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CELLULAR RESPONSE TO TENSION IN HAMSTER BUCCAL EPITHELIUM

by

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Submitted in partial fullfillment of the requirement

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Abstract

by

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The objective of this study was to elucidate the relationship between epithelial mitotic activity and mechanical tension.

Twenty 30 or 31 day old male Golden Syrian Hamsters were used. They are randomly divided into four groups. Each group comprised five animals. In four of the five animals in each group, the left cheek pouch was stretched by the injection of fast-setting latex material. The contralateral pouch was left unstretched as control site. The fifth animal in each group was sham-operated by suturing the left pouch opening. The

contralateral side was left untouched.

The animals were subjected to two, four and seven days of mucosal stretching. In one group the experimental period was ten days but tension was released on the seventh experimental day, thus allowing for a three day recovery period.

In order to assess the mitotic rate of buccal epithelium, autoradiography was used by administration of tritiated thymidine twenty-four hours before sacrifice, in a dose of 1.0 μCi/gm of body weight.

Interpretation of data was based on inter- and intragroup comparisons and comparisons between unstretched and sham-operated animals regarding (1) epithelial mitotic index, (2) epithelial thickness, (3) basal cell density.

The conclusion of my results is that mechanical tension decreased basal cell density, increased epithelial mitotic activity temporarily, but did not alter epithelial thickness significantly. The basement membrane might have lengthened during stretching thus providing increased space for newly formed cells. Following removal of the mechanical tension, there were significant signs of recovery. Basal cell density, epithelial thickness, and epithelial mitotic activity at experimental sides were similar to those of unstretched sides. The findings of this project suggest that mechanical tension is, under certain conditions, one stimulus by which the growth of capsular matrices

may be influenced.

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DEDICATED

to

Mrs. Hwang-Tang Chen

My mother, whose unselfish love and sacrifice have made my education possible. I will always love and thank you.

IN MEMORIUM

Chin-San Chen, M. D., Ph.D.

My father, who died on May 25, 1975. His kindness and generosity have been remembered by his patients and friends. I will always be proud to be your daughter.

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INTRODUCTION

At the present time orthodontic treatment of dentofacial anomalies is mostly empirical. However, a better understanding of the nature of growth control mechanisms will provide a more rationale approach to orthodontic therapy.

Researchers have attempted to elucidate these mechanisms by formulating and testing different hypotheses or theories. In 1960, Moss and Young proposed the functional matrix hypothesis, which was based on the previous works of van der Klaauw (1946, 1948, 1951, 1952). According to Moss (1969), bone is secondarily responsive to a chain of control factors targeted at contiguous tissues. The genetic and epigenetic determinants of craniofacial skeletal development resides within the functional matrix, which includes soft tissue capsules and the enclosed spaces, and not in the bone itself. Determined by functional demands, the primary volumetric increase of functional spaces is the result of a compensatory increase in the size of the capsular matrices. Growth of the enveloping capsule is produced by mitosis of both its epithelial and mesenchymal cellular elements and the consequent increase in intercellular materials, which results in an expansion of the capsule as a whole. Later, Moss (1971) suggested that epithelium has intrinsic properties to control the functioning spaces. Thus, growth of the facial skeleton is due to mitotic activity in the epithelial lining of the capsular

matrix. He has also proposed a mechanism to explain the developmental regulation of functional matrices (1971, 1972, 1975). The volumeric enlargement growth of functioning spaces, is the result of epithelial mitosis, neurotrophically regulated via afferent nerves. He argued that the primary site of genetic regulation resides within the central nervous system. Via neurotrophic processes, the CNS constantly monitors the genetically controlled metabolic activity and phenotypic expression of all innervated cells and tissues. Solow and Kreiborg (1975) have also suggested that growth and the ultimate size and shape of facial osseous structures are closely associated with the soft tissue draping. They hypothesized that relief of tension of soft tissues might potentially allow for alterations in skeletal craniofacial development.

Several types of appliances used in orthodontic treatment potentially affect the oral mucosa by generating biomechanical tension. Bite opening appliances stretch the buccal mucosa mainly vertically and palatal expanders stretch the palatal mucosa horizontally. Appliances which have buccal shields incorporated will stretch the mucosa three-dimentionally. The effect of such treatment procedures on non-skeletal tissue is little known. Based on the contention regarding the primary role of soft tissue in craniofacial growth, the aim of functional treatment is to affect soft tissue and thus secondarily hard tissue. It seems reasonable to argue that manipulating soft

tissue matrices ought to be a primary concern in the treatment of skeletal maloccusions. Mechanical tension may influence local control factors and stimulate epithelial mitosis. During certain abnormal growth condition, my supposition is that such stimulation can be therapeutically advantageous. Thus, the present experiment is in attempt to elucidate the cellular response to induced tension in buccal epithelium.

Statement of Thesis

The objective of this research project was to investigate the relationship between epithelial mitotic activity and mechanical tension.

In an experimental group of hamsters, mechanical tension was applied by the injection of fast-setting latex material into the cheek pouch. In order to assess mitotic rate of buccal epithelium, autoradiography was used by administration of a radioactive isotope (tritiated thymidine) twenty-four hours before sacrifice.

Data were collected regarding (1) epithelial mitotic index, (2) epithelial thickness, and (3) basal cell density. Interpretation of data was based on inter- and intragroup comparisons and comparisons between unstretched and sham-operated animals.

Literature Review

Epithelium is a unique tissue. It has no supporting stroma within itself and it may be subjected to considerable compressive or tensile forces without damage. This tissue maintains its cohesiveness by intercellular adhesion. The presence of folding cellular membranes and communicating intercellular channels (Tucker, 1968) and the microfilament system in epidermal cells (Cloney, 1969) makes the tissue deformable and hence resistent to applied forces. Microfilament bundles of epithelial cells are only found extending across the luminal and basal ends of the cells. In this respect they contrast desmosomal tonofilaments and microtubules, each of which can curve in a variety of directions throughout the cell (Wessell, 1971).

Gibson et al. (1965) stated that considerable extension of relaxed skin can take place with relatively little loading. During this phase, the fibers of the collagen network become successively aligned in the direction of the stretching force. When the majority of the fibers are arranged parallel to the line of stretch, further extension in that direction is resisted by the fibers themselves and very little additional extension is obtained, even with an increase in load. The elastic fibers in the dermis form a secondary network interconnected with that of collagen and probably act as stores of the energy required to return the collagen network to the relaxed state. Interstitial fluid is displaced from the network as the fibers are being re-

oriented. It has been shown that the epithelium makes no significant contribution to the mechanical properties and behavior of skin at high stress levels (Kydd, 1982). Presently, it is not known whether this is true also at low levels of stress.

Numerous experiments investigating factors that stimulate and regulate cell proliferation have been done.

Hormonal actions on mitosis have been demonstrated *in vivo* (Bullough, 1955; Waymouth, 1954; Fell, 1955) as well as *in vitro* (Allen, 1937, 1956; Burkhart, 1942; Drasher, 1952). There is agreement that insulin promotes proliferation while thyroid hormone inhibits growth. Based on work previously cited, Swann (1958) concluded that there are two main types of response to mitogenic hormones; short and long latent period responses. He suggested that long latent period response is an inductive effect. The latent period and cellular hypertrophy represent the period of switch-over and synthesis of mitotic protein and other materials for division. The short latent period response (e.g., epithelium), may be explained by a general stimulation of energy metabolism.

Somatomedin, a hormone-dependent growth factor in serum, has been shown to be able to stimulate the incorporation of amino acids into protein, uridine into RNA, and thymidine into DNA and also promote cell replication in HeLa cell cultures

(Salmon and Daughaday *et al.* , 1957, 1966, 1967, 1970, 1971). Van Wyk *et al.* (1973, 1975) hypothesized that growth hormone stimulates skeletal growth and protein anabolism through the mediation of somatomedins, which are potent insulin-like substances.

Based on the enhanced mitotic rate during wound healing, Abercrombie (1957) suggested the existance of a "wound hormone" that stimulates mitoses. This concept contrasts the works by Bullough (1961, 1962, 1972) on chalones, which are endogenous tissue-specific mitotic inhibitors. They inhibit epidermal mitotic activity by reducing the proportion of the basal cells that are in the mitotic cycle and the speed at which this cycle is completed. They also act to reduce the speed at which the distal post-mitotic cells age and die. Bullough formulated a negative feedback theory of mitotic control based on an epidermal chalone system. However, this theory still remains untested.

