Light and Electron Microscopic Analysis of Controlled Injury to Follicular Unit Grafts

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BACKGROUND. **Careful manipulation of hair units is essential for a good yield of transplanted hair.**

OBJECTIVE. **To analyze the morphology of dissected follicular units submitted to crushing, stretching, bending, and drying.**

METHODS. **Follicular units were either crushed, bent, stretched with forceps, or left drying on surgical gloves for 3 minutes. The specimens were fixed and prepared for observation with light microscopy or transmission and scanning electron microscopy.**

RESULTS. **No alterations were detected in follicular units that had been crushed, bent, or stretched. Major damage occurred in samples that were left to dry on gloves.**

CONCLUSION. **Letting the follicular unit dry appears to be the worst mishandling to which the follicular units may be subjected during routine hair transplantation.**

HAIR TRANSPLANTATION employing follicular units results in better aesthetic appearance and increased economy of the donor area.[1] However, new problems arose with this technique due to the increased fragility of the follicular units as compared to larger minimicrografts cut to size.[2]

There has been much discussion on the importance of proper handling of the grafts during surgery.[3] The manner of harvesting, cutting, and grafting should be studied in order to evaluate the importance of each step on the success of hair transplantation.

In 1984, Norwood and Shiell,[4] summing up the technical and physiological problems involved with hair transplanting failures, concluded that there was an "X factor" leading to poor growth of hair.[4] In 1994, Greco[5] concluded that iatrogenic trauma could be responsible for poor hair growth. He then assumed that a human factor (H-factor) occurred during follicular manipulation. Most of the reports on poor hair growth somehow implicate rough handling of follicular units during dissection and extended time of dissected units spent on long transplant sessions.[6–10]. Several authors blame the use of alcohol-containing saline, which is used for producing tumescence as well as for storage of grafts during surgery.[11–13] Increased research to better understand the physiology of the specimens used in hair transplantation is needed.

The purpose of this study was to determine some of the factors responsible for poor graft yield,[2] such as crushing, bending, stretching, and letting the grafts dry during surgery. Dissected samples were submitted to controlled trauma, compatible with usual handling of follicular units during everyday routine. We analyzed the samples with light microscopy, and with scanning and transmission electron microscopy in order to detect possible lesions produced by trauma. Whereas most samples were analyzed just after trauma, we attempted to retrieve and analyze some samples 1 year after grafting.

Materials and Methods

Harvesting Follicular Units

Twelve male patients, ages 32–63 years, were enrolled in the study. The samples were collected during routine follicular unit transplantation after obtaining patient's informed consent.

After anesthetizing the donor area, an ellipse of skin of variable length and measuring approximately 1 cm in width was dissected using a scalpel. The ellipse was further dissected with razor blades under a stereo microscope to obtain individual follicular units surrounded by as little connective tissue as possible.

Treatment of the Follicular Units

One group of follicular units was kept as intact controls. The other specimens were submitted to one of the following treatments: bending, crushing, stretching, or drying on surgical gloves for about 3 minutes. Bending, crushing, and stretching were done with forceps, using forces greater than

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those employed during routine surgical procedures. At least 3 follicular units were used for each group.

Preparation of Specimens for Microscopy

Control, untreated samples, and treated samples were fixed with a solution of 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 hours. After rinsing in the same buffer, the samples were fixed for 2 hours with 2% osmium tetroxide and washed with phosphate-buffered saline.

For scanning electron microscopy the specimens were dehydrated in a graded series of ethanol and dried with a CPD-010 critical point drier. The specimens were then glued to aluminum stubs, coated with gold using MED-030 sputtering equipment, and observed with a scanning electron microscope.

For transmission electron microscopy the samples were dehydrated with a graded series of ethanol and embedded with Spurr's resin. Two-micrometer sections were stained with toluidine blue for light microscopic observation. Thin sections stained with lead citrate and uranyl acetate were observed with a transmission electron microscope.

