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A Sequential Electron Microscopic Healing Study of Grafted Palatal Mucosa

Robert Alan Weinstein
Loyola University Chicago

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A SEQUENTIAL ELECTRON MICROSCOPIC
HEALING STUDY OF
GRAFTED PALATAL MUCOSA

BY

ROBERT ALAN WEINSTEIN D.D.S.

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
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Finally, my brother Steve deserves special thanks.

DEDICATION

to my wife Carolyn

without whose lasting patience
and understanding, I know this
work would not have come to be

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CHAPTER I

INTRODUCTION

The preservation of a wide variety of tissues by freezing and storage at low temperatures for subsequent use in surgical procedures has been used extensively in recent years. It has been repeatedly observed, through various techniques, that damage does in fact occur to tissues by the freezing, glycerolization and thawing processes themselves. This tissue injury is reversible, however, as proven by its viability after various types of transplant procedures.

It is the purpose of this study to demonstrate first a technique for successfully freezing and storing human palatal mucosa. Subsequently, ultrastructural changes that occur in the glycerolized, frozen and thawed mucosa will be examined. Finally, a sequential electron microscopic healing study will be performed to determine histologic changes in the palatal tissue autografts. A concomitant light microscopic healing survey of the same tissue will be used for supplemental comparisons.

CHAPTER II

REVIEW OF THE LITERATURE

A) Clinical Characteristics of Palatal Mucosa:

The palatal mucosa and gingiva have been classified by Orban and Sicher¹ as masticatory mucosa. This is differentiated from lining and specialized oral mucosa on the basis of clinical and histological differences. Masticatory mucosa is generally coral pink in color and tightly fixed to the underlying periosteum and therefore immovable. The palatal epithelium is uniform in character throughout and exhibits a thick keratinized layer. Orban² and Lund³ describe four distinguishable regions of the hard palate according to the structure of the submucous layer. The first consists of the gingival region adjacent to the teeth and alveolar processes. The second region is similar histologically and consists of the palatine raphe. This area lies in the midline and extends from incisive papilla to soft palate. The anterolateral area or fatty zone is the third region and lies between the median raphe and gingiva. Finally, there is the posterolateral or glandular zone which also lies between the median raphe and gingiva. Posterior to this zone lies the soft palate.

The anterior palate is characterized by the presence

of five or six transverse folds or palatine rugae. These may vary in number and prominence. Although palatal tissue is normally pink in color, different shades may exist due to variable melanin pigmentation. In addition, Dummett⁴ feels the color may vary with the degree of epithelial cornification, the depth of epithelialization, and the degree of vascularity.

B) Ultrastructure and Histology of Palatal Mucosa:

The entire palate is covered by stratified squamous epithelium. Varying degrees of keratinization may exist in different areas of the palate. Four distinct layers of epithelium can be distinguished.

Stratum basale

The stratum basale or basal cell layer consists of low columnar cells with elongated nuclei that lie perpendicular to the basal surface of the epithelial tissue. The undulant double membrane which encloses the nucleus is perforated by numerous pores. Outside the nucleus the cytoplasm contains different inclusions. Mitochondria are dispersed throughout the cytoplasm but seem to be more numerous in the immediate perinuclear region.^{5,6} Characteristic rough surfaced endoplasmic reticulum is well developed, but sparse as compared with the large number of free ribosomes present throughout the cytoplasm.^{6,7}

The Golgi apparatus is also well developed and usually evident at opposite poles to the nucleus.⁸ Lipid vacuoles, various types of vesicles, glycogen granules and melanosomes are interspersed between loosely woven bundles of tonofilaments throughout the cytoplasm.^{5,9} These bundles of tonofilaments or tonofibrils cross the cytoplasm in various directions and are randomly arranged. They often are seen connecting with the attachment plaques of the desmosomes and sometimes change directions perpendicularly close to these attachment plaques.^{5,7} Charles and Smiddy¹⁰ postulate that the tonofilaments course from one desmosome to another within the cell.

The intercellular spaces are rather wide and clearly seen even at low magnification. The intercellular material filling these spaces is probably of a mucopolysaccharide nature and obviously plays a role in intercellular adhesion. The epithelium, however, is not a rigid assembly of units but a population of cells capable of independent migration. The function of this intercellular material as a lubricant in facilitating the sliding of cells past one another has been suggested. The cell peripheries have cytoplasmic processes of different sizes and shapes which frequently interdigitate.^{7,12} Desmosomes have a rather even distribution around the cells. The structure of desmosomes has become well known. They consist of lamellar

structures which parallel adjacent cell membranes. Tonofilaments converge upon the attachment plaques which back the inner leaflet of the cell membrane. Between these attachment plaques are four parallel layers of low electron density. These are separated by three more osmiophilic layers which present themselves as two lateral dense lines and a central dense line.^{5,6,11,12} This central zone is felt to be the intercellular contact layer.¹²

Additional attachment devices consisting of hemidesmosomes, tight junctions (zonula occludens), and intermediate junctions (zonula adherence) are also frequently observed. Dense thickenings which occur at intervals in the dermal portion of the basal cell membrane are termed hemidesmosomes. These devices have alternating light and dense zones, with no adjacent counterpart in the lamina densa. The tight junctions in the stratum basale are usually short and follow a straight or slightly wavy course. This junctional complex is one in which the outer membrane leaflets of adjoining cells come together and fuse.¹² Farquhar and Palade¹⁴ suggest these areas, in addition to representing attachment devices, influence diffusion conditions of the intercellular spaces and facilitate ion exchange between adjoining cells. Intermediate junctions consist of strict parallelism of adjoining cell membranes

with a constant intercellular space (approx. $200\text{\AA}$), and may span 0.2 microns in length.¹⁵ Their function is obscure, but it is possible they may represent temporary intercellular attachment zones.

The basal epithelial cells are frequently interrupted by so-called clear cells. The intercellular space between the clear cell and basal cell is narrow ($200-300\text{\AA}$) and lacks both desmosomes and tight junctions.⁷ The only attachment devices are of the intermediate type. There are no tonofilaments in the cytoplasm and the chromatin rich nucleus has deep infoldings.⁷ Many small vesicles, pre-melanosomes, and melanosomes represent much of the cytoplasmic contents. Zelickson⁶ states these cells produce melanin which they impart to the surrounding epithelial cells.

Stratum spinosum

The stratum spinosum lies above the basal cell layer and is several cell layers thick. The cells nearest the basal cell layer are polyhedral in shape. The cells approaching the stratum granulosum are more flattened and elongated. In contrast to the basal cells, the plasma membrane of the spinous cells is richly supplied with desmosomes and numerous tight and intermediate junctions.⁵ The intercellular spaces are as wide or even wider than those observed in the stratum basale, with frequent inter-

digitation of cytoplasmic projections.

The nuclei are generally round and exhibit more infolding than those in the basal layer--especially as the cells migrate toward the surface.¹⁶ Tonofibrils are abundant and distributed much more uniformly than in the basal cells.^{7,16} Tonofilaments similarly attach to desmosomes. Perinuclear zones are generally free of these filaments, as it is here that the cell organelles are found. In this area, large vesicles and endoplasmic reticulum are present in a random distribution. There are numerous ribosomes throughout the cytoplasm. Mitochondria are also most abundant in these perinuclear regions. The Golgi apparatus is not clearly defined.⁶

Projections of clear cells are seen interspersed between the epithelial cells and have similar characteristics as described for those in the basal layer. Mature melanin granules, however, cannot be identified with certainty.¹⁶ Thilander⁷ frequently found numerous rod-shaped bodies in these cells; they have a striated appearance and in some instances seem to be continuous with a vesicle or the endoplasmic reticulum. No function has been determined for these bodies.

The spinous cells nearest the stratum granulosum contain distinctive granules different from other cellular inclusions. They appear throughout the cytoplasm but are

most numerous near the distal cell surface. Frithiof and Wersäll¹⁷ describe these granular type bodies as being ovoid or rounded in section, and designate them "membrane-coating granules." More recently they have been designated "microgranules."¹¹ Under high magnification these granules have a highly ordered structure bound by a limiting membrane.¹⁸ A lamellar structure is seen with parallel dense layers separated by a lighter zone. Their function is not known but it has been postulated they are of mitochondrial origin.¹⁷

Stratum granulosum

The cells of the stratum granulosum continue the process of flattening begun in the stratum spinosum. At the most distal aspect they are almost as flat as keratinized cells, with their axes parallel to the surface. Tonofilaments are numerous throughout the cell, especially in the deeper levels, but they are not as distinct as they were in the two lower layers.⁶ Membrane-coating granules, identical to those in the distal spinous layer, become more prevalent in the stratum granulosum.¹⁸ In the cells beneath the stratum corneum, they can be seen in close association with infoldings of the cell membrane and sometimes in the extracellular space.¹⁸

The most distinguishing feature of this layer are the

keratohyaline granules it contains. These are electron dense, irregularly shaped granules which display no internal structures or evidence of any bounding membrane.¹¹ Their number and size vary considerably from cell to cell. They often are closely associated with tonofibrils, frequently forming where two or more tonofibrils merge.^{6,7} An intimate association of these granules with ribosomes has also been described.¹¹ As the upper level of cells is approached, the keratohyaline granules become more numerous, while the cell nucleus and organelles seemingly disintegrate. This is true of mitochondria, endoplasmic reticulum, free ribosomes, and identifiable tonofilaments.⁶ Vacuolization is common to the upper level of these cells with disintegration of nuclei and organelles.⁷

As compared with the stratum spinosum the intercellular spaces are narrower, but attachment devices such as desmosomes, tight and intermediate junctions, are more plentiful.⁶ At the junction of granular cell plasma membranes with stratum corneum, the attachments appear thicker and more dense. Here the structure of desmosomes change and they show a composite structure.⁷