Prostaglandins (Kischer, 1967) and epidermal growth factors extracted from submandibular glands of adult male mice (Cohen, 1973) have been demonstrated to drastically increase mitotic activity and keratinization. Brophy (1959) reported that the first sign of regeneration of injured skin was a vast accumulation of glycogen in basal cell cytoplasm. It was only when this had subsided that mitotic activity took place. Later, Meyer *et al.* (1974) found that mitotic activity (1974) and

lactate dehydrogenase activity increased in buccal epithelium in Zinc-deficient rats. The role of cyclic AMP in local growth control mechanism was pointed out by Pastan (1972). He concluded that cyclic AMP is acting as a second messenger between a hormone and its effects within the cells and also controls the activity of genes.

Neurotrophism has been shown to be associated with the regulation of mitosis. Gutman (1964) defined neurotrophism as a non-impulse transmitting neural function that involves axoplasmic transport, and provides for long-term interaction between neurons and innervated tissues that homeostatically regulate the morphological, compositional, and functional integrity of those tissues. Overton (1950) placed Amphibian epidermis in culture with grafts of central nervous tissue, and observed a sharp rise in the mitotic index with a well defined latent period. Mione (1964) reported that Amphibian limb regeneration is initiated by epidermal proliferation, which occurs only after reinnervation and intimate neuroepithelial contact. Recently, Brockes (1984) has studied the mitotic growth factors and nerve dependence of limb regeneration. He claimed that a monoclonal antibody to new blastema cells has provided evidence that Schwann cells and muscle fibers contribute to the blastema, i.e. the progenitor cells of the regenerate, and identified blastemal cells whose division is persistently dependent on the nerve. The

findings of these investigations are very convincing, though the nature of the neurotrophic substances and the processes of their introduction into the target tissue are not determined at present.

In addition to the biochemical stimulation of mitoses, there is increasing evidence for local biomechanical induction of cell proliferation.

It has been suggested that the rates of cell formation and maturation play an important role in the adaptive response of the epidermis to mechanical stimulation (Mackenzie, 1974). Friction, a mitotic stimulus, results in epidermal thickening and increases both cell size and number of cells. Francis et al. (1977) applied rubefacient onto the ears of guinea pigs. They reported that hyperemia may contribute to the enhanced epidermopoiesis observed.

In vitro experiements focusing on the relationship between mitotic rate and tension have been reported. Maroudas (1972) attached single cells to individual beads of varying diameter, and placed them on a soft agar surface, over feeder cells. Based on photomicrographic data, Maroudas observed that cells on bigger beads were associated with cellular multilayers while small beads progressively failed to support growth. Mean value for growth failure of cells ranged from 5% on 55 μm beads to 52% on 25 μm beads. The investigator postulated that the cells have to spread sufficiently to tense themselves, and that tension

stimuates the cell cycle. Supporting evidence for this view was provided by Curtis and Sheer (1978). They grew sheets of fibroblasts from chick embryos, cultured on nylon mesh which was anchored to a polyestyrene ring in a culture dish containing tritiated thymidine. The cells were mechanically stressed for one hour by deforming the mesh with the aid of piezoelectric ceramic element. The results showed that mechanical stress of cells leads to an increase in mitotic frequency and in the proportion of cells in S phase.

Lorber and Milobsky (1968) stretched the skin to a varying degree in living rats by the insertion of stainless steel pins beneath the epidermis. By means of autoradiography, labeled epidermal cells were followed for 36 hours. The authors found that slight stretching initially increased the labeling of basal cells compared with control areas. With further tension a point was reached where the percentage of labeled cells decreased. Marked tension consistently prevented labeling.

In order to assess the histologic changes of keratinized mucosa under tension, Philipsen et al. (1973) placed different diameter polyethylene tubes into the esophagus of guinea pigs for ten minutes before sacrifice. The dilated esophagus resulted in a uniform flattening of the epithelium without discontinuity of the tissue. The epidermal papillae were evened out. The length of the basement membrane of the dilated esophagus was found to be

considerably greater than in control animals.

Francis and Marks (1977) investigated dermal factors responsible for inducing epidermal hyperplasia. A subcutaneous implant of a silastic gel-filled testicular prosthesis was placed into the guinea pig flank skin to cause "non inflammatory" stretching. The opposite flank was sham-operated as control. By autoradiography, it was observed that there was a significant increase in the germinative cell population and a slight increase in dermal thickness at the stretched sites on the second experiemental day. However, labeling indices, as well as epidermal thickness, had returned to normal on day 7. Thus no sustained epidermal hyperplasia was found.

A well-designed experiment to investigate the effects of mechanical stretch on epidermis was done by Squire (1980). Stretching was accomplished by implanting a small spring into the backskin of hairless mice, by which also degree of tension could be measured. To identify any effect due to surgical trauma, sham-operated animals were used, in which the implanted springs did not exert any tension. Squire reported that there were significantly higher mitotic indices after one day in both experiemental and sham-operated animals while a slight decrease of epidermal thickness was observed. However, after two days, both the mitotic index and thickness of the epidermis of the stretched skin were greater compared to sham-operated and control skin. Significant differences were still maintained after four days.

At this time the stretched epidermis showed a hyperplastic response with a thickening of all cell layers. It appears that tension due to stretching increased the mitotic activity of the epidermis leading to an increased progenitor cell population and subsequent tissue hyperplasia.

In order to eliminate tension generated from masticatory movements, Meyer (1984) fed rats with a soft diet for five weeks. She observed reduced mitotic activity and atrophy in oral epithelium.

The present project has several aspects distinguishing it from previous studies. Most experiments investigating the effects of tension on tissue entail tissue immobilization and fairly extensive surgical prosedures which result in tissue damage. Use of the hamster cheek pouch in this project has the following advantages :(1) the stretched tissue remains functional and (2) the surgical procedure is reduced to a minimum to avoid inflammatory and traumatic tissue reactions. In contrast to previous studies investigating the response of skin to tension, this experiment focused specifically on the response of the buccal mucosa. The outer surface of the epithelium was used as a reference unit to assess mitotic activity in this porject, a method which has been reported to be a most reliable one (Korring _et al_., 1972). However, most of the reported experiments have used the basal cells or basement membrane as reference

units. Furthermore, and unlike previous studies where tension was constantly present, the present project also investigated the response of the tissue following removal of mechanical tension.

MATERIAL and METHOD

SAMPLE

Twenty 30 or 31 day old male Golden Syrian Hamsters, with body weights ranging from 57 to 79 gm, were used. They were fed commercial rat chow and kept in standard laboratory housing at a constant temperature of 70°F. To eliminate the effect of the estrous cycle on mitotic activity, only male hamsters were used (Bullough, 1943).

The Golden Syrian Hamster was chosen as the experimental animal for the following reasons :

1) It is a relatively hardy animal, that could be expected to withstand the physical and psychological stress of the experiment.
2) Ease of handling.
3) The cheek pouch of the hamster offers a unique experimental site for tissue stretching. This can be accomplished with minimal surgery. The risk for inflammation is minimized and immobilization of tissues can be avoided.
4) The pouch wall has an extensive capillary network, and thus good blood circulation can be expected at the experimental site.

GROUPING and EXPERIMENTAL PERIOD

The sample was divided into four groups according to

different experimental periods. Following application of mechanical tension, groups I, II, III were sacrificed at 2, 4, 7 days, respectively. In group IV tension was released by removal of the stretching device at the 7th experimental day, whereafter the animals were sacrificed on day 10.

Each group comprised five animals. In four animals, the left cheek pouch was stretched and the right pouch was left untouched as control site. The fifth animal in each group was sham-operated by suturing the left pouch orifice. The contralateral side was left untouched.

EXPERIMENTAL PROCEDURE

The finger of a latex glove, sealed with cyanoacrylate and into which a piece of latex tubing had been inserted, was utilized as the stretching device. Under anesthesia, this device was inserted into the left cheek pouch with the tubing sticking out of the pouch opening. The distended pouch (Figure 1) is about 5 cm in length and 1 cm in width (Magalhaes *et al.*, 1965), thus 3.5 c.c. to 4 c.c. rubber base impression material, Citracon, was injected through the tubing to moderately stretch the pouch (Figure 2).

After the impression material was set , the tubing was cut and the circular muscle of the pouch was sutured to the masseter muscle to close the pouch opening. In order to prevent soft tissue laceration, 4-0 silk was used and about 4 mm of tissue was picked up in each stitch (Figure 3).

