammation around the treatment sites at any time. The tumescence noted immediately after injection yielded to a palpable fullness of the area at the 1-week follow-up visit, which persisted throughout the duration of the study. At early time points, PRFM induced only a minimal to mild inflammatory response in the dermis, which resolved within 1 week.

Histologically, at 1 week, activated fibroblasts, new collagen deposition, and angiogenesis in the mid- to deep dermis was evident and were accompanied by focal areas of mild lymphocytic infiltrates (**Figure 1**). In addition, the fibrous septae between the subdermal fat became thickened and more cellular. Over time, the inflammatory infiltrates resolved, and dermal changes became more prominent and were accompanied by more obvious angiogenesis. By 3 weeks after injection, there were wide areas of neocollagenesis and angiogenesis (**Figure 2**). Also noted in the dermis was the presence of small, clear cells with eccentric nuclei that resembled adipocytes. Immunohistochemical staining for adipophilin confirmed that these new adipocytes were actively packaging lipid globules. In addition, the more superficial fat cells in the subcutis also were positive for adipophilin, also indicating an anabolic state, while mature adipocytes (including those deeper in the subcutis and thus further from the site of treatment) did not stain positively for adipophilin. At 10 weeks, the fibroblast response appeared less active and fairly quiescent, while new collagen and blood vessels were still evident in the dermis (**Figure 3**). Adipocytes in the dermis and the subdermis were also present and were more uniform in size and stained strongly for adipophilin. No areas of dysplasia were noted in any specimen at any time.

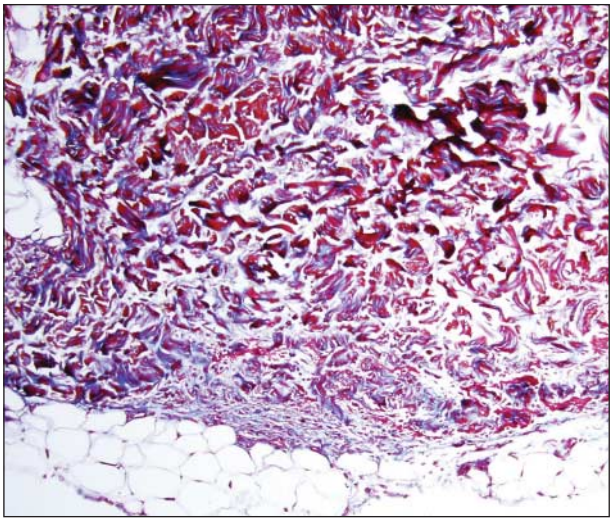

Figure 1. Biopsy at 7 days of skin treated with platelet-rich fibrin matrix. Blue staining is typical of type II collagen (trichrome stain, original magnification ×10).

COMMENT

Platelet preparations have been identified as a useful source of autologous growth factors, and many preparations and devices have been promoted for clinical use. However, these various systems differ greatly in their final product. The most commonly studied isolate is PRP, which can differ greatly between systems, chiefly in platelet concentration, red blood cell contamination, white blood cell content, volume, and method of platelet activation. These differences affect the timing of platelet growth factor release, the fibrin substrate on which healing occurs and the degree of associated inflammation. In a study comparing the effects of PRP to autologous blood clot on fibrovascular tissue ingrowth into porous polyethylene implants,[2] PRP was associated with less neutrophil and macrophage implant infiltration than autologous blood clot at 2 days and significantly more endothelial cells and fibroblasts at 7 days. However, by 14 days and thereafter, no significant cellular differences were noted between autologous blood clot and PRP-treated implants.

Platelet-rich plasma systems can generate a product with an elevated concentration of growth factors up to 80 to 180 times the normal value. However, the optimal concentration of growth factors has not been established; Kakudo et al[14] have described increased adipose-derived stem cell (ADSC) counts and dermal fibroblast proliferation in vitro when grown in the presence of 5% PRP, with growth rates declining in the presence of higher concentrations. Oh et al[15] have shown that transfer of human fat into nude mice underwent less weight and less volume loss over a 10-week period when mixed with activated PRP in a 3.3:1 (fat to PRP) ratio and histologically was more vascularized and demonstrated less vacuolization and fibrosis than fat treated with saline. Clinical

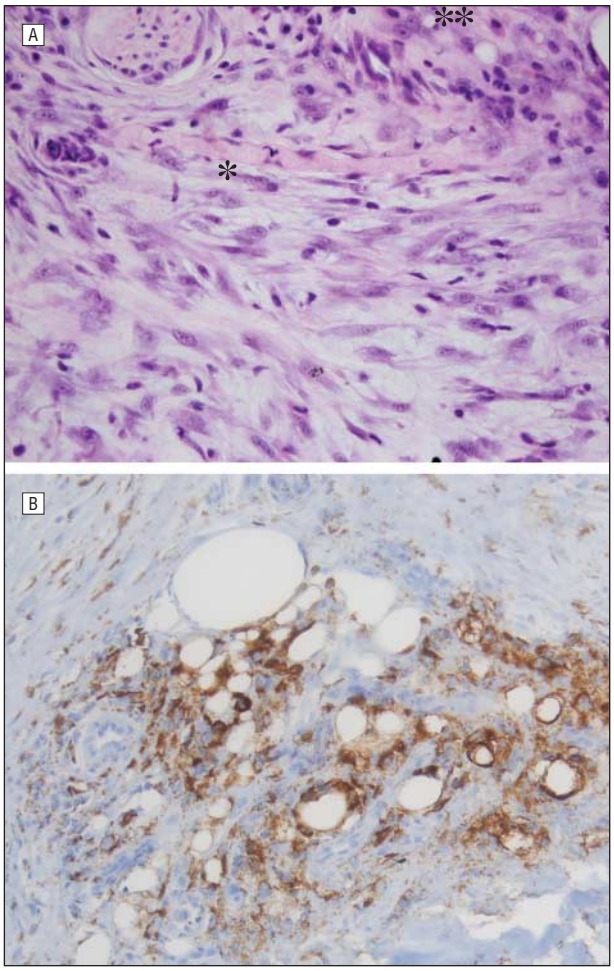

Figure 2. Biopsy at 19 days and immunohistochemical staining. A, Biopsy at 19 days shows reactive fibroblasts, new blood vessel formation (*), and lipid-containing cells (**) (hematoxylin-eosin, original magnification ×40). B, Immunohistochemical staining demonstrates active production of adipophilin, a protein associated with differentiating adipocytes (adipophilin stain, original magnification ×20).

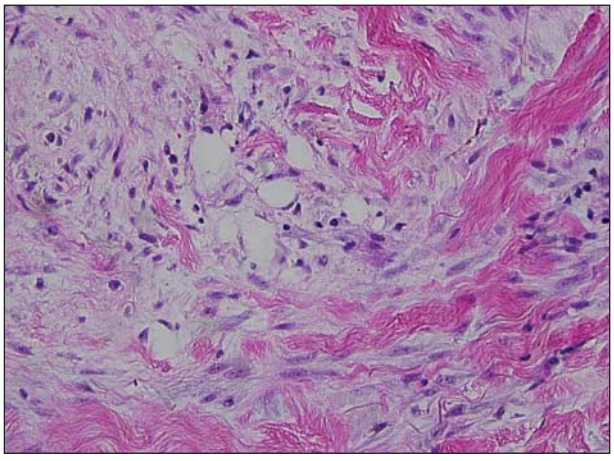

Figure 3. Biopsy at 10 weeks shows less reactive fibroblasts and more mature collagen, blood vessels, and adipocytes in the dermis (hematoxylin-eosin, original magnification ×40).

results with PRP have been equivocal, however, in promoting epithelialization of wounds.[3,16] On the basis of in vitro results, Kakudo et al[14] have suggested that an essential factor overlooked by standard PRP applications is the need to release platelet growth factors at the appropriate concentrations, pointing out that not only is the effect of PRP concentration dependent, but also that transforming growth factor β, while acting as a mitogen for mesodermal derivatives such as fibroblasts, can inhibit cellular proliferation, especially of cells of ectodermal origin.