Results

Light Microscopy

Examination of follicular units showed the main components of the hair as well as the surrounding tissues, such as connective tissue, adipose tissue and sebaceous glands (Figures 1 and 2). Analysis of light microscopic sections did not show noteworthy modifications when crushed, stretched, or bent follicles were compared to intact untreated samples. It was even difficult to find the areas that had been crushed or bent; as well, very few signs of structural damage due to stretching could be observed. On the other hand, damage was observed in samples that were allowed to dry on the surgical gloves. As compared to control samples (Figure 3), nuclear modifications were prominent in dried samples,

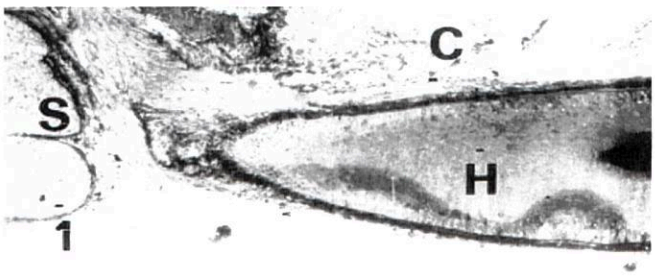

Figure 1. Low-magnification light micrograph of an untreated, control follicular unit showing the hair (H), surrounding connective tissue (C), and part of a sweat gland (S). (Toluidine blue; magnification 55×.)

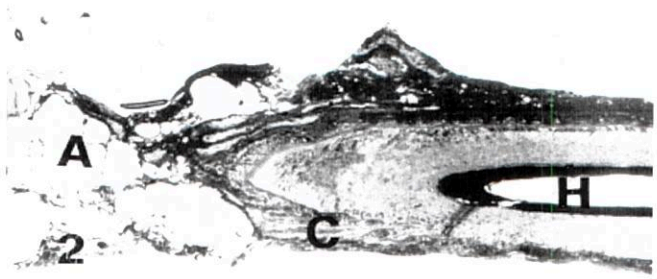

Figure 2. Low-magnification light micrograph of a dried follicular unit. H: hair; C: surrounding connective tissue; A: adipose tissue. (Toluidine blue; magnification 55×.)

consisting of darkly stained nuclei (Figure 4). The cytoplasm of some cells of dried samples had vacuoles of different sizes (Figure 4).

Scanning Electron Microscopy

With the scanning electron microscope it was possible to analyze the connective tissue that covered the lateral surfaces of the follicular units, the surface of the epidermis, and the protruding hairs. The crushed and stretched follicular units did not show damage and their morphology was very similar to that of undamaged specimens (Figure 5). The collagen fibrils of the connective tissue of the lateral surfaces of the units presented normal features and arrangement (Figure 6). In dried specimens, however, the lateral surfaces were compact and hyaline, and it was not possible to recognize the collagen fibrils and other components of the connective tissue as well as fat cells (Figure 7).

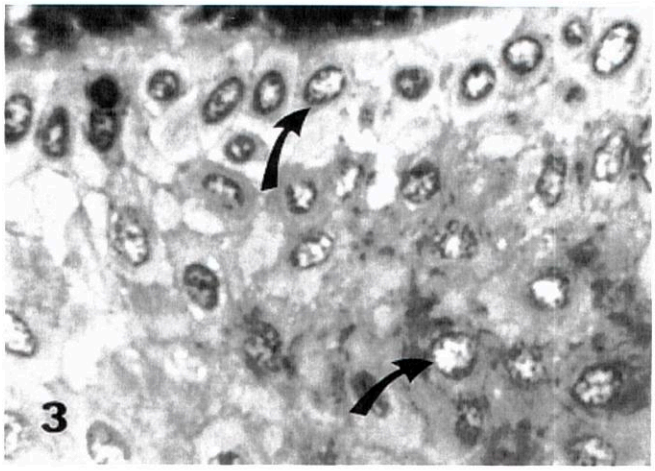

Figure 3. Part of the epithelium of the hair bulb of an intact follicular unit. The nuclei (arrows) show normal patterns of chromatin. The basement membrane is on the top of the figure. Light micrograph. (Toluidine blue; magnification 600×.)

