Preservation of Human Gingiva

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PRESERVATION OF HUMAN GINGIVA

BY

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DEDICATION

I dedicate this book to Gerry Chazan, whose help and understanding these past years have helped to make me who I am.
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CHAPTER I
INTRODUCTION

At the present time a wide variety of tissues are being successfully preserved and stored at low temperatures for future use in research and in therapy. Surgeons have often been in need of skin or oral mucosa for grafting purposes to treat burn victims or to repair congenital or acquired defects. The main problem has always been procurement of adequate amounts of suitable tissue. The ideal solution is tissue banks which serve to supply the various disciplines with tissue on demand. Blood banks for example, or eye banks for corneal tissue have been functioning successfully during many years. Their therapeutic impact has been tremendous.

It is the purpose of this project to demonstrate a technique for freezing and storing human gingiva. Ultrastructural changes that occur in the glycerolized, frozen and thawed gingiva were examined. In order to demonstrate its viability, the glycerolized, frozen and thawed gingiva
was transplanted, autogeneically, in vivo. A sequential histopathologic healing study was performed to determine the changes in the grafted tissue.
CHAPTER II
REVIEW OF THE LITERATURE

A) Classification of the oral cavity and its covering epithelium:

Anatomically, the oral cavity is divided into two parts—the oral vestibule, or vestibulum oris, which is the smaller outer portion; and the oral cavity proper, or cavum oris proprius, which is the larger, inner portion. The oral vestibule is the space between the lips and cheeks in the periphery and the teeth and alveolar processes centrally. The oral cavity proper, inside the arches of the teeth, contains the tongue, which is movably attached to its floor.

Orban and Sicher\(^3\) developed a classification of oral epithelium relating clinical and histologic differences found in various specific zones of the oral mucosa to underlying structures. This classification has subsequently been accepted by the Committee on Nomenclature of the American Academy of Periodontology.\(^4\)

The oral mucosa is described by Orban and Sicher\(^3\) as being of three different types, according to the behavior of the mucous membrane and its fixation to underlying parts.
During mastication some parts of the oral mucous membrane are subjected to heavy forces of pressure and friction. These parts, gingiva and the covering of the hard palate, may be termed masticatory mucosa. The second type of oral mucosa, lining mucosa, is merely the protective lining of the oral cavity. It consists of the mucosa of the lips and cheeks, the vestibule, the upper and lower alveolar processes peripheral to the gingiva, the floor of the mouth, the inferior surface of the tongue, and the mucous membrane of the soft palate. The third type of oral mucosa is the covering of the dorsal surface of the tongue and is termed specialized mucosa.

B) Clinical characteristics of gingiva.

Clinically, the gingiva is defined as that part of the oral mucous membranes which is attached to the teeth and the alveolar process of the jaws.

The free gingiva is the most coronal portion and ends, in a healthy state, in a knife-edged margin. The free gingiva is not attached to the tooth but forms a tight "cuff" which may be displaced by gently probing to reveal the gingival sulcus. The sulcus is bounded laterally by the tooth and sulcular epithelium, coronally by the sulcular orifice, and apically by the "epithelial attachment" to the tooth.
The attached gingiva is fixed immovably to periosteum of the alveolar process and is separated from the lining or alveolar mucosa by a scalloped line, the muco-gingival junction. This line occurs on the vestibular surfaces of the upper and lower jaws and the inner surface of the lower jaw. It is not apparent in the palate.  

The gingiva is normally pink, sometimes with a greyish tinge depending on the thickness of the stratum corneum. There may be melanin pigmentation present in varying degrees and patterns.

C) Histology of Gingiva.

The gingiva is covered by stratified squamous epithelium. This epithelium is usually cornified to varying degrees.

**Basal cell layer**

The basal cell layer consists of low, columnar shaped cells arranged perpendicular to the surface. The cells appear to be laterally compressed resulting in a perpendicular orientation of the nucleus with respect to the basement membrane.

Recent investigations utilizing electron microscopy have shown that the nuclei of the basal cells are round
or oval and bounded by a double membrane perforated by nuclear pores. In the cytoplasm rough endoplasmic reticulum may be seen to be continuous with the nuclear membrane. These cells also contain free ribosomes, a Golgi complex and mitochondria; the latter are numerous and tend to concentrate close to the nucleus. Lipid droplets, various types of vesicles, glycogen granules and melanosomes are seen. Tonofilaments are present in the cytoplasm; they course in bundles (tonofibrils) that are usually arranged perpendicular to the surface of the cell. The tonofibrils end at their union with an attachment plaque located along the cell membrane. The attachment plaques line up opposite each other along interdigitations of the cell membranes of adjacent cells. These paired attachment plaques are called desmosomes.

In the basal layer, along the surface of the cell membrane and in juxtaposition with the basement membrane, there are attachment plaques with no adjacent counterparts in the basement membrane. These structures are called hemidesmosomes and are believed to be, in part, the method by which epithelial cells adhere to the basement membrane.
Stratum spinosum

The stratum spinosum is above the basal cell layer and is several cell layers thick. It is oriented parallel to the epithelial surface, the cells are polygonal in shape and flatten out as they approach the stratum granulosum. The cytoplasm of the cells stains less basophilic than that of the cells of the basal layer and the nuclei are centrally located. 5

Electron microscopy of the stratum spinosum reveals cells structurally similar to basal cells but with fewer organelles, particularly free ribosomes. The tonofilaments are much more numerous. 13

The cells of this layer are connected by intercellular bridges along adjacent cell surfaces. These areas represent desmosomes and in the stratum spinosum they are most numerous and better developed. 5

Stratum granulosum

The stratum granulosum is located immediately above the stratum spinosum and is several layers thick. The cells are spindle shaped for flattened and lie with their axes parallel to the surface. The nuclei appear
small and pyknotic. \textsuperscript{5} Intercellular bridges are indistinct by light microscopy but electron microscopy demonstrates the presence of desmosomes and their associated tonofilaments. \textsuperscript{13} Keratohyalin granules first appear in the stratum granulosum and increase in size and number as the stratum corneum is approached. \textsuperscript{14}

Zelickson's \textsuperscript{15} studies of human epidermis concur with those of Listgarten \textsuperscript{13} in human gingiva with regard to continuing loss of cellular organelles associated with synthetic activity as the cornified layers of cells are approached. The more keratohyaline granules in the cell the fewer the organelles. Lamellar granules appear in this layer and are called membrane coating granules \textsuperscript{15}, they may be intra- or extra-cellular.