An alternative solution has been to isolate platelets and use the plasma as more than a carrier vehicle. Platelet-poor plasma (PPP) has been shown to be mitogenic for mesenchymal and adipose derived stem cells and for adult osteocytes, fibroblasts, endothelial cells, and adipocytes. Kakudo et al[14] have shown that both PRP and PPP, when activated with calcium and thrombin, can induce proliferation in vitro of ADSCs and dermal fibroblasts. The effect of activated PPP potentially may be mediated by the effect of polymerized fibrin. Platelet-rich fibrin matrix captures 60% to 90% of the platelets in a sample of whole blood and allows the platelets to be suspended in almost the entire volume of plasma. This suspension thus harnesses the positive effects of PPP as well as those of platelet-released growth factors. The activation of platelets with calcium chloride induces a more physiologic and more sustained growth factor release from platelets.[17]

Anitua et al[17] investigated the effects of application of a plasma rich in growth factors, which is very similar to the PRFM used in our study, with few white blood cells and slightly concentrated platelets in plasma. These workers noted enhanced fibroblast proliferation and secretion of hyaluronic acid from fibroblasts when treated with plasma rich in growth factors and believed the reduction in leukocytes relative to whole blood and PRP reduced the proinflammatory effects of proteases and acid hydrolase released from white blood cells.

Cervelli and coworkers[10] demonstrated that treatment of ADSCs with PRFM increased cellular proliferation at 4, 6, and 8 days but did not increase intracytoplasmic lipid accumulation in these cells.

Platelet-rich fibrin matrix has been used in orthopedic,[18] vascular,[3] and oral and maxillofacial surgery[19] in a variety of applications. In each, PRFM has been shown to induce healing through a process of angiogenesis associated with tissue-appropriate cellular proliferation. Bone[4] as well as soft-tissue regeneration has been induced in both in vitro and in vivo studies. Refractory venous leg ulcers have been successfully treated with topical application of PRFM.[3] Clinical use in facial plastic surgery has also been described.[11-13]

In vitro studies of PRFM have shown a platelet recovery of 60% to 90%, an average platelet lifespan of 7 days after PRFM formation and sustained release of platelet-derived growth factor, vascular endothelial growth factor, insulinlike growth factor 1, and transforming growth factor β for up to 7 days.[20,21] Studies have shown that the media from cultures of PRFM increase proliferation of endothelial cells and fibroblasts as well as mesenchymal stem cells.[22] There have been several clinical reports of mixing PRFM with autologous fat to enhance the success of cosmetic autologous fat transfer as well as to treat chronic venous leg ulcers,[6-10] and Torio-Padron et al[23] and

Schoeller et al[24] have shown enhanced survival of preadipocytes when coinjected with fibrin.

In a prospective clinical trial,[11] PRFM injected into the deep dermis or immediate subdermis produced clinically significant improvement in deep nasolabial folds within 14 days which was sustained throughout the 12 weeks of the study. Since that study, the use of PRFM has been expanded to other indications, including soft-tissue (dermal and subdermal) augmentation for rhytids, folds, depressions, and acne scar effacement and to accelerate wound healing after rhytidectomy, rhinoplasty and autologous fat transfer and around implants.[12,13] The present study was initiated to better elucidate the mechanism of rapid and sustained volume enhancement after injection of PRFM.

Our early findings corresponded well with our clinical observations, with minimal inflammatory cellular reaction and significant fibroblasts activation and collagen production, correlating well with the lack of clinically apparent inflammation in the presence of visible improvement as early as 7 days after treatment. Angiogenesis was also apparent within the first few weeks of treatment, also in agreement with previous in vitro work. However, the presence of lipid-sequestering cells within the dermis was unexpected, and the association of these cells with activated fibroblasts suggested a common etiology. Immunohistochemical staining for adipophilin was highly positive in these cells at 19 days; adipophilin is a protein found very early in differentiating adipocytes, where it binds and packages cytosolic lipid droplets peripherally.[25] It would appear that the adipocytes noted within the dermis represent redifferentiation of existing activated fibroblasts, but less likely, they may be derived from mesenchymal stem cells. In addition, adipocytes in the superficial subcutis also stained positively for adipophilin, indicating an adipose anabolic state.

The process of fibroblast activation and collagen deposition became less prominent after approximately 6 weeks, although adipophilin staining was present throughout the 10 weeks of the study. Despite this, adipogenesis was also much less prominent by the end of the study period, as was angiogenesis. Throughout the course of the study, there was no evidence of granuloma formation, abscess formation, excessive scarring, epidermal hyperplasia or dysplasia.

CONCLUSIONS

In this study we have documented and described the histological changes induced by injections of PRFM into the skin. Platelet-rich fibrin matrix injection leads to development of new blood vessels, activation of fibroblasts with neocollagenesis and adipogenesis within the dermis, and induction of an anabolic state in subcuticular adipocytes. Interestingly, a substantial portion of patients treated clinically with PRFM describe their skin as "softer" after approximately 8 to 12 weeks. It is possible that this description is related to the development of small collections of adipocytes within the dermis. Our findings support the use of PRFM for dermal and subdermal soft-tissue augmentation in conjunction with surgery (with or without implants) and, in particular, as an adjunct to autologous fat transfer. Acceleration of angiogenesis would, in theory, lead to vascularization of a greater portion of the transplanted fat and yield greater fat retention. In addition, positive staining for adipophilin suggests that existing and induced adipocytes were stimulated into an anabolic state. This, in addition to promotion of rapid revascularization, may be a mechanism for enhancement of adipocyte survival after autologous fat grafting. We are currently investigating the optimal ratio of fat to PRFM in promoting this survival. Angiogenesis, collagen deposition, and adipogenesis appear to be the histologic basis of volume augmentation after PRFM injection in the face.

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Author Contributions: Drs Sclafani and McCormick had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Sclafani and McCormick. *Acquisition of data:* McCormick. *Analysis and interpretation of data:* Sclafani and McCormick. *Drafting of the manuscript:* McCormick. *Critical revision of the manuscript for important intellectual content:* Sclafani and McCormick. *Obtained funding:* McCormick. *Administrative, technical, and material support:* Sclafani and McCormick. *Study supervision:* Sclafani and McCormick.
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ORIGINAL ARTICLE

The Healing Effects of Autologous Platelet Gel on Acute Human Skin Wounds

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Objective: To compare the healing of full-thickness skin punch wounds treated with topical autologous platelet gel (APG) vs conventional therapy (antibiotic ointment and/or occlusive dressings) in healthy volunteers.

Methods: A prospective, single-blind, pilot study comprising 80 full-thickness skin punch wounds (4 mm diameter) was conducted on the thighs of 8 healthy volunteers. With each subject serving as his or her own control (5 punch sites per leg), APG was applied topically on one thigh, and an antibiotic ointment and/or a semiocclusive dressing was applied on the other thigh. Healing was monitored for spontaneous wound closure by clinical assessment and by digital photographs over 6 months. Over 35 days, 64 serial dermal biopsy specimens (6 mm diameter) were analyzed (using hematoxylin-eosin, Mason trichrome, CD-34, and Ki-67 stains) to measure differences between treated and control sites for cellularity, granulation formation, vascularity, epithelialization, and cellular replication.

Results: Over a 42-day period, the APG-treated sites had statistically increased wound closure compared with controls by visual clinical assessment and by digital planimetry photographic measurements ($P \leq .02$). On day 17, the percentage of closure was 81.1% ± 2.5% (mean ± SE) for the APG-treated sites and 57.2% ± 5.9% for the control sites. Also, the APG wound closure velocities were significantly faster than those of the controls ($P = .001$). Histologically, over time, the APG-treated sites had similar cellularity, cellular replication, granulation tissue, vascularity, and epithelialization compared with controls. However, when the platelet count in the gel was more than 6 times the baseline intravascular platelet count in some subjects, epithelialization and granulation formation appeared 3 days earlier in the APG-treated group. Furthermore, in vitro testing of supplemental APG showed increased endothelial cell proliferation compared with controls ($P < .04$).