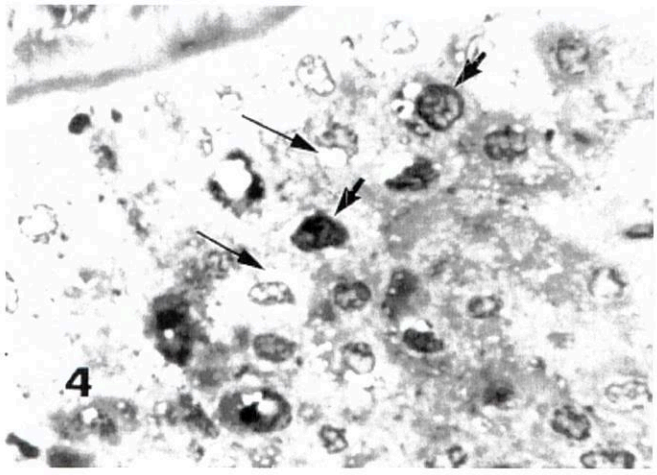

Figure 4. Epithelium of the bulb of a dried follicular unit. Several cells have a darkly stained nucleus (short arrows) and vacuoles in their cytoplasm (long arrows). The basement membrane is on the top of the figure. Light micrograph. (Toluidine blue; magnification 600×.)

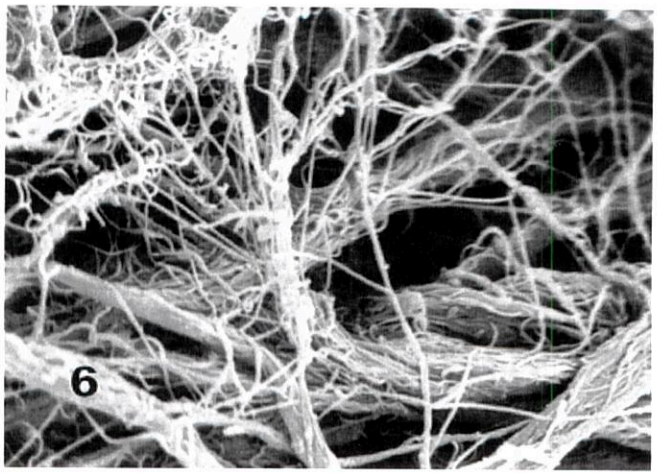

Figure 6. Scanning electron micrograph of the connective tissue of the lateral surface of a control follicular unit showing collagen fibrils. (Magnification 2900×.)

Transmission Electron Microscopy

Only sections of intact and dried hair units (at least three specimens per patient) were observed with a transmission electron microscope, as the specimens submitted to other traumas did not show any structural damage with light microscopy. In sections of dried samples, the general organization of the tissues was preserved. There were, however, clear nuclear and cytoplasmic alterations in dried specimens as compared to untreated specimens. Whereas in intact specimens the chromatin of epithelial cells of the units was organized in clumps that were apposed to the inner nuclear envelope (Figures 8 and 9), in the nuclei of treated specimens the chromatin was formed of very irregular clumps as well as by bridges that crossed the interior of the nuclei (Figure 10). Sometimes a dense band of chromatin was apposed to most of the inner nuclear envelope (Figure 11). The cytoplasm of epithelial cells had many vacuoles which were not present in normal cells, as well as a frequent widening of the perinuclear endoplasmic reticulum cisternae (Figures 10

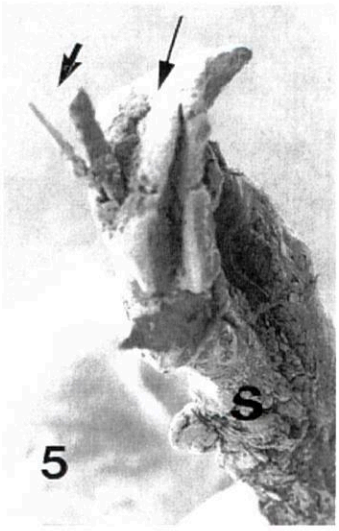

Figure 5. Scanning electron micrograph showing an undamaged follicular unit. Observe the connective tissue of the lateral surfaces of the dissected follicular unit (S), the surface of the skin (long arrow), and two protruding hairs (short arrow). (Magnification 30×.)

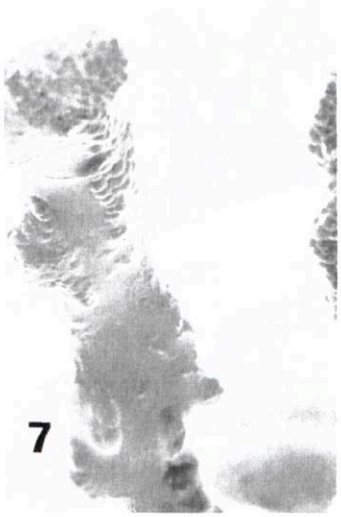

Figure 7. Scanning electron micrograph of a dried follicular unit. The surface of the lateral connective tissue is smooth and does not show any structural detail. (Magnification 26×.)