Anesthesia

One hundred and fifty mg/Kg body weight of Ketamine HCl ("Ketaset", Bristol Lab., Syracuse, New York 13201) with ten mg/Kg body weight of Xylazine HCl ("Rompun, Haver Lockhart Lab., Shawnee, Kansas 66201) was given peritoneally in the right posterior abdominal quadrant to prepare animal for surgery (Curl and Peters, 1983).

Radioactive isotope labeling

Tritiated thymidine, which is incorporated into the cell nucleus during DNA synthesis, was used to assess mitotic activity of epithelial cells.

Twenty-four hours prior to sacrifice, each animal recieved a intraperitoneal injection of ring-labeled tritiated thymidine (21 Ci/ mmol, Amersham Co., Illinois 60005) in a dose of 1.0 μCi/gm of body weight. All injections were administered between 5:00 p.m. to 7:00 p.m. to minimize the effect of diurnal variation on cell proliferation (Bullough, 1948).

Sacrifice and Specimen Preparation

The animals were perfused with 10% buffered formalin by an intracardiac route. One hundred mg/kg body weight of Pentobarbital ("Diabutal", Diamond Lab., Des Moines, Iowa 50304) was injected intraperitoneally to prepare animals for perfusion. Animals were immobilized on a metal plate, and the chest cavity was opened to expose the heart. A hypodermic needle, gauge

25G 5/8", was inserted through the wall of the left ventricle in the direction of the aorta. The right atrium was punctured to drain the blood, and administration 10% neutral formalin under a constant pressure of 70 mmHg, was continued until clear formalin flowed from the puntured wound (Figure 4).

After perfusion, both cheek pouches were removed and immediately pinned on Sylgard to prevent tissue shrinkage. Specimens were additionally fixed in 10% buffered formalin for 24 hours. A tissue block, 3mm by 2mm, was excised from the middle part of the pouch, dehydrated in a graded series of ethanol 50%, 70%, 80%, 90%, 95% to 100%), 15 minutes in each solution.

Methacrylate embedding medium (JB-4, Polysciences, Inc., Paul Vally Industrial Park Warrington, PA. 18976) was used. The dehydrated tissues were infiltrated in catalyzed solution A overnight then embedded in solutions A and B mixed. The embedded tissue blocks were allowed to polymerize completely overnight; and then re-embedded to orient tissue so that tissue could be cut perpendicular to the epithelial surface. The cured blocks were sectioned on a microtome (The Sorvall Type JB-4 "Porter-Blum" Microtome) with a glass knife at 3 microns.

Autoradiography Procedure for Light Microscopy

Basically the technique described by Kopriwa and Leblond (1962) was followed.

(1) Coating Technique

The slides with the labeled specimens were dipped in Kodak

NTB2 emulsion in the dark room where a save light was on.

The following sequence was employed for emulsion coating:

1) Melt emulsion in a water bath held constant at 41°C.
2) Pour the emulsion into triangular staining jar to the level where the tissues on the slide were covered, but the slide label was not.
3) Dip and withdraw the slide vertically without tilting.
4) Let the emulsion air-dry for 2 hours by keeping the slides vertically on a plastic rack on moist tissue paper.

(2) Exposure

After drying the coated slides were stored in light proof black plastic boxes which contained 4 tissue paper bags with Drierite. The box was wrapped in several layers of alumminum foil and kept standing vertically in a refrigerator (4°C). The optimal duration of exposure time was estimated to be 25 days.

(3) Processing

After 25 days of exposure, the slides were transferred to glass slide trays and processed in the dark room where a save light light was used. Sequence of procedures was described below :

1) Develop in freshly prepared Kodak Dektol developer at 17° C for 2 minutes and 30 seconds. Agitate gently every 30 seconds.
2) Rinse in stop bath at 17°C for 15 seconds.

3) Fix in Kodak rapid fixer at 17°C for 3 minutes.

4) Wash thoroughly in running filtered tap water for 20 minutes.

5) Dry in well-ventilated area whereafter the preparations are ready for staining.

(4) Hematoxylin-Eosin Staining

The slides were arranged in a metal slide tray and stained by bringing the tray through the following steps,

1) Place in Gill's Hematoxylin #3 for 45 minutes.

2) Rinse in distilled water ten times.

3) Place in acid water (10 drops 1 M HCl in half gallon distilled water) and agitate gently for 2 minutes.

4) Place in Scott's tap water substitute for one minute and egitate gently, followed by distilled water rinse.

5) Place in 0.5% Eosin for 3 minutes.

6) Wash with distilled water five times.

7) Air dry.

After drying, the slides were dipped in xylene for 2 minutes, and mounted with Permount. When dry, the slides were ready for examination with oil immersion microscopy.

Quantitative Studies

The autoradiograms were examined at 630X magnification in order to evaluate the cellular response of hamster buccal epithelium to tension.

Two tissue blocks were examined from each pouch and 15-20

sections from each tissue block were arranged on each slide. Six readings were taken randomly from each slide. All data recording was done in a double blind format.

Three quantitative studies were done as described below :

1) Mitotic activity assessment

Tritiated thymidine incorporated into the cell nuclei during DNA synthesis, and these cells may be identified in autoradiography by the presence of reduced silver grains in the film overlying the nuclei. To be counted as a significantly labeled cell in this project, a cell with a minimum of eight grains was required.

Karring and Loe (1972) reported that the number of mitoses relative to a reference unit (1 mm square) of the outer surface of the epithelium was found to be the most reliable method for assessing mitotic activity in stratified squamous epithelium. Thus, the mitotic index in this research was expressed as the number of labeled basal cell per mm length of the outer surface of the epithelium.

2) The thickness of Malpigian layer of the epithelium

This was measured from the basement membrane to the junction between the granular and keratin layers with a calibrated eyepiece. The measurements were taken perpendicular to the basement membrane.

3) Basal cell density

Cell density was expressed as the number of basal cells per mm length of the basement membrane. The criteria used to defining a basal cell were : (1) cell membrane in contact with basement membrane, and (2) visible nucleus also required.

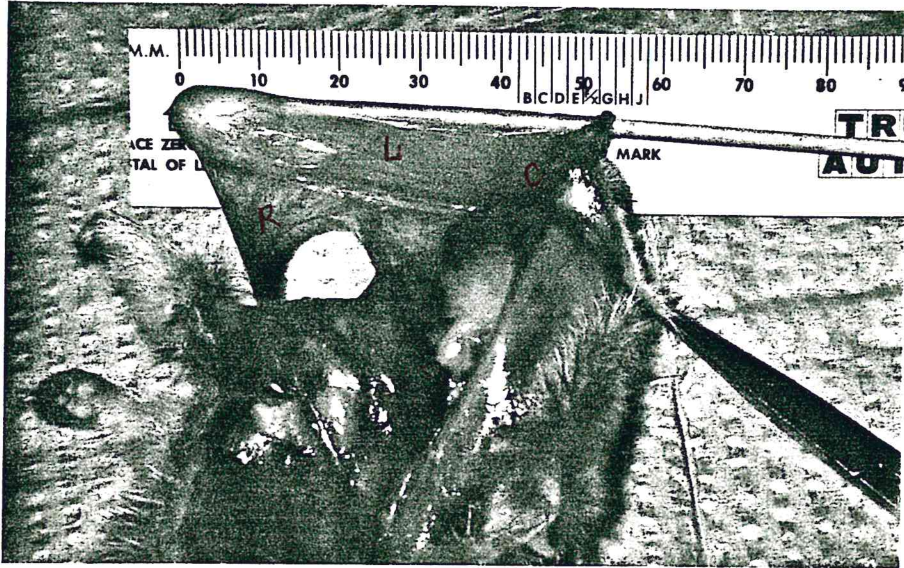
Figure 1 -- Anatomy of the cheek pouch.

C.: Circular Muscle.

L : Longitudinal Muscle.

R : Long Retractor Muscle.

Figure 2 -- Procedure for insertion of stretching device.