**Stratum Corneum**

The stratum corneum is composed of flat, elongated cells. The cells may be completely keratinized with no discernible nuclei or parakeratinized with the nuclei still present in the cell. \textsuperscript{5}

Electron microscopy reveals that the cells of the stratum corneum are sharply demarcated from those of lower layers. They appear shrunken, flattened out, and primarily
composed of densely packed tonofilaments. Cytoplasmic organelles, when present, are not easily recognizable. The desmosomes undergo a change in this layer, they lack the discrete dense attachment plaque on either cell membrane seen in other layers. The desmosomes have a central dense layer with a more electronlucent layer on either side. The uppermost cornified cells appear to separate from underlying cells by rupture of the desmosomes.

Basement membrane and epithelio-connective tissue junction

Linz has described a distinct PAS positive undulating line separating the epithelium of the gingiva from the underlying connective tissue. Electron microscopy confirms the presence of a basement membrane. It is described as a dense fibrillar lamina interposed as a sheet between the basal cell layer and the connective tissue and is referred to as the "basement lamina" or "lamina densa." The 200 Å to 800 Å space seen between the lamina densa and the cell membranes of the basal cells has been termed the lamina lucida and is filled with fine filaments and a diffuse substance which may
be a cementing substance.

The attached gingiva has the greatest number of epithelial ridges and penetrations into the connective tissue. Emslie\textsuperscript{17} claims that the epithelio-connective tissue junction of the masticatory mucosa is adapted to provide a better mechanical bond between the epithelium and the connective tissue in areas of stress. Meyer and Gerson\textsuperscript{10} agree with this concept. In relating the structural features of the palatal mucosa to mechanical stress, they state that masticatory mucosa is equipped by well known devices to resist mechanical forces. In the lamina propria, the tightly packed bundles of collagen fibers provide firm attachment to the underlying bone. The anchoring of the epithelium to the connective tissue is secured by the deep, closely spaced interdigitations of the epithelial ridges and the connective tissue papillae\textsuperscript{10}.

Lamina propria and submucosa

The connective tissue of the attached gingiva consists of dense collagenous tissue. It appears to consist of two layers—a) a papillary layer directly
below the epithelium, and b) a deeper reticular layer made up of dense collagen fibers which are continuous with the periosteum of the alveolar bone. The papillary layer of the attached gingiva is characterized by long connective tissue projections into the epithelium. As the attached gingiva blends into the alveolar mucosa these projections become shorter until they ultimately disappear.

Connective tissue proper

The connective tissue of the marginal gingiva consists of a dense system of collagen fibers. Orban claims that the coarse bundles of collagen fibers are responsible for the surface stippling of the gingiva. These fibers have been topographically classified by Goldman as the "gingival fiber apparatus". Glickman explains their function as bracing the marginal gingiva against the tooth, providing rigidity to withstand forces of mastication tending to displace the tissue, and to unite the marginal gingiva with cementum and adjacent attached gingiva.

The connective tissue of the gingiva contains blood vessels of varying size. The loose connective tissue surrounding vascular channels has been shown to contain
pluripotential connective tissue cells. These cells have been called "adventitial" cells by Marchand\textsuperscript{20} and "polyblasts" by Maximow\textsuperscript{21,22}. These cells have been described as being present in loose connective tissue but a clear definition of them is lacking as to source, structure, and function\textsuperscript{23}. Toto\textsuperscript{24,26} has speculated that these cells give rise, locally, to plasma cells, foreign body giant cells and osteoclasts. Stirling\textsuperscript{27} describes cells of hematogenous origin as giving rise to mesenchymal tissue. Ross\textsuperscript{28} however, has clearly demonstrated that the fibroblast is produced locally, from undifferentiated mesenchymal cells.

It is very difficult to demonstrate the presence of a submucosa in the connective tissue of the gingiva. If any is present it is fused to the lamina propria and periosteum, so that a uniform network of dense, collagenous connective tissue extends from bone to epithelium\textsuperscript{5}.

D) Intraoral Grafting

The use of free gingival grafts, now a widely utilized clinical procedure for correcting various periodontal defects, is a recent innovation. The development of the technique is an outgrowth of many years of successful skin grafting in the practice of medicine.
Gorney et al\textsuperscript{29} were the first ones in successfully applying free grafting techniques intraorally in 1942 utilizing skin from the thigh to perform vestibular deepening. It was not until 1964 that tissue from intraoral sites was used for the same procedure, obviating the necessity for procuring skin\textsuperscript{30}.

Nabers\textsuperscript{31} was the first periodontist recorded as having used free gingival grafts in dentulous patients in mucogingival surgery. His technique was very simple, utilizing resected gingival tissue.

It was not until 1968 that the principles of successful grafting of oral mucosa were elaborated by Sullivan and Atkins\textsuperscript{32}. Coordinating information and principles laid down by plastic surgeons using skin they classified masticatory mucosa grafts as full thickness—consisting of deep layers of lamina propria and epithelium, and partial thickness—thinner grafts of epithelium with some underlying connective tissue. The principles for a successful "take" according to Sullivan and Atkins\textsuperscript{32} and Converse\textsuperscript{33} are: 1) hemostasis of the recipient bed, 2) initial pressure to establish plasmatic circulation, and 3) immobilization of the graft.