Conclusion: This pilot study provides preliminary evidence that topical APG may hasten wound closure in full-thickness dermal wounds in healthy individuals.

Trial Registration: clinicaltrials.gov Identifier: NCT00199992

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INNOVATIVE DEVICES FOR PROCESSING autologous blood to concentrate platelet-rich plasma (PRP) into autologous platelet gel (APG) have recently become available.[1] Currently, APG is being used clinically in reconstructive, cosmetic, orthopedic, cardiovascular, oral maxillofacial, and dermatologic surgery in an attempt to improve tissue healing.[2-6] It is believed that platelets have concentrated levels of naturally occurring growth factors and other substances that have the potential to accelerate healing (**Table 1**). The use of APG to reduce ecchymosis and edema has received mixed reviews in clinical reports, and its clinical use remains controversial.[6-8]

To investigate whether topical APG can accelerate acute skin healing in healthy individuals, we conducted a prospective study testing APG on full-thickness skin punch wounds in healthy subjects. Full-thickness dermal punch wounds were selected for the acute skin-healing model because the model is minimally invasive, technically straightforward to create, and easily followed up over time.[9,10] It also has minimal discomfort and low potential morbidity for subjects with reduced healing variability, which often limits healing measurements.[11] **Figure 1** schematically shows the healing steps of this full-thickness dermal wound model over time.

METHODS

After institutional review board review and approval, subjects were recruited on a voluntary basis to participate in the study. Volunteers eligible for the study were healthy men and women older than 21 years who were willing to follow instructions and be seen for 13 visits over 6 months. Informed consent, medical history, physical examination, and vital signs were

obtained. In the study, 80 full-thickness skin punch wounds (4 mm diameter) were made on the lateral thighs of 8 healthy volunteers (10 punch wounds per subject). In each subject, APG was applied to one thigh (5 punch sites), while the contralateral side (5 punch sites) served as the control. Therefore, each subject served as his or her own control to control for variables of nutrition, healing response, health status, and tissue oxygen level.

Individuals who were diabetic, were keloid or scar formers, had a collagen vascular disease or a bleeding disorder, or were taking an anticoagulant or a steroid medication over the last month were excluded from the study. Individuals who had an allergy to local anesthetic or bacitracin were also excluded. Women of childbearing potential had to have a negative pregnancy test result within 1 week of the study and were required to use a reliable method of birth control during the study.

PUNCH WOUND PROCEDURE

On day 0, the lateral aspect of the upper part of the thigh was shaved and disinfected with 70% alcohol and allowed to dry. After the administration of local anesthesia with 1% lidocaine, five 4.0-mm–diameter skin punch biopsies (Fray Products Corp, Buffalo, NY) were performed along the upper lateral thigh area (5 cm below the greater trochanter prominence) in a linear alignment (3 cm apart). Wound sites on each leg were labeled A, B, C, D, and E. Site E was the most proximal site on the leg. Hemostasis was obtained with 10 minutes of pressure to avoid cautery. The patients were seen in follow-up on days 1, 7, 10, 14, 17, 21, 24, 28, 31, 35, and 42 and 6 months later. They were instructed regarding proper wound care, eg, how to keep the area clean, while the wounds healed by secondary intention or were closed with suture after biopsy. In phase 1, APG was applied 1 time, on day 0. In phase 2, APG was applied twice, on days 0 and 7 (**Table 2**).

APG PREPARATION

To prepare the APG, two 60-mL aliquots of anticoagulated blood (13% anticoagulant citrate dextrose formula A) were obtained from each subject by venipuncture before the punch biopsies were performed. Each aliquot was processed by an autologous platelet separator (Magellan Autologous Platelet Separator; Medtronic Inc, Minneapolis, Minn) to yield 5 mL of PRP from each aliquot, thereby obtaining a total of 10 mL of PRP from each subject. One milliliter of PRP was used for platelet cell count analysis (Cell Dyn 1700 Hematology Analyzer; Abbott Diagnostics, Abbott Park, Ill) (**Figure 2**). An autologous serum dispenser kit (Magellan Autologous Serum Dispenser Kit; Medtronic Inc) was used to create approximately 1.3 mL of autologous thrombin-rich serum from 2 mL of PRP. The APG was created at the wound site by codispensing the remaining PRP and the thrombin-rich serum using an autologous serum dispenser kit and a 5-cm cannula tip (Magellan 2″ Cannula Tip; Medtronic Inc).

PHASE 1 (GROUPS 1 AND 2)

On day 0, APG was applied topically to the tested skin punch biopsy site, and the control received bacitracin and/or a semiocclusive dressing. Baseline platelet counts were obtained on all of the blood samples before autologous blood processing and after APG preparation.

Group 1

Autologous platelet gel (0.2 mL) followed by white petrolatum (USP) ointment (Topco Associates, Skokie, Ill) was applied topically to each treated site. For the control group, a topical antibiotic (500 U/g of bacitracin zinc ointment [USP]; Walsh Dohmen Southeast, LLC, Birmingham, Ala) was applied to the control wounds. All wounds were subsequently covered with a semiocclusive dressing (Tegaderm Coverlet; Beiersdorf-Jobst Inc, Rutherford College, NC).

Table 1. Substances Released From the α Granules of Platelets During Wound Healing

Platelet-derived growth factor
Basic fibroblast growth factor
Vascular endothelial growth factor
Transforming growth factor β1
Transforming growth factor α
Epidermal growth factor
Thrombospondin
Platelet thromboplastin
Coagulation factors
Serotonin
Histamine
Platelet-activating factor
Hydrolytic enzymes
Endostatin (antiangiogenic)

Group 2

Autologous platelet gel (0.2 mL) followed by semiocclusive dressing was applied topically to each treated site for 7 days. For the control group, the same semiocclusive dressing was applied to the control wounds for 7 days.

PHASE 2 (GROUPS 3 AND 4)

Phase 2 was identical to phase 1; however, in addition, APG (0.2 mL) was applied to each treated punch wound site for a second time on day 7. On days 0 and 7, 120 mL of blood was drawn and centrifuged with the autologous platelet separator to obtain 10 mL of PRP, and 2 mL of the PRP was used to make 1.3 mL of APG in the same manner as in phase 1. The skin biopsies in phase 2 were performed later because a second APG dose was administered on day 7. Group 3 was similar to group 1 and different only in that APG was applied twice. Group 4 was similar to group 2 and only different in that APG was applied twice.

MEASUREMENT OF HEALING PARAMETERS

In phases 1 and 2, the following wound healing parameters were measured and recorded on days 1, 7, 10, 14, 17, 21, 24, 28, 31, 35, and 42:

1. The remaining open wound area was measured at each punch site.
2. The time required for full closure of the dermal punch biopsy wound by secondary intention was determined.
3. In phase 1, on days 7, 10, 14, and 35, a second set of paired full-thickness skin punch biopsy specimens (6 mm diameter) (APG treated vs control) were obtained after a local anesthetic (1% lidocaine) was administered, and the incisions were closed with 3-0 nylon suture (at sites A, B, C, and D on each leg). The 6-mm-diameter skin specimens were placed in 10% buffered formalin for later histologic analysis. In phase 2, on days 10, 14, 17, and 35, a second set of paired full-thickness skin punch biopsy specimens (6 mm diameter) were obtained (at sites A,