M.M.
0 10 20 30 40 50 60 70 80
B C D E F G H J
MARK
R
L
C

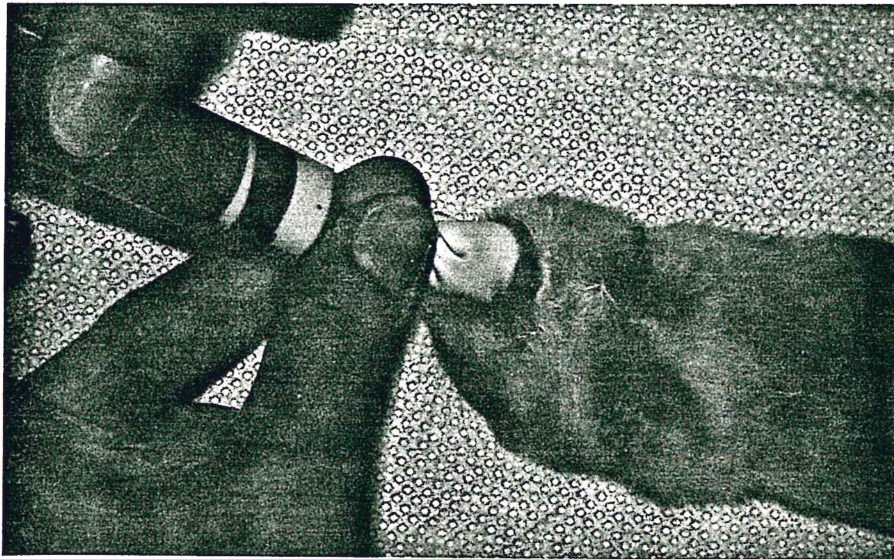


Figure 3 -- Comparison of control, stretched and sham-operated sides.

C : Control Side

X : Stretched Side

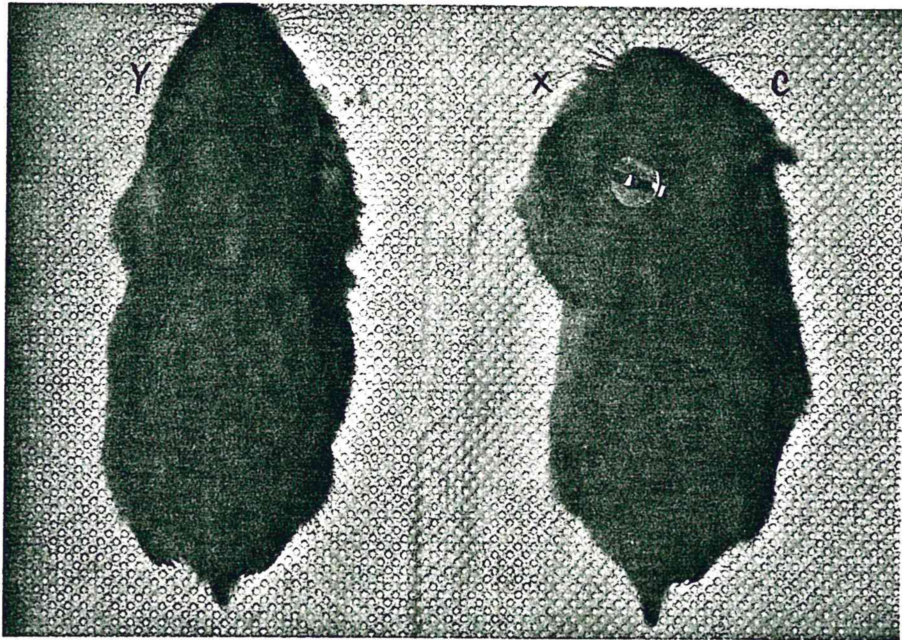
Y : Sham-operated Side

Figure 4 -- Procedure for perfusion. The animals were perfused with 10% buffered formalin by intra-cardiac route.

N : A hypodermic needle (Gauge 25G 5/8")

A : Aorta

R : Rib Cage


Y
X
C

A
R

RESULTS

Body Weight Change

Data on body weight were collected daily. Comparison of initial body weight revealed that there was no significant difference ($p > 0.05$) among the four groups (Table II). Mean value of daily body weight change (Table I) for each of the four groups was calculated. It was found that during the first two days after insertion of the stretching device, the animals lost on the average about 3 grams per day. The third and fourth days after the operation, very small weight gains were observed, thereafter the animals steadily gained about 1 to 2 grams per day. The sham-operated animals did not show weight loss following suturing of the pouch opening. Each of them gained about 1 to 2 grams per day.

Weight of the Stretching Device

After the animals were killed, the stretching devices were removed and weighted. Weight data for these were analyzed statistically by an ANOVA. The results did not show significant differences among the four groups at the 5% level of confidence (Table III).

Histologic Descriptive Data (Figure 5,6,7,8)

In the stretched tissue, it was found that collagenous fibers in the submucosa were aligned parallel to the direction of stretching, while in the unstretched tissue the collagenous

fibers were arranged multidirectionally.

The basal cells of the epithelium following stretching were cuboidal in shape and oriented perpendicular to the basement membrane. In contrast, the basal cells in the unstretched tissue were slightly slender and angulated to the basement membrane.

In the experimental pouch of the group IV animals, in which the stretching device was removed on day 7 and the animals were sacrifice at day 10, the morphology of the basal cells were similar to those of the unstretched site. On the other hand, the submucosal collagenous fibers had mixed characteristics of both stretched and unstretched tissue.

The histologic pictures of sham-operated animals were similar to those of control animals.

There were no signs of inflammation in either stretched or sham-operated tissue.

Statistical Analysis of Data Regarding Tissue Response

In order to assess the influence of mechanical tension on epithelial growth, intra- and intergroup comparisons of (1) mitotic index, (2) epithelial thickness and (3) the basal cell population were made. Means of 12 readings, which were randomly taken from each pouch, were statistically analyzed (Table IV, VIII, XI, XIV) according to the following steps:

(1) Intergroup comparison

One-way analysis of variance (ANOVA) was used to compare the

unstretched sides of the four groups.

(2) Intragroup comparison

(a) Paired two-tailed t-tests were performed to compare the unstretched and stretched sides within each of the four groups.

(b) Two sample two-tailed t-tests were used to compare the unstretched and sham-operated sides within each of the four groups.

Biometry of Mitotic Index

Analysis of variance revealed no significant difference (p 0.05) between the control sides of the four groups (Table V). Mitotic index of the stretched sides in group I was significantly higher than that of the contralateral control sides. Even though the mean values of mitoic indices of the stretched sides in groups II, III and IV were lower than those of the contralateral control sides, there were no significant differences at 5% confidence level (Table VI). Comparison about mittic index between control and sham-operated sides revealed no significant differences ($p > 0.05$) in groups I, II and III. However, a statistically significant difference ($p < 0.05$) was seen in group IV (Table VII).

Biometry of Epithelial Thickness

The results showed there was no significant difference

($p > 0.05$) between control sides of the four groups (Table IX). Further, no statistically significant differences were found between control and stretched sides (Table X), and between control and sham-operated sides (Table VII).

Biometry of Basal Cell Density

Intergroup comparisons with an ANOVA revealed significant differences ($p < 0.05$) among unstretched sides of the four groups (Table XII). Statistical analysis also showed significantly decreased basal cell density on the stretched sides in all of the four groups in comparison with their contralateral control sides (Table XIII). No significant difference was found between control and sham-operated sides in any of the four groups (Table VII).

In brief, there was a significant increase in the epithelial mitotic index after administration of mechanical tension for two days. However, this phenomenon was not maintained. On day 4, 7, and 10, no statistically significant differences were found between stretched and unstretched sides. The epithelial thickness did not show significant changes under stretching condition. The basal cell density observed at the stretched sides was significantly lower than that at the unstretched sides. However, after removal of mechanical tension for three days, there was no significant difference between experimental sides and control

sides. A comparison between control and sham-operated animals regarding (1) mitotic index, (2) epithelial thickness, (3) basal cell density revealed that there was no significant difference between any of the groups except for group IV. The mitotic index in this group was found to be significantly different, being higher on the sham-operated side.