Although Sullivan, et al\textsuperscript{34} published a subsequent paper
on the histology of the grafted tissue and recipient site, an earlier paper by Gargiulo and Arrocha\textsuperscript{35} described the sequential healing of free gingival grafts in humans. Gargiulo and Arrocha's work confirmed other studies performed in skin by Clemmesen\textsuperscript{36,37} who clearly demonstrated nourishment of the grafted skin by a plasmatic circulation. By day four, in both gingiva and skin, there was invasion of the graft by budding cepillaries and a "take" was evident. Subsequent biopsies of gingiva\textsuperscript{35} demonstrated continued incorporation of the grafted tissue with some concomitant loss of superficial epithelial cells. The thirty day biopsy showed that the gingival graft was indistinguishable from mature tissue.

These histologic studies were confirmed by Oliver et al\textsuperscript{38}, in 1968 and again by Gargiulo and Brackett\textsuperscript{39} in 1970, who also examined the effect of the procedure on the underlying osseous structure and discovered a thickening of the labial plate of bone beneath the grafted tissue.

\textbf{E) Preservation of epithelial tissue}

The likelihood that skin might be frozen and thawed in vitro and be in a functional state was suggested by the work of Mider and Morton\textsuperscript{40} who used it as control material
for freezing experiments with tumors. Adult rat skin was frozen to -74°C, both rapidly (3-5 min.) and slowly (20 min.), thawed in 8-12 min. in 30°C water and transplanted allogeneically into subcutaneous tissue of adult rats. The grafts were removed after ten days and histologic evidence of viability was demonstrated by the presence of mitotic figures. Less cellular damage was seen in the skin grafts that were frozen slowly.

Briggs and co-workers, following through on the work of Mider and Morton developed further the techniques used for obtaining, freezing, thawing, and autogeneically transplanting mouse skin. In their studies they found that a slow freeze (15-35 min.), quick thaw (1-2 min.) method gave the best results. None of their methods were as successful as their grafting of fresh skin. They accepted the idea that some cellular damage to skin and connective tissue cells occurred by formation of ice crystals during processing.

Webster, from his extensive experimental work dating back to 1932, reported a total of 23 patients upon whom refrigerated skin grafts have been applied (all but 5 autogeneically). He reported success with transplantation of skin stored up to 21 days at 4°C. (autogeneic transplants).
In 1945 Sturmia and Hodge\textsuperscript{43}, heavily influenced by studies in the preservation of plasma, reported that conditions necessary for preservation of human skin were essentially rapid freezing, maintenance at a temperature below -15°C, and rapid thawing followed by warming to 37°C. Forty-one autogeneic split-thickness skin grafts were frozen in citrated plasma and preserved in the frozen state from one to sixty-one days at temperatures of -20°C to -25°C. The frozen and thawed grafts applied to three patients resulted in 80.5% permanent takes. Thirty-four control grafts of fresh skin in the same patients resulted in 86.4% takes. The results do not appear to be affected by the time of storage of the grafts.

Medawar and Billingham\textsuperscript{44} investigated the conditions of freezing and thawing that would result in the least possible damage to the various cellular and fibrous tissue constituents of skin in rabbits. Quick freezing was achieved by chilling a copper strip carrying the grafts as rapidly as possible to a temperature of -150°C. Slow freezing was carried out by placing the copper strip carrying the graft in a bath at -79°C for 15 min. and then transferring it to a bath at -150°C. for a further 15 min. The strip was then
plunged into a bath of isopentane chilled in liquid air to
-150°C. Quick thawing was achieved by plunging the copper
strip into Ringer's solution at 27°C. for 1-2 min. Slow
thawing took at least five minutes. Before being subjected
to one or the other freezing procedures, the grafts were soak-
ed for one hour at room temperature in either Ringer's solution
or in a 15% w/w solution of glycerol in Ringer's solution.
The use of glycerol followed the lead of Polge, Smith, and
Parkes46 who found that it protected fowl spermatozoa from
the fatal effects of quick freezing.

Medawar and Billingham44 assessed the degree to which
the skin had resisted its treatment invitro on the follow-
ing criteria: 1) survival of the grafted epithelium demon-
strated by growth over surrounding raw area and by survival
of graft tissue itself, 2) survival of epidermal melanoblasts
as shown by regeneration of a crop of pigmented hairs 3)
preservation of fine fibrous organization of the dermis,
as shown by the permanent conservation of a distinct graft
margin, the regeneration of a crop of hairs of normal
density and orientation, and the preservation of the overall
appearance that distinguished ear skin sharply from the skin
of the general integument of rabbits.
Their findings were summarized as follows: 1) rabbit ear skin can withstand either slow or rapid freezing down to the temperature of liquid air, 2) slow freezing gives better protection than rapid freezing but pretreatment with glycerol solution protects skin against some of the harmful consequences of rapid freezing. Epidermal melanoblasts do not withstand rapid freezing unless they have been protected by glycerol solution, 3) rapid thawing gives better preservation than slow thawing. Malpighian cells of the epidermis survive slow thawing after impregnation with glycerol solution but not after impregnation with Ringer's solution, 4) skin, slowly frozen to -79°C. after impregnation with glycerol solution and stored for four months, was indistinguishable, on transplantation, from a freshly removed graft. The storage of skin at -79°C. does not result in progressive deterioration during the time of storage.

Smith\textsuperscript{45}, in 1954, describes the impact of the cryoprotective agent glycerol, in appropriate concentrations, on the preservation of tissues at low temperatures. In referring to the work of Polge, Smith, and Parkes\textsuperscript{46} and later of Billingham and Medawar\textsuperscript{44} she indicated that,
with the aid of glycerol, a wide variety of cells and tissues could be preserved for long periods at low temperatures for future use in experimental, diagnostic, or therapeutic medicine.

Work done by Rubinstein and Trobaugh\(^4\) describes a technique for preservation of hematopoietic tissue and describes histologic changes in the frozen and thawed tissue at the ultrastructural level.