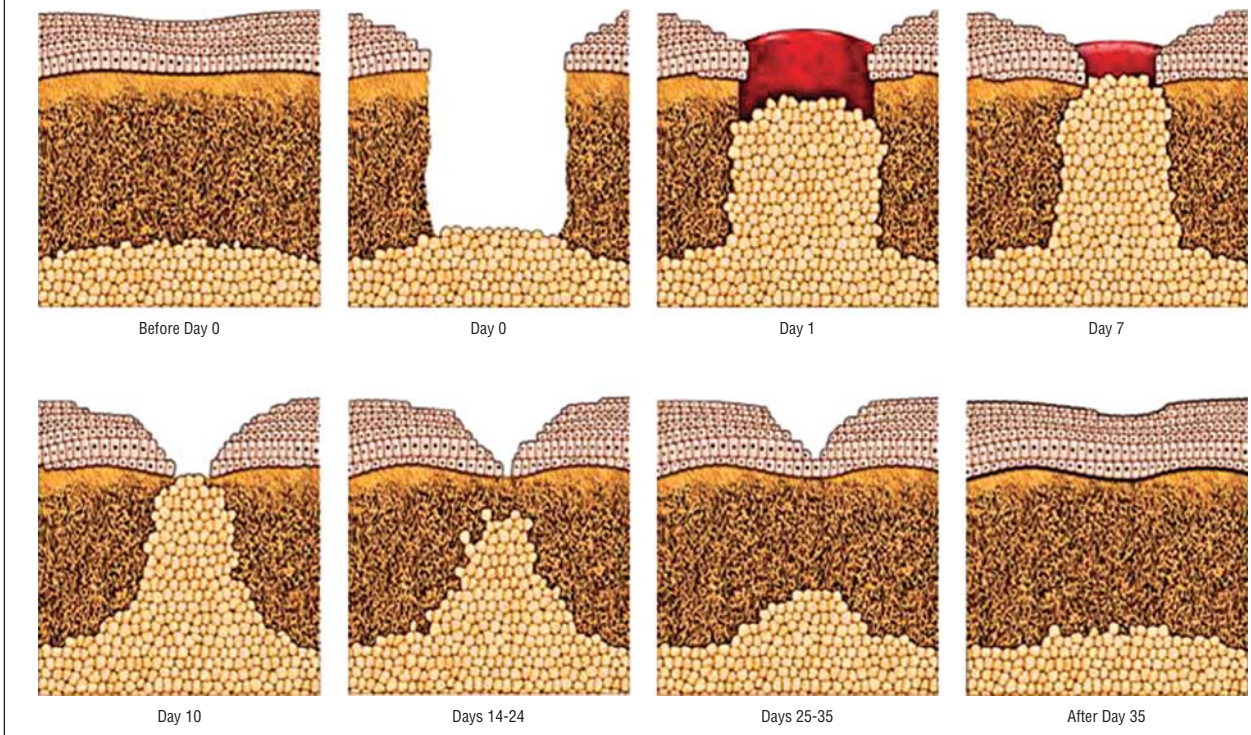

Figure 1. Schematic drawing of the skin wound healing model over time. On day 0, a full-thickness skin punch wound was created, resulting in adipose tissue prolapsing to the skin surface. On days 14 through 24, an epithelial and granulation tissue bridge formed. On days 25 through 35, the wound closed by secondary intention.

Table 2. Study Design of the Groups

Phase 1: APG applied topically as a 1-time dose to a 4-mm–diameter skin punch biopsy site
Group 1: APG (1-time dose) with white petrolatum ointment vs bacitracin ointment
Group 2: APG (1-time dose) with semiocclusive dressing vs semiocclusive dressing alone
Phase 2: APG applied topically as a 2-time dose (second dose given 7 d later) to a 4-mm–diameter skin punch biopsy site
Group 3: APG (2-time dose) with white petrolatum ointment vs bacitracin ointment
Group 4: APG (2-time dose) with semiocclusive dressing vs semiocclusive dressing alone

Abbreviation: APG, autologous platelet gel.

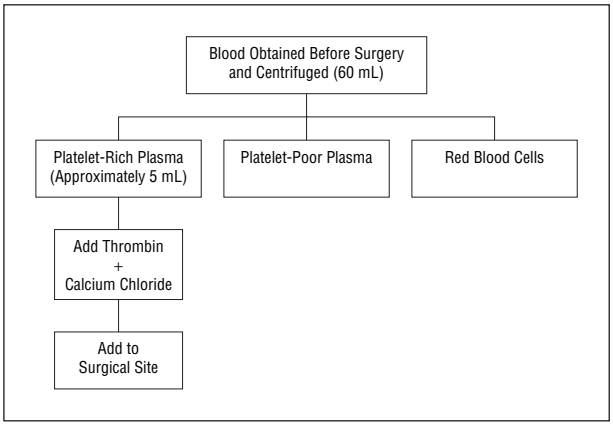

Figure 2. Autologous platelet gel preparation.

B, C, and D on each leg) and then prepared and analyzed by the same methods as in phase 1.

At 6 months, the remaining punch wound sites (site E) that did not undergo biopsy were evaluated clinically to assess for scar size, color, and contour after healing by secondary intention.

TIME REQUIRED FOR COMPLETE WOUND CLOSURE

Clinical assessment was performed and standardized wound photographs were taken with a digital camera (Olympus 3040; Olympus, Melville, NY) with an adapter attachment through an optical microscope (OPMI 1; Carl Zeiss, Jena, Germany) at all sites on days 1, 7, 10, 14, 17, 21, 24, 28, 31, and 35 and after 6 months. The photographs were later evaluated by blinded observers, who determined and recorded the time to achieve full closure. For statistical comparisons between treated and control sites, analysis of variance with repeated measures was used.

HISTOLOGIC MEASUREMENTS

Four serial biopsies (6 mm diameter) on sites A, B, C, and D were performed on each leg (treated and control sites) on days 7, 10, 14, and 35 in phase 1 and on days 10, 14, 17, and 35 in phase 2. The biopsy specimens were fixed in 10% buffered formalin for at least 24 hours. They were then embedded in paraffin and prepared in 4 × 6-µm transverse paraffin sections and

mounted on slides for evaluation of the following parameters under various stains: degree of angiogenesis on CD-34 stain; degree of cellular replication on Ki-67 stain; connective tissue production and turnover on Mason trichrome stain; and epithelialization on hematoxylin-eosin stain.

The extent of angiogenesis and connective tissue present was assessed by the amount of specific stain seen under high (×400) and low (×20) power in representative areas and scored in a blinded fashion by a histopathologist on a scale of 1 though 4: 1, no staining seen; 2, minimal staining seen; 3, moderate staining seen; and 4, excessive staining seen.

APG GROWTH FACTOR PROFILE

To investigate the change in growth factor levels in the preparation of PRP for APG, enzyme-linked immunosorbent assay kits (Quantikine Immunoassay Kit; R&D Systems, Minneapolis, Minn) were used to measure and compare the differences between growth factor concentrations in the initial whole blood samples and the resulting PRP used for the preparation of APG. Briefly, the steps of the technique were as follows: Initial blood samples from 9 different healthy volunteers were taken and centrifuged at 200*g* for 15 minutes to separate out the red blood cells, while the PRP samples were centrifuged at 150*g* for 5 minutes to remove any remaining red blood cells. The clear supernatants containing the platelets were treated with mammalian protein extract reagent (M-PER; Pierce Biotechnology, Rockford, Ill) to lyse all platelets and to cause them to release their growth factors. The resulting suspension was centrifuged at 14 000*g* for 15 minutes to remove cellular debris. The supernatant was used to assay for the growth factor of interest using the 96-well plate provided in the kit with a microtiter plate reader (SpectraMax; Molecular Devices Corp, Sunnyvale, Calif). Tumor growth factor β1 required an activation step before it was assayed in the 96-well plate. Activation of latent tumor growth factor β1 was achieved by the addition of a mixture of 2.5N acetic acid and 10M urea. The reaction was stopped after 10 minutes by reversing the acidified samples with a mixture of 2.7N sodium hydroxide and 1M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid).