Stratum corneum

The stratum corneum is composed of flat elongated cells oriented parallel to the epithelial surface. In

palatal mucosa, this layer is usually five to eight cells thick. These cells are sharply demarcated from those of lower layers, as the transition is quite abrupt. The cells appear shrunken, flattened out, and are primarily composed of densely packed filaments in an osmiophilic, amorphous substance.⁷ Oval vacuoles are randomly dispersed throughout the cytoplasm. No discernible organelles are present. The intercellular space is very narrow and follows a more linear pattern, parallel to the surface, than in the deeper epithelium. Desmosomes and tight junctions occur at short intervals, but have a modified structure. The plasma membranes are thick with no discernible attachment plaques in areas of desmosomal formation. The cytoplasm subjacent to these modified desmosomes sometimes shows a small concentration of dense amorphous material.¹² In the lower stratum corneum, where cell borders are more distinct, striated intercellular material can be seen in continuity with the dense intercellular part of the modified desmosomes.¹⁸ This material is similar in appearance to the membrane-coating granules found intracellularly in both stratum spinosum and stratum granulosum. Occasionally, the intercellular spaces at the tissue surface are closed by a modified desmosome or tight junction.^{7,12} In other instances, the uppermost cornified cells appear to separate from neighboring

cells by rupture of the desmosomes.⁵

The epithelio-connective tissue junction

The palatal epithelium is separated from the underlying connective tissue by a basal lamina. This structure is 400-700Å thick and consists primarily of a finely granular or sometimes filamentous substance.⁷ It is undulating in nature, following the numerous invaginations of connective tissue into the overlying epithelial layer. Interposed between this osmiophilic basal lamina and the cell membranes of the basal cells is a clear homogenous zone 300-500Å in width which has been named the lamina lucida.¹² It is filled with fine filaments and a diffuse substance which may be a cementing substance.¹⁹ This zone is interrupted at irregular intervals by filamentous structures extending from hemidesmosomes on the basal cell membranes to the lamina densa.⁵ The hemidesmosomes are about 200Å thick and consist of attachment plaques on the basal cell membrane with a peripheral density in the lamina lucida, appearing as an osmiophilic line. This line in the lamina lucida is located 200-250Å from the attachment plaque and gives the area a lamellar appearance.¹²

Immediately below the lamina densa, Meyer¹¹ describes a scalloped network of densely staining fibrils, 200-400Å in diameter. These fibrils display a banding pattern,

though without regular repeating units as seen in collagen. As these "anchoring fibrils" approach the basal lamina, they branch into smaller groups of filaments ($20\text{\AA}$ in diameter) which fan out into a spray. These filaments then enter the basal lamina and appear to contribute to its filamentous network. Some filaments seem to traverse the basal lamina and the lamina lucida, reaching the plasma membrane of the basal cell. This relationship is more pronounced at the sites of hemidesmosomes.

On the connective tissue side of the basal lamina, the anchoring fibrils are in intimate relationship with collagen fibers of the lamina propria. Collagen fibers running toward epithelium take on a course parallel to the basal lamina, passing through spaces or tunnels formed by the anchoring fibrils.¹¹

The epithelio-connective tissue junction with attachment devices and undulating pattern, is an adaptation to provide a better mechanical bond between epithelium and connective tissue in areas of stress. Meyer and Gerson²⁰ claim that structural features of palatal mucosa equip this tissue to resist mechanical forces. Thilander and Bloom¹² state that the number and position of these attachment devices, coupled with the degree of keratinization, determine how effectively the mucosa can resist penetration of different substances from the tissue surface.

Submucosa and connective tissue

The structural aspects of connective tissue and submucosa vary in different areas of the hard palate. The marginal areas and median palatine raphe are zones in which the submucosal layer cannot be differentiated from the lamina propria or periosteum.² In these areas, fibrous bands of collagen bundles run at right angles to the surface epithelium, fixing lamina propria directly to periosteum. In the paired areas between palatine raphe and palatine gingiva, these bands become separated by a distinct submucosal layer. Fat lobules are packed into the spaces in the anterolateral zones, whereas minor salivary glands occupy the posterior aspects. Sicher²¹ states that the fat and glands provide a resilient cushion for wide areas of the palatine mucosa. Schour²² describes the function of this cushion as to distribute local pressures over a wider area in the same manner as the skin over the palms and soles.

Numerous cells of different function and structure are interspersed between intercellular substances and fibrillar elements. These cells, including fibroblasts, histiocytes, mobile macrophages, mast cells, lymphocytes, neutrophils, and plasma cells, will vary in occurrence and appearance in different areas of the palate. The fibroblast

is the principal structural cell of the lamina propria and warrants more detailed discussion. It is the most frequently found cell and carries out the major cellular functions of the tissue. It is an elongated or star-shaped cell having long cytoplasmic processes, and its nucleus generally contains a prominent nucleolus. It is most frequently observed in close association with surrounding collagen bundles. Ultrastructural features reveal numerous mitochondria, a well marked Golgi complex, large amounts of granular endoplasmic reticulum and free ribosomes.

The fibroblast is believed to be the connective tissue cell responsible for biosynthesis of collagen ground substance, mucopolysaccharides, and elastin; and its organelles provide the morphologic framework for the synthesis, aggregation, and secretion of these substances.²³

A voluminous mass of loose connective tissue is interposed between periosteum and submucosa proper in the posterolateral hard palate. This tissue contains the anterior branches of palatine nerves and the greater palatine artery which exits the major palatine foramen in this area.^{21,24}

C) Intraoral Grafting:

The first reported grafting procedures in order to establish an increased buccal sulcus in the mandibular vestibule were performed by Moskowicz²⁵ in 1916 and Esser²⁶

in 1917. In this technique, an extra-oral pocket was formed in the submental region and a skin graft molded around a dental compound stent was inserted. About ten days later, an incision was made on top of the stent so it could be removed intraorally. This procedure created a skin-lined pouch that constituted the new labial sulcus. Within a few years this technique was improved by Pickerill,²⁷ utilizing entirely an intraoral approach. Gillies,²⁸ Waldran,²⁹ and Kilner and Jackson³⁰ also used this technique in cases of insufficient labial sulcus in the maxilla. Various other grafting techniques have been reported since this time for extension of atrophic edentulous ridges in order to provide better denture retention.^{31,32,33}

It was not until 1964 that Propper³⁴ utilized tissue from an intraoral site for a similar procedure, obviating the necessity of creating a wound in another area of the patient's body. In 1967, Goldberg³⁵ pointed out the advantages of mucosal grafts for vestibuloplasty: 1) all surgery is intra-oral, 2) scar formation is minimal, 3) the grafted tissue will cushion the dentures, and 4) healing time is shortened and discomfort minimized. In 1968, Sullivan and Atkins³⁶ elaborated on the principles of a successful "take" of grafted oral mucosa: 1) hemostasis of the recipient bed, 2) initial pressure to establish circulation, and 3) immobilization of the graft.

The healing process of split thickness skin grafts has been divided into two phases by Clemmensen.^{37,38} The first phase consists of plasmatic circulation which takes place before the blood supply is restored. The vessels of the free graft are filled with fluid from the recipient bed by hydrostatic dynamics (capillary action). This early plasmatic circulation prevents the drying of the graft and keeps the vessels open, permitting communications from the underlying vessels to be established. The plasmatic phase ensues for about the first 48 hours after graft placement. The second phase begins with evidence of some blood flow into the grafted tissue. At the end of 4 or 5 days, blood flow is established in all vessels.³⁸ Sequential healing studies of free gingival grafts by Gargiulo and Arrocha³⁹ confirmed these findings. Under light microscopy, they found that sequential biopsies of the transplanted tissue demonstrated continued incorporation of the graft into the surrounding tissue, with some concomitant loss of superficial epithelial cells.

Korman's work involving a light microscopic healing study of frozen, thawed and grafted oral mucosa, revealed delayed regeneration of the grafted tissue, especially in the more superficial epithelial layers. This delay in regeneration was attributed to cryoinjury from freezing, thawing and glycerolization of the mucosal tissue. Maloney et al⁴¹

reported the absence of inflammatory changes in canine mucosal grafts after 17 days. Thirty day biopsies from several histologic studies of grafted oral mucosa have shown the graft to be indistinguishable from the surrounding mature tissue.^{39,40,42}

D) Preservation of Epithelial Tissue:

The preservation of epithelial tissue by cold storage has been reported as early as 1903, by Wentscher.⁴³ He used refrigerated free skin as autografts on laboratory animals as autografts. Successful transplantation was accomplished after refrigeration (0° - 4° C.) from 7 to 14 days. This idea was elaborated upon by Carrel,⁴⁴ who successfully transplanted various animal tissues after they had been stored by refrigeration (-1° C. to $+7^{\circ}$ C.) for periods ranging from 1 day to 11 months. He concluded that vaseline was the most satisfactory media for preserving these grafts under refrigeration.

Deliberate refrigeration of skin removed at elective operations for later clinical use was performed by Brown and McDowell⁴⁵ in 1943. They reported the case of a successful full-thickness graft on the neck, previously stored in an ice-box at 3° to 5° C. for 48 hours. More extensive use and refinement of technique was presented one year later by Webster.⁴⁶ He reported success with autogenous

transplantation of skin stored up to 21 days at 4°C.

The concept that skin might be frozen and thawed in vitro to become functional as a transplant was suggested by the work of Mider and Morton.⁴⁷ They utilized rat skin as control material for freezing experiments with tumors. Methyl cellosolve-carbon dioxide mixtures were used for both rapid (3-5 min.) and slow (20 min.) freezing to -74°C. Thawing was done either at room temperature, 20°C., or in a water bath at 30°C., and took 8-12 min. The thawed tissue was then inoculated allogeneically into the subcutaneous tissue of adult rats. Light microscopic preparations of the grafted tissue at ten days revealed mitotic figures among the epithelial cells, which along with the general appearance of the tissue, indicated viability. Less evidence of cellular damage was seen in the skin grafts that were frozen slowly.

Briggs and Jund⁴⁸ claimed that the experiments of Mider and Morton were inconclusive; that frozen and thawed skin remained functional, since no skin grafts proper were made. They developed methods of obtaining, freezing, thawing, and autogeneically transplanting mouse skin with success. They concluded that slow freezing (15-35 min.) to -78.5°C. with rapid thawing (1-2 min.) to 30°C. gave the best results. The postulation was made that the "bound"

water content of the cells might have accounted for the reduction of the ice crystal formation that is known to cause irreversible damage during freezing.

In 1945, Strumia and Hodge⁴⁹ reported 80.5% take of autogenous frozen and thawed split thickness skin grafts as compared with 86.4% takes of fresh skin in the same patients. Their freezing techniques were based on previous experimental work on the preservation of plasma in the frozen state. It was found that under conditions of rapid freezing, maintenance at a temperature below -15°C . and rapid thawing, followed by warming to 37°C ., all the physicochemical properties of complex colloids were retained. Instantaneous freezing at very low temperatures was not felt to be essential. Their results did not appear to be affected by the time of storage of the grafts, which ranged from one to sixty-one days.

Billingham and Medawar⁵⁰ investigated frozen and thawed rabbit skin with two objectives in mind: 1) to determine those conditions of freezing and thawing that result in the least possible damage to the various cellular and fibrous constituents of skin; and 2) to determine the degree to which skin will withstand dehydration in the frozen state. Their methods involved the use of thin copper strips to which the split thickness skin grafts

adhered to with vaseline. Quick freezing was achieved by plunging the copper carrier with graft below the surface of an isopentane bath chilled in liquid air to a temperature below -150°C . Slow freezing involved placing the copper strip with graft in a bath at -79°C . for 15 minutes, and then transferring it to a bath at -150°C . for 15 minutes more. Quick thawing was achieved by plunging the metal graft-carrier bearing the frozen grafts directly into 37°C . Ringer's solution. Slow thawing took 5 minutes in a cold air chamber surrounded by crushed ice.