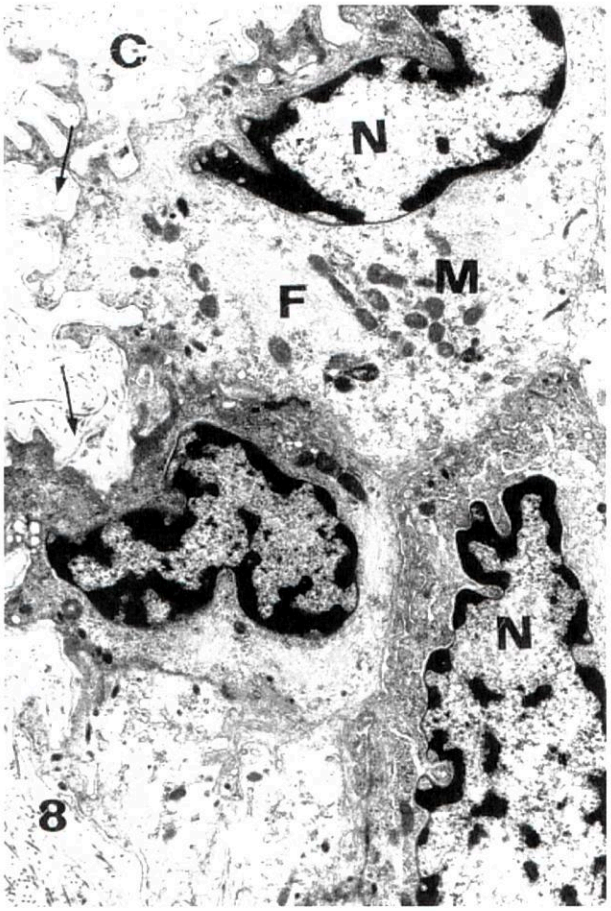

Figure 8. Part of the basal layer from the hair follicle of an undamaged follicular unit. Several epithelial cells are seen. The nuclei (N) have irregular profiles and contain well-distributed chromatin, mainly apposed to the internal nuclear envelope. The cytoplasm of these cells contains mitochondria (M) and intermediate filaments of the cytoskeleton (F). C: connective tissue; arrows: epithelial basement membrane. Transmission electron micrograph. (Magnification 7200×.)

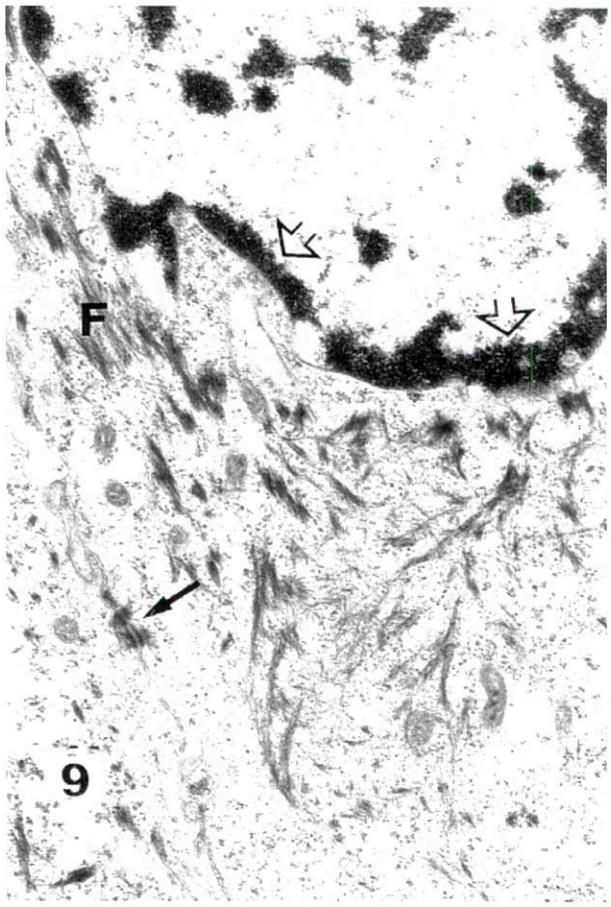

Figure 9. Higher magnification of epithelial cells from the bulb of an undamaged follicular unit. Most heterochromatin (short arrows) is apposed to the inner face of the nuclear envelope. Ribosomes and bundles of intermediate filaments (F) predominate in the cytoplasm. Long arrow: desmosomes. Transmission electron micrograph. (Magnification 14,000×.)