EFFECT OF APG ON ENDOTHELIAL CELL REPLICATION IN VITRO

To determine the effect of APG on endothelial cell replication, a cell proliferation assay (CellTiter 96; Promega Corp, Madison, Wis) was performed using human microvascular endothelial cells derived from the dermis. The number of cells was counted at 24, 48, 72, and 96 hours. The APG group was compared with controls consisting of basal medium, basal medium with serum growth factors, basal medium with platelet-free plasma gel, and basal medium with thrombin.

The steps of the technique were as follows: Thrombin was added to PRP in 3 wells of a 48-well plate to form APG in the APG-treated group. Similarly, thrombin was added to platelet-free plasma to form platelet-free plasma gel. The human microvascular endothelial cells derived from the dermis were trypsinized, resuspended in microvascular endothelial cell medium 2 (Clonetics EGM-2MV; Cambrex BioScience, Walkersville, Md), and then counted using a trypan blue exclusion. A total of 3750 cells were added to each well and incubated at 37°C and 5% carbon dioxide for 4 hours to facilitate adhesion of the cells to the culture plate. For each condition, 3 replicate wells were used. After the incubation period, the EGM-2MV was removed from all the wells and the wells were rinsed twice with Hanks balanced salt solution. Basal medium 2 (EBM-2; Cambrex BioScience) was added to all the rinsed wells except for the group that received basal medium with serum growth factors (EGM-2MV). The plate was placed in an incubator at 37°C and 5% carbon dioxide until the number of cells was to be counted.

Analysis of variance with repeated measures was used to determine whether there were statistical differences between treatments at each time point. *P* values between specific treatment pairs were calculated using *t* tests.

RESULTS

The study included 4 men and 4 women (age range, 21-58 years). Over the 6-month course of the study, there were no dropouts and every subject went to every follow-up visit (12 follow-up visits per subject, for a total of 96 follow-up visits for all subjects). Among the 8 subjects, who had 80 dermal punch wounds, no infections were evident. The patients tolerated the APG treatment well, with no serious adverse events. On day 0, 1 patient had persistent oozing on 1 control skin punch site and required brief electrical cauterization for hemostasis. Sixty-four serial skin biopsy specimens were obtained serially from the 80 full-thickness punch wounds for histologic analysis, and the remaining sites were monitored for spontaneous wound closure by secondary intention (site E). The advantages of this skin punch model were that each subject served as his or her own control and the sites were easily accessible for measurements and photographic analysis over 6 months.

WOUND CLOSURE MEASUREMENTS OVER TIME

According to visual clinical measurements of wound closure over a 42-day period, the APG-treated sites had statistically increased wound closure compared with the control sites ($P<.001$, using analysis of variance with repeated measures) (**Figure 3**). On day 7, the percentage of closure was 14.0% ± 1.1% (mean ± SE) for the APG-treated sites and 7.0% ± 1.1% for the control sites. On day 14, the percentage of closure was 73.9% ± 2.9% for the APG-treated sites and 49.6% ± 3.6% for the control sites. On day 17, the percentage of closure was 81.1% ± 2.5% for the APG-treated sites and 57.2% ± 5.9% for the control sites by simply pooling data from all available sites at each visit (**Figure 4**).

According to digital planimetry photographic measurements, the APG-treated sites had significantly increased wound closure compared with control sites over a 42-day period ($P=.02$, analysis of variance with repeated measures). In matching the visual clinical wound closure assessments with the digital photographic planimetry measurements, a strong correlation of these measurements was evident (correlation coefficient ≥.90) (**Figure 5**).

TIME REQUIRED FOR COMPLETE WOUND CLOSURE

Regarding the specific time required to achieve complete wound closure, the APG-treated wounds had a tendency to have a higher percentage of complete closure compared with the control wounds on days 24 and 28 by clinical assessment and by photographic digital

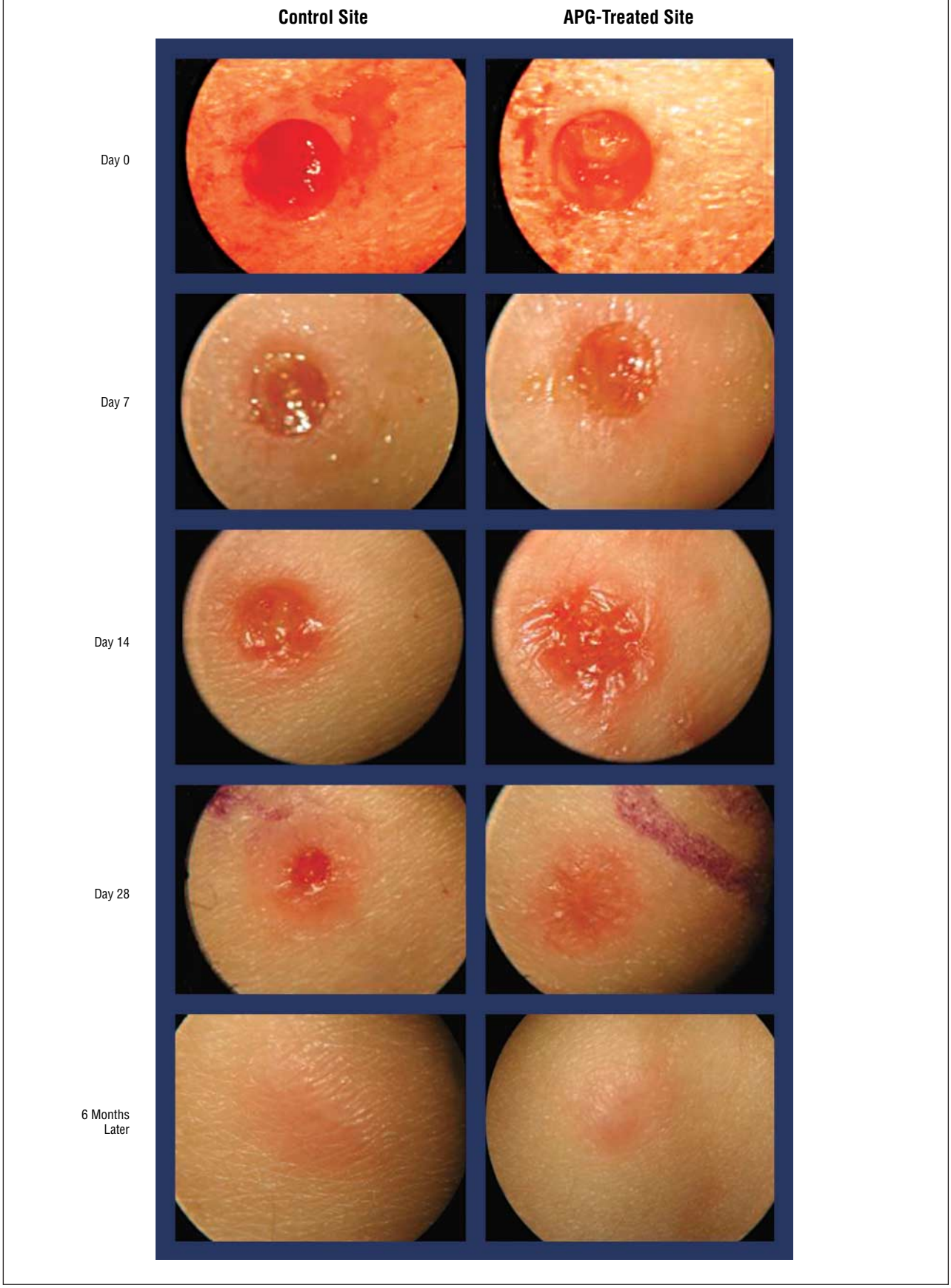

Figure 3. Clinical photographs of a representative wound site allowed to heal by secondary intention over time, showing the differences between the control site and the autologous platelet gel (APG)-treated site (site E) in subject 1 on days 0, 7, 14, and 28 and after 6 months.

