- Have a correct handling of all the procedures for selecting the best indications.
- Do not use any techniques that are unscientific, unreliable, or inadequately established.
- Be well aware of complications, the better to avoid or correct them.
- Examine the patient every year to control the evolution of the alopecia process and to determine if any additional refinements are required.

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16 Follicular cell implantation: Research update on "hair cloning"

Jerry E. Cooley

Ten to fifteen years ago, most hair restoration physicians believed that "hair cloning," or cultured follicular cell implantation, would have become a practical reality by now. Several decades of basic animal research established the seeming inevitability that cell therapy would become a successful treatment for androgenetic alopecia, with the attendant hope for unlimited hair. Yet, the current situation suggests that cell-based treatments are no closer to successful realization than when the author last reviewed this topic 10 years ago.[1] The present review will cover published research over the past 10 years. The basic rationale and conceptual framework for using cell therapy to treat hair loss has been discussed in prior reviews.[2–4]

Although clinical trials are underway using cultured follicular cells, no published results in humans are available at the time of this writing. Furthermore, because of the inherent commercial potential of research findings, it can be assumed that much more is known than is publicly available. While knowledge of the cellular and molecular mechanisms underpinning hair growth has progressed substantially over the past 10 years, it appears that this has not yet translated into a clinically useful method to treat hair loss in humans using cell therapy.

The basis for cell therapy began in the fundamental research of normal mammalian hair growth. It has been established that hair growth occurs because of the dynamic interaction between epidermal and mesenchymal cell populations within the hair bulb. Using the rat whisker as a model, Oliver showed that the spherical aggregate of mesenchymal cells in the bulb known as the dermal papilla (DP) could induce new follicles when removed from the whisker and subsequently implanted into skin which normally lacks follicles.[5] Carrying this research further, Jahoda showed that rat whisker dermal papilla that had been cultured in vitro could induce new hair growth when implanted into incisions in the rat ear.[6] This research, published in the mid-1980s, initiated the race to develop cell-based treatments for hair loss. In the ensuing years, several commercial ventures were launched based on the work of Oliver and Jahoda.

Over the past 10 years, we have seen two major commercial ventures fail which were based on using implanted cultured autologous DP in humans. While some increased hair growth was claimed, it was largely agreed that simply implanting cultured DP in humans did not result in clinically meaningful hair induction (i.e., no follicular neogenesis occurred). What has become increasingly clear is that methods that routinely induce new hair follicles in mice and rats are largely unsuccessful in humans.

A third commercial venture is based on using not cultured dermal papilla, but the adjacent mesenchymal dermal sheath "cup" cells (DSC). This is based on the research of McElwee, who showed that implantation of cultured DSC cells from mouse whiskers could be injected into the ear, resulting in "colonization" of the dermal papilla of ear hair follicles with whisker cells programmed to produce thicker hair.[7] Early reports from clinical trials using cultured DSC in humans suggest it is safe and may produce some hair thickening. Rather than causing true follicular neogenesis, it appears that implantation of DSC cells may simply lead to the thickening of existing hair.

It is important to note here that no published reports exist of reproducible de novo hair follicle induction in humans (i.e., the formation of entirely new hair follicles in alopecic skin). What the current commercial ventures mentioned above do appear to show is that implantation of cultured follicular cells may result in clinically apparent thickening of native hair follicles. Whether these implanted cells incorporate into the structure of the native follicle and survive cycling or whether they merely secrete stimulatory factors that transiently promote thicker hair is an important distinction. If it is the former, then such a treatment may represent a true breakthrough in alopecia treatment with long-lasting or permanent effects. If it is the latter, then the expense of cell-based treatment may not justify the temporary benefi s. Furthermore, the increasing popularity of injecting platelet-rich plasma (PRP) for hair loss,[8,9] as well as an injectable treatment under development containing growth factors secreted by cultured neonatal fibroblasts,[10] may produce similar gains at a fraction of the expense, without the burdensome regulatory requirements of cell therapy.