and 11). No obvious alterations were seen in the cytoskeleton of dehydrated specimens.

Similar alterations were observed in cells of the connective tissue: as compared to the fibroblasts of untreated follicular units (Figure 12), in those of dried specimens the chromatin of fibroblast nuclei had many irregular clumps or sometimes the whole nucleus was occupied by a dense clump of chromatin (Figure 13). Vacuolization of endoplasmic reticulum cisternae was also common in dehydrated connective tissue cells (Figure 13). No obvious alteration of collagen fibrils was seen.

Discussion

Our results showed that some of the manipulations to which the follicular units were submitted did not affect substantially the cellular structure and the arrangement of the cells as a tissue. Specifically, crushing, stretching and bending did not result in visible histologic damage or impaired yield of the grafts. Of course, the treatment we imposed on the dissected follicular units was reasonable, as rough treatment would certainly cause morphologic and functional alterations.[14,15] Although no morphologic evidence was observed following crushing and bending of hair follicles, it is not possible to affirm that biological effects of these treatments did not occur.

The only treatment that effectively influenced the structure and vitality of the follicular units was letting the samples dry on the surgical gloves. The cytoplasmic and especially the nuclear alterations of cells submitted to drying are characteristic of cell death. Although some authors have stated that drying samples facilitates the introduction of the follicular units into the recipient

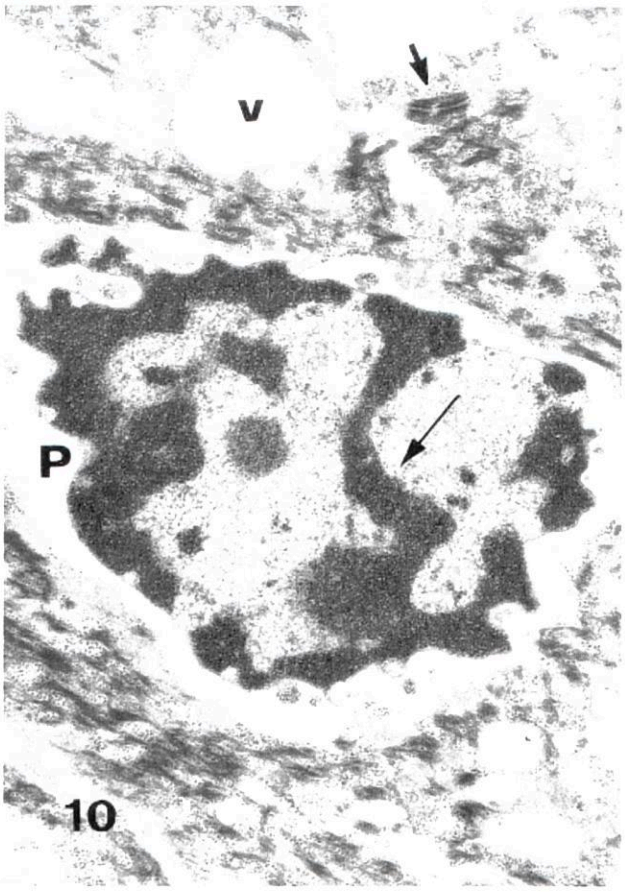

Figure 10. Epithelial cell from a dried follicular unit. The heterochromatin (long arrow) forms a bridge within the nucleus. The perinuclear cisterna (P) is wide and the cytoplasm has many vacuoles (v). Arrow: desmosome. Transmission electron micrograph. (Magnification 14,000×.)