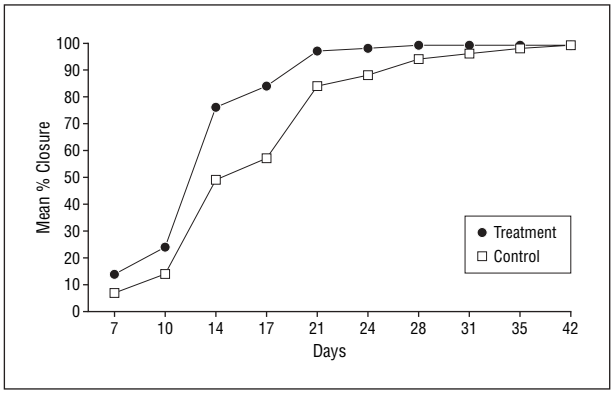

Figure 4. Clinical assessment of wound closure over time (sites D and E). The autologous platelet gel (APG)-treated sites had increased wound closure compared with the control sites over a 42-day period ($P<.001$). Specifically, on day 14, the percentage of closure for the APG-treated sites was 80% compared with 50% for the control sites.

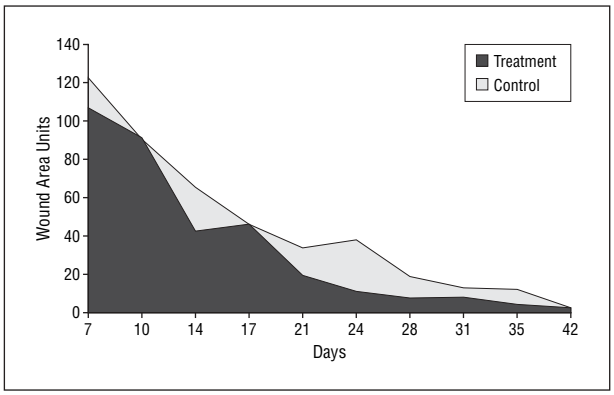

Figure 5. Total open wound surface areas of autologous platelet gel (APG)-treated sites vs control sites measured by digital planimetry over time. The APG-treated sites had a steeper slope (closure rate) between days 10 and 14.

measurements. Specifically, on day 21, 10 (63%) of the 16 APG-treated sites had full closure compared with 5 (31%) of the 16 control sites ($P=.13$, χ^2 test). On day 24, 13 (81%) of the APG-treated sites had full closure compared with 7 (44%) of the control sites ($P=.07$, χ^2 test). On day 28, 14 (88%) of the APG-treated sites had full closure compared with 9 (56%) of the control sites ($P=.06$, χ^2 test). However, because of the small sample size of each subgroup on each day, the *P* values were not significant (**Figure 6**). Among the punch wound sites that did not undergo biopsy (site E), the average time for the APG-treated wounds to achieve 100% closure was 29.75 days compared with 35.38 days for the control wounds.

HEALING CLOSURE VELOCITY

To quantify the wound healing closure velocity from day 0 to day 35 from the planimetry measurements, a biostatistician used a quantitative model that was based on an earlier proposed wound healing trajectory model for skin wounds. The wound healing trajectory approximately follows an exponential course.[12] Thus, the wound healing closure velocity from day 0 to day 35 from the planimetry measurements was modeled with an exponential decay formula with a single time constant as follows: $f(t)=A^{*}exp(-\lambda^{*}t)$, where *A* is the initial open wound area, *exp* is the exponential function, λ is the rate of wound closure correlating with the slope of the curve, and *t* is the time in days. This formula correlated well with the closure rates seen for the control and treated sites over time. It incorporated all open wound area data points for a patient-treatment combination. **Figure 7** shows an example of a healing trajectory curve drawn from the exponential decay formula from all data points of the open wound area in 1 subject. Using this formula, the calculated healing curve closely matched the wound closure rate.

The APG-treated sites appeared to heal faster than the control sites (with the APG-treated sites showing a steeper

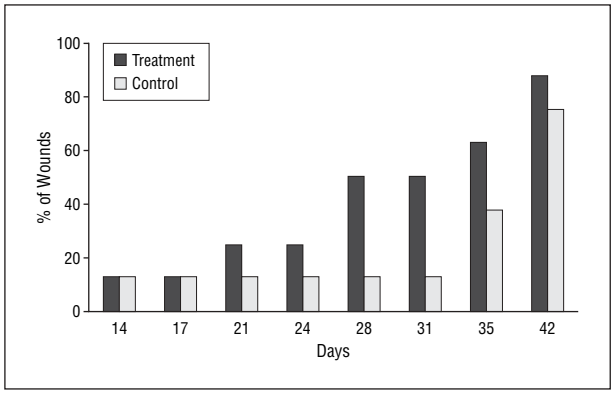

Figure 6. Percentage of wounds that reached full closure according to planimetry measurements (by days).

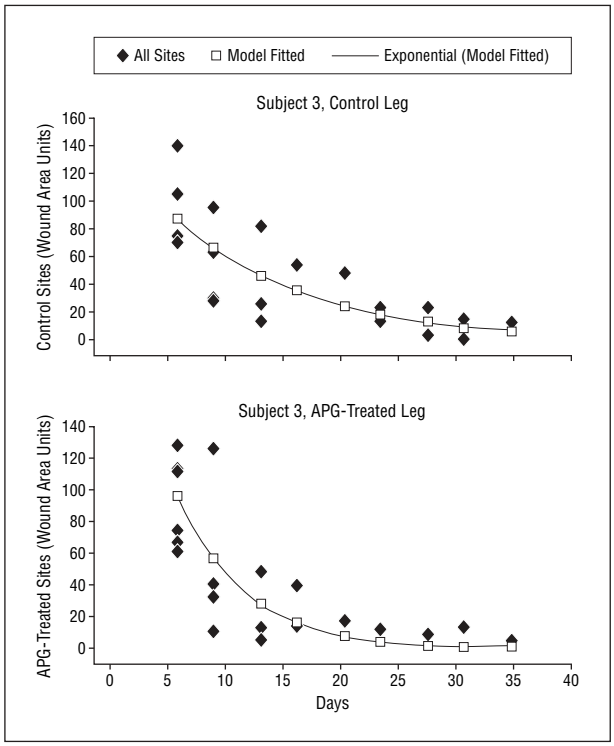

Figure 7. Wound healing trajectory depicted by the wound closure velocity in subject 3. The top graph (control wounds) shows the control data points of the open wound area over time (diamonds). The curved line with squares depicts the calculated wound healing closure velocity. The bottom graph (autologous platelet gel [APG]-treated wounds) shows the APG open wound area sites over time (diamonds) in the same subject, with the calculated wound healing closure velocity curve (squares).

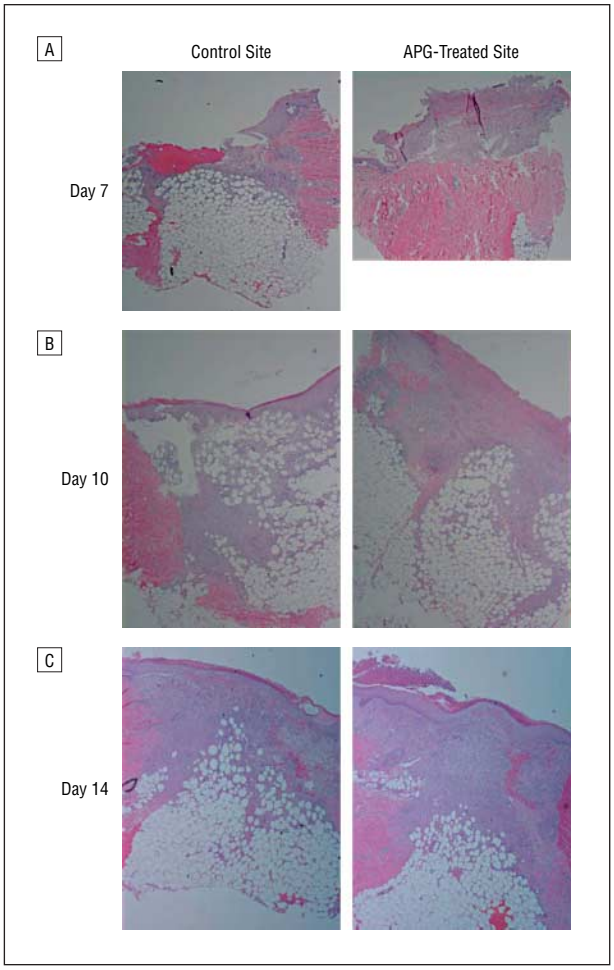

Figure 8. Subject 3. Clot formation and epithelial encroachment over the wound on day 7 (A), granulation tissue bridge formation over the wound on day 10 (B), and formation of a thicker granulation tissue bridge over the autologous platelet gel (APG)-treated wound on day 14 (C) (hematoxylin-eosin, original magnification ×20).

