The University of Manitoba

An Animal Model System for *In Vivo* and *In Vitro* Studies of Scar Tissue in Cleft Palate: A Histological and Biochemical Study

by

Greg Vigneux

A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirements for the

Degree of Master of Science

Department of Preventive Dental Science

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ABSTRACT

The long-term objective of this project is to understand the factors involved in regulating the remodelling of oral cavity connective tissue during normal growth and during repair following palatal wounding. Scar tissue formation following cleft palate repair and its consequences on maxillary growth and dental occlusion are of prime interest in this study.

The experimental animal model which was developed for the examination of palatal scar tissue, was the eighteen day old Sprague-Dawley male rat. Palatal surgery was performed using a high speed dental handpiece with a surgical burr (#4) in the region between the maxillary first molars. The remodelling process was accomplished by using light microscopy and (^{3}H)-proline autoradiography. Palatal epithelialization was completed by the fourth post-operative day and collagen synthesis was seen to be active in the regions of epithelium, connective tissue and bone. Intramolar width in experimental animals was found to be less than in control animals of the same age. The general body weight of the experimental rats was significantly less than the corresponding control rats.

Biochemical analysis consisted of an *in vitro* model which was developed using a Trowell-type culture dish in an incubation chamber. Palatal samples in organ culture

were individually labelled with (^{14}C)-glycine in a time-course study and total collagen production was measured. New collagen production was found to be greater in the anterior portion of the palate when compared to the posterior palate in both control and experimental explants.

Type III collagen production was studied using (^{14}C)-glycine in a time-course study in vitro. The ratio of type III collagen production in palatal scar tissue to type I collagen was found to be significantly greater in experimental rats than in control rats at the 99% level of confidence. With time, the type III collagen ratio was found to decrease in both experimental and control rat palates.

The pattern of synthesis of different collagen types could be instrumental in the identification of the important cell populations involved in palatal scar formation. After their identification, one could then proceed to examine the regulatory mechanisms involved and thus lead to the prevention of scar tissue in the palate. The prevention of this scar tissue could lead to elimination of the growth disturbances of the maxilla and dental malocclusions, which are seen in most repaired cleft lip and palate patients.

DEDICATION

My parents, Mary and Ted Vigneux, have always been supportive in my educational endeavours. I appreciate their constant support and positive reinforcement.

As well, I am very grateful to Beth, who provided kindness and support through the final half of my stay here.

These three individuals have provided me with sound advice and I'd like to dedicate this thesis to them.

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CHAPTER I

INTRODUCTION

The craniofacial anomalies of cleft lip plus cleft palate (CL/P) and cleft palate (CP) alone pose severe handicaps and life-long implications for the afflicted individual. The members of many health disciplines are brought together in an attempt to resolve existing as well as anticipated problems of the cleft palate child. Cosmetics, plastic and maxillo-facial surgery, nutrition counselling, speech and hearing therapy, dentistry, orthodontics, and special education are but a few of the special needs that a cleft palate child will require. Throughout life these children can suffer from speech and hearing incompetencies which together with socially unacceptable facial esthetics result in severe limitations in their social and educational development. This creates a lowered potential in these individuals to contribute to society which is indeed difficult to measure.

This health problem is especially acute in the province of Manitoba and epidemiologic studies have indicated an incidence of 1.98 per 1000 live births which is significantly higher than that of other populations which average 1.25 per 1000 live births (Welch, 1978). Both Mennonite (Welch, 1978) and native Indian groups (Emmanuel et al., 1973; Lowry and Renwick, 1969; Welch, 1978) have a higher incidence of CP and CL/P and the significant presence of these groups contribute to the higher incidence found in Manitoba. The problem has been recognized only recently and rehabilitation programs have

been created in 1980 to ensure that multidisciplinary treatment is both available and adequately delivered to these patients.

Current therapy in North America involves surgical lip closure very soon after birth and subsequent primary palate closure around one year of age (Ross and Johnston, 1972). This creates acceptable esthetics as well as facilitates feeding procedures. Initial speech development can occur in the improved, although not ideal environment. The secondary palate is closed usually by the age of two years. This creates a situation which often leads to an abnormal growth and development of the facial and dental complex. Facial asymmetries and disproportions, and dental malocclusions with esthetic and functional problems often arise and worsen with age (Ross and Johnston, 1972).