ANIMAL RESEARCH AND STUDY MODELS

These commercial ventures have their origin in basic research conducted on rats or mice. The seminal studies by Oliver and Jahoda were based on manipulating dissected rat vibrissa (whisker) which later proved cumbersome and limiting as research methods became more advanced. Over the years, more refined models have been developed which have allowed researchers to study the intricate inner workings of follicular neogenesis. Interestingly, the follicular neogenesis models that have been developed to study hair growth are now being retooled for cell therapy,

with the goal of creating hair follicles in vitro first and then implanting these into areas of alopecia.

For many years, the silicon chamber model, pioneered by Ulrich and Yuspa[11] was the dominant model used by researchers in this area. Dermal and epidermal cells, either fresh or cultured, could be combined in full-thickness wounds on the backs of immunodeficient mice and covered with a bell-shaped silicon chamber that confines and protects the cells; after 1 week, the chamber is removed and new hair growth is apparent within 3 weeks. There are numerous variations on this technique, all of which can consistently and successfully produce hair growth via follicular neogenesis (Figure 16.1).

Qiao created a "flap graft" model that dispensed with the need for the chamber, allowing implantation of trichogenic dermal and epidermal cells below a flap of host skin.[12] This model still required the implantation, and subsequent removal, of a silicone sheet. Stenn developed a "patch assay" that consisted of injecting dermal and epidermal cells subcutaneously into immunodeficient mice. The resulting "hair ball" consists of hair bulbs located centrally with hair growth occurring radially outward.[13]

Almost all in vitro models have relied on mouse, usually embryonic, as the source of cells. It has been noted by researchers that successful hair follicle induction using mouse or rat cannot necessarily be translated into success using human cells. Although Stenn and colleagues reported success with their "patch assay" using adult human dermal cells, it is important to note that the epidermal cells were from human fetuses and the host was immunodeficient mice.[14]

One report stands alone in using only adult human cells and tissue. Krugluger described a human skin organ model in which injections of human DP and epithelial cells resulted in follicle induction and growth of vellus-type hair.[15] However, no follow-up studies from this group, or using this technique, have been reported in the many years since its original publication.

Figure 16.1 **Hair growth via follicular neogenesis can be consistently achieved in mice by a variety of methods using combinations of mesenchymal and epithelial cells. In this case, cultured dermal papilla and neonatal keratinocytes were combined in a protected chamber on the back of an immunodeficient mouse, followed several weeks later by abundant hair growth.**

CELL CULTURING CONDITIONS

Early experiments clearly showed that culturing conditions determined the success of dermal papilla-induced follicle morphogenesis. Cultured dermal papilla gradually lose their inductive ability as they are passaged and expanded in culture. Yoshizato showed that the presence of keratinocytes or keratinocyte conditioned media could keep cultured DP inductive through many passages.[16] Subsequent to this, it was found that the factors present in conditioned media were soluble WNT proteins excreted by keratinocytes[17] whose primary function appears to be keeping beta-catenin in the DP active, which is essential for maintaining inductive potential.[18]

In addition to the presence of soluble WNT factors to keep beta-catenin in cultured DP active, it has been found that three-dimensional culture conditions favor subsequent follicle induction over standard two-dimensional techniques.[19] Several reports have shown that cultured DP cells are more inductive when coaxed to aggregate into spheres rather than as the standard monolayers present in two-dimensional culture.[20–22] There is a growing consensus that these "3D" culture systems are superior to older "2D" methods.

CELL IMPLANTATION TECHNIQUE

While the techniques used by various researchers can be rather confusing, they can basically be divided into four distinct approaches (Figure 16.2). When the first reports of successful follicle induction in animals using cultured cells were released in the mid-1980s, it was assumed that not only would success in humans follow shortly, but that the envisioned treatment would consist of injections of dissociated cells directly into the scalp, where they would induce the formation of new follicles. Over the past 10 years, it has become increasingly clear that injection of dissociated dermal cells, with or without epidermal cells, is largely unsuccessful in inducing new follicle formation in humans.