The only reported work done on frozen oral mucosa was that of Goggins and Gibson\(^4\). They treated human gingiva with the protective agent dimethylsulfoxide, slowly froze it at a rate of 1°C./min. to \(-30°C\) and then at 10°C./min. to \(-150°C\), at which temperature they stored it for fourteen months. Utilizing a quick thaw (1-2 min.) they were able to grow the cells in tissue culture and determine enzyme activity. Cell migration in culture was evident within 7 days. Histochernically, enzyme activity was demonstrated for individual dehydrogenases, acid and alkaline phosphatases, and non-specific esterases. The histochemical demonstration of active enzymes in the tissues after storage would presuppose that they were present in the tissue before freezing and therefore cannot be construed to be a test for viability in itself.
F) **Cryoinjury**

The retention of tissue structure and function has been shown to be dependent on the rate and manner of freezing and thawing, as well as on the concentration and nature of the cryoprotective agents used.49

A number of mechanisms have been proposed to explain cryoinjury to animal cells. Factors implicated include: pretreatment with cryoprotective agents50, intracellular ice crystal formation51, increases of intracellular electrolyte concentration52, and the effect of osmotic stress on membranes53.

Sherman50, using mouse skin and freeze-thaw techniques described by Medawar and Billingham44, demonstrated that pretreatment with 5% or 15% solutions of glycerol or DMSO could, in itself, cause cellular damage which contributed to the loss of several mouse skin transplants. The 5% solutions caused less damage than the 15% solutions. Steere51 used freeze-etching to study changes occurring in the freezing of red blood cells, dog heart and intestinal epithelium of monkeys. He found the technique particularly suitable for showing membrane disruption by ice crystal formation. He stated that the use of glycerol in increasing
concentrations up to 40% decreased damage to membranes by decreasing the amount of ice crystals formation. He also noted that sometimes, even if severe damage occurred to a cell, there may be little apparent damage to cells immediately adjacent to the damaged one.

Farant, et al\textsuperscript{52}, demonstrated that glycerol lowers the amount of ice in equilibrium with the residual intracellular solution at any temperature during freezing. As a result, the build up in the concentration of electrolytes and other solutes at any temperature during freezing is less marked. They speculate that the direct action of high concentrations of solutes and osmotic swelling during thawing are two of the possible factors that damage cells during slow freezing.

Meryman\textsuperscript{53} studied freezing injury to red blood cells. He concluded that the suspension of cells in solutions of non-penetrating solutes (i.e. NaCl) of progressively increasing concentration creates a progressively increasing stress which is related to the osmotic loss of cell liquid phase and an associated cell volume reduction. At some limiting volume, the stress becomes sufficient to cause a sudden loss of membrane integrity and an influx of extracellular solution. The limiting stress appears to be unrelated to the absolute concentration or identity of the
extracellular solute (provided it is non-toxic), and unrelated to the character of the intracellular solvent or the concentration of intracellular solutes. It is related to the proportion of cell liquid phase removed. There is evidence for a progressive development of stress up to the critical level. There is also evidence that the loss of permeability, when it occurs, is basically reversible but that immediately subsequent events can render it irreversible.

According to Meryman, hypertonic stress is undoubtedly the principal cause of freezing injury in living cells when ice formation is extracellular. He favors the hypothesis that the stress of hypertonic suspensions results from a resistance of the cell to unrestrained volume reduction and that the failure of the cell to shrink freely results in an osmotic gradient across the cell membrane, with an inward osmotic gradient balanced by the mechanical resistance of the cell. The specific mechanism whereby this pressure gradient causes a change in membrane permeability and the circumstances resulting in the transformation of this basically reversible process into irreversible membrane damage remains to be determined.
CHAPTER III
MATERIALS AND METHODS

All materials used in conjunction with any surgical procedure were sterilized and a sterile surgical procedure was adhered to at all times.

1. Procurement of Tissue:

Four patients were selected who required free gingival grafts in the mandibular anterior region of the mouth as part of their total treatment plan. The procedures were explained to all the participants and all were willing to be part of the study.

At an appointed time all four patients were anesthetized by local infiltration in the region of the greater palatine foramen. A strip of palatal gingiva approximately 2 cm. by 5 mm. by 1 mm. was secured from each patient (fig. 1 & 2). A 3 mm. strip was removed from one end of the tissue as fresh tissue control and placed immediately in cold 2.5% buffered glutaraldehyde for the electron microscopic study. The remaining strip was placed in a specimen bottle containing cold Hank's solution. A dressing was placed over the surgery area, Empirin compound #3 was prescribed for analgesia, and the patients were reappointed for three days later.
2. Methods of Freezing and Thawing:

The following procedures, with a slight modification, were already used to achieve successful transplantation of murine bone marrow. One hour after they were excised, each fragment of tissue to be frozen was removed from the bottle containing pure Hank's solution and was immersed in a separate bottle containing 15 ml. of a 12% solution of glycerol in Hank's balanced salt solution.**

Five minutes later each strip of tissue and 1 ml. of its medium were transferred to a freezing ampule* which was sealed by means of the flame of a propane burner.

A separate ampule containing protective agents (standard proportion) and a fragment of mucosa was stoppered with a sterile rubber vacutainer stopped, and a Saber Cu-constantan thermocouple*** was forced through the stopper. One tip of the thermocouple was immersed in the solution and the other one was connected to an Electronik III strip chart recorder.****

* Wheaton Glass Co.

** The glyceralization, freezing and thawing procedures were carried out at the Section of Hematology, Dept. of Medicine of Rush-Presbyterian St. Luke's Medical Center, Chicago, Illinois.

*** Linda C. Div. Union Carbide

**** Honeywell Lab Products
The tissue was frozen to \(-12^\circ\text{C.}\) at a rate of \(2^\circ\text{C.}/\text{min.}\) by flooding the cooling chamber of a Linde BF-4-1* freezing chamber with vaporized Nitrogen. Immediately after the release of the heat of fusion, the suspension was further cooled to \(-100^\circ\text{C.}\) at the same rate of \(2^\circ\text{C.}/\text{min.}\). The freezing ampules were transferred immediately to a liquid Nitrogen tank \((-196^\circ\text{C.})\) where they were stored for three days.