It has been suggested that many of the orthodontic and dental problems stem from the lack of normal remodelling capacity in the soft connective tissues of the lip and palate which were closed surgically very early in life (Pruzansky, 1955; Ross and Johnston, 1972). Certain deformities of the maxilla occur fairly frequently, such as medial collapse of the upper alveolar arch or what is seen clinically as maxillary arch constriction. Disturbance in the downward growth of the maxilla resulting in maxillary retrognathism has been shown (Foster, 1962). The cause for such deformities has been blamed on

surgery and the resulting scar tissue which produces a tight upper lip and a growth restricting effect (Pruzansky, 1955). This tension has also been seen in palatal scar tissue after surgical repair of an isolated cleft palate (Foster, 1962).

Scar tissue from the lip and palate has been shown to contribute to severe mid-face underdevelopment and the resulting complex malocclusions (Ross and Johnston, 1972). It also limits the long range stability of orthodontic corrections which require extensive periodontal, oral and facial connective tissue remodelling. Constriction of surgical wounds in palatal mucoperiosteum has been demonstrated in young animals (Kremenak and Searls, 1971). As this phase of healing was seen to coincide with the onset of skeletal growth aberrations of the maxilla, it was proposed that contraction in the soft tissue wounds was causally related to the ensuing developmental deformities of the adjacent facial skeleton (Kremenak et al., 1967; Olin et al., 1974). Pruzansky as early as 1955 summarized the problem which we have today when he stated that "where excessive surgical manipulation produces tight, unyielding labial musculature, binding scars across the palate, and arrests in growth of the maxilla, arch expansions are not possible or if achieved, are difficult to maintain".

CHAPTER II

LITERATURE REVIEW

A. Skeletal and Dental Abnormalities Common in Cleft Lip and Palate Individuals

Children with oral clefts present dental problems that may be present at birth or in early infancy. It is not uncommon to observe the eruption of a primary incisor near the margin of the cleft at birth or shortly thereafter. As the infant grows, the following dental problems are observed: (1) an increase in the number of congenitally missing teeth, especially in the posterior segments of both the maxillary and the mandibular arches; (2) supernumerary teeth; (3) fused teeth and irregularities of tooth size; (4) malformed teeth; (5) malpositioned teeth; (6) delayed eruption of teeth, especially the maxillary canine on the side of the cleft; and (7) overeruption of the mandibular anterior teeth (Jolleys, 1954; Hagerty and Hill, 1963; Graber, 1949; Sidhu et al., 1982).

The common treatment problems in the cleft palate patient are: (1) management of the free-floating premaxilla of bilateral clefts; (2) lack of bone base over which to move teeth; (3) pseudomandibular prognathism due to over-closure; (4) dental crossbites; and (5) crowding in a constricted maxillary arch.

Some cephalometric characteristics associated with the surgically treated cleft lip and palate patient may be stated briefly to consist of the following: (1) a slight overall decrease in size; (2) moderate retrusion of the midface and the maxillary anterior dentoalveolar area; (3) downward and backward displacement of the anterior region of the mandible associated with an

increase in the size of the gonial angle (Lynch and Peil, 1966).

B. Growth and Development of the Maxilla in Cleft Lip and Palate Individuals

A review of the literature has revealed a number of contrasts in growth and development of the maxilla in children with repaired cleft lip and/or cleft palate as compared with those in normal children. There is some evidence to suggest that the maxilla is wider than normal at birth but significantly narrower by the age of three years in a cleft population (Fish, 1973). Wider maxillae may also be associated with clefts of the lip (Nakamura et al., 1972). Findings on the association between palatal clefting and maxillary length are not all in agreement. Some data indicates decreased maxillary length (Krogman et al., 1975); other data suggests no difference (Fish, 1973; Crabb and Foster, 1977). Palatal clefting may also be associated with more posterior positioning of the maxilla (Bishara, 1973) or with more posterior and superior positioning of the maxilla (Hayashi et al., 1976). The rate of maxillary growth and concomitant changes in configuration have also been studied (Foster et al., 1977). Peak maxillary arch growth in both length and width appears to occur between six and seven years in males and between seven and eight years in females, while peak changes in configuration appear to occur in both sexes between five and six years. A number of these studies have also provided data on the position and configuration of the alveolar segments in unilateral clefts of the lip and palate (Mapes et al.,