Prior to being subjected to the slow or rapid freezing procedure, the grafts were soaked for one hour at room temperature in either Ringer's solution or in a 15% w/w solution of glycerol following the lead of Polge, Smith, and Parkes,⁵¹ who found that glycerol protected fowl spermatozoa from otherwise fatal effects of vitrification or "snap freezing".

The clinical assessment upon which Billingham and Medawar,⁵⁰ and later Billingham⁵² determined how well rabbit skin was able to resist their freezing procedures was based on the rate of growth of the transplanted graft, its degree of pigmentation, the normality of its crop of hairs, the survival of sebaceous glands, and restored integrity of the fine structure of the collagenous endoskeleton of the graft.

Their findings were summarized as follows: 1) rabbit ear skin can withstand either rapid or slow freezing down to the temperature of liquid air; 2) slow freezing gives better preservation than rapid freezing, but impregnation with glycerol abates some of the harmful consequences of rapid freezing. Epidermal melanoblasts do not withstand rapid freezing unless first protected by glycerol; 3) rapid thawing gives better preservation than slow thawing; and 4) skin slowly frozen to $-79^{\circ}\text{C}.$, after impregnation with glycerol solution and stored for four months was indistinguishable, on transplantation from a freshly removed graft.

Smith,⁵³ from extensive experimental work dating back to 1949, elaborated upon the cryoprotective agent glycerol used in appropriate concentrations for the preservation of tissues at low temperatures. It was an accidental discovery when Polge, et al.,⁵¹ in 1949, found that glycerol had the remarkable property of protecting the spermatozoa of the domestic cock against the otherwise fatal effects of freezing and thawing. This discovery, in addition to investigations carried out on mammalian tissues by Billingham and Medawar,⁵⁰ and Blumenthal and Walsh,⁵⁴ indicated that with the aid of glycerol a wide variety of cells and tissues could be preserved for long periods of time at low temperatures for future use in various aspects of medicine.

Work done by Rubinstein and Trobaugh⁵⁵ describes a technique for preservation of hematopoietic tissue. In addition, the histologic changes in the frozen and thawed tissue at the ultrastructural level is described.

The first reported work done on frozen oral mucosa was that of Goggins and Gibson⁵⁶ in 1968. Human gingival tissues obtained at the time of periodontal surgery were fragmented and suspended in a balanced salt solution containing 7.5% dimethylsulfoxide, a cryoprotective agent. A slow freeze was then instituted at a rate of 1°C./min. to -30°C. , and then at 10°C./min. to -150°C. It was stored at -150°C. for fourteen months. Utilizing a quick thaw (1-2 min.), they were able to grow these gingival cells in tissue culture. Cell migration was evident within seven days in culture and generally a monolayer of epithelial-like cells was clearly demonstrated by the tenth day. Enzyme histochemical studies were carried out on other fragments that were refrozen on dry ice. Enzyme activity was clearly demonstrated for individual dehydrogenases, acid and alkaline phosphatases, as well as non-specific esterases. In general, the oxidative enzymes showed greatest activity in the deeper layers of epithelium with decreasing activity toward the surface. The histochemical demonstration of active enzymes in the tissues after storage

would presuppose that they were present before freezing and therefore could not be construed to be a test for viability in, and by, itself. On the other hand, complete loss of enzyme activity could indicate loss of viability.

Korman,⁴⁰ in 1972, studied clinical and histologic regeneration of frozen and thawed human oral mucosa used as autogenous grafts for periodontal procedures. The tissues were placed in ampules containing Hank's balanced salt solution with 12% glycerol, and a slow freeze initiated at the rate of 2°C./min. to -100°C. . The freezing ampules were then transferred immediately to liquid nitrogen (-196°C.) and stored for three days.

Rapid thawing (1-2 min.) was accomplished by plunging the ampules into a 40°C. water bath. After deglycerolization and grafting procedures, biopsies were taken at specified intervals to evaluate the regeneration under the light microscope. Sequential biopsies of the grafted tissues showed similar patterns of regeneration studied by other workers⁵⁷ on fresh gingival grafts. However, a retarded lack of maturation in epithelium and connective tissue was noted, especially in the earlier specimens. The more obvious deviations were seen in the superficial epithelial cells, while basal cell and connective tissue progressed similarly to that of normal controls. A con-

clusion was made that freezing and thawing of glycerolized gingiva apparently accounted for the deviations from normal, seen by sequential histologic examination.

E) Cryoinjury:

Significant abatement in cellular damage by freezing has been attained since 1949 when Polge, Smith and Parkes⁵¹ discovered that living cells could be frozen and thawed without harm if glycerol was introduced into their suspending medium. This glycol is a water binder as one mole of glycerol ties up three moles of water. Many other cryoprotective agents, including other glycols and various sugars, are also effective water binders, but glycerol is still widely favored because of its relative nontoxicity in moderate concentrations and relative ease with which it passes through most cell membranes.⁵⁸ The 5-15% concentration that is generally used does not prevent all ice from forming, but it does serve to reduce the concentration of solutes and rate of ice crystallization to a tolerable degree.⁵⁸ In his work with human erythrocytes, Lovelock⁵⁹ proposes that the buffer effect of including glycerol in the suspending medium is to reduce the range of critical temperatures in which damage takes place. He proposes a colligative mechanism of protection whereby the presence of glycerol in the suspending medium lowers the concentration

of salt in equilibrium with ice at any temperature below freezing; when the glycerol is able to penetrate the cells, this also applies to the natural salts inside them. If enough glycerol is present, the salt concentration does not rise to a critically damaging level until the temperature is so low that the rate of damage is also low enough to be tolerable.

Meryman⁶⁰ cites that many cryoprotective compounds are known to penetrate the cells which they protect, while other compounds can enter cells without apparent damage at room temperature. However, after freezing and thawing, with temperature rises and progressive separation of ice, the latter compounds provide little cryoprotection due to their lack of affinity for water. The most important characteristics of solutes that protect living cells from freezing temperatures is most probably affinity for water. Ease of penetration is another important factor.

Most cells live in contact with a dilute aqueous medium, both intra- and extracellularly. Water is the solvent for suspended solutes such as proteins, lipids, salts, hydrogen ions, and other substances. The effects of freezing are transmitted to the cell through this medium.⁵⁸ When living cells are frozen, the transition of water to ice provides increased concentration of these dissolved

substances in the remaining liquid. It is this increased concentration of intracellular components that is deleterious to the cell and often causes irreversible damage. A number of mechanisms have been described to explain injury to living cells after freezing and storage at low temperatures. Lovelock,⁵⁹ with specific reference to erythrocytes, proposes that it is the concentration of salt resulting from the freezing out of water that produces hemolysis. He demonstrated that whenever the extracellular concentration exceeded 0.8M NaCl, hemolysis began; even when the relationship between salt concentration and freezing temperature was altered by the addition of cryoprotective agents.

Lovelock⁵⁹ further explains that the destructive action of exposure to concentrated salt solutions is not instantaneous and the survival of rapidly frozen and thawed cells is attributed to this factor. He suggests salt concentration may cause damage in the form of a "lyotropic" effect on the lipoprotein constituents of the cell membrane because of the cholesterol and phospholipids appearance in the supernatant when erythrocytes are suspended in excessively high salt concentrations.

Doebbler and Rinfret⁶¹ have shown through biochemical analysis that cells recovered physically intact after rapid

freezing have altered electrolyte composition. Total cation concentration remains relatively unchanged, while sodium increases and potassium decreases. They suggest some enzymatic damage occurs in the cell membrane rendering the usual cation transport processes ineffective.

Meryman⁶² also studied freezing injury to red blood cells, and postulated the mechanism of freezing injury is due to osmotic stress rather than the toxic effects of increased salt concentrations. He concluded that suspension of cells in solutions of non-penetrating solutes (e.g. NaCl) of progressively increasing concentration creates a progressively increasing stress which is related to the osmotic loss of cell liquid phase and 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 is related to the proportion of cell liquid phase removed and not the absolute concentration or identity of the intra- or extracellular solute (provided it is non-toxic). There is evidence for a progressive development of stress up to the critical level.

Meryman⁶² favors the hypothesis that the stress of hypertonic suspensions results from resistance of the cell to unrestrained reduction in volume. This 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.

Physical damage to the cell by ice crystal formation, both within the cell and throughout its suspending medium, has been proposed by Mazur.⁶³ From his work involving the freezing of various microorganisms at different rates, he suggests that death is not the result of high concentrations of solutes. If it were, one would expect longer exposures to produce greater damage, but the reverse actually occurs. Slow cooling is less harmful than rapid cooling, even though slow cooling results in the longer exposure. Moreover, even with rapid cooling the damage occurs almost entirely within the first minutes of exposure and does not increase significantly with time. He proposes the physical consequences of exposure to low temperatures that are responsible for cell death fall into two major categories: those derived from changes in kinetic energy of molecules or temperature itself, and those dependent upon phase changes; specifically the freezing of water and the melting of ice.

Steere⁶⁴ used freeze-etching to study changes occurring in the freezing of red blood cells, dog heart, and intestinal epithelium of monkeys. He found this technique

particularly suitable for showing membrane disruption by ice crystal formation. The use of glycerol in increasing concentrations up to 40% revealed decreased damage to membranes by decreasing the amount of ice crystal 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 ones.

Subsequent studies by Mazur⁶⁵ and Litvan⁶⁶ attribute cryoinjury to differences in vapor pressure of water across the cell membrane resulting from the supercooled intracellular water as the temperature decreases. Each proposes an alternative mechanism by which this difference in vapor pressure attains equilibrium. Both mechanisms suggest cellular damage in the form of protein denaturation.

Cell cryoinjury, manifested by ultrastructural and related functional alterations of mitochondria has been studied by Sherman,⁶⁷ using mouse kidney. He considers injury to be induced directly by physical distortion, increased electrolyte concentration, or other events during the formation and dissolution of ice. The proposition is made that freezing and thawing may elicit deleterious consequences indirectly through an initial effect on another organelle or group of organelles based on their

inherent variations in cryosensitivity, and their interrelationships in form and function.

Persidsky⁶⁸ suggests that lysosomes may play a crucial role in the mechanism of cell cryoinjury, due to their high sensitivity to environmental changes. A lysosome specific agent, trypan blue, was chosen for his experiments because of its known ability to concentrate selectively in lysosomes and to inhibit their enzymes. This dye was injected into the bone marrow of rats before testing the ability of the marrow to recover after cryopreservation. Based on incorporation of ^{14}C -1 glycine, and growth in tissue culture, bone marrow cells from treated animals showed on the average, three times greater recovery than cells from untreated animals. It was concluded that the striking improvement in cell preservation resulted directly from the inhibition of lysosomal enzymes by trypan blue. Consequently, it may follow that activation of lysosomal enzymes by freezing and thawing can be a primary cause of cell cryoinjury.