TABLE I

Weight Data; Initial Weight, Final Weight and Weight Change/Day

Group	#	Initial Weight (Grams)	Mean (Grams)	SEM	Final Weight (Grams)	Mean (Grams)	SEM	Weight Change Per Day
I	1	75.0			72.1			
	2	68.1			65.7			
	3	66.2	69.28	1.95	62.0	66.15	2.13	-1.562
	4	67.8			64.8			
II	1	71.2			65.9			
	2	78.9			74.9			
	3	60.4	69.25	3.90	51.9	63.00	4.89	-1.512
	4	66.5			59.3			
III	1	61.9			62.1			
	2	64.8			66.7			
	3	64.1	66.20	2.67	61.8	66.52	3.19	+0.046
	4	74.0			75.5			
IV	1	69.9			75.8			
	2	69.3			69.0			
	3	71.8	68.70	1.72	79.3	73.42	2.49	+0.472
	4	63.8			69.6			

TABLE II

ANOVA (one-way) for Initial Body Weight of the Four Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value
Model	3	25.641907	8.54730225	0.29328724
Error	12	349.717316	29.1431097	
Corrected Total	15	375.359223		

Critical value of "F" for $\alpha = 0.05$ and degree of freedom 3, 12 is 3.49

TABLE III

ANOVA (one-way) for Weight of Stretching Device for the Four Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F
Model	3	2.40249991	0.800833305	1.28906779
Error	12	7.45499945	0.621249954	
Corrected Total	15	9.85749936		

Critical value of "F" for $\alpha = 0.05$ and degree of freedom 3, 12 is 3.49

Table IV

Data on Mitotic Index

Group	#	Control Side Xi	Control Side Mean	Control Side SEM	Experimental Side Xi	Experimental Side Mean	Experimental Side SEM
I	1	1)			1)		
	2	5.40			10.80		
	3	6.75	6.30	0.45	10.80	11.70	0.899
	4	6.75			13.50		
II	1	7.65			8.10		
	2	4.95			0.90		
	3	9.80	8.30	1.296	7.20	4.725	1.738
	4	10.80			2.70		
III	1	9.80			2.10		
	2	5.40			4.20		
	3	6.75	7.40	0.924	7.80	4.05	1.344
	4	7.65			2.10		
IV	1	5.40			2.25		
	2	7.20			2.25		
	3	10.80	7.20	1.273	7.20	4.725	1.738
	4	5.40			6.75		

Mitotic Index: Number of Labeled Basal Cell Per MM Epithelial Surface

SEM: Standard Error of Mean Sample

1) Due to technical problems, the data of this animal was considered invalid.

Table V

ANOVA (one-way) for Mitoic Index: Unstretched Sides of the Four Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value
Model	3	7.01333308	2.337769	0.5037820
Error	11	51.044999	4.64045453	
Corrected Total	14	58.0583329		

Critical value of "F" for α= 0.05 and degree of freedom 3,12 is 3.49

The zero data from the number one animal of group I was used in this analysis.

TABLE VI

Paired-Comparison t-Test for Mitotic Index: Unstretched and Stretched Sides within Each of the Four Groups

Group	#	Control	Experi.	Mean	SEM	T
I	1	1)	1)			
	2	5.40	10.80			
	3	6.75	10.80	-5.40	0.779	6.9282
	4	6.75	13.50			
II	1	7.65	8.10			
	2	4.95	0.90			
	3	9.80	7.20	-3.575	1.776	-2.012887
	4	10.80	2.70			
III	1	9.80	2.10			
	2	5.40	4.20			
	3	6.75	7.80	-3.35	1.995	-1.67947
	4	7.65	2.10			
IV	1	5.40	2.25			
	2	7.20	2.25			
	3	10.80	7.20	-2.588	1.367	-1.892721
	4	5.40	6.75			

Critical value of "T" for $\alpha = 0.05$ and degree of freedom 3 is 3.18

1) Due to technical problem, the data from this animal was considered invalid.

TABLE VII

t-Test for Mitotic Index , Epithelial Thickness and Basal Cell Density: Unstretched and Sham-operated Sides within each of the Four Groups

	Mitotic index		Epithelium Thickness		Basal Cell Density	
Group	T Value	df	T Value	df	T Value	df
I	-2.0696	2	-0.2138	3	-0.2057	3
II	0	3	-0.1841	3	-0.0507	3
III	0	3	-0.8741	3	0.2371	3
IV	-6.5447	3	-0.1639	3	0.1233	3

Critical value of "T" for $\alpha = 0.05$ and degree of freedom 3 is 3.18

Critical value of "T" for $\alpha = 0.05$ and degree of freedom 2 is 4.30

TABLE VIII

Data on Epithelial Thickness

		Control side			Experimental Side		
Group	#	Xi(μ)	Mean(μ)	SEM	Xi(μ)	Mean(μ)	SEM
I	1	42.33			67.85		
	2	35.85			50.80		
	3	35.71	38.47	1.24	44.44	50.86	6.06
	4	44.18			40.34		
II	1	41.40			38.22		
	2	39.82			37.96		
	3	41.00	41.98	0.81	40.74	39.88	1.10
	4	44.18			42.59		
III	1	44.58			48.42		
	2	42.86			38.36		
	3	41.53	44.04	1.48	55.76	42.42	6.22
	4	41.66			27.12		
IV	1	40.34			48.68		
	2	37.06			46.58		
	3	51.86	44.50	3.76	39.16	43.66	2.34
	4	55.16			40.21		

SEM: Standard Error of Mean Sample

TABLE IX

ANOVA (one-way) for Epithelial Thickness: Unstretched Sides of the Four groups

Source	Degree of Freedom	Sum of Squares	Mean Squares	F Value
Model	3	90.890152	30.2967173	1.19598194
Error	12	303.985031	25.3320859	
Corrected Total	15	394.875183		

Critical value of "F" for $\alpha = 0.05$ and degree of freedom 3, 12 is 3.49

TABLE X

Paired-Comparison t-Test for Epithelial Thickness: Unstretched and Stretched Sides within Each of the Four Groups

Group	#	Control	Experi.	Mean	SEM	T
I	1	44.33	67.85			
	2	35.85	50.80			
	3	35.71	44.44	10.84	5.7564	1.8831
	4	44.18	40.34			
II	1	41.40	38.22			
	2	39.82	37.96			
	3	41.00	40.74	-1.7225	0.5986	-2.8776
	4	44.18	42.59			
III	1	44.58	48.42			
	2	42.86	38.36			
	3	41.53	55.76	-0.2425	6.1146	-0.0396
	4	41.66	27.12			
IV	1	40.34	48.68			
	2	37.06	46.58			
	3	51.86	39.16	-2.4475	6.5892	-0.3714
	4	55.16	40.21			

Critical value of "T" for $\alpha = 0.05$ and degree of freedom 3 is 3.18

TABLE XI

Data on Basal Cell Density

		Control Side			Experimental Side		
Group	#	Xi	Mean	SEM	Xi	Mean	SEM
I	1	110.25			72.45		
	2	105.52			92.40		
	3	101.32	108.67	2.31	83.25	85.78	5.11
	4	112.88			95.02		
II	1	124.95			86.62		
	2	123.90			86.62		
	3	113.92	118.55	2.50	74.02	86.54	5.04
	4	112.88			98.70		
III	1	119.70			86.10		
	2	114.45			88.72		
	3	105.60	114.83	2.73	96.60	87.15	4.00
	4	113.40			77.18		
IV	1	110.22			100.80		
	2	86.10			116.55		
	3	95.02	99.85	4.46	92.92	101.46	5.27
	4	102.38			93.45		

Basal Cell Density: Number of Basal Cell Per MM Basement Membrane

TABLE XII

ANOVA (one-way) for Basal Cell Density: Unstretched Sided of the Four Groups

Source	Degree of Freedom	Sum of Squares	Mean Square	F Value
Model	3	918.046203	306.015401	5.91231778
Error	12	621.107483	51.7589569	
Corrected Total	15	1539.15369		

Critial value of "F" for $\alpha = 0.05$ and degree of freedom 3, 12 is 3.49

TABLE XIII

Paired-Comparison t-Test for Basal Cell Density: Unstretched and Stretched Sides Within Each of the Four Groups

Group	#	Control	Experi.	Mean	SEM	T
I	1	110.25	72.45			
	2	105.52	92.40			
	3	101.32	83.25	-21.6375	5.5225	-3.9180
	4	112.88	95.02			
II	1	124.95	86.62			
	2	123.90	86.62			
	3	113.92	74.02	-32.4225	6.1046	-5.3111
	4	112.88	98.70			
III	1	119.70	86.10			
	2	114.45	88.72			
	3	105.60	96.60	-26.1375	6.1319	-4.2625
	4	113.40	77.18			
IV	1	110.22	100.80			
	2	86.10	116.55			
	3	95.02	92.92	2.5000	9.4653	-0.2641
	4	102.38	93.45			