1974; Wada and Miyazaki, 1975; Bishara et al., 1976; Crabb and Foster, 1977). It remains unclear whether the forward growth of the maxilla is normal or whether both arches are more posteriorly positioned though normal in relationship to each other. These studies use cephalometric landmarks in their analysis of maxillary position. The points of reference are difficult to locate reliably and can be changed significantly by a slight movement in head position. The studies have also failed to consider such surgical variables as timing of surgery, the surgeon, the type of operation, and the number of operations which have been performed. All of these variables might well affect facial growth (Ross and Johnston, 1972).

C. Mandibular Growth in Cleft Lip and Palate Individuals

The studies of the growth and development of the mandible in individuals with clefts of the lip and/or palate indicate that clefting is associated with retrognathism but not micrognathism (Krogman et al., 1975), a more obtuse gonial angle (Krogman et al., 1975; Horowitz et al., 1976; Hayashi et al., 1976; Vora and Joshi, 1977; Bishara et al., 1976, 1978), and lingual inclination of the lower incisors (Bishara et al., 1976, 1978; Vora and Joshi, 1977). However, Fish (1973) found no significant differences in any of the mandibular dimensions between subjects with and without clefts in the birth-to-three-year age range. It is clear from the disagreements noted on mandibular form and development in both normal and cleft-palate individuals, that further study is needed

in this area. Mandibular measurements in one study (Fish, 1973) used intra-dental arch measurements while others (Bishara et al., 1976; Krogman et al., 1975) have used cephalometric landmarks. This could account in part for the differences found. The sample size varied from study to study as did the population sample which was surveyed. Krogman et al. (1975) had a sample of 59 cleft palate cases and 43 cleft lip and palate cases. These patients had undergone repair by one surgeon and represented a sample from the Lancaster Institute of Pennsylvania. Bishara et al. (1976) used a sample of 20 individuals with unoperated clefts of the lip and/or palate from Kerala, India. The use of standardized techniques might reveal some important mandibular characteristics within individual populations of repaired cleft lip and palate populations.

D. Facial Proportions in Cleft Lip and Palate Individuals

Bergersen (1972) reported that skeletal maturation is significantly correlated with the facial growth spurt in normal male adolescents from at least five years prior to the onset of the adolescent growth spurt to one year following its onset. Nakamura et al. (1972) found that facial growth rates appeared to be the same in children with cleft deformities as in normal children. Krogman et al. (1976) found that individuals with repaired cleft palate exhibited increased upper and lower facial height, while Hayashi et al. (1976) found that upper facial height was reduced while lower facial height was increased. Horowitz et al. (1976)

indicated that upper anterior facial height is reduced while lower anterior facial height is increased. The authors found posterior facial height to be similarly affected; upper posterior facial height was found to be reduced while lower posterior facial height was found to be increased. In a study of 25 Indian children with surgically-repaired cleft palate, Vora and Joshi (1977) found upper anterior facial height to be unaltered, while lower anterior facial height was increased.

The obvious lack of unanimity of facial proportions in cleft-palate individuals clearly indicates the need for further research on this question. Part of the disagreement may result from differences in methodology, particularly in the selection of measurement points, or from racial differences. Very little data seems to be available on the patterns and rates of facial growth in normal and cleft-palate individuals. Finally, if growth rates are not significantly different in normal and cleft-palate individuals (Nakamura et al., 1972), but facial proportions do differ significantly (Hayashi et al., 1976; Horowitz et al., 1976; Vora and Joshi, 1977), then the facial characteristics of the two groups must be different to begin with. This particular issue has not been addressed in the literature.

E. Types of Cleft Lip and Cleft Palate

Defective development of the embryonic primary palate may result in clefts of the lip and anterior maxilla. Clefts of the hard and soft palate result from defective development of the

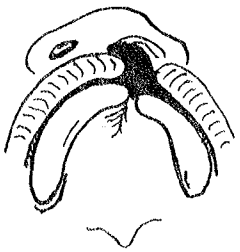
embryonic secondary palate and frequently occur in individuals with clefts of the lip (and anterior maxilla). Almost all clefts of the lip with or without cleft palate have different embryological, epidemiological, and etiological backgrounds than clefts involving only the hard and soft palate (Ross and Johnston, 1972).