CHAPTER III

MATERIALS AND METHODS

The ultrastructure of frozen and stored human palatal mucosa used as autogenous free graft material for mandibular vestibuloplasty was studied through the course of the healing process. Concomitant clinical and light microscopy evaluations were also performed. All materials used in conjunction with any surgical procedure were sterilized and sterile surgical procedure was adhered to at all times.

1. Procurement of Tissue

Four edentulous patients presenting with atrophy of the mandibular anterior alveolar ridge were selected (fig 1). Mandibular vestibuloplasty with an autogenous graft was required as part of their total treatment plan. The procedures were thoroughly explained to all the participants and all were willing to be part of the study. Written consents were signed in all cases. An identical procedure was carried out on all four patients.

At an appointed time, the patient was anesthetized by local infiltration of the anterior two-thirds of the hard palate. By suprapariosteal dissection, a horseshoe shaped strip of palatal tissue measuring approximately

1 cm in width by 4 cm in length was secured (fig 2). Two 2 mm³ fragments of tissue were taken from one end of the graft as fresh tissue controls. One fragment was immediately placed in cold 2.5% buffered glutaraldehyde for the electron microscopic study, while the other was placed in 10% formalin for subsequent evaluation by light microscopy. The remaining donor graft was then "de-fatted," and placed in a specimen bottle containing cold Hank's balanced salt solution. The patients maxillary prosthesis, lined with Surgicel* was placed for hemostasis.

2. Methods of Freezing and Thawing

Approximately 15 minutes after excision, the graft 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 12% solution of glycerol in Hank's balanced salt solution. The specimen in the suspending medium was then taken to another facility for the subsequent freezing procedure.**

The strip of graft and 5 ml of its medium were transferred to a freezing ampule*** approximately one hour

* Johnson & Johnson

** The freezing procedures were carried out at the Section of Hematology, Dept. of Medicine of Rush-Presbyterian St. Lukes Medical Center, Chicago, Illinois

*** Wheaton Glass Co.

after the initial surgery. This ampule was sealed by the flame of a propane torch and then placed in a Linde BF-3* liquid nitrogen freezing unit.

A separate ampule containing protective agents (standard proportion) and a fragment of mucosa was also placed in this unit. This ampule was stoppered with a sterile rubber vacutainer stopper, and a Saber Cu-constantan thermocouple** was forced through the stopper. One tip of the thermocouple was connected to an Elektronik III Strip Chart Recorder.***

The tissue was frozen to -12°C . at a rate of $2^{\circ}\text{C}/\text{min}$. by flooding the cooling chamber of the freezing unit with vaporized nitrogen. Immediately after the release of the heat of fusion, the suspension was cooled to -100°C . at $2^{\circ}\text{C}/\text{min}$. The frozen ampules were immediately transferred to a liquid nitrogen tank (-196°C .) where they were stored for one month.

One hour prior to transplantation, the frozen ampule was brought to Loyola University in a container filled with frozen CO_2 (-79°C .). Immediately prior to the graft stage of the vestibuloplasty procedure, the ampule was immersed in a 40°C . water bath and thawed for 1-2 minutes.

* Honeywell Lab. Products

** Linde C. Div. Union Carbide

*** Honeywell Lab. Products

3. Deglycerolization

After thawing the ampule was cut open and its contents poured into a sterile 100 ml beaker. Then, at two minute intervals, the following amounts of sterile 35% glucose and 6% dextran solutions were added to the beaker:

2.5 cc glucose
3.75 cc dextran
11.25 cc dextran
15 cc dextran
12.5 cc dextran

Two 2mm³ sections were again sectioned from the strip graft and fixed for both electron and light microscopy as described previously. Following procurement of these biopsies, the graft was immediately transplanted to the recipient bed.

4. Preparation of the Recipient Bed

A surgical technique similar to that described by Hall and O'Steen⁶⁹ was employed. With the patient under a running intravenous sedation, bilateral mandibular nerve block anesthesia with 2% xylocaine (conc. epinephrine 1:100,000) was given. In addition, the anesthetic solution was infiltrated into buccal, labial, and lingual tissues, serving to "balloon" the tissues for ease of dissection and to provide hemostasis. A mucosal incision was then made on the buccal aspect of the alveolar crest, just anterior to the retromolar pad. This incision was

extended along the junction of the loose and attached gingiva to the retromolar pad of the opposite side. A suprapariosteal dissection of buccal and labial tissue was then performed, stripping all muscle attachments, excepting the inferior-most attachment of mentalis muscle. Both mental nerves were exposed bilaterally and left intact. A similar suprapariosteal dissection was carried out in the anterior lingual area with stripping of the superior belly of the genioglossus muscle. Lowering of the anterior floor of the mouth was accomplished by placement of four evenly spaced catgut sutures placed through the free lingual tissue, under the mandible and attaching to corresponding areas of the dissected free labial tissue margins. The free labial and buccal tissue margins were then sutured to the periosteum at the most inferior aspect of the dissection (fig. 3).

5. Placement of the Graft

A prefabricated clear acrylic splint (fig. 4), pre-soaked in clear merthiolate solution, was then filled with a soft periodontal dressing, and an impression of the surgical area was taken. Green stick dental compound was used to extend the splint in the necessary areas. The graft was secured to the periosteum with intermittent catgut sutures (fig. 5). With graft in place, the acrylic splint

with corrected lining and extension was then placed over the surgical area and secured with two 25 gauge stainless steel circummandibular wires. The patients were discharged and placed on appropriate antibiotic and analgesic therapy.

6. Post-Operative Care and Biopsy Procedure

Two small biopsies (2mm x 2mm x 2mm) of the graft during the healing stages were taken from each patient (i.e. patient #1 was biopsied at 0 and 12 days; #2, at 2 and 15 days; #3, at 5 and 25 days; and #4, at 9 and 30 days). The acrylic splint was not removed until one week post-op, and thus it was necessary to provide a hole in the anterior region of the splint (fig. 6) for obtaining biopsy tissue from patients #2 and #3 (the 2 and 5 day biopsies). These biopsies were excised with a #11 Bard Parker knife and Allis forceps.

7. Processing Tissue for Electron Microscopy

All tissues to be examined under the electron microscope (fresh as well as frozen and thawed) were trimmed into 1mm^3 fragments. These fragments were fixed in 2.5% buffered glutaraldehyde for at least one hour and post-fixed in buffered OsO_4 for 60 minutes. The tissues were then dehydrated in 70%, 95%, and 100% alcohols and propylene oxide (1-2 epoxypropane), respectively. Epon 812

was used as the embedding medium. The tissue blocks were sectioned with a diamond knife attached to an MT-1 Porter-Blum Ultratome. The sections, 550-650^o in thickness, were picked up in 300 mesh copper grids. They were then stained with a saturated solution of uranyl acetate for 3 minutes, washed in distilled water for one minute and post-stained with 0.2% lead citrate for 3 minutes.

9. Processing Tissue for Light Microscopy

The biopsies (2mm x 2mm x 2mm) to be prepared for light microscopy were immediately fixed in 10% formalin solution. They were then embedded in paraffin and sections of 7-10 microns in thickness were cut and stained with hematoxylin and eosin.

CHAPTER IV

FINDINGS

A) Clinical and Light Microscopic Findings:

Due to technical difficulties, especially the size of the specimen, many of the biopsies (days 2, 5, 9, and 15) prepared for light microscopy did not show epithelium upon examination. A previous sequential light microscopy healing study of frozen and thawed oral mucosal tissue, by Korman,⁴⁰ will be utilized for the description of the above mentioned missing biopsies and subsequent correlation with the light microscopic and ultrastructural changes observed in this healing study.

Zero day post-frozen graft

Immediately after thawing, the graft was identical in appearance, texture, and consistency to the same fresh tissue (fig. 2).

A histologic section of the zero day post-frozen graft is seen in fig. 7. The section is cut tangentially, showing both epithelium and some underlying connective tissue. The epithelium shows generalized hydropic degeneration with notable perinuclear edema throughout all levels. The stratum granulosum shows an average thickness with typical keratohyaline granules. The least demonstrable

light microscopic changes are seen in the lower spinous and basal cells.

The basal lamina appears thicker than normal. The underlying connective tissue reveals well preserved fibroblasts, but collagen bundle formations are amorphous in appearance. Capillaries, devoid of erythrocytes, are present in the connective tissue.

Two day post-frozen graft

Clinical assessment of the two day graft was difficult since the acrylic stabilization splint was still in place with only a small hole provided for obtaining a biopsy specimen (fig. 8). The graft, however, was fairly well adherent to the recipient bed. It was shiny in appearance and white in color. Areas of overlying necrotic tissue were evident and easily wiped away with a cotton-tipped applicator.

Histologically, the stratum corneum is almost entirely absent. The cells of the stratum granulosum are pale in appearance with deficient uptake of the hematoxylin stain by their nuclei. The stratum spinosum and stratum basale have the same pale appearance (fig. 9). The basal lamina, although still amorphous in appearance, is disrupted throughout. Acute inflammatory cells can be seen in the connective tissue. The amorphous appearance of

the collagen fibers persists, with fibroblasts interspersed throughout.⁴⁰

Five day post-frozen graft

Again, adequate visualization of the graft tissue at this stage was impaired due to the overlying acrylic stabilization splint. The donor tissue, however, was quite adherent to the recipient bed. The graft remained shiny in appearance and white in color. Areas of necrosis were still evident, and at this stage, scattered areas of what appeared to be granulation tissue, red in color and more bulbous in appearance, were dispersed intermittantly about the graft area (no clinical photograph available).

Histologically, the epithelium appears to have almost completely degenerated (fig.10). Only a few cells in the deeper layers of epithelium can be distinguished as structurally intact (fig. 11). The overall appearance of the epithelium, except for the basal layer, gives a "Swiss cheese" effect, with nuclei either faded, shrunken, or absent.⁴⁰

There is a semblance of basement membrane in only a few areas. Acute and subchronic inflammatory cells are present in the connective tissue. Collagen tissue at this stage appears to be regenerating and some healthy appearing fibroblasts can be identified. There is some endo-

thelial proliferation, but few red blood cells present inside the vessels.⁴⁰

Seven day post-frozen graft

A biopsy was not scheduled to be taken at seven days, however, since the acrylic splint was removed at this time, a clinical photograph was taken to show the first full view of the entire graft after placement of the splint at surgery (fig. 12). The graft still appeared whitish in color and well adherent to the recipient site. It was surrounded by numerous areas of granulation tissue which covered the exposed periosteal tissue provided by the surgery. The white area seen overlying the genial tubercles was probably due to the irritation produced by the acrylic splint.

Histologically, Korman⁴⁰ describes evidence of regeneration in the epithelium. This is seen primarily in the basal layer where individual cells are discernible with some semblance of organization. Mitotic figures can also be seen in the basal layer (fig. 13). However, there is a disruption in maturation such that cell layers, other than the basal cell layer, are not clearly differentiated (fig. 14).