Critical value of "T" for α= 0.05 and degree of freedom 3 is 3.18

TABLE XIV

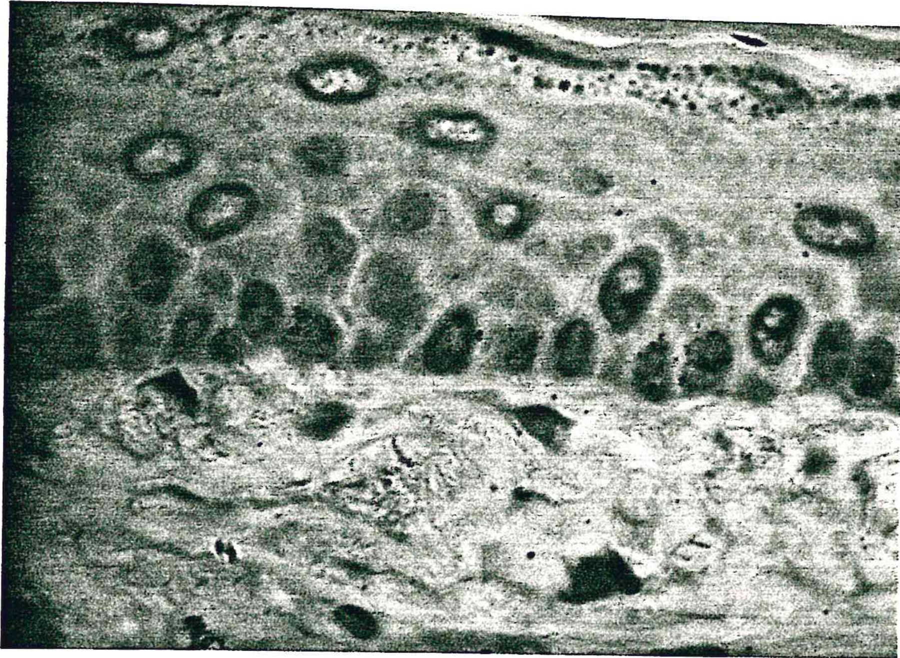
Data on Mitotic Index, Epithelial Thickness and Basal Cell Density of Sham-Operated Sides

Group	Mitotic Index	Epithelial Thickness	Basal Cell Density
I	5.25	37.04	107.10
II	3.60	39.29	98.70
III	2.10	45.10	124.95
IV	7.65	36.50	116.02

Figure 5 -- Autoradiography of Group I.

A : Stretched Side

B : Unstretched Side



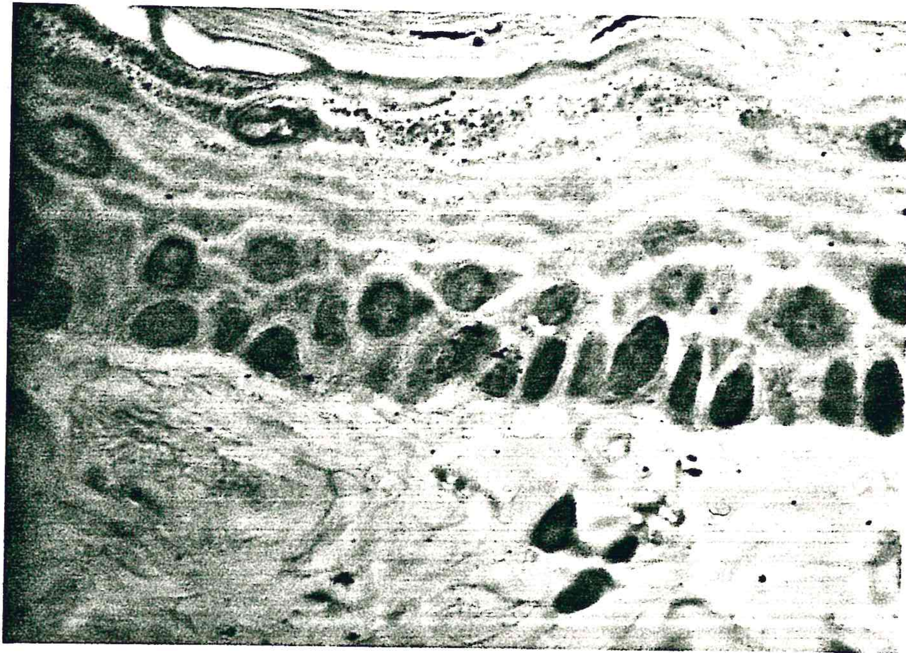
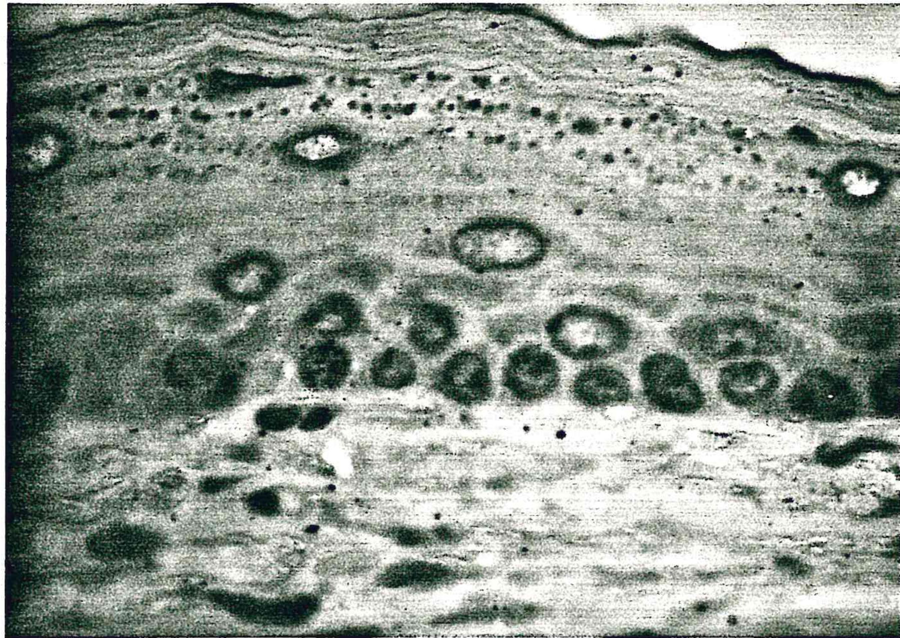


Figure 6 -- Autoradiography of Group II.

A : Stretched Side

B : Unstretched Side



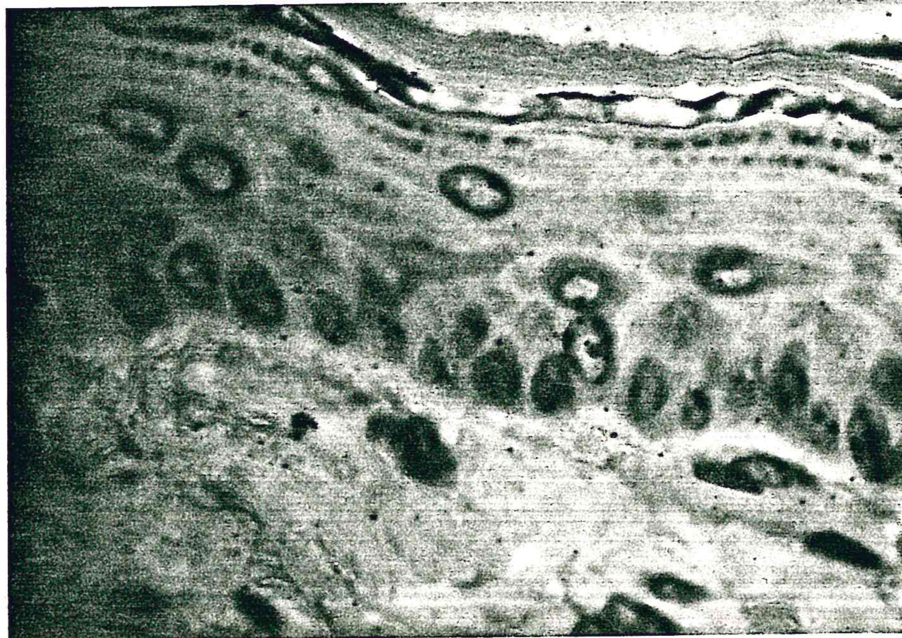
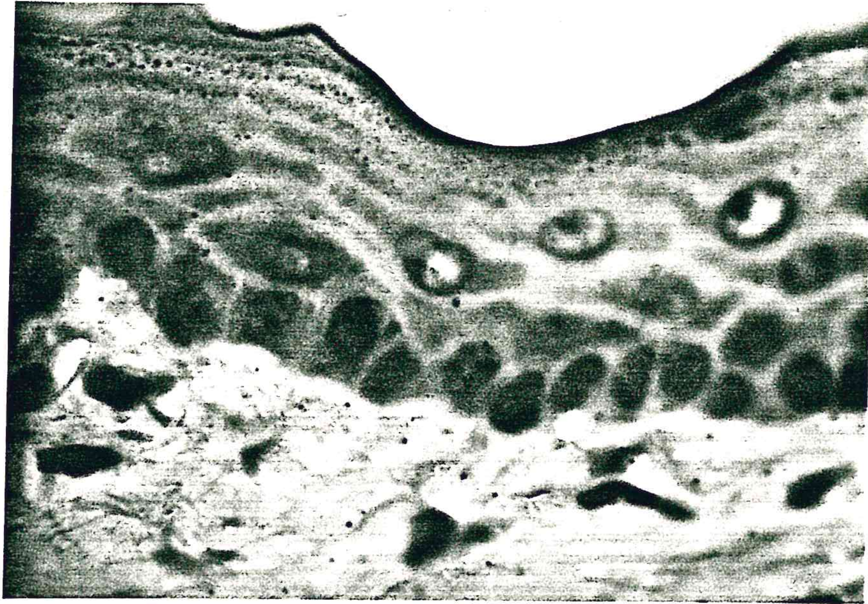


Figure 7 -- Autoradiography of Group III.