Many reports using animal models have appeared focusing on modifying and augmenting the method of culture and implantation. The rationale is that by optimizing the culture and delivery of cells, follicle induction will be successful in humans. One report describes the comparative success of different implantation techniques on hair regeneration when using cultured DP alone (no epidermal cells). Cultured rat DP placed directly beneath the host epidermis using the "hemi-vascular sandwich" technique showed superior hair induction.[23] These researchers claimed that this technique allowed use of dissociated DP cells alone, and that success was due to

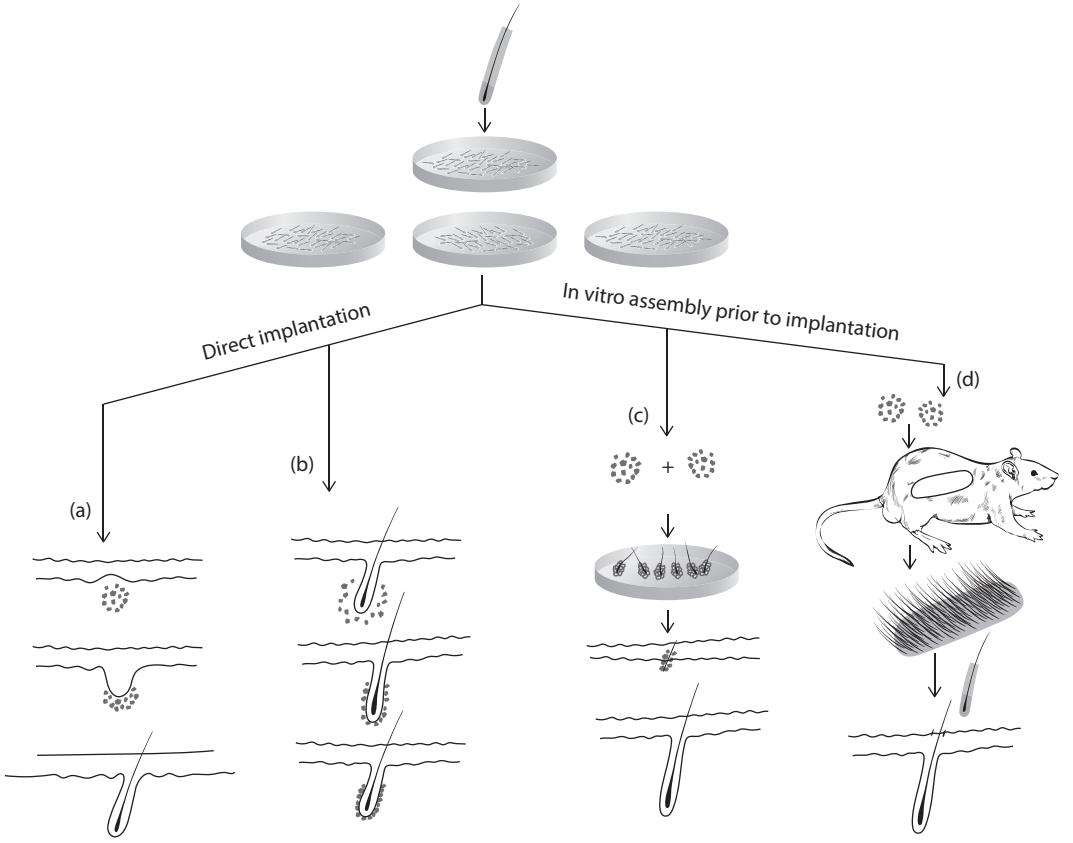

Figure 16.2 **Treating hair loss using follicular cell implantation may mean different approaches depending on the research team. Initially, the simplest approach that was tried involved injecting dissociated cultured dermal papilla (with or without keratinocytes) in an effort to stimulate follicular neogenesis (a). This approach has so far proved unsuccessful in humans. Animal evidence has suggested specific types of mesenchymal cells at the base of the follicle, called dermal sheath cup cells, can be injected into the skin and will attach to preexisting follicles, conferring the properties of the injected cells on the native follicles (b). This approach is currently in clinical trials. Another approach is to take cultured epithelial and mesenchymal cells and combine them using sophisticated in vitro incubation methods, spurring the creation of "proto-hairs," which can then be implanted (c). Perhaps the most promising technique to date involves taking cultured cells and implanting them into immunodeficient mice to serve as intermediate hosts. Once follicular neogenesis has occurred, mature hair follicles are harvested and implanted similar to modern-day hair transplantation techniques (d). See text for more detailed explanations of each approach.**

contact between DP cells and host epidermal cells as well as better oxygenation of the implanted cells.