Cells in the superficial layers have pale staining nuclei and foamy appearing cytoplasm. Capillary formation and chronic inflammatory cells are seen in the underlying

connective tissue.

Nine and ten day post-frozen grafts

Clinically at nine days, the graft tissue appeared to be more blended into the surrounding tissue, although it was still white in appearance and rough in texture. The graft is seen at the depth of the vestibule in fig. 15. Superior and lateral to the graft, more reddened areas can be observed. These areas represent granulation tissue overlying exposed periosteum left at surgery.

Histologically, at ten days, the post-frozen graft in Korman's⁴⁰ study shows lacking of maturation in all sections of epithelium. The cell layers are indistinct. Cells in the upper epithelial 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 keratohyline granules can be observed. The basal layer, however, shows mitotic activity (fig. 16). The basal lamina is well defined in certain areas and appears to be lifting away from the lamina propria. The connective tissue appears young and proliferative. Capillaries can be seen in some sections and few inflammatory cells are present in the connective tissue.

Twelve day post-frozen graft

At twelve days, clinical inspection of the graft shows good incorporation into the surrounding tissues with some difficulty in determining the graft margins. The color of the graft has turned from white, as previously described, to coral pink and more closely simulates the color of surrounding tissues. The graft surface still remains rough in texture. Some areas of granulation tissue are still noticeable (fig. 17).

A tangential cut of epithelium and connective tissue at twelve days is seen in fig. 18. Intracellular edema persists to a mild degree throughout the epithelial layers with interspersed pyknotic nuclei. Widening of intercellular spaces is evident, especially above the basal layer. Some mitotic figures are seen in the basal cells. The connective tissue exhibits increased vascularity with areas of amorphous appearing collagen. Chronic inflammatory cells are prevalent throughout the connective tissue. The epithelium is mildly acanthotic.

Fourteen and fifteen day post-frozen grafts

At fourteen days, Korman⁴⁰ describes the upper layers of epithelium as showing large globules of eosinophilic material (fig. 19), which probably stem from a coalescence of the eosinophilic material described at day ten. The

stratum spinosum and basal layer are well defined. Mitotic activity is seen in the basal layer (fig. 20). The connective tissue continues to be young and proliferative, with many capillaries and sub-acute inflammatory cells present.

Clinically, at fifteen days, the graft site is partially covered with areas of granulation tissue. More granulation tissue than seen at twelve days is present, and represent the only areas left of inflammation; for the remainder of the graft is healthy in appearance with graft margins difficult to distinguish (fig. 21).

Nineteen day post-frozen graft

Clinically, the graft is well attached to the underlying and surrounding tissues. Some blebs of granulation tissue still prevail. The graft is now smooth in texture and healthy in color. Palatal rugae formation can be observed in the midline (fig. 22).

Histologically at nineteen days, the epithelium shows signs of mild dyskeratosis (fig..23). Pyknotic nuclei and signs of intracellular edema are still prevalent. Parakeratosis in the stratum corneum is observed. Mitotic figures are still numerous in the basal cell layer (fig.24). In several areas, the basal lamina appears to "dip" between basal cells and continue into the intercellular spaces (fig. 24, arrow). The connective tissue shows less

inflammatory cells than seen at fourteen days. However, amorphous appearing collagen is still present.

Twenty three day post-frozen graft

Clinically, the graft appears well healed and blended into the surrounding tissue (fig. 25). Good differentiation of cell layers throughout the epithelium is present histologically, but some dyskeratosis still prevails. Basal cells still show numerous mitotic figures. The connective tissue shows some signs of chronic inflammation (fig. 26).

Thirty day post-frozen graft

At thirty days, healthy palatal tissue lies in the mandibular vestibule surrounded by healthy appearing oval mucosa and distinct rugae formation can be detected (fig. 27).

Histologically at thirty days, the epithelium appears normal with all cell layers intact in the absence of pathologic changes (fig. 28). Parakeratosis of the stratum corneum, keratohyaline granules in the stratum granulosum, and well developed spinous and basal cell layers are all evident. The basement membrane is well defined. The connective tissue is still young though less cellular and vascular than the fourteen day specimen. Few inflammatory cells are present.

B.. Sequential Ultrastructural Healing Study

Zero day fresh graft

An electron micrograph of fresh palatal tissue shows epithelium at the level of stratum spinosum (fig. 29). This polyhedral shaped cell presents a normal architecture. The nucleus is surrounded by an intact double membrane (NM). The chromatin (cr) pattern is well organized. The cytoplasm (C) is rich in ribosomes (r) and tonofilaments (t), which coalesce into tonofibrils (T). Numerous round mitochondria (m) with well defined cristae (ci) seem to be more concentrated in the perinuclear regions. Only a few profiles of granular endoplasmic reticulum (er) can also be observed in the cytoplasm. The plasma membrane (P) is well preserved, showing numerous interdigitations between adjacent spinous cells, and abundant attachments including desmosomes (d) and tight junctions (tj), all morphologically intact.

Fig. 30 shows a higher magnification of a spinous epithelial cell from the fresh graft. Tonofibrils (T), ribosomes (r), and mitochondria are unremarkable. The latter are surrounded by intact membranes and contain well preserved cristae (ci). Desmosomes (d) showing the typical lamellar structure are seen paralleling adjacent cell membranes. Tonofilaments (t) converge upon the attachment

plaques of the desmosomes in a perpendicular direction. Several tight junctions (tj) also serve as attachments in the intercellular space.

The epithelio-connective tissue junction with a portion of one basal cell is observed in fig. 31. The basal cell is clearly separated from the underlying connective tissue by the undulating, somewhat homogeneous, basal lamina (bl). Numerous hemidesmosomes (hd) project into the basal lamina on the epithelial side. The basal cell shows a normal cytoplasmic array of free ribosomes (r) and tonofibrils (T). Several round mitochondria (m) are present adjacent to a profile of dilated endoplasmic reticulum (er). On the connective tissue side, numerous transversally cut collagen bundles (Co) are evident, as well as fragments of a few fibroblasts (F).

Fig. 32 shows a low power magnification of fresh palatal connective tissue. Depicted in the center of the electron micrograph is a fibroblast (F) cut in cross section. It shows a normal nucleus (N) and chromatin pattern (cr) with an intact double nuclear membrane. Numerous mitochondria (m) are present in the cytoplasm (c) with well preserved cristae (ci) formations. Ribosomes and fibrillar elements are dispersed throughout the cytoplasm. The plasma membrane (P) is intact. Adjacent to the

fibroblast are collagen bundles (Co) cut in tangentially and in cross section.

Zero day post-frozen graft

Fig. 33 is a low power electron micrograph of epithelial cells at the level of stratum spinosum. The nuclei (N) of these cells appear shrunken and pyknotic, with a low nuclear-cytoplasmic ratio. They are in different states of preservation. The nucleus of cell #1 has a double nuclear membrane which appears to have undergone cytolysis. The chromatin (cr) is abnormally clumped and part of the nuclear contents appears to have extruded into the cytoplasm. The nucleus of cell #2 shows similar types of chromatin abnormalities, but with better preservation of the nuclear membrane (NM).

The cytoplasm of these epithelial cells show obvious changes in the organelles. All cells clearly demonstrate disrupted profiles of endoplasmic reticulum (er). Some mitochondrial outlines (m) are observed with no evidence of organization or cristae. Areas of clumped ribosomes (r) and tonofibrils (T) are also evident. There is a difference of staining quality between cells, with cell #2 being obviously more osmiophilic. The cytoplasm of cell #2 is in general, more homogeneous in nature. The intercellular spaces between some cells are widened, and

show a decreased number of desmosomes in areas (arrows).

Fig. 34 shows a high magnification of an epithelial cell at the spinous level, with a fragment of an adjacent cell. Abnormalities throughout the cytoplasm and nucleus are similar to those described for fig. 33, with more easily discernible clumping of their contents. A distinction between chromatin clumps and nucleoli cannot be made within the nucleus. Outlines of mitochondria (m) lack any cristae structure. Clumping of ribosomes (r) and tonofibrils (T) is more pronounced in the more osmophilic cell (#1). Some desmosomes (d) seem to have a well preserved lamellar structure with convergence of tonofilaments (t) onto their respective attachment plaques. Other desmosomes, however, have undergone degeneration (arrow).

Fig. 35 is a high power electron micrograph of the intercellular space between spinous epithelial cells. Most obvious are the quite distinct desmosomes (d) with well preserved architecture. A lamellar type structure with seven distinct zones is clearly demonstrated. A well preserved tight junction (tj) may also be observed. The intercellular spaces (ics) are markedly widened.

Fig. 36 is a low power view of connective tissue. Several fragments of fibroblasts (F) are seen containing

sharply defined nuclei (N), but no clear evidence of intact plasma membranes. The nuclear morphology of these cells is quite disrupted with clumping of chromatin similar to that seen in the epithelial cells (arrow). One cell shows a distinguishable nucleolus (n). Cytoplasmic organelles are scattered in a haphazard fashion among and between cells with disruption of their normal architecture and continuity. There is evidence of inter- and intracellular edema. Collagen bundles (Co) cut both transversely and longitudinally, are interspersed throughout. They are fairly well preserved, but sparse. Fragments of a degenerated macrophage (M) are also observed.

Two day post-frozen graft

An epithelial cell from the stratum spinosum is seen in fig. 37. The nucleus (N) is pyknotic but intact with a fairly well preserved double nuclear membrane. Perinuclear edema can be observed. Clumping of chromatin (cr) is evident, especially adjacent to the nuclear membrane. Numerous mitochondrial shells (m), lacking cristae or internal structure, are observed, especially in the perinuclear regions. Some disrupted profiles of endoplasmic reticulum (er) are also seen. Tonofibrils (T) are distributed throughout the cytoplasm and remain in a fairly good state of preservation. The ribosomes (r) are extremely

sparse. The intercellular spaces (ics) are widened with some well preserved attachment devices. A difference in staining quality is noted between cells #1 and #2.

Fig. 38 is a low power electron micrograph of connective tissue at day two. Collagen fibers (Co) cut transversely and longitudinally are sparsely interspersed throughout. Most notable is a clearly distinguishable capillary (Ca) with a well preserved endothelial cell (E). The nucleus (N) of this cell is normal in appearance, with double membrane and well preserved chromatin pattern. In the lumen of the capillary, there are remnants of degenerated blood cells. Extravasated into the connective tissue space are two poorly preserved neutrophils (Ne). Two fibroblasts (F), with abnormal nuclear and cytoplasmic architecture, can be seen lining the capillary.