One hour prior to transplantation, the frozen ampules were brought to Loyola University in a container filled with frozen \(\text{CO}_2\) \((-79^\circ\text{C.})\). Immediately prior to transplantation, the ampules were immersed in a \(40^\circ\text{C.}\) water bath and thawed for 1-2 minutes.

3. Deglycerolization:

After thawing the ampules were cut open and their contents poured into separate beakers. Then, at two minute intervals, the following amounts of sterile 35% glucose and 6% Dextran solution were added to each beaker:

- 0.5 cc. glucose
- 0.75 cc. Dextran
- 2.75 cc. Dextran
- 3.00 cc. Dextran

*Honeywell Lab. Products
Approximately 2 mm. were trimmed from the strip of gingiva and fixed for electron microscopy in the manner described in Section 7 of this chapter.

The grafts were immediately transplanted to the recipient site.

4. Preparation of the Recipient Site:

The following surgical techniques were previously described by Gargiulo and Brackett. The recipient site was in all cases the mandibular anterior region of the mouth. The teeth in the area were previously scaled and a gingivectomy was performed when needed to eliminate any residual pocket depth. A split thickness apically positioned flap exposed the connective tissue bed of the recipient site. The flap was sutured with 0000 silk and hemostasis achieved with the aid of saline-moistened 2x2 gauze sponges and pressure (fig. 3 & 4).

5. Placement of the Graft:

The graft was held in place by loop sutures not penetrating the graft. Pressure was applied to the graft for several minutes with moist 2x2 sponges. A piece of Adaptec* was draped over the graft and recipient bed. Additional Orban dressing** was then placed over the wound area so as to cover the wound completely and immobilize the graft. The patients were

*Johnson and Johnson
**Sargent Drugs
prescribed Pen Vee K* for one week and Empirin Compound with 1/2 grain of codeine for analgesia (fig. 5 & 6).

6. Post Operative Care and Biopsy Procedure:

Small biopsies (2mmx5mmx1mm) of the graft were obtained for histologic study from the four patients (i.e. patient #1 was biopsied at 0 and 7 days, #2 and 2 and 10 days, #3 at 4 and 14 days, and #4 at 0 and 30 days). Dressings were changed and replaced such that each patient was dressed for no less than 2 or more than 3 weeks. The biopsies were excised with a #15 Bard Parker and Orban Knives and were immediately fixed in a 10% formalin solution. The specimens were embedded in paraffin and sections of 7-10 microns in thickness were cut and stained with hematoxylin and eosin.

7. Processing Tissue for Electron Microscopy:

All tissues (fresh as well as frozen and thawed) were trimmed into 1mm³ fragments. These fragments were fixed in 2.5% glutaraldehyde for at least one hour, buffered OsO₄ for 60 minutes. The tissues were then dehydrated in 70%, 95% and 100% alcohols and propylene oxide. Epon 812 was used as embedding medium. The tissue blocks were sectioned with a diamond knife attached to an MT-1 Porter Blum ultratone. The sections, 550-650 Å in thickness, were stained with a saturated solution of uranyl acetate and 0.2% lead citrate for three minutes each.

*Wyeth Laboratories
CHAPTER IV
FINDINGS

A. Sequential Histopathologic Healing Study.

The zero hour graft appears identical, clinically, to a graft of fresh gingiva (fig. 7). The biopsy consists of a piece of the grafted tissue approximately 2 mm. in width, 4 mm. in length, and 1 to 2 mm. in thickness. The sections are cut tangentially and are composed almost entirely of epithelium, with some underlying connective tissue (fig. 8). The epithelium shows generalized hydropic degeneration. The stratum corneum is within normal limits. The stratum granulosum shows an average thickness and typical keratohyaline granules. The lower spinous and basal cells show the least demonstrable light microscopic changes (fig. 9).

The basement membrane appears thicker than normal. The fibroblasts in the connective tissue are well preserved but the collagen bundles are amorphous in appearance. Capillaries, devoid of red blood cells, are present in the connective tissue in some sections.

The two day graft is clinically firmly adherent to the recipient bed. It is shiny in appearance and white in color.
(no clinical photograph is available). Histologically, the stratum corneum is almost entirely absent in all sections. The cells of the stratum granulosum are pale in appearance, the nuclei not taking the hemotoxylin stain well. The stratum spinosum and basal layer have the same pale appearance (fig. 10). The basement membrane, at this time, is still amorphous where present (fig. 11). Acute inflammatory cells are present in the connective tissue. The amorphous appearance of the collagen continues but fibroblasts are present throughout.

The clinical appearance of the four day graft is shiny, and it is firmly bound down to the recipient bed (fig. 12). The biopsy is sectioned tangentially (fig. 13). The epithelium appears to have almost completely degenerated. Only a few cells in the deeper layers of epithelium in some sections can be distinguished as structurally intact (fig. 14). The overall appearance of the epithelium, except for the basal layer, gives the effect of "swiss cheese", with nuclei either faded, shrunken, or absent.

There are few areas where a semblance of a basement membrane remains (fig. 15). Sub-chronic inflammatory cells are present in the connective tissue. The collagen seems to be regenerating and some plump young fibroblasts can be identified. There is some endothelial proliferations, but few
red blood cells are present inside the vessels.

Clinically, **seven days** post-operatively, the graft is well incorporated into the recipient bed but can be clearly delineated from the surrounding tissue (fig. 16). Histologically, the epithelium is regenerating beginning from the basal layer, but there is a disruption in maturation such that cell layers, other than the basal cell layer, are not clearly differentiated (fig. 17). Chronic inflammatory cells are present in the connective tissue. The cells in the superficial layers of epithelium have pale staining nuclei and foamy appearing cytoplasm. Some perinuclear edema is still evident. The basal cells are viable and have large nucleoli (fig. 18, 19). Mitotic figures may be seen in many sections. Fig. 19 shows mitotic figures in the basal or lower spinous layer in late prophase or early metaphase.