TOWARD A NEW PARADIGM

Current research appears to be leading toward a treatment paradigm where dermal and epidermal cells are combined for a period of time *before* implantation. Since it is becoming clear that implantation of cultured cells does not lead to follicle neogenesis in vivo, researchers are attempting to create the follicles in vitro and then transplant these. In a method reported by Qiao, mouse dermal and epidermal cells were cocultured prior to implantation, resulting in the formation of primitive "proto-hairs" which could be subsequently transplanted, resulting in growth of mature, cycling hair.[24] It bears repeating that this involved animal cells, not human.

Along these lines, Lindner described the creation of "neo-papilla" using cultured human DP and components of extracellular matrix, followed by coculture with human keratinocytes and melanocytes. This resulted in the formation of in vitro follicles with vellus-like hair shaft growth.[25]

Similarly, Tsuji and colleagues reported that cultured DP and epidermal cells could be combined in a collagen gel to create a "bioengineered follicle germ," which could then be transplanted along with a fine suture which serves

as a guide for follicle directionality and connection to the outside epidermis, thus preventing cyst formation.[26] When transplanted into the hairless skin of mice, these bioengineered "hair germ" grafts took root and produced hair, and analysis of these hair follicles showed all correct layers of normal follicles and accessory structures including connection with surrounding host tissue (i.e., arrector pili muscles and nerve fibers).[27] Normal hair cycling occurred, confirming the presence of necessary stem cell populations within the new hair follicles.

Furthermore, these researchers reported similar success using adult human follicle stem cells (DP and bulge region-derived epithelial cells), which may represent an important breakthrough. In a variation of their method, they implanted the bioengineered hair germ into the subrenal capsule of mice, providing a protected, vascularized space for induction to occur. After 2 weeks, mature hair follicles were seen in clusters and could be harvested for subsequent transplantation as one- and two-hair follicular units.[28] They suggested that these bioengineered hair grafts could be used in a manner similar to follicular unit transplantation (FUT). One might envision a treatment where the patient's follicles are shipped to the lab, where the cells are dissected out and multiplied in cell culture using the latest techniques in 3D culture, then combined in surrogate mice where follicular neogenesis occurs, after which the follicles are harvested and shipped back to the surgeon where they can be implanted via the FUT technique.

Finally, mention should be made of the latest developments in stem cell research which may affect follicular cell implantation for hair loss. Research models, as mentioned, frequently use neonatal keratinocytes which are rich in stem cells. In an autologous cell therapy for hair loss, adult keratinocytes will be needed, and in particular those with "bulge" stem cell properties. Xu recently reported the ability to generate these bulge stem cells from induced pluripotent stem cells using specified cellular signaling factors.[29] And Vacanti recently reported the ability to create pluripotent stem cells from somatic cells using acidic culture conditions.[30] This breakthrough suggests that nonstem somatic cells have the potential to convert into stem cells if given the right cues. Together, these research findings suggest that the requisite population of stem cells can be created through exogenous signals and culturing conditions prior to combining them for in vitro follicular neogenesis. While exciting, it should also be noted that, if successful, such maneuvers will greatly increase the complexity of a follicular cell implantation procedure, meaning many more years of testing and development. One major concern is that these implanted stem cells are not tumorigenic.

CONCLUSION

Follicular cell implantation for hair loss remains an exciting possibility, but true hair multiplication is a long way off from practical reality in the clinic. Cell-based treatments may come online first as "hair thickening" treatments, where periodic scalp injections produce modest clinical gains similar to finasteride and minoxidil. True hair follicle neogenesis in humans has proven far more complex than that which has been routinely carried out in rats and mice, but intricate models are now being developed which keep alive the dream of unlimited hair.