Orofacial clefts are the most common of all facial malformations, occurring in all major racial and ethnic groups. Clefts occur in families from every social, educational and economic level and are particularly distressing, because most of these children are otherwise normal.

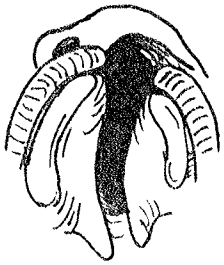
There is great variation among facial clefts and therefore there must be a practical and convenient method of classifying them for communication and research purposes. To the basic scientist interested in the etiological and developmental factors related to facial clefts, there are two major groups. The first group includes those clefts involving the lip and anterior maxilla, regardless of whether the cleft involves portions of the remaining hard and soft palate. (Figure II-1 cleft lip, unilateral and bilateral cleft lip and palate). Only the more complete forms are illustrated, but minor degrees of involvement occur. The second major group of facial clefts includes those involving hard and soft palate only (Figure II-1 - cleft palate). The most complete form is illustrated, although the cleft may be limited to the soft palate. The following abbreviations from Ross and Johnston (1972) will be used:

CL - Clefts involving the lip (and alveolus).

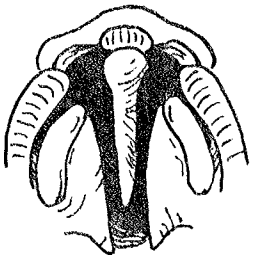
CLP - Clefts involving the lip and palate.



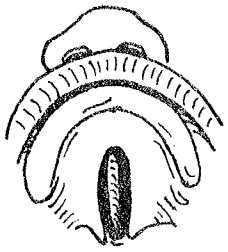
Cleft Lip



Cleft Lip and Palate unilateral



Cleft Lip and Palate bilateral



Cleft Palate

FIGURE II-1 Variations in clefts of the lip and palate at birth. Adapted from Ross, R.B. and Johnston, M.C., Cleft Lip and Palate, Williams and Wilkins, Baltimore, 1972. p. 2.

CL(P) - Clefts involving the lip with or without cleft palate.

CP - Clefts involving the hard and soft palate only.

CL/P - All types of clefts involving the lip and/or palate.

F. Facial Growth in Surgically Repaired Cleft Lip and Palate

(1) Surgical Variables

In a cleft involving the maxillary complex, there is great variability in the amount of tissue present, in the relationship of the parts and in the individual's growth potential (Ross and Johnston, 1972). Variations in the surgical procedures are important and include the following partial list. The timing of the surgery has been stated to be crucial since some believe that early repair of the hard palate interferes more with midface growth than surgery performed later in childhood (Bernstein, 1968; Graber, 1949). The type of operation seems to have a differential effect on facial growth and the most satisfactory procedures have been those which report to be least traumatic (Jolleys, 1954; Ross and Johnston, 1972; Slaughter and Brodie, 1949).

The evidence available indicates that multiple surgical procedures to the hard palate cause severe maxillary growth disturbances (Slaughter and Brodie, 1949; Chapman and Birch, 1965).

Ross and Johnston (1972) reported that the surgeon was the most important factor. Their review indicated that maxillary arch contraction was much less in the patients of one particular surgeon, although the cases considered had essentially the same operations. Moreover, this surgeon had average results in his first

few years, but had consistently superior results thereafter.

It is also important to consider the type of cleft lip and/or palate under repair. Most of the literature groups unilateral and bilateral cleft lip and palate patients as one entity in their sample size. Differences in growth of the face could exist between these populations as well as differences when compared to individuals with isolated cleft palate. Isolation on maxillary growth and midfacial growth is of prime interest to the dentist and orthodontist. However, the interrelationship with growth of the nose, growth of the sinuses, growth of the pharynx, and growth of the musculature must always be kept in mind.

(2) Results Indicating Reduced Midface

Numerous investigators have noted that cleft individuals who have undergone surgery in childhood exhibit differences in craniofacial relationships as compared with non-cleft individuals (Jonsson and Stenstrom, 1978; Ortiz-Monasterio et al., 1959).

Some people believe that traumatic surgery involving the vomero-premaxillary suture (VPS) contributes to impaired midfacial growth (Friede and Morgan, 1976).