The basement membrane, where present, appears thicker than normal. There is great proliferative activity in the connective tissue. Many young fibroblasts are present and endothelial proliferation is at its height. Several foreign body giant cells are seen in figure 19.

The **ten day graft** appeared clinically to be attached and well defined (fig. 20). Histologically, maturation of the epithelium seems to be lacking in all sections (fig. 21) as
in the seven day specimen. Cell layers are indistinct. The cells in the upper layers are pale staining and some cells have intracellular globules of eosinophilic material. Other cells in the upper layers have shrunken or missing nuclei and foamy, pale cytoplasm. No keratohyaline granules are present. The basal layer shows mitotic activity. A mitotic figure in telophase can be seen in figure 22.

The basement membrane is well defined in certain areas and appears to be lifting away from the lamina propria, but this is probably artifact. The connective tissue is young and proliferative. Capillaries can be seen in some sections. There is hemorrhage in the area. Few inflammatory cells are present in the connective tissue.

Clinically, at fourteen days, the graft is well incorporated into the underlying tissue and appeared to be whiter than the surrounding area (fig. 23). Histologically, in the upper layers of epithelium globules of eosinophilic material seen at 10 days have coalesced into larger globules (fig. 24). The stratum spinosum and basal layer are well defined. Mitotic activity is evident on many sections in the basal layer. A cell in late anaphase or early telophase may be seen in fig. 25.

The basement membrane is indistinct (fig. 26). The connective tissue continues to be young and proliferative, with
many capillaries and sub-acute inflammatory cells present.

The thirty day graft has the clinical appearance of mature gingiva (fig. 27). The epithelium is within normal limits, it shows a parakeratotic surface, a stratum granulosum with some keratohyaline granules, a well developed stratum spinosum and basal cell layer (fig. 28).

The basement membrane is well defined (fig. 29). The connective tissue is still young, though less cellular and vascular than the fourteen day specimen. Few inflammatory cells are present.

B. The Ultrastructure of Fresh Gingiva.

1) Ultrastructure of epithelium:

Only cells of the stratum spinosum were identified on the sections examined.

Figure 30 shows a low power view (6,650X) of epithelium at the level of the spinous layer. The architecture of the tissues is normal. The nuclei (N) with their double nuclear membranes (NM) are intact. The chromatin pattern (cn) is normal and nucleoli (n) are present. The cytoplasm (C) shows well defined mitochondria (M), numerous tonofibrils (T), and ribosomes (R) and some profiles of granular endoplasmic reticulum (ER). The plasma membrane (PM) cannot be distinguished.
Desmosomes (D) are numerous and intercellular bridges (ICB) and intercellular spaces (ICS) are uniform in appearance.

Figure 31 shows a higher power (16,800X) view of an epithelial cell in the spinous layer. The nucleus (N) and double nuclear membrane (NM) are intact. The chromatin pattern (cn) is normal and nucleoli (n) are present. Numerous mitochondria (M) with partially intact double membranes (DM), tonofibrils (T), and ribosomes (R) are present in the cytoplasm (C) of the cell. Profiles of rough endoplasmic reticulum (ER) are seen. The plasma membrane (PM) can be distinguished in part. Numerous desmosomes (D) and intercellular bridges (ICB) are present. The intercellular spaces (ICS) are not well represented in this section.

2) Ultrastructure of the Connective Tissue:

Figure 32 shows a low power (16,800X) electronmicrograph of connective tissue. The fibroblast (F) is cut in cross section. It shows a normal nucleus (N) and chromatin pattern (cn) and an intact double nuclear membrane (NM). Numerous mitochondria (M) are present in the cytoplasm (C) with partially intact double membranes (DM). Ribosomes (R) and cytoplasmic fibrils (CF) are also seen. The plasma membrane (PM) is intact. Collagen bundles cut in cross section (CB) and tangentially (!) are present.
Figure 33 is a higher power (33,250X) electron micrograph of fresh connective tissue. It shows a fibroblast (F) cut in cross section with an intact nucleus (N), normal chromatin pattern (cn) and double nuclear membrane (NM). Mitochondria (M) may be seen surrounded by a double membrane (DM). Numerous profiles of rough endoplasmic reticulum (ER) are present. Ribosomes (R) and cytoplasmic fibrils (CF) are also seen in the cytoplasm (C). An intact plasma membrane (PM) surrounds the cell. Collagen bundles (CB) are present extracellularly and are cut longitudinally (L), tangentially (!), and in cross section (CB).

C. The Ultrastructure of Glycerolized and Deglycerolized, Frozen, and Thawed Gingiva.

1) Ultrastructure of the Epithelium and the Epithelio-Connective Tissue Junction:

The stratum corneum and stratum granulosum could not be identified on any of the sections examined. The stratum granulosum was probably destroyed and part of its content released since isolated membrane-coating granules (MCG) were seen and are shown in figure 34 (98,000X). These granules are abnormal in appearance, the membranes being disrupted.

Figure 35 shows a lower power view (6,650X) of epithelium at the level of the spinous layer. An abnormal architecture
of the cells is present. The nuclei (N) appear shrunken and pyknotic. In all nuclei the nuclear membrane (NM) is disrupted, the chromatin (cn) is homogenous and the nucleoli (n) are absent. The cytoplasm (C) of the cells is clumped with no distinct cellular organelles. The plasma membranes (PM) are absent in all cases. The desmosomes (D) that remain vary from normal to degenerate in appearance. There is widening of the normal intercellular bridges (ICB).

Figure 36 shows a high power (16,500X) view of disrupted intercellular bridges (ICB). Some desmosomes (D) appear normal in structure (D) while others are not. There is no evidence of tonofibrils (T) associated with the desmosomes (D).