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17 Platelet-rich plasma and stem cells

Gilbert Amgar, Joseph Greco, and Fabio Rinaldi

INTRODUCTION

Autologous cellular therapy is a medical paradigm that allows the safe use of our own cells to enhance surgical and nonsurgical procedures in the treatment of hair loss diseases.

Regenerative growth factors and stem cells in our body modulate the rate of repair that other healing cells perform. Think of them as the maestro leading the orchestra into a beautiful musical piece, first directing the horns to play, then calling the strings to join in, all the time, regulating the flow, depth, and tempo.

PLATELET-RICH PLASMA (PRP)

Currently, autologous platelet-rich plasma (PRP) has been used in diverse indications for more than 30 years with effective results. The first publication on this subject goes back to 1975[1] and deals with the use of platelets as physiological adhesives in corneal surgery. Since then, the literature has widely supported and covered their functions and effects. Most specialties were interested in PRP when a repairing process was necessary: Osseous regeneration in dental surgery,[2] healing of ulcers in diabetic patients,[3] large-scale burns,[4] in orthopedics,[5] in rheumatology, in sports medicine,[6,7] and in plastic surgery (cosmetology). There have been numerous reviews of published literature regarding autologous cellular therapy use in wound care, and Carter et al. published a 10-year systemic review and meta-analysis that included strict protocol inclusion of 24 peer-reviewed published papers from 8577 citations on acute and chronic wounds.[8] In 1996, biology showed a positive effect of growth factor on the hair follicle.[9] Since that time, this process has increasingly been used by physicians.

Definition: Physiology

Platelets are small particles without nucleus, coming from the fragmentation of the megacaryocytes. Their life span is about 10 days. They contain granulations. The morphology of the platelets changes when they become activated: They became round, with pseudopods, and they release alpha granules, within which those growth factors are stored. More than 60 factors have been identifi d.[10] The secretion begins 10 minutes after activation, and 95% of the factors are secreted in the first hour.

The most important growth factors for hair applications are (Table 17.1):

Table 17.1 Most Important Growth Factors for Hair Applications

Growth Factors	Area	Effect
PDGF	Epithelial cells and sebaceous glands of hair follicles	Hair canal formation
VEGF	Vascular plexus in dermal papilla	Improve follicle vascularization
EGF	Epithelial cells and fibroblast	Improve the ratio of anagen High doses of EGF can induce regression of the follicle
IGF 1	Keratinocytes	Slow down apoptosis
FGF	Keratinocytes and endothelial cells	Promote the anagen phase
NGF	Perifollicular neurogenic	Modulating effect on the hair depending on the receptor with which it interacts

- PDGF (platelet-derived growth factor) stimulates the growth of dermal mesenchyme.[9]
- VEGF (vascular endothelial growth factor) increases vascular permeability. The hair follicle is an avascular structure. The growth of hair depends on the vessel capillaries that form the vascular plexus in dermal papilla.[11]
- EGF (epidermal growth factor) stimulates mitosis on epithelial cells and fibroblasts, and improves the ratio of anagen. EGF inhibits the entry in the catagen phase, promoting the anagen phase.[12]
- IGF-1 (insulin-like growth factor) regulates at the cellular level the expression of a powerful messenger anti-apoptotic capable of preventing cell death.[13]
- FGF (fibroblast growth factor) stimulates the proliferation and differentiation of keratinocytes and endothelial cells.[14]
- NGF (nerve growth factor) stimulates hair growth and slows apoptosis. NGF has a modulating effect on the hair depending on the receptor with which it