Fragments of two fibroblasts (F) can be seen in fig. 39. Clumping of chromatin in both nuclei (N) as well as homogeneous cytoplasmic contents can be observed. Both cells show a clear zone partly surrounding the nucleus representing perinuclear edema. No mitochondria or profiles of endoplasmic reticulum are observed. In cell #2 there are two large vacuoles containing osmiophilic material. These structures are consistent with "lipid bodies (LB)."

Five day post-frozen graft

Fig. 40 is a low power view of epithelial cells at the spinous level. Signs of intercellular and perinuclear edema are still evident at this stage. The nuclei (N) exhibit a partly well preserved double nuclear membrane (NM), but still show clumping of chromatin, especially adjacent to the nuclear membrane (arrow #1). The cytoplasmic contents are still poorly preserved. Empty vesicles which are compatible with mitochondrial remnants (m), and scattered profiles of swollen endoplasmic reticulum (er) can be seen. Only a few, short tonofibrils (T) can be distinguished. Clumps of a granular material (arrow #2) can be seen throughout the cytoplasm. At this magnification, the structure of these clumps is not clear, but we might suspect a ribosomal origin. The intercellular spaces are only slightly widened and fairly good preservation of desmosomes is detected.

Fig. 41 is a medium power view of the epithlio-connective tissue junction at day five. The basal lamina (bl) is well defined with poorly preserved hemidesmosomes (hd) spaced irregularly along its epithelial border. Collagen bundles (Co) in cross-cut and longitudinal sections are seen in the connective tissue and are in a poor state of preservation. On the epithelial side, the inter-

cellular spaces (ics) are still widened, but desmosomes, although few in number, appear intact. The nuclei and cytoplasmic contents show clumping and degeneration.

Fig. 42 is an electron micrograph of a fibroblast (F) seen at medium power. The nucleus (N) is well defined and intact with some perinuclear edema evident. It is difficult to distinguish nucleoli from clumped chromatin material (arrow) within the nucleus. Cellular organelles as granular endoplasmic reticulum (er) and mitochondria (m) are disrupted, showing signs of degeneration. Clumping of cytoplasmic ribosomes is quite evident. The cell is well outlined by an intact plasma membrane (P). Collagen fibers (Co) and dispersed elements of degenerated cells surround the fibroblast.

Nine day post-frozen graft

A low power magnification of transversely cut basal epithelial cells (epithelial ridges) is shown in fig. 43. These cells are surrounded by a distinct basal lamina (bl). Well defined plasma and nuclear membranes are evident. Clumping of chromatin (cr) within the nucleus is less pronounced than that seen in the five day specimen. Mitochondria (m), which are few in number, still show signs of disruption. A section of one neutrophil (Ne) in a fairly good state of preservation is seen.

Collagen bundles (Co), cut in various sections, are evident in the connective tissue in between the epithelial ridges. Myelin bodies (mb), representing whorls of disrupted membranes, are present within the cytoplasm.

Fig. 44 shows transversely cut epithelial cells from rete ridges and a fibroblast (F). Within the fibroblast the cytoplasm appears to be rich in profiles of dilated endoplasmic reticulum (er), and a few mitochondria (m) in various degrees of preservation are present. Transversely cut collagen (Co) bundles are interspersed between all cells. The epithelial cells are surrounded by a distinct basal lamina (bl), however, no hemidesmosomes can be distinguished. Only one epithelial cell (#1) showed a section of its nucleus, which contained clumped chromatin. The plane of section of this particular cell must have included part of the nucleus and the underlying cytoplasm as well, since degenerated membranes and a "lipid body (LB)," which are normally seen in the cytoplasm, are seen in the center of the nucleus. The cytoplasm of this cell demonstrates a few profiles of dilated granular endoplasmic reticulum (er) and cross sections of what appear to be smooth endoplasmic reticulum (ser). The latter structures are distributed throughout the cytoplasm. The cytoplasm of the other epithelial cells show lipid bodies (LB), myelin bodies (mb), numerous ribosomes, tonofila-

ments (t), and granular endoplasmic reticulum (er).

Twelve day post-frozen graft

Fig. 45 is a low power magnification of basal epithelial cells and their junction with connective tissues at twelve days. The nuclei are of normal size and contour, showing a double nuclear membrane and nucleoli (n). The chromatin (cr) material is clumped only in a few areas and appears fairly well organized. The cytoplasm appears homogeneous at this magnification with evidence only a few organelles. Mitochondria with some apparent inner-structure can be seen. Interspersed throughout the cytoplasm are numerous dark staining areas appearing to be clumps of tonofibrils, which can often be seen in close association with what appears to be desmosomes. Areas of probable desmosome (d) formation are intermittently spaced along the plasma membranes. There is very little interdigitation between cells. The intercellular spaces (ics) are filled with a homogeneous, osmiophilic substance. This substance appears similar in consistency to the basal lamina (bl); both the basal lamina and the intercellular substance appear to be continuous. The underlying connective tissue is barely distinguishable in this micrograph.

A high power electron micrograph of basal epithelial

cells at the level of the intercellular space is represented in fig. 46. The cytoplasm (C) of the epithelial cells is relatively homogeneous, with an increased number of tonofilaments (t), and few ribosomes (r). A most interesting finding, and not previously reported in palatal tissue, is the formation of vesicles (v) lining the plasma membrane. They measure approximately $600\text{\AA}$ in diameter. The vesicles are interrupted by areas of increased electron density, where the beginning of desmosome formation is suggested (arrow #1). These vesicles could be related to those found in the nine day specimen, although the latter were dispersed throughout the cytoplasm (fig. 44). In the otherwise homogeneous intercellular spaces (ics), increased areas of electron density are noted; corresponding to the electron dense areas in the cytoplasm of adjacent cells (arrow #2). An electron dense line running through the central portion of the intercellular space can be distinguished in one instance (arrow #3). This line may correspond to the "intercellular contact layer" of the desmosome, as described by Listgarten.⁵

Fig. 47 is an electron micrograph of the epithelio-connective tissue junction at 12 days. The epithelial cell nucleus is similar to those described in fig. 45.

In this electron micrograph, the above mentioned vesicular formations (v) are seen lining the plasma membrane adjoining the basal lamina (bl). Similarly, vesicles cease to exist where electron dense areas in the cytoplasm abut the plasma membrane. This suggests the beginning formation of hemi-desmosomes. The basal lamina is quite distinct and homogeneous. On the connective tissue side, longitudinal sections of collagen bundles (Co) can be observed in a few areas displaying distinct periodicity. More superficial layers of an amorphous and indistinct substance are noticeable.

Fig. 48 is a tangential section of a fibroblast within the connective tissue of the 12 day post-frozen graft. Most prominent are the two nucleoli (n) within the nucleus. The chromatin material shows areas of condensation and clumping, primarily adjacent to the nuclear membrane. A double nuclear membrane is intact along with a well-defined plasma membrane (P). The cytoplasm shows numerous profiles of dilated endoplasmic reticulum (er), and a few mitochondria in a poor state of preservation. Numerous ribosomes are also present throughout the cytoplasm.

Fifteen day post-frozen graft

Several cells at the level of the stratum spinosum

are seen under low power in fig. 49. The nucleus (N) with double nuclear membrane and chromatin material are well preserved. Some areas just adjacent to the nuclear membrane show a moderate increase in electron density of chromatin (arrows). Prominent nucleoli (n) are observed in one cell. The cytoplasm is rich in ribosomes and tonofibrils which appear well organized. Numerous mitochondria (m) in different states of preservation (or regeneration) line the nuclear membrane. Some show evidence of cristae formation while most have a vacuolated appearance. No profiles of endoplasmic reticulum can be distinguished at this magnification. The intercellular spaces (ics) show marked widening with numerous well defined desmosomes.

Fig. 50 represents a transverse cut across several basal cells apparently at the level of an epithelial ridge. The nucleus of one cell is quite distinct (N), showing an intact double nuclear membrane and clumping of chromatin material, especially notable along the nuclear membrane. The cytoplasm of these cells appears complex with numerous organelles. Small vesicles (approx. $600\text{\AA}$ in diameter) are noted to be interspersed throughout the cytoplasm in all the cells (v). These vesicles appear similar to profiles of smooth endoplasmic reticu-

lum. Similar vesicles line up along the plasma membranes (P). As seen in the twelve day sections, when areas of electron density appear along the plasma membranes -- representing possible desmosomal formation -- the vesicles disappear (arrow #1). In many instances, the above described vesicles are intimately associated with the granular endoplasmic reticulum (arrow #2). The amount of endoplasmic reticulum is increased in these cells when compared to that seen in normal epithelial cells.

Poorly preserved mitochondria (m) and several lipid bodies (LB) are also seen in different areas of the cytoplasm. The intercellular spaces (ics) are widened and filled with a homogeneous material assumed to be the basal lamina (bl). Some areas of hemidesmosomal formation (hd) are noted.

The epithelio-connective tissue junction is seen in fig. 51. The basal cells show increased amounts of rough endoplasmic reticulum and vesicles as described in fig. 50. However, more areas of condensation of fibrillar elements (presumably the beginning formation of attachment devices) are evident along the plasma membranes (P) and overlying the basal lamina (bl). Subjacent to the basal lamina is a fibrillar type material, possibly representing pre-collagen (pCo).

Nineteen day post-frozen graft

Fig. 52 shows a low power magnification of epithelial cells at the spinous layer. The nucleus (N) seen is well preserved with normal appearing nucleoli (n) and double nuclear membrane (NM). The chromatin material shows a normal distribution. Throughout the cytoplasm (C) there is an abundance of tonofibrils (T) and ribosomes (r). Only a few mitochondria (m) can be observed at this power. There are numerous normal appearing and moderately widened intercellular spaces (ics).

Fig. 53 is a high power electron micrograph of the intercellular space at the level of stratum granulosum. At this level, desmosomes (d) are quite distinct with a clearly defined lamellar architecture. A thick condensation of tonofibrils abut the attachment plaques of the desmosomes. In several areas (arrows) the tonofilaments (t) are gathered into dense fibrils, taking on somewhat of an organized structure. A keratohyaline granule (khg), typical of this layer is also noted.

After numerous preparations of tissue from nineteen days, no sections of connective tissue elements or basal cell layer were obtained.

Twenty-three day post-frozen graft

Fig. 54 is an electron micrograph of a spinous

epithelial cell. The nucleus (N), chromatin material (cr) and double nuclear membrane are intact, showing good organization. The ribosomes are plentiful and dispersed more uniformly than in the nineteen day specimen. Mitochondria (m) are numerous, showing good inner structure with cristae formation. Granular endoplasmic reticulum (er) is sparse and still lacking clarity. The intercellular spaces with numerous desmosomes (d) and some tight junctions (tj) are normal in appearance.