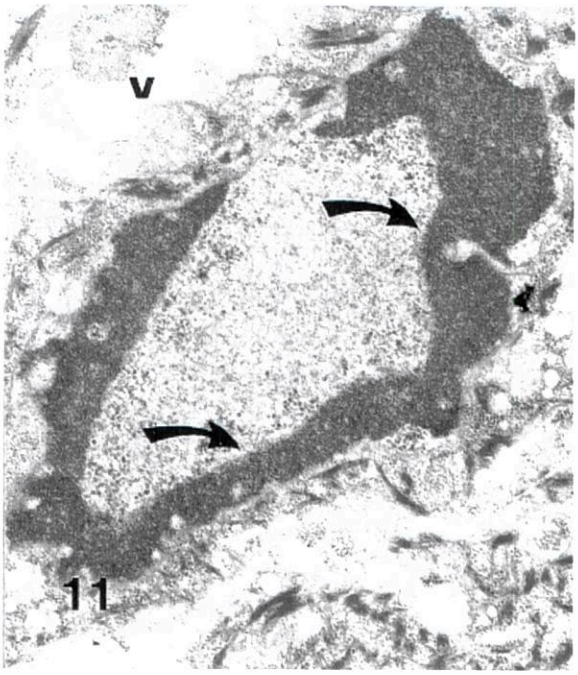

Figure 11. Epithelial cell from a dried follicular unit. Observe vacuoles (v) in the cytoplasm and the atypical arrangement of the heterochromatin (arrows). Transmission electron micrograph. (magnification 14,000×.)

sites due to their stiffness, the advantages of this procedure should be weighed against the disadvantages of the damage that results from drying, as this may cause death of a large population of the cells of the grafts.

Our attempts to retrieve dried follicular units that were grafted 1 year before were disappointing, as we could not detect the remains of the follicular structure. The profoundly altered morphology of dried specimens explains the lack of growth observed 1 year later.

Drying of biological material in atmospheric air results in great damage to the organic structures because of the high surface tension of water. The sudden decrease of surface tension when water evaporates at the surface of the follicular unit has several consequences, primarily the intense shrinking of the sample as a whole, as well as severe disruption of the structure of the cells and extracellular matrix that constitute the follicular unit. Thus drying biological structures must be done using adequate methodology. A good example of this is lyophilization, used for several purposes such as food preservation, vaccine preparation, or study of macromolecules, which must be done under vacuum; on the other hand, controlled drying as is done for sample preparation for scanning electron microscopy results in very good preservation of biological structures.

When large minigrafts (six hairs) are used, even the loss of a few hairs still results in some visible success. However, today, with individual follicular units gradually substituting for large minigrafts, poor growth of each planted graft produces absolutely no growth in that particular area. One of the main reasons for this is the fact that when large minigrafts are mishandled, only some follicular units are damaged and many of those remaining are able to grow after grafting. After drying of large minigrafts, only peripheral follicular units may be damaged, while the protected internal follicular units are preserved. However, when individual follicular units are used, damage results in complete failure of that particular graft. For this reason, care in handling follicular units is much more critical than in other previously used methods.

We suggest that care must be taken to offer adequate protection to the hair grafts against trauma, especially avoidance of sample drying during any stage of the grafting procedure.

Figure 12. Connective tissue of an undamaged follicular unit showing part of a fibroblast surrounded by collagen fibrils (*). N: nucleus; C: cytoplasm. Transmission electron micrograph. (Magnification 25,000×.)