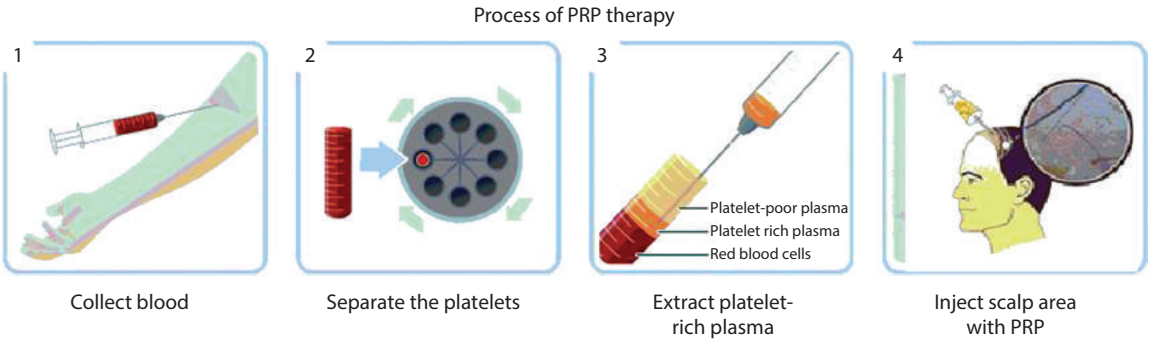

Figure 17.1 **General process for PRP extraction.**

interacts.[13] NGF acts also as a stress mediator. This is certainly a good way to explain the stress and hair loss correlation.[15]

Preparing procedure

The procedure to obtain PRP begins by doing a blood sample on an anticoagulated tube (Figure 17.1). Then tubes are centrifuged for about 5 to 10 minutes at 600–1200 G. (Speed and time depend on the kit protocol.) According to their density, the red cells go down in the tube (Figure 17.2). On the upper part, we find the plasma and the platelets. We usually talk about PRP when we have a concentration three to seven times normal standards.

The patient must sign an informed consent describing the procedure, the contraindications, the expected benefi s, and the possible side effects.

The contraindications are cervicofacial cancer history, hematologic diseases, pregnancy, active local infection, and anticoagulant treatment. We have to pay attention to history of allergies because activated platelets also release histamine.

Application fields

In 2012, an in vivo animal study conducted by Li showed that the injection of activated PRP into the skin of mice once every 3 days for 3 weeks greatly stimulated hair growth. According to the results, PRP increased hair growth and hair follicle survival due to its promotion of cell proliferation and its anti-apoptotic properties. Thus, PRP can potentially prolong the anagen phase of the hair growth cycle.[16]

For humans, the three principal applications fields are androgenic alopecia (AGA), alopecia areata, and surgical procedures.

ANDROGENIC ALOPECIA

The prevalence of progressive AGA approaches 50% of Caucasian men and women beyond the age of 40; whereas in Asian, Native American, and African American men the prevalence is lower and AGA is less severe.[17]

Four studies bring us interesting results concerning PRP and androgenic alopecia.

The first from Rinaldi and Co[18] is an in vitro study realized on 50 patients. Twelve follicles were taken from each patient, four follicles were divided in PRP, four in Ringer solution, and four in standard solution. Then, the mitotic activity was measured. The result shows a significant increase of mitotic activity and reduction of apoptotic process in the PRP group.