A : Stretched Side

B : Unstretched Side



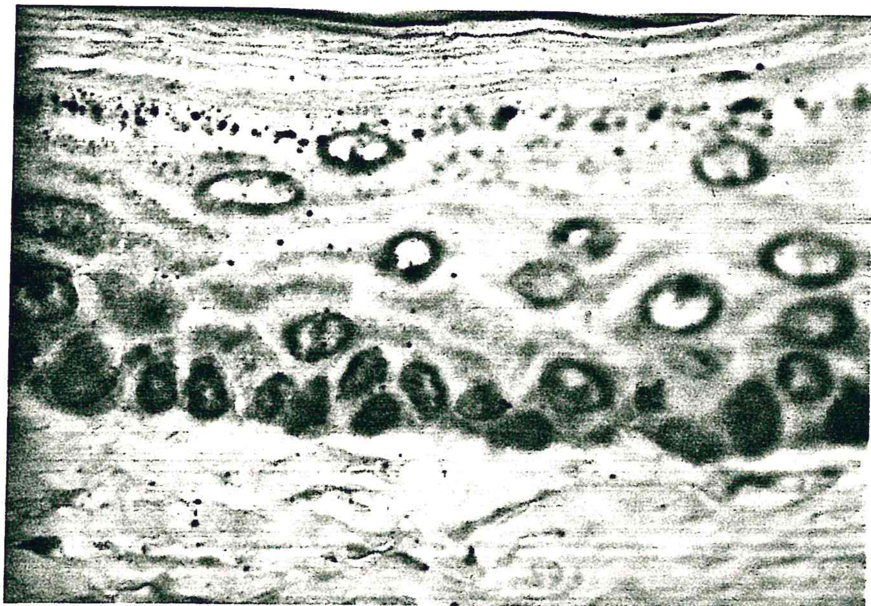
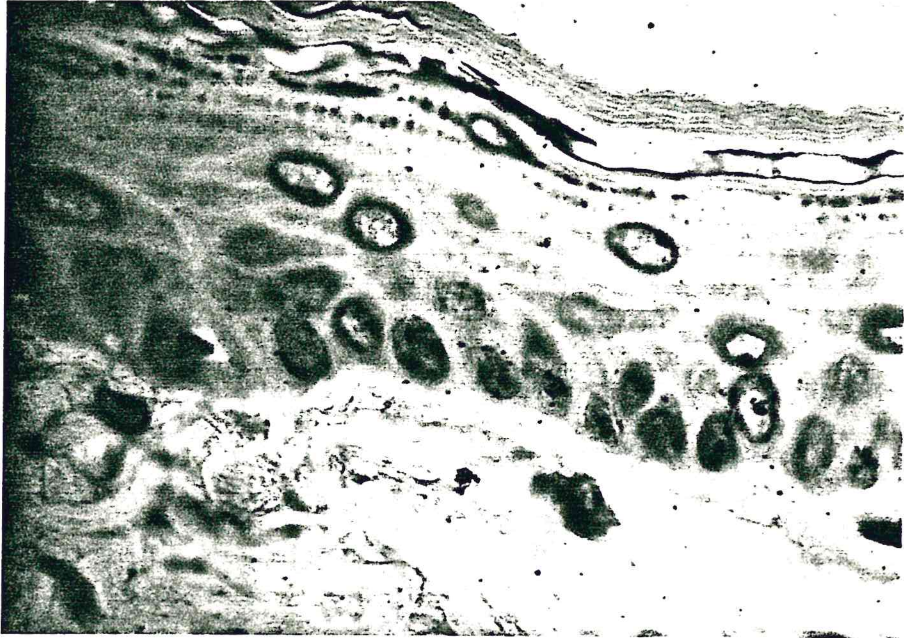
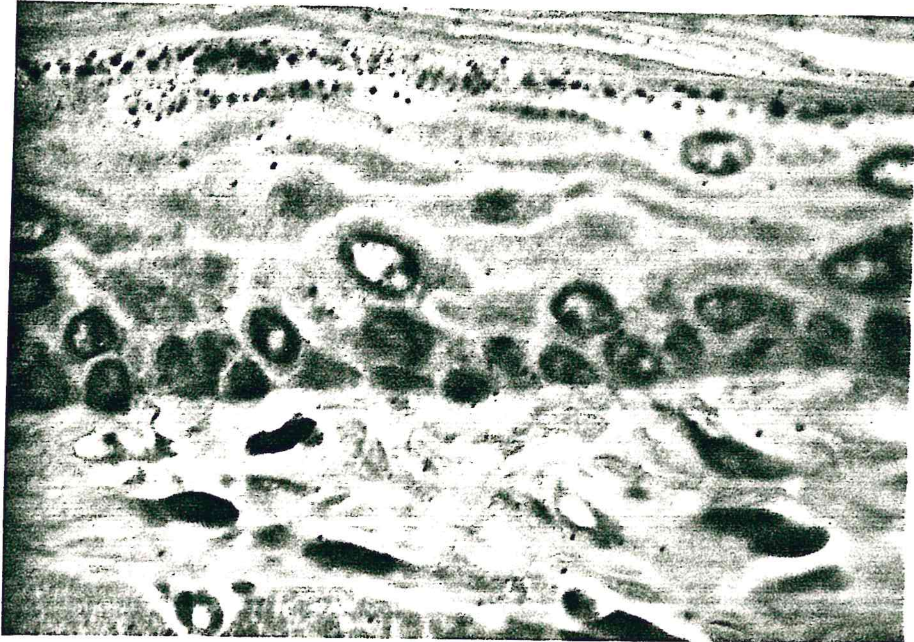


Figure 8 -- Autoradiography of Group IV.

A : Stretched Side

B : Unstretched Side





DISCUSSION

This research is a model for studying the relationship between epithelial mitotic activity and mechanical tension. Autoradiography was used to assess mitotic rate of buccal epithelium. Tritiated thymidine was chosen in this project because (1) tritium emits beta particles with an average size of 1.5 microns and thus readily compatible with body tissues (Fukuyama et al., 1961), (2) DNA is the only labeled substance present in significant amount in sections following thymidine-H administration (Leblond, 1959), and (3) its radioactive index is a relatively reliable indicator of the rate of cell proliferation. Warburton (unpublished data) reported that 96% of mitotic figures gave a radioautographic reaction in 6-μ sections. However, I believe that, with adquate exposure, the efficiency of detection of labeled nuclei would be increased in sections of 3-μ thickness.

It was found that neither autoradiograms from the stretched nor the contralateral unstretched tissues of animal number 1 in group I, show any labeled cells. After double-check by re-sectioning the tissue blocks and repeating the autoradiography there were still no labeled cells observed. Obviously, the problem was not caused by failure of the autoradiography procedure, but rather by the injection technique. Possibly this negative result was due to either the injection of tritiated thymidine into intestinal tract or bladder and

excreted before absorption. Alternately, tritiated thymidine might have leaked due to animal movement during injection. The data from this animal was considered invalid.

In the present study, the differences between control and sham-operated sites were not significant. Thus, observed tissue responses were not due to inflammatory or traumatic reactions resulting from the surgical procedures but rather due to the tissue stretching.