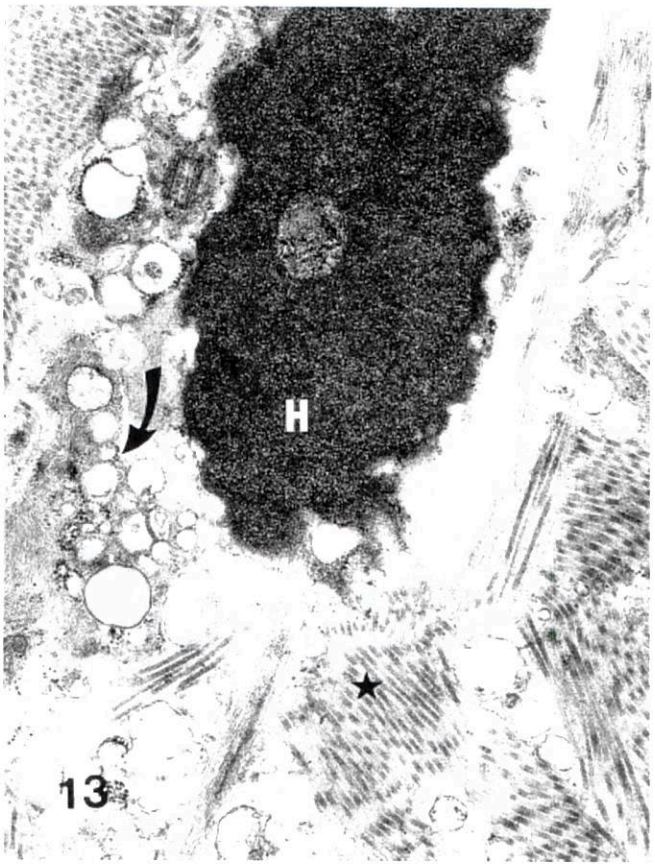

Figure 13. Part of a fibroblast from a dried follicular unit. Darkly stained heterochromatin (H) occupies the whole nucleus. The cell membrane is absent and organelles (arrows) are seen free in the extracellular space. Collagen fibrils (*) surround the cell. Transmission electron micrograph. (Magnification 18,000×.)

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Commentary: H_2O and the X-Factor

In 1984, Dr. Richard Shiell conceived of the term "X-factor" to describe the unexpected poor growth in a small percentage of patients undergoing hair transplantation. Drs. Norwood and Shiell hypothesized that this X-factor might be due to an immunologically mediated rejection producting a localized ischemia to the grafts. The original concept of an X-factor was for it to be a diagnosis of exclusion, after "all other known causes of poor growth" had been eliminated. However, the quest to elucidate all of these "known" causes has proven to be a formidable challenge.

A major obstacle to sorting out the various causes of poor growth is that the hair transplant procedure itself is constantly changing. The problems that affected the large grafts of the early 1980's, producing phenomena such as "doughnutting," no longer apply to the small graft transplants in vogue today. However, megasessions, with their long operating time, reliance on a large surgical team, and use of more delicate grafts, introduce a new set of porblems, all potentially manifesting as poor growth.

Significant progress has been made in recent years with regard to reducing the number of elements that might be lumped in the X-factor category. Greco, in 1994, greatly increased our awareness of the problems in dealing with small grafts with his introduction of the term "H-factor" to describe iatrogenic contributions to poor growth and his focus on mechanical trauma as a major culprit. He offered the logical explanation that as grafts became smaller (and the surface area:volume ratio larger) they would be more subject to a host of insults that included crushing, squeezing, bending, drying, and warming.

To help understand poor growth, Dr. Limmer examined the time that grafts were kept outside the body, Drs. Cooley and Vogel implicated trauma to the dermal papillae, and Dr. Kim experimented with follicular transection. Dr. Seager and the other follicular unit enthusiasts (myself included) felt that even the act of separating intact hair follicles from their naturally occurring groups might impair growth.

The X-factor, a general term for the unknown that elicits a visceral discomfort in physicians and scientists alike, was being attacked from all sides, but the more numerous the hypotheseis, the more difficult it became to design studies to isolate each issue, and the more difficult it was to ascertain which of the ideas were clinically important. Dr. Gandelman appears to have taken a giant stride in solving the problem. Although his light and electron microscopic analyses of injured grafts will only temporarily circumvent the need for the bilateral controlled human studies that will ultimately be necessary to confirm his basic science research, the implications of his work are enormous. It is the kind of stuff that causes us to smack ourselves on the heads and say, "Why didn't I think of that?"

It appears that Dr. Gandelman has identified a common denominator for many different forms of graft injury. Not only does desiccation disrupt cell membranes and destroy intracellular structures, but poorly hydrated grafts are more subject to the many mechanical and thermal stresses that we endlessly obsess about, namely, crushing, squeezing, bending, and warming.

This study does not address the issue of whether intact follicular units may have a greater susceptibility to the effects of dehydration than larger grafts, or more resistance than very thin one- and two-hair micrografts, but then it doesn't really matter. It should be a relatively simple task to keep all of our grafts well hydrated to prevent this important form of injury. Then while our grafts are soaking safely, we will have time to decide which of the less-important factors to fret about next.

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