Figure 17.2 **Once centrifuged.**

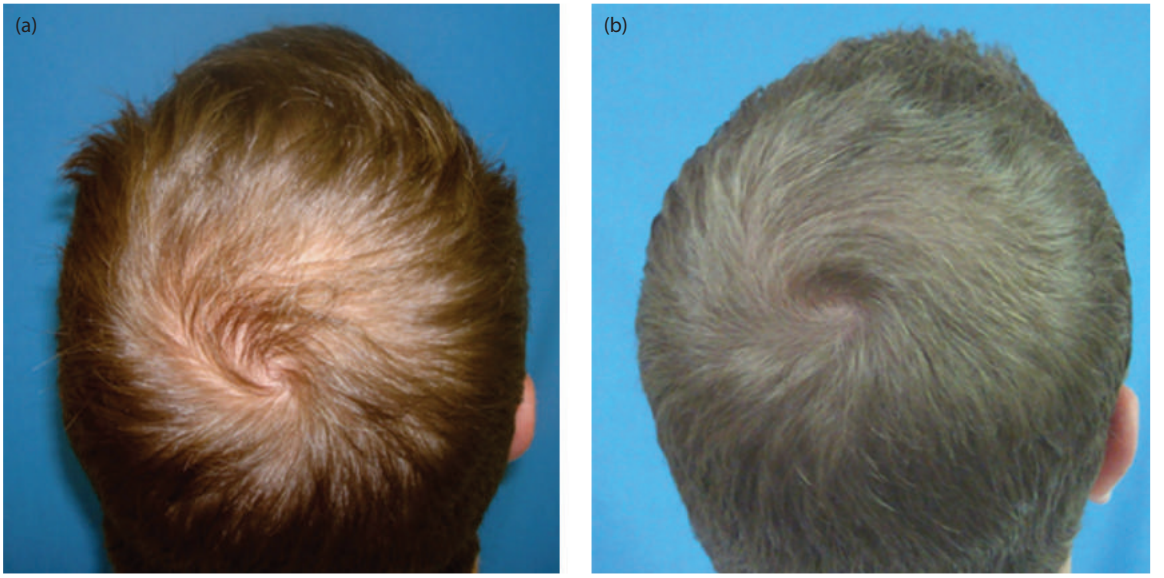

Figure 17.3 **Male androgenic alopecia. (a) Before and (b) 8 months after PRP session. (Courtesy of Dr. Greco.)**

The study by Kiyosawa and Co[19] was conducted on 26 volunteers with thin hair who received five local treatments. They were evaluated for 12 weeks. Areas were photographed and digitally measured. The result shows that PRP improves the density 16% at 12 weeks.

In another study, Greco and Brandt[20] show that traumatizing and infusing growth factors into the scalp reversed miniaturization over an 8-month period when compared to control. Ten hair samples were taken from each patient, five patients in the control group (CG) and five patients from the treatment group (TG). Hair diameter was measured with a Starrett micrometer.

The CG demonstrated a 2.8% average decrease in hair shaft diameter at 4 months and 3.5% decrease at 8 months. (This is the classical evolution of androgenic alopecia.) An increase of 9.7% in average hair shaft diameter at 4 months and then 6.1% at 8 months was observed in the TG (Figure 17.3).

A recent evaluation conducted by Amgar and Bouhanna brings objective parameters for the human scalp[21] (Figures 17.4 through 17.6). Seventy patients (male and female) were included. They all got digital measurement (Figure 17.7) (tattoo mark, hair density, caliber, and terminal/vellus ratio) and standard photographs. Blood, 60 cc, was drawn, and 10 cc of PRP was processed without any activation. The scalp was first traumatized with a 1.5 mm micro needling roller. Patients got two sessions with 3 months between them. At 3 months, they observed a density variation of 19.7%. That means [(third-month density–initial density)/initial density]. At 6 months,

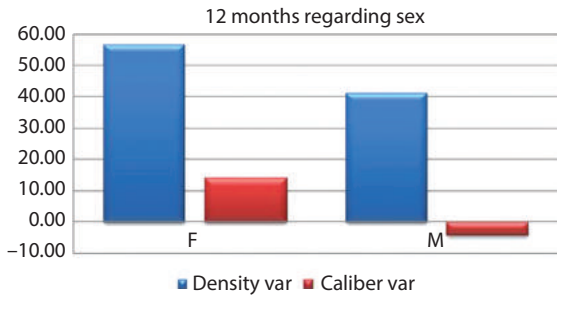

Figure 17.4 **Twelve months density and caliber variation in male and female study—two PRP sessions with 3 months between them.**

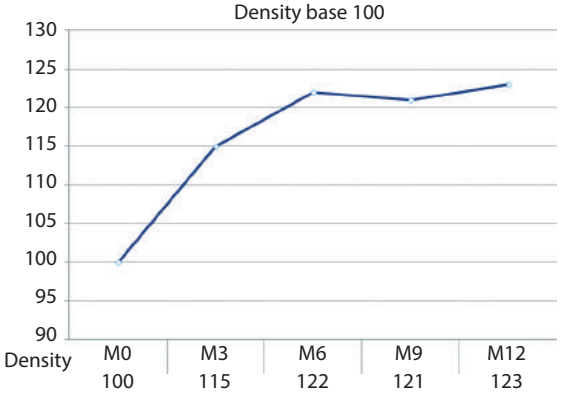

Figure 17.5 **Twelve months density variation in base 100.**