The epithelio-connective tissue junction is represented in fig. 55. The nucleus of cell #1 shows some signs of degeneration, noted by clumping of chromatin material (cr). The nucleus of cell #2 is more normal in appearance. Tonofibrils, ribosomes and mitochondria (m), in different states of preservation, fill the cytoplasm of these cells. Especially notable are numerous profiles of granular endoplasmic reticulum (er) seen in cell #1. The plasma membranes are intact and well defined. Interposed between these two epithelial cells and the basal lamina (bl) is another epithelial cell (#3), cut longitudinally. Vesicles (v), similar to those seen at fifteen days are noted throughout the cytoplasm of these three cells. In some areas they line the plasma membranes. The basal lamina is homogeneous and is lined

on the epithelial side by irregularly spaced hemidesmosomes (hd). On the connective tissue side there are cross-cut sections of collagen fibers (Co) which appear slightly degenerated.

Thirty day post-frozen graft

Fig. 56 is a low power electron micrograph of spinous epithelial cells. Both cells show well defined nuclei with double nuclear membrane and well organized chromatin (cr). In one cell, a well preserved nucleolus (n) is evident. The cytoplasm of each cell shows a normal pattern of ribosomes and tonofibrils. Mitochondria (m) are numerous, however, some lack cristae formation. The intercellular spaces are within normal limits and show numerous interdigitations with adjacent cells. Desmosomes (d) are well preserved but fewer in number than those seen in previous biopsies.

Fig. 57 depicts a fibroblast within the connective tissue of a thirty day post-frozen graft. It is well preserved and shows an intact nucleus (n) and well-defined nuclear membrane. Cytoplasmic organelles, including mitochondria (m), and rough endoplasmic reticulum (er) are clearly discernible and in a good state of preservation.

CHAPTER V

DISCUSSION

Glycerolization, freezing, thawing, deglycerolization, and subsequent autogenous transplant of palatal tissue is a successful procedure, as indicated in our studies by the maintenance of a viable donor graft in its recipient bed. This procedure, however, is not without damage to cells and interstitial tissue. Ultrastructural studies of fresh glycerolized and deglycerolized cells reveal disruption of most cellular membranes with extrusion of cellular contents, swelling, and disruption of cellular organelles; and homogenization or coarse clumping of chromatin material and other protein constituents. These alterations have been confirmed by other investigators.^{40,59,62,64-67}

Freezing damage to both connective tissue and epithelial cells was quite evident in our zero day post-frozen grafts, although it appeared in varying degrees even among neighboring cells. Disruption of the cell nuclear membranes in addition to homogenization and clumping of cell proteins (ribosomal and chromatin) as we have observed, has been explained by several theories. At slow cooling rates, as we utilized, ice crystal formation is restricted to extracellular spaces.^{62,70}

Litvan⁶⁶ provides an explanation for the damage we have observed. He assumes that during the freezing process, intracellular water remains liquid-like (supercooled) below 0°C. and therefore its vapor pressure is greater than that of ice. Upon cooling, extracellular ice forms and a vapor pressure difference is created. This initiates extracellular water movement, thus creating a nonequilibrium state within the cell. Litvan⁶⁶ suggests that equilibrium is restored by the gradual release of bound water (desorption) and subsequent excretion of the free water from the cell. The chief cause of cellular damage is probably due to dehydration and consequent denaturation. Glycerol, by increasing the viscosity of intra- and extracellular fluids, improves survival rate by diminishing the water migration rate and changes in partial pressures.

Mazur⁶⁵ similarly theorizes that intracellular water remains undercooled as the temperature is lowered below 0°C. and recognizes resulting changes in vapor pressure. He suggests that equilibrium is restored by water migration out of the cell leading to increased intracellular salt concentration that causes protein denaturation and cellular damage.

Considerable ultrastructural damage probably occurred

during glycerolization of the tissue.^{71,72} Since glycerol freely permeates the cell membrane, it changes the osmotic gradient and the elastic forces seeking to return cellular membranes to their original position.⁷² If the osmotic gradient is too great, rupture of membranes may occur. Certainly this can account for distention of mitochondria and other membrane bound organelles seen in our study (fig. 37).

Sherman⁶⁷, in his studies of cryoinjury to mitochondria of mouse renal cortex, found freezing damage in the form of mitochondrial swelling, change to globular shape, precipitation and loss of matrix, loss of dense granules, and irregularity and loss of cristae. Similar damage to all mitochondria was not observed. He suggests, concurring with findings by other authors,^{60,73} that differential sensitivity to factors causing cryoinjury varies from animal to animal, organ to organ, tissue to tissue, cell to cell, and even between cells of the same type within the same animal. He also showed that pretreatment with cryoprotectives alone before freezing produced alterations, primarily in reduction of cristae.

Using osmotic shock, streptolysins, and cycles of freezing and thawing, Bendall and deDuve⁷⁴ found lysosomal membranes more sensitive to this type of insult than mito-

chondrial membranes. Mellors⁷⁵ and co-workers showed that when lysosomal membranes are injured, their hydrolytic enzymes are released with subsequent damage to mitochondria in the form of swelling, uncoupling of oxidative phosphorylation, and coincident physical alterations in cristae and matrix. Furthermore, Allison and Paton⁷⁶ have shown that activated lysosomal enzymes, released when lysosomes are injured within cells, are capable of damaging chromosomes as well as mitochondria. Certainly, our post-frozen grafts showed ultrastructural changes as described above.

Nuclear damage with clumping of chromatin, in addition to cytoplasmic ribosomal damage, as we noted in all post-frozen cells, can be accounted for by denaturation of protein from increased salt concentrations, action of hydrolytic lysosomal enzymes or desorption of "bound" water -- all mentioned above. This damage, however, was apparently reversible, for at 9 days, nuclear chromatin clumping appeared less severe. More granular endoplasmic reticulum was also evident at this time; although still in a disrupted state, ribosomal lining of these membrane-bound organelles was more distinct and organization more evident. Complete integrity with a normal appearance of these structures varied in different tissue layers at different stages throughout the healing process.

Sequential healing studies of frozen, thawed and grafted oral mucosa, performed by Korman⁴⁰ at the light microscopic level, showed signs of morphologic regeneration in seven days. This was observed at the level of the stratum basale, the cells of which showed architectural integrity and numerous mitotic figures. The upper levels of epithelium, however, presented signs of degeneration through fourteen days. Our five day ultrastructural survey also showed signs of degeneration while our nine day specimens revealed the beginning of a regenerative process. We must then assume that the mucosa begins its recovery phase from freezing damage at least one week after the grafting procedure.

It must be emphasized that with an ultrastructural study as we have performed, a concomitant light microscopic analysis must be carried out. The ultra-high magnification provided to us by the electron microscope enables us to observe more intricate changes within each cell, e.g., membrane structures, integrity and position of organelles, and minute alterations of nuclear and cytoplasmic contents. On the other hand, light microscopy gives us a broader picture of changes within groups of cells and neighboring tissues. Specific degenerative processes, cellular damage and timing of repair may then

differ by each method of analysis.

In our ultrastructural studies, the initial signs of recovery were evidenced as early as nine days by a decrease in the clumped appearance of both ribosomes and chromatin material. These signs became more pronounced by 15 days, at which time abnormally large amounts of granular endoplasmic reticulum were observed in the basal epithelial cells. This is compatible with a recovered potential of protein synthesis.

The vesicular formations observed at 12, 15, and 23 days were only found in basal cells. The highest concentration of these vesicles was found in and about the increased profiles of granular endoplasmic reticulum at 15 and 23 days, and along the plasma membranes at days 12 and 15. The vesicles lining the plasma membranes may play a precursor role in intercellular adhesion. This is more likely when we realize the increased amount of plasma membrane surface area provided by the vesicles (fig. 46). Further substantiation of this phenomenon can be seen where these vesicles disappear and a more sophisticated type of intercellular attachment - in the form of a desmosome - develops. It may also be postulated that these vesicles later develop into the numerous interdigitations observed between mature cells, creating

additional bonding.

The vesicular structures which appeared in the cytoplasm of the basal cells at days 12, 15, and 23 were interspersed, and without an organized distribution. At times they were observed in close association with small, longitudinally cut tubules. It is possible that these vesicles represent profiles of smooth endoplasmic reticulum cut in cross section and consequently play a role in some type of transport or storage mechanism of protein.

Sandborn et al ⁷⁷, in their investigations upon numerous types of rat tissue, found cytoplasmic microtubules (cross-cut sections appearing as small vesicles) in all tissues examined. The description of these tubules was similar to those seen in our study. They were found throughout the cytoplasm, often in close association with cytoplasmic organelles, and frequently in contact with the plasma membranes. They suggest several possible functions of these microtubules: 1) supportive; 2) contractile; and 3) serving to transport fluids and suspended solids. They conclude that these microtubules may serve as a connecting channel between cytoplasmic organelles and the plasma membrane, creating a continuous membrane system. This concept of a microcirculatory system, functioning as a transport device between organelles and

plasma membrane, seems applicable to the basal cells we observed in an active state of regeneration.

It may be suggested in several of our fifteen day specimens (figs. 50 and 51) that the cells we term basal cells are in fact clear cells within the basal layer, as described by Thilander.⁷ What appear to be melanosomes and numerous vesicular structures are dispersed throughout the cytoplasm of these cells. However, clear cells lack sophisticated intercellular attachments as desmosomes. Comparing these cells with clearly defined basal cells in the twelve and twenty-three day specimens (figs. 47 and 55), similar desmosomal formations are evident along the plasma membranes where electron dense structures appear when the vesicles lining the plasma membrane cease to exist. With apparent desmosomes evident along the plasma membranes we must then assume that these are in fact basal cells, possibly with melanin imparted to them from adjacent clear cells, as suggested by Zelickson.⁶

Our basal cells from days nine to twenty three, showed few interdigitations between cells which were separated by wide spaces filled with a homogeneous type of cementing substance. This intercellular substance was frequently continuous with the basal lamina. Increased demand for nutrient supply by actively regenerating

basal cells may also account for these findings. Widened intercellular spaces in direct continuity, via the basal lamina, with the underlying connective tissue and capillary endings would serve to facilitate transport of metabolites and increased levels of oxygen to these cells.

The lack of interdigitations between cells and increased amounts of intercellular mucopolysaccharides could also serve to facilitate the sliding of cells past one another. Irreversibly damaged cells at higher levels requiring replacement would possibly dictate a more rapid migration of cells from lower levels.

At 23 days, ultrastructural regeneration was still evident in the basal cells. Spinous cells at this time appeared normal with only a mild degree of clumped chromatin material and abnormal concentrations of ribosomes in the cytoplasm. The intercellular spaces still appeared widened to a moderate degree. Light microscopic sections from this stage of the healing process appear almost normal with widened intercellular spaces the only evidence of abnormality. Both ultrastructural and light microscopic findings at 30 days were indistinguishable from normal keratinized oral mucosa.

In our clinical studies, graft adaptation and take was clearly evident seven days post-operatively (fig. 12).