The most obvious result in this research was the significant rise of mitotic index after application of mechanical tension for two days. This is in accord with previous reports regarding the effect of stretching on mitotic index, either _in vivo_ (Lorber _et al_., 1968; Francis _et al_., 1977; Squire, 1980) or in cultured cells (Curtis _et al_., 1978). It has been suggested that cell formation and maturation play an important role in the adapative response of the epidermis to mechanical stimulation (Mackenzie, 1974). Francis _et al_. (1977) proposed that the increased size of the epithelial germinative pool causes lengthening of the epidermis, thereby relieving tension. Lengthening of the basement membrane during stretching was reported by Philipsen _et al_ (1973). Squire (1980) also found that skin increased in length under mechanical tension. He suggested that increased mitotic activity of epidermis due to tension leads to an increased progenitor cell population. In my project, I was

not able to assess possible length change of the basement membrane. Thus, it was not possible to estimate whether the epithelial progenitor cell population increased or decreased.

A number of related factors may explain the significant increase in the mitotic index of stretched tissue on day 2. Stroker and Rubin (1967) first proposed the term "density dependent inhibition of growth" to explain why cell proliferation decreases dramatically with increasing local cell density in cell cultures. Zetterberg _et al_. (1970) have shown similar results. Maroude (1972) observed that normal cells do not grow unless attached to a substratum. He suggested that mitosis has "anchorage dependence" in normal cells. He postulated that the cells have to spread sufficiently to tense themselves, and that tension stimulates the cell division cycle.

Cell density and contact surface area between cells and substratum are considered factors controlling cell division. Two hypotheses have been suggested to explain these. The first states that direct cell-to-cell contact, perhaps involving the interaction of a receptor with its ligands on an adjacent cell, provides a negative signal for growth (Dulbecco, 1970). Garber (1976) suggested the cell surface as a site for growth control. She proposed that the cell surface, containing a mosaic of receptors, mediates morphogenetic interactions and profoundly influences pathways of cyto-differentiation through molecular identification in the intimate contact between cells. The second

hypothesis states that growth ceases at a confluent cell density because of the establishment of a diffusion boundary layer which maintains the supply of critical nutrients of growth factors below a critical level (Stroker, 1973). The action of diffusion boundary layers has been interpretated as evidence that increase in rate of exchange of metabolites between cell and medium stimulates the cell cycle.

In the present project, it could possibly be argued that a decreased cell density in stretched tissue reduced the negative signals for growth and disturbed the diffusion boundary. The increased contact surface area between the basal cell and the basement membrane facilitates uptake of nutrients and growth factors by the basal cells. Thus, mitotic activity was increased after the application of mechanical tension for two days. Negative signals for growth could possibly involve the release of chemical mediators following receptor-ligand interaction, which might change the cell membrane permiability or inhibit the synthesis of materials necessary for division.

Chalones, which are specific endogenous tissue-specific mitotic inhibitors, have been proposed to maintain epithelial homeostasis through a feedback mechanism (Bullough, 1961, 1962, 1972). However, this theory still remains untested.

An interesting finding was observed in my research. The mitoic index of stretched tissue declined to a normal range

on day 4 , while the basal cell density was maintained at a significantly lower level than that of unstretched tissue. This finding contrasts to the previous described "density dependent inhibition of growth" and "anchorage dependence of growth".

Curtis _et al_. (1978) provided evidence that the cell cycle is not controlled by the diffusion rate. Rather, they suggested that increased tension within the cell stimulates the cell cycle, possibly by involvement of the microfilament system. Folkman and Moscona (1978) suggested that cell shape may be important in growth control. Folding of cellular membranes in epithelial cells were noted by Porter and Bonneville (1963) and which they considered to be a kind of membrane "storage". Tucker (1968) suggested that folding of epithelial cell membranes forms a mechanism to comply with physiological and mechanical requirement for expansion and contraction. Microfilament bundles of epithelial cells extending across the luminal and basal ends of the cells have been found to contribute to the change of cell morphology (Wessell, 1969). In stretched tissue, the folded cellular membrane may distend and the cell shape change in order to achieve a balance of adhesive forces between basal cells and between basal cells and basement membrane (Folkman and Moscona, 1978). Cell shape has been shown to be tightly coupled with DNA synthsis and growth in nontransformed cells. Incorporation of ^{3}H -thymidine has been found to be inversely proportional to the height of the cell (Folkman and Moscona, 1978). In my study, the

morphology of epithelial cells on the stretched sides were more cuboid than those on the unstretched sides. Lorber _et al_. (1968) suggested that increased mitotic activity was a means of restoring the shape of the cells from the alteration induced by stretching. I suggest that the shape of the basal cells was altered in order to equalize the multidirectional adhesive forces and to transmit mechanical tension to underlying tissues. The change of cell shape might, to a certain degree, activate the genes controlling the synthesis of materials necessary for division. When the mechanical tension was released, the mitotic activity of basal cells dropped to a normal range.

The effect of neurotrophism might be involved in the change of epithelial mitotic activity in the present project. When tissue is stretched, the central nervous system receives information through activation of local mechanoreceptors. Under the commands of CNS, efferent nerves innervating blood vessels of viscera and muscles might alter local blood supply and vascular permiability or depolarize the basal cell membrane.

It had been suggested that disrupting normal micropotentials can stimulate epidermopoiesis (Francis, 1977). However, it is not clear whether the micropotentials are generated by neural mediators or by direct mechanical disturbance of electric charges at the surface of the cellular membrane. They could also be generated by liberated chemical mediators

following the cell recognition process.

In the present experiment, stretched epithelium did not show signs of hyperplasia. This finding is in agreement with that of Francis et al. (1977). However, Squire (1980) claimed that tension leads to an increased progenitor cell population and subsequent tissue hyperplasia. The contol of epithelial thickness is multifactorial. The rate of mitosis is not the main factor determining epithelial thickness. Cell migration also plays an important role. Lorber (1968) has reported that any degree of tension usually inhibites cell ascension. This seems to be a reasonable explanation why the epithelial thickness did not show significant change in my study.

Following a three day recovery period after removal of mechanical tension, I found no significant differences in basal cell density between experimental and unstretched sites. It is reasonable to assume that the basement membrane was lengthened during stretching, thus decreasing the basal cell density. The increased area offered space for newly formed cells and prevented cell crowding that otherwise might be the result of increased mitotic activity.

The interpretation of my results is that mechanical tension decreased basal cell density, increased epithelial mitotic activity temporarily but did not alter epithelial thickness significantly. The basement membrane might have lengthened during stretching thus providing space for newly formed cells. There

were significant signs of recovery following removal of the mechanical tension. The basal cell density, epithelial mitotic activity and epithelial thickness at experimental sites were similar to those of unstretched sites.

The findings in this project have shown that mechanical tension is one stimulus by which the growth of capsular matrices may be influenced. The result should encourge continued research regarding growth control of capsular matrices. A significant breakthrough in orthodontic treatment would be reached if one could therapeutically regulate the growth of specific soft tissues and thus guide the size and shape of the associated hard tissue.

SUMMARY

Several types of appliances used in orthodontic treatment potentially affect non-skeletal tissue by generating biomechanical tension. The effect of such treatment procedures on buccal mucosa is little known. The present project is a model for studying the relationship between epithelial mitotic activity and mechanical tension.

The experiment was carried out on twenty 30 or 31 day old male Golden Syrian Hamsters. The sample was randomly and equally divided into four groups according to different experimental periods. In each experimental group, the left cheek pouch was stretched by injection of fast-setting latex material. The contralateral pouch was left untouched as control site. Each group comprised one sham-operated animal by which the tissue response to tension could be distinguished from the poxxible effects of inflammatory or traumatic reaction.

Tritiated thymidine was given twenty-four hours before sacrifice. Autoradiography was performed in order to assess the epithelial mitotic rate.

Data were collected regarding (1) epithelial mitotic index, (2) epithelial thickness, (3) basal cell density. Interpretation of data was based on the inter- and intragroup comparisons and comparisons between unstretched and sham-operated animals.

The most obvious result in this project was a significnat rise in epithelial mitotic rate after administration of

mechanical tension for two days. However, this phenomenon was not maintained. Data from days 4, 7, and 10, showed that the mitotic indices of stretched tissues declined to a normal range. Under stretching condition, the basal cell density was significantly decreased while the epithelial thickness did not show significant change. The basement membrane might have lengthened during stretching thus providing increased space for newly formed cells.

After three days following the removal of stretching devices, there were significant signs of recovery. The epitheial mitotic activity, epithelial thickness and basal cell density of experimental sites were similar to those of control sites.

The finding of this project suggested that mechanical tension is, under certain conditions, one stimulus by which the growth of capsular matrices may be influenced.

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