Figure 37 is a high power (33,250X) electronmicrograph of part of a basal cell with its associated intercellular bridges (ICB). The nucleus (N) shows some clumping of chromatin material (cr). The double nuclear membrane (NM) can be distinguished in part. The mitochondria (M) are round and numerous. Some of them show an intact double membrane (DM) and a few intact cristae (Cr). The ribosomes (R) appear well preserved as well as the tonofilaments (T) which are abundant but not associated with the desmosomes. The desmosomes (D) are preserved to varying degrees, some appear intact (!).

Figure 38 is an electronmicrograph (16,500X) of a basal
cell at the epithelio-connective tissue junction (J) showing very few short profiles of granular endoplasmic reticulum (ER). The basement membrane (BM) of the epithelio-connective tissue junction is present but no definite inner structure can be seen. The interdigitations of the epithelial cell with the lamina propria are well represented.

2) Ultrastructure of the Connective Tissue:

Figure 39 shows a low power (16,500X) electronmicrograph of connective tissue. The fibroblast in this section appears to be well preserved. The nucleus (N) and its chromatin pattern (ch) are intact as is its double nuclear membrane (NM). The cytoplasm (C) is surrounded by a partially intact plasma membrane (PM). The cytoplasmic organelles will be described in figures 41 and 42. The collagen bundles (CB) appear to be separated.

Figure 40 shows a high power (33,250X) view of a portion of a fibroblast. It shows a normal nucleus (N) enclosed by an intact double nuclear membrane (NM). In the cytoplasm profiles of granular endoplasmic reticulum (ER) are present. There are round and elongated mitochondria (M) surrounded by a partially intact double membrane (DM). Some cristae are well represented (!). Cytoplasmic fibrils (CF) are numerous and well organized, running longitudinally through the cyto-
plasm of the cell. Ribosomes (R) are present.

Figure 41 is a high power (98,000X) electronmicrograph of a portion of the cytoplasm of a fibroblast. The Golgi apparatus (G) is partially dilated. Myelin bodies (MB), which are whirls of disrupted membranes, are present. Dilated profiles of smooth endoplasmic reticulum (SER) can be seen.

Glycerolization, freezing, and thawing of the tissue must have accounted for all the ultrastructural alterations previously described.
CHAPTER V
DISCUSSION

That gingiva can be glycerolized, frozen, thawed and deglycerolized and still maintain its viability is proven by the success of our free gingival grafts. In our clinical studies, graft adaptation and take was clearly evident seven days post-operatively (fig. 16) and total repair was accomplished by thirty days post-operatively (fig. 27). Our clinical findings correlate well with the biopsy specimens at seven days (fig. 19) and thirty days (fig. 28).

The regeneration of normal gingiva seen at 30 days in our study did not follow the same course as that seen in other studies\textsuperscript{34-39}. The deviations from normal seen in our 0 day biopsy (fig. 8) can be due only to the effect of glycerolization, freezing, thawing, and deglycerolization. The hydropic degeneration of epithelium and the changes in the connective tissue and basement membrane resemble the 2-day biopsy in the study done by Gargiulo and Brackett\textsuperscript{39}.

By seven days, mitotic activity is seen in the basal layer of the epithelium (fig. 19), but there is a definite lack of maturation of the epithelial layers (fig. 17) not reported by other workers\textsuperscript{35,38,39}.  

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This lack of maturation is seen in the epithelium of the ten day specimen (fig. 21) as well. The beginning of the appearance of globules of eosinophilic material intracellularly in the upper layers of epithelial cells suggests a dystrophy of acid mucopolysaccharide synthesis. The small globules of mucopolysaccharide present at ten days have coalesced into large globules in the epithelium of the fourteen day specimen (fig. 24). Toto has described this phenomenon as mucopolysaccharide keratin dystrophy of the oral epithelium. He observed that the material was formed in the prickle cell layer. Some injury must have occurred to the generation of cells surviving our experiment which resulted in the synthesis of this material as the cells move to the surface.

The connective tissue appears to lag behind in its repair when compared with the repair described in both skin and gingiva by other workers. Vascular proliferation and invasion of the graft tissue was not observed in the four day specimen (fig. 15). The connective tissue at seven days, however, is highly vascular and proliferative and from that point, no difference in repair of connective tissue can be observed when compared to the repair described by others.

By thirty days formation of attached gingiva was complete.
Considerable ultrastructural damage probably occurred during glycerolization of the tissue.\textsuperscript{50,54} Since glycerol freely permeates the cell membrane, it changes the osmotic gradient between the cell and the extracellular fluid. An influx of fluid to balance the difference occurs to a point where there is an equilibrium between the osmotic gradient and the elastic forces seeking to return cellular membranes to their original position.\textsuperscript{54} If the osmotic gradient is too great, rupture of membranes may occur. Certainly this can account for the distension of Golgi, mitochondria and other membrane bound organelles seen in our study (fig. 41).

Another damaging factor to consider prior to freeze-thaw is that of tissue hypoxia. One would expect that cells furthest from the terminal capillaries would suffer the most from hypoxia and severe disruption was indeed seen in superficial layers of epithelium (fig. 31).

As pointed out by Meryman\textsuperscript{53}, hypertonic stress is probably the principle cause of freezing injury to membranes when ice formation is extracellular since glycerolization reduces the formation of intracellular ice crystals substantially\textsuperscript{51}. There may be direct damage to membranes and additional cellular damage may occur if lysosomes are damaged and their contents leak out.\textsuperscript{55}
During tissue thaw, water molecules released from extracellular lattices will move directly into the cell. The result is dilation of the vesicles, the extent of which is a direct function of the extent of membrane damage incurred during freezing (fig. 41)\textsuperscript{54}. Obviously, the extent of damage to intracellular membranes will, to a large degree, determine the subsequent viability of the cell.