This concurs with findings by other authors.^{39,40} At 19 days, complete clinical healing was not evidenced in our study while other authors,³⁹ utilizing similar grafts, report complete clinical healing at 14 days. We must attribute the delayed healing response of our grafts to tissue injury from the glycerolization, freezing and thawing processes.

CHAPTER VI

SUMMARY

The aim of this project was to study, on an ultrastructural level, the sequential healing phenomena which take place after freezing and thawing human palatal autografts. Palatal free grafts were obtained from four human subjects for use in mandibular vestibuloplasty. Two small biopsies of each graft were taken immediately after procurement and were labelled fresh tissue controls. All biopsies were prepared for both light and electron microscopy. The remaining tissues were then glycerolized, frozen in liquid nitrogen at controlled rates, and stored for approximately one month. The tissues were then thawed, deglycerolized and transplanted autogenously to the anterior mandibular region for the purpose of vestibular extension. Immediately prior to transplantation, small biopsies were again taken from each segment of tissue. These were designated zero day post-frozen grafts. Two biopsies were taken alternatively from each patient at 2, 5, 9, 12, 15, 19, 23 and 30 days.

All grafts were successful with complete clinical healing evident at 23 days. A sequential study by light microscopy showed initial tissue damage from freezing with complete repair by 23 days. The sequential untrastructural

study also revealed initial cryoinjury to cells, but on a more intricate level involving specific cellular elements. Complete regeneration was not evident by 23 days, however, at the ultrastructural level. Spinous cells at thirty days appeared normal, however basal cells from the thirty day specimen were not seen. There is indication for further investigation to determine completion of regeneration on the ultrastructural level. The electron microscopic technique has served to more intricately elucidate regeneration of frozen and thawed tissue at the cellular level.

CHAPTER VII

CONCLUSIONS

Human palatal mucosa may be glycerolized, frozen, stored, thawed and autogenously transplanted with success. Although disruption and tissue damage was observed both on a light and electron microscopic level, this was not clinically significant as all grafts were successful.

Ultrastructural and light microscopic changes observed in our biopsy specimens were attributed to glycerolization, freezing and thawing processes. As evidenced primarily by our ultrastructural study, regeneration of grafted epithelium was effected via the basal cell layer. The formation of vesicular structures and alterations in both the basal lamina and intercellular substances may play a significant role in this regenerative process. The electron microscope has served to elucidate changes in regenerating cells not previously described by light microscopy.

CHAPTER VIII

ILLUSTRATIONS

Table of symbols for illustrations:

C	-	cytoplasm
Ca	-	capillary
ci	-	cristae
Co	-	collagen
cr	-	chromatin material
CT	-	connective tissue
E	-	endothelial cell
er	-	granular endoplasmic reticulum
F	-	fibroblast
hd	-	hemidesmosome
ics	-	intercellular space
LB	-	lipid body
M	-	macrophage
m	-	mitochondria
mb	-	myelin body
N	-	nucleus
n	-	nucleolus
Ne	-	neutrophil
NM	-	nuclear membrane
P	-	plasma membrane
pCo	-	pre-collagen
r	-	ribosomes
ser	-	smooth endoplasmic reticulum
T	-	tonofibrils
t	-	tonofilaments
tj	-	tight junction
v	-	vesicles

Fig. 1

Preoperative Surgical Site

Fig. 2

Donor Tissue from Palate

Fig. 3

Recipient bed before
placement of graft

Fig. 4

Acrylic stabilization
splint

Fig. 5

Graft sutured to place in
recipient bed

NOTE: hole provided in splint
for obtaining early biopsy
material

Fig. 6

Acrylic splint in place

Note: hole provided in splint
for obtaining early biopsy
material

Fig. 7

0 day biopsy (100X)

Note: generalized hydropic
degeneration with perinuclear
edema

Fig. 8

2 day graft

2 day graft

Note: good appearance of stratum
keratinized epidermis spinosum and
papillary dermis basal lamina

Fig. 9

2 day biopsy (250X)

Note: pale appearance of stratum basale and stratum spinosum and amorphous disrupted basal lamina

Fig. 10

5 day graft (100X)

Note: marked degeneration of
epithelium with increased fi-
broblastic activity

Fig. 11

5 day biopsy (250X)

Note: extreme perinuclear edema
with numerous light staining
nuclei

Fig. 12
7 day graft

Note: graft intact amidst an
abundance of granulation
tissue

Fig. 13

7 day biopsy (250X)

Note: mitotic figures in basal
layer and foreign body giant
cells in connective tissue

Fig. 15

7 day graft

Note: areas of

Fig. 14

7 day biopsy (100X)

Note: disruption in maturation
of epithelium and highly proli-
ferative connective tissue

Fig. 15
9 day graft

Note: areas of
granulation tissue in
basal layer

Fig. 16
10 day biopsy (250X)
Note: mitotic activity in
basal layer

Fig. 17

12 day graft

showing intracellular edema
and swelling of intracellular
spaces

Fig. 18
12 day biopsy (40X)
Note: intracellular edema
and widening of intracellular
spaces

Fig. 19

14 day biopsy (40X)

Note: large globules of eosinophilic
material in immature epithelium

in basal cell layer

Fig. 20
14 day biopsy (250X)
Note: mitotic activity
in basal cell layer

Fig. 21
15 day graft

Note: areas of granulation
tissue about graft

Fig. 22

19 day graft (100x)

Note: blebs of granulation tissue

Fig. 23

19 day biopsy (100X)

Note: mild dyskeratosis
and intracellular edema

between basal cells (arrow)

Fig. 24

19 day biopsy (250X)

Note: areas where basal
lamina appears to "dip"
between basal cells (arrow)

Fig. 25

23 day graft (100X)

Note: graft tissue is edema
blended well into the
surrounding oral mucosa layer

Fig. 26

23 day biopsy (100X)

Note: some perinuclear edema
still evident and numerous
mitotic figures in basal layer

Fig. 27

30 day graft (100x)

Note: palatal rugae formation in grafted tissue

basaloid of stratified squamous

Fig. 28

30 day biopsy (100X)

Note: normal appearing
epithelium with para-
keratosis of stratum corneum

Fig. 29
0 day fresh biopsy (16,800X)
Stratum spinosum

Fig. 30

0 day fresh biopsy (33,250X)

Stratum spinosum

Fig. 31

0 day fresh biopsy (33,250X)

Epithelio-connective tissue junction

Note: well-defined basal lamina

Fig. 32
0 day fresh biopsy (16,800X)
Fibroblast

Fig. 33

0 day post-frozen biopsy (16,800X)

Stratum spinosum

Note: difference in staining
quality between cells #1 and #2

Fig. 34

0 day post-frozen biopsy (33,250X)

Stratum spinosum

Note: clumping of chromatin
material and desmosomal degenera-
tion (arrow)

Fig. 35
0 day post-frozen biopsy (98,000X)
Stratum spinosum
Note: highly ordered structure of
desmosomes and tight junction

Fig. 36
0 day post-frozen biopsy (6,650X)
Connective tissue
Note: disrupted plasma membranes
of fibroblasts and remnants of
macrophage (M)

Fig. 37

2 day post-frozen biopsy (33,250X)

Stratum spinosum

Note: perinuclear edema and
widened intercellular spaces

Fig. 38

2 day post-frozen biopsy (6,650X)

Connective tissue

Note: Endothelial cell (E) and
Capillary (Ca)

Fig. 39

2 day post-frozen biopsy (16,800X)

Connective tissue

Note: 2 fibroblasts(F) lacking distinct organelles and exhibiting perinuclear edema(especially F₁)

Fig. 40

5 day post-frozen biopsy (6,650X)

Stratum spinosum

Note: different staining characteristics of the cells and clumping of chromatin material

Fig. 41

5 day post-frozen biopsy (16,800X)
Epithelio-connective tissue junction
Note: intact basal lamina with poorly
preserved hemidesmosomes(hd)

Fig. 42

5 day post-frozen biopsy (16,800X)

Fibroblast

Note: indistinguishable nucleoli
(arrow) and clumping of chromatin
material

Fig. 43

9 day post-frozen biopsy (6,650X)

Basal epithelium

Note: section traverses the epithelial ridges. Basal cells are surrounded by a distinct basal lamina(bl)

Fig. 44

9 day post-frozen biopsy (6,650X)

Basal epithelium

Note: basal lamina(bl) and rich profiles of granular endoplasmic reticulum(er) in basal cells

Fig. 45

12 day post-frozen biopsy (6,650)

Basal epithelium

Note: basal lamina(bl) is continuous with intercellular spaces(ics). Near homogeneous cytoplasm exists throughout the epithelial cells.

Fig. 46

12 day post-frozen biopsy (98,000X)

Stratum spinosum-intercellular space

Note: Presence of vesicles, apparent desmosomal formation (arrows 1 & 2), and beginning of possible intercellular contact layer (arrow 3)

Fig. 47

12 day post-frozen biopsy (33,250X)
Epithelial-connective tissue junction

Note: hemidesmosome formation(hd),
vesicles lining plasma membrane(v),
and smooth endoplasmic reticulum(ser)

Fig. 48

12 day post-frozen biopsy (16,800X)

Fibroblast

Note: nucleoli(n) and clumping of
chromatin(cr)

Fig. 89

15 day post-frozen biopsy (16,800X)

Stratum spinosum

Note: perinuclear organelles, widened intercellular spaces(ics), and condensation of chromatin material(arrows)

Fig. 50

15 day post-frozen biopsy (16,800X)

Basal epithelium

Note: desmosomal formation (arrow 1)
and association of vesicles (v) with
endoplasmic reticulum (arrow 2)

Fig. 51

15 day post-frozen biopsy (33,250X)

Epithelio-connective tissue junction

Note: Hemidesmosome formation(hd) and
pre-collagen(pCo) within the connective
tissue

Fig. 52

19 day post-frozen biopsy (6,650X)

Stratum spinosum

Note: widened intercellular spaces(ics)

Fig. 53
 19 day post-frozen biopsy (98,000X)
 Stratum granulosum-intercellular space
 Note: highly organized desmosomes(d),
 areas of tonofilament condensation,
 and keratohyaline granule(khg)

Fig. 54

23 day post-frozen biopsy (33,250X)

Stratum spinosum

Note: good organization of cellular components

Fig. 55

23 day post-frozen biopsy (16,800X)

Epithelial-connective tissue junction

Note: distinct basal lamina(bl), hemidesmosome formation(hd), and many profiles of endoplasmic reticulum(er)

Fig. 56
30 day post-frozen biopsy (16,800X)
Stratum spinosum
Note: few intercellular attachments

Fig. 57

30 day post-frozen biopsy (16,800X)

Fibroblast

Note: good organization of cellular components

CHAPTER IX
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APPROVAL SHEET

The thesis submitted by Robert A. Weinstein, 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 theses is, therefore, accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 18, 1973
DATE

Robert A. Weinstein
SIGNATURE OF ADVISOR