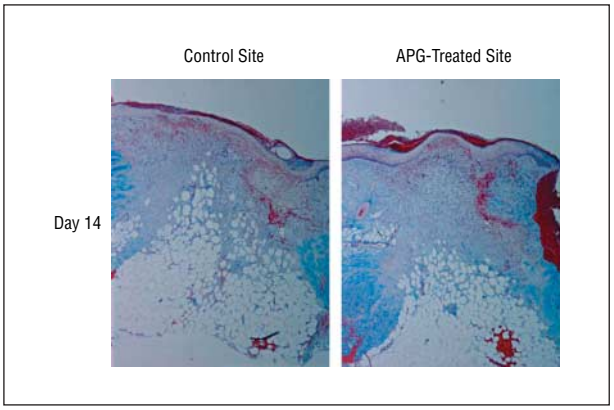

Figure 9. Subject 3. A slide similar to that seen in Figure 8C shows mature (dark blue) and immature (light blue) collagen in granulation tissue bridge forming over the wound on day 14 (Masson trichrome, original magnification ×20).

Table 3. Growth Factor Assays (ELISA)*

Growth Factors	Initial Blood Sample (60 mL)	PRP (6 mL)
PDGF-AB, ng/mL	10.2 ± 1.4	88.4 ± 28.8
PDGF-AA, ng/mL	2.7 ± 0.5	22.2 ± 4.2
PDGF-BB, ng/mL	5.8 ± 1.4	57.8 ± 36.6
TGF-β1, ng/mL	41.8 ± 9.5	231.6 ± 49.1
VEGF, pg/mL	83.1 ± 65.5	597.4 ± 431.4
bFGF, pg/mL	10.7 ± 2.9	48.4 ± 25.0
EGF, pg/mL	12.9 ± 6.2	163.3 ± 49.4

Abbreviations: APG, autologous platelet gel; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; PDGF, platelet-derived growth factor; PRP, platelet-rich plasma; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor.

*Values are given as mean ± SD (adapted with permission from Medtronic Inc, Minneapolis, Minn). Blood samples were obtained from 9 healthy subjects (different volunteers from outside the wound punch study); APG was made by combining PRP with thrombin.

healing slope, with a qualitative shift of the curve to the left, compared with the control sites). This faster healing curve was most pronounced before day 14. In looking at the overall healing velocity and incorporating all time points by comparing the decay parameters obtained from the exponential formula between the APG-treated sites and the control sites, the treated sites had a statistically significant faster healing velocity than the control sites (paired *t* test, $P = .001$). Thus, the rate of wound closure was especially faster in the APG-treated sites compared with the control sites within the first 14 days.

HISTOLOGIC FINDINGS

Histologically, some early qualitative trends were seen in the dermal biopsy specimens when the platelet count in the gel concentrate was more than 6 times the baseline intravascular platelet count. In some subjects who had a more than 6-fold increase in platelet count in the gel concentrate, the APG-treated site showed more epithelialization over the adipose tissue on day 7 than its matched-paired control site (**Figure 8**A). By day 10, more granulation tissue was also present earlier in the APG-treated sites than in the control sites (Figure 8B). On day 14, a thicker granulation tissue bridge was present in some of the APG-treated sites (Figure 8C and **Figure 9**). On day 17, both control and APG-treated sites were very similar in the amount of epithelialization and granulation tissue that was present. In evaluating cellular replication by Ki-67 staining and new vessel growth by CD-34 staining at the peripheral edge of the wound on day 17, the treated and control groups were found to be similar histologically.

APG GROWTH FACTOR PROFILE ANALYSIS

The enzyme-linked immunosorbent assay was used to measure growth factor concentrations in initial blood and PRP samples from 8 different healthy volunteers. **Table 3** shows a 4-fold increase in basic fibroblast growth factor (FGF) levels to a 12-fold increase in epidermal growth factor levels after PRP preparation. Thrombin and PRP are combined to make the APG.

It was noted that the concentrations of any given growth factor in the platelets in the initial blood samples varied from

donor to donor. Also, the relative concentrations of the various growth factors (growth factor profile) within a donor varied across the donor population. On average, it was observed that the growth factor concentrations in the samples increased as the number of platelets increased, although variability was high in some cases. Thus, irrespective of the growth factor concentrations in the initial blood sample from a given donor, there was at least a 4-fold increase in the growth factor concentration in the APG that was prepared using the PRP obtained.

EFFECT OF APG ON ENDOTHELIAL CELL REPLICATION IN VITRO

Using the endothelial cell proliferation assay on human microvascular endothelial cells derived from the dermis, a statistically significant increase in endothelial cells cultured in the presence of APG was seen at 48, 72, and 96 hours later in comparison to their respective controls of basal medium, basal medium with serum growth factors, basal medium with platelet-free plasma gel, and basal medium with thrombin ($P = .05$ at 24 hours; $P = .04$ at 48 hours; $P = .03$ at 72 hours; and $P = .01$ at 96 hours) (**Figure 10**).

The basal medium served as a negative control, while the basal medium with serum growth factors was the positive control based on well-established cell culture techniques. In the case of the negative control, it was observed that cells initially proliferated, probably because of their exposure to serum growth factors before the experiment (cells were not serum starved before the experiment). However, over 72 and 96 hours, the basal medium alone was not sufficient to sustain the cells, and their number decreased. In contrast, the addition of serum growth factors in the positive control was sufficient to maintain the cell count over the entire 96-hour period. Because the APG provides a 3-dimensional matrix into which the endothelial cells can potentially proliferate, the platelet-free plasma gel was used to assess the 3-dimensional effect of the gel and the contribution of the free plasma levels of growth factors. Therefore, the difference between the cell counts in the presence of APG and the counts in the presence of platelet-free plasma gel could be attributed to the growth factors provided by the activated platelets in the APG. The basal medium with thrombin group showed that the addition of thrombin alone did not have a positive effect on endothelial cell proliferation.

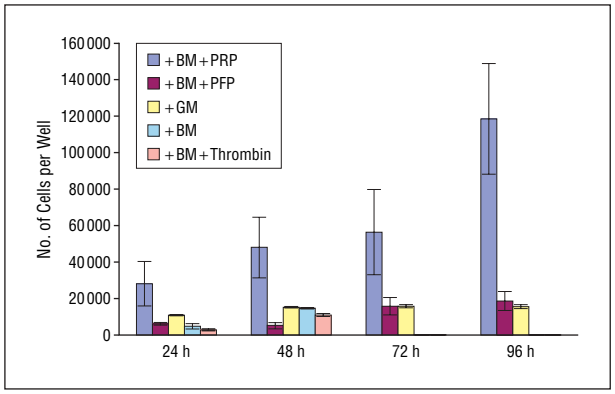

Figure 10. Endothelial cell proliferation assay on human microvascular endothelial cells derived from the dermis at 24, 48, 72, and 96 hours (adapted with permission from Medtronic Inc, Minneapolis, Minn). Autologous platelet gel (APG) was compared with controls consisting of basal medium without serum growth factors (BM), BM with serum growth factors (GM), BM with platelet-free plasma (PFP), and basal medium with thrombin; APG was made by combining platelet-rich plasma (PRP) and thrombin; and PFP was made by combining PFP and thrombin. At 48, 72, and 96 hours, the differences were statistically significant ($P = .05$ at 24 hours; $P = .04$ at 48 hours; $P = .03$ at 72 hours; and $P = .01$ at 96 hours). Error bars represent SD.