While recovery of the tissue following freezing and thawing is observed grossly, all cells examined showed varying degrees of changes, especially in the mitochondria, endoplasmic reticulum, and cell membranes. The more superficial epithelial cells were most severely affected (fig. 35), while basal cells (fig. 37 & 38) and connective tissue cells and collagen (fig. 39 & 40) look normal as compared to our controls of fresh gingiva (fig. 30-33). No explanation for this variable resistance from cell to cell and from layer to layer is offered though it has been observed by other workers in other tissues treated in a similar manner.\textsuperscript{51,54}

The fact that cellular ultrastructural changes in the upper layers of the gingival epithelium may be compatible with tissue recovery is evident by the success of our gingival grafts. This success, with tissue subjected to extremely
harsh treatment, is in itself sufficiently encouraging for us to speculate that perhaps other tissues, bone for example, may be preserved in a like manner and later used to repair defects caused by disease or congenital abnormalities.
CHAPTER VI
SUMMARY

Four strips of palatal gingiva were obtained for use as free gingival grafts. A small fragment of each of the fresh tissue strips was resected and prepared for electron microscopic study (controls) and the remaining tissue was glycerolized and frozen. When thawed, another small sample of each gingiva was resected and prepared for electron microscopic study; the remaining grafts were transplanted immediately to the mandibular anterior region of four patients at Loyola University Dental School as a therapeutic procedure (autogenic transplants). The grafts were successful and biopsies were taken alternatively from the different patients at 0, 2, 4, 7, 10, 14 and 30 days post-operatively. The biopsy specimens were prepared for light microscopy and stained with H & E. An evaluation of the ultrastructural changes in the frozen and thawed tissue was made. It showed that ultrastructural damage occurred to the superficial layers of the epithelium. Basal cells and connective tissue elements were indistinguishable from the controls of fresh gingiva.
CHAPTER VII
CONCLUSIONS

There is no way to determine tissue viability with a strictly morphologic approach. What appeared fairly well preserved under light microscopy showed considerable disruption when seen under the electron microscope. Whatever disruption was seen, with light or electron microscopy, was not clinically significant since all our grafts were successful.

Freezing and thawing glycerolized gingiva must have accounted for the deviations from normal seen in the light and electron microscopic sections. It is evident that there were less alterations of the ultrastructure of the basal cells and fibroblasts than of the more superficial layers of the epithelium. Ultrastructurally well preserved basal cells and fibroblasts seem to be the cells responsible for the success of our frozen and thawed free gingival grafts.
Fig. 1. Donor site

Fig. 2. Donor site: Tissue Removed
Fig. 2. Donor site: Tissue Removed
Fig. 3. Area requiring a graft to increase the amount of attached gingiva.
Fig. 4. Recipient bed prepared
Fig. 5. Graft held in place by looped sutures
Fig. 6. Dressing in place over graft

Fig. 7. 0 day graft
Fig. 7. 0 day graft
Fig. 8. 40X 0 day biopsy
note: perinuclear edema
Fig. 9. 250X 0 day biopsy
Note: perinuclear edema, amorphous basement membrane and collagen fibers
Fig. 10. 40 X 2 day biopsy
note: acute inflammation, ghostlike appearance of epithelium
Fig. 11. 250 X 2 day biopsy

Note: presence of fibroblasts in connective tissue and ghostlike appearance of epithelium
Fig. 12. 4 day graft
Fig. 13. 40X 4 day biopsy
note: "swiss cheese" appearance of epithelium
Fig. 14. 250X 4 day biopsy
note: perinuclear edema and light staining of nuclei of all but a few epithelial cells
Fig. 15. 100X 4 day biopsy
note: increased capillary activity and fibroblastic activity
Fig. 16. 7 day graft
Fig. 17. 40X 7 day biopsy
note: immature epithelium, vascular, active connective tissue

Fig. 18. note: highly proliferative connective tissue and presence of foreign body giant cells
Fig. 18. 100X 7 day biopsy

note: highly proliferative connective tissue and presence of foreign body giant cells
Fig. 19. 250X 7 day biopsy
note: mitotic figures in lower spinous and basal layer and foreign body giant cells in connective tissue
Fig. 20. 10 day graft
Fig. 2L 40X ten day biopsy
note: immature epithelium and presence of globules of eosinophilic material. Young and proliferative connective tissue
Fig. 22. 250X 10 day biopsy
note: mitotic figure in basal layer
Fig. 23. 14 day graft
Fig. 24. 40X 14 day biopsy
note: large globules of eosinophilic material in immature epithelium
Fig. 25. 250X 14 day biopsy
Note: mitotic figure in basal layer
Fig. 26. 100X 14 day biopsy
note: young proliferative connective tissue
Fig. 27. 30 day graft

Fig. 28. 40X 30 day biopsy

Note: normal gingival architecture with parakeratotic surface
Fig. 28. 40X 30 day biopsy

Note: normal gingival architecture with parakeratotic surface
Fig. 29. 250X 30 day biopsy
   note: presence of basement
   membrane and mature connective
tissue
Fig. 30. Fresh gingiva 6,650X
Fig. 31. Fresh gingiva: spinous cell
16,800X
Fig. 32. Fresh gingiva: fibroblast
16,800X
Fig. 33. Fresh gingiva: fibroblast
33,250×
Fig. 34. Membrane coating granule
98,000X
Fig. 35. Experimental gingiva
6,650X
Fig. 36. Experimental gingiva: desmosomes 16,500X
Fig. 37. Experimental gingiva: basal cell
33,250X
Fig. 38. Experimental gingiva: Basal cell
16,500X
Fig. 39. Experimental gingiva: fibroblast 16,500X
Fig. 40. Experimental gingiva: fibroblast
33,250X
Fig. 41. Experimental gingiva: fibroblast 98,000X
CHAPTER VIII

BIBLIOGRAPHY


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APPROVAL SHEET

The thesis submitted by Michael H. Korman, D.D.S., has been read and approved by a committee from the Department of Oral Biology.

The final copies have been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is, therefore, accepted in partial fulfillment of the requirements for the Degree of Master of Science.

5/17/72
DATE

[Signature]
SIGNATURE OF ADVISOR