COMMENT

The purpose of this pilot study was to compare the healing differences of full-thickness skin punch wounds with topical APG and conventional therapy in healthy volunteers. We found that APG-treated sites showed improved healing over a 42-day period compared with conventional therapy. Specifically, by visual clinical measurements over 42 days, APG-treated wounds demonstrated higher wound closure rates than control wounds. These clinical findings strongly correlated with the separate blinded digital planimetry photographic measurements. Also, the wound healing closure velocity within 14 days of wounding was faster in the APG-treated sites than in the control sites.

When the platelet count in the gel concentrate was more than 6 times the baseline intravascular platelet count in a subgroup of subjects, APG appeared to have a greater histologic effect by accelerating epithelialization and granulation tissue formation. This histologic finding may be because a higher concentration of growth factors was more available to influence healing from the higher number of platelets delivered. Histologically, it appears that APG had its greatest effect on healing early on (<14 days after wounding), and by 17 days, the control wound caught up in the histologic analysis. This finding correlates with a recent study on a rodent model showing that the effect of platelet gel on healing appears to occur in a transient fashion within 14 days of administration.[13] Our preliminary findings indicate that APG has the potential to improve healing in acute full-thickness dermal wounds in healthy subjects. These findings are consistent with earlier reports suggesting that platelet releasate may actively promote acute dermal wound healing.[2,14]

What are the possible mechanisms of enhanced healing of APG in full-thickness skin punch wounds? One explanation is that both granulation tissue formation and epithelialization were enhanced by APG. This enhancement was histologically observed in some subjects when the platelet levels in the gel concentrate were more than 6-fold higher than the intravascular platelet levels. Another explanation is that wound contraction was enhanced by APG, allowing faster wound closure. Previous studies have reported that platelet-derived growth factor and transforming growth factor β1 (both of which are found in APG) can transform fibroblasts into myofibroblasts and cause myofibroblasts to contract.[15,16] Also, wound contraction could be enhanced by calcium, which is added during the platelet gel preparation. Further investigations are required to determine whether these mechanisms play a role in improved healing.

COULD APG BE USEFUL IN ACUTE SURGICAL WOUNDS?

The long-standing goal in surgery is to achieve optimal wound healing after surgery. Through the ages, it has been generally assumed that normal acute wound healing is the best outcome that can be expected. However, is it possible to make normal wounds heal faster? We realize that attempting to improve the normal healing of healthy subjects remains controversial. One point of view may ask, "If a normal wound is going to heal anyway, what is the point of accelerating it?" There are at least 2 possible answers to this question:

1. "If it is possible to hasten normal wound repair, it should improve quality of life in surgical patients during their postoperative recovery." In fact, a previous study has suggested that acute wound healing can be enhanced by growth factor therapy. In a clinical study by Cohen and Eaglstein,[11] topical application of a single isolated growth factor, recombinant human platelet-derived growth factor (becaplermin), was shown to enhance the closure of acute dermal wounds in healthy subjects.Therefore, using growth factors to make normal wounds heal faster is an interesting possibility and could change the way we manage normal surgical wounds.
2. "It may be theoretically possible to prevent problem wounds from developing in postoperative patients by the administration of APG during surgery to those who are prone to poor healing." This prophylactic use of APG would be a scenario similar to administering prophylactic antibiotics in the head and neck region in a clean-contaminated surgical case to reduce the risk of postoperative wound complications from infection.[17] The prophylactic use of growth factors to prevent tissue injury has shown some promise in both animal and human studies. In the FGF family, basic FGF (FGF-2) was shown to reduce radiation injury and to reduce adverse postsurgical healing in irradiated skin in the porcine model.[18] In humans, keratinocyte growth factor 2 (palifermin) is a recent FDA-approved product that is used to reduce the severity and duration of oral mucositis in patients who receive chemoradiation therapy for hematologic cancers.[19] Thus, a prophylactic approach using growth factors to prevent wound complications is an intriguing possibility that could alter how we manage compromised surgical wounds in the future.

COULD APG BE EFFECTIVE IN CHRONIC WOUNDS?

If specific growth factors are deficient or dysfunctional in chronic wounds, could adding APG improve chronic wound healing? The following characteristics make a chronic wound different from an acute healing wound: chronic wounds are thought to have increased proteases, increased proinflammatory cytokines, decreased protease inhibitors, and decreased growth factors.[20] Also, FGFs and transforming growth factor β concentrations are down-regulated in chronic wounds and are significantly lower than those in acute wounds.[18,21] By adding APG as a growth factor source to the wound, the increased protease activity within the chronic wound would have to be counteracted to prevent the breakdown of the growth factor proteins contained in APG.

Over the last 15 years, clinical trials have studied the topical effects of exogenous recombinant growth factors on chronic extremity skin wounds due to diabetes and vascular insufficiency. Results of these clinical studies have been both promising and disappointing. Several studies have shown promise in using platelet releasate to improve the healing of diabetic neuropathic foot ulcers.[22] It is important to emphasize that wound healing is dependent not only on the growth factor environment, but also on nutrition, status of infection, wound care, and oxygen level of the tissues.[23,24]

Chronic nonhealing wounds are the result of multiple causes (eg, diabetes, radiation exposure, ischemia, persistent infection, and neoplasia), with each chronic condition having its own pathologic process. Thus, the treatment of each chronic condition should be tailored to achieve optimal therapy, eg, surgical debridement, moist dressings, control of infection, and proper nutrition. To improve the healing of chronic diabetic neurotrophic foot ulcers, the application of a single growth factor, platelet-derived growth factor, was most effective when used in conjunction with good wound care.[25] Therefore, growth factor therapy should be considered an adjunctive means of therapy and not a replacement for standard wound care.

LIMITATIONS

Several limitations of our study should be noted. This was a pilot study, and the APG application and initial clinical measurements were performed in a single-blinded fashion. However, the photographic planimetry data and histologic measurements were determined in a blinded fashion. Even so, the initial wound clinical closure measurements statistically correlated strongly with the blinded photographic planimetry data.

Another limitation of our study is that the platelet count in the gel concentrate varied among the subjects. When the platelet count in the APG was more than 6 times the subject's baseline platelet count, the beneficial effects of APG became more histologically apparent and consistent (5 of 8 subjects had platelet gel concentrates greater than 6 times the baseline intravascular platelet levels). Therefore, in the future, if consistently elevated levels of platelets greater than 6 times the baseline platelet count levels can be achieved by APG processing, more beneficial results in wound healing may be seen.

One concern with supplemental growth factors is that they could increase the risk of malignancy or excessive scarring. However, to our knowledge, no studies to date have reported any evidence of malignant transformation using supplemental growth factors when there is no preexisting genetic mutation. Also, we know of no clinical study in which growth factor therapy has resulted in an increased propensity to keloid or hypertrophic scar formation. In previous studies, the effects of supplemental growth factors seem to occur in a transient manner on their target cells.[20]

CONCLUSIONS

By increasing our understanding of APG on acute full-thickness skin wounds, new growth factor interventions may improve soft tissue healing in surgical patients. If APG treatment accelerates acute dermal healing, it could prevent and reduce some postsurgical wound healing complications commonly seen in patients who are susceptible to poor healing.

In this pilot study, APG appeared to enhance wound closure in acute full-thickness dermal wounds in healthy subjects. Furthermore, the wound closure velocity of APG-treated wounds was greater than that of control wounds. It appears that when the platelet count in the gel is more than 6 times the baseline intravascular platelet count, the effects of the gel are more pronounced histologically. Further investigations are needed to confirm the consistency of these results. If further studies support these findings, APG treatment during surgery could have a useful impact on the enhancement of postoperative dermal wound healing in surgical patients.

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