Maxillary growth after palatal surgery was studied in Sweden and it was concluded that reducing the amount of scar tissue by covering raw surfaces with autogenous full-thickness skin graft was one way to reduce maxillary growth impairment after palatal surgery (Jonsson and Stenstrom, 1978).

Most surgeons agree that the underdevelopment of the maxilla which develops in a number of cleft palate patients is due to the

surgical manipulation at the time of closure of the cleft. This clinical impression is strengthened by the report of Ortiz-Monasterio and co-workers (1959; Sidhu et al., 1982) who studied adult unrepaired cleft palate patients by cephalometric techniques and found normal maxillary development in all patients.

Cephalometric studies of facial bone growth of human cleft palate patients demonstrate that traumatic or repeated operations on the lip and palate may retard growth of the maxilla (Slaughter and Brodie, 1949; Stark, 1962). Some authors feel that the vascular supply for subperiosteal bone growth may be impaired by extensive or repeated elevation of the periosteum (Stark, 1962). However, Sarnat (1958), in experimental studies with monkeys, removed the oral mucoperiosteum unilaterally, ligated the descending palatine artery unilaterally and resected the hard palate on one side only to find no growth disturbance.

The binding effect on the maxilla of a repaired cleft lip has been noted by some authors (Pfeifer and Schuchardt, 1964; Johanson and Ohlsson, 1965). Some people have advocated bone grafts for repositioning of the maxillary segments and stabilization in infancy. However, others feel that primary bone grafting to the alveolar process and hard palate in addition to the soft tissue repair will cause pronounced maxillary growth retardation (Friede, 1977; Friede and Johanson, 1980). This midfacial growth arrest will result in anterior and lateral crossbites as well as concave facial profiles in most cases by adulthood.

Many authors have alluded to the possible retardation of growth in the palate from mechanical limitation due to postopera-

tive scarring (Lynch and Peil, 1966; Moss, 1966; Kremenak et al., 1970). The postoperative limitation of scars has been recognized in burns and other traumatic deformities and may be one of the more important factors causing retarded maxillary growth in postoperative cleft palate patients.

Moss (1966) felt that any significant postoperative fibrosis might cause a mechanical restriction of maxillary growth. Such fibrosis would prevent the normal passive and secondary spatial relocation of the facial bones (as well as of the orofacial capsule) as it relates to the continued primary expansive forces of the orofacial matrices. In an operational sense, excessive scarring would produce a synostosis.

Extensive mobilization of septal soft tissue could reduce the growth potential of the vomero-premaxillary suture (VPS) by temporary blood supply deprivation or by formation of scar tissue which may have long-term effects as speculated by Jolleys (1954), Fuchs (1967), Sarnat and Wexler (1968), Latham et al. (1975) and Friede (1978).

Palatal repair has been discussed extensively and some have stated that facial growth in children with cleft lip and palate is abnormal mainly because of surgery to the palate (Ross and Johnston, 1972). The mechanism causing growth retardation is thought to be a continuous band of scar tissue extending from the pterygoid palates over the tuberosities and finally along the alveolar process. This fibrosis could gradually inhibit the forward drift of the dental arch. Dentoalveolar growth may be distorted as well, creating retrusion and narrowing of the dental

arch. Harvold (1961) observed that the premaxilla in bilateral cleft lip and palate patients retained its protrusive position longer in cases with minimal palatal scar tissue. Palatal fibrosis and thus growth inhibition is thought to develop particularly after the so-called push-back repair where areas of maxillary bone are left denuded by posterior and medial displacement of palatal mucoperiosteal flaps (Dingmar and Grabb, 1971; Blocksma et al., 1975). Comparison between patients with a two-stage palatal closure and push-back repair revealed the former to be a less traumatic method from the point of view of facial growth (Slaughter and Pruzansky, 1954).

It is well recognized that other parameters such as the timing of the procedure, the extent of undermining and denudation of the palatal shelves are also significant factors in determining the final effect on growth (Pruzansky, 1954; Palmer et al., 1969; Longacre, 1972; Hotz and Perko, 1974; Blocksma et al., 1975). The dynamics of facial growth within an environment of changing muscle forces, scar tissue creation and changing occlusal patterns could be altered significantly in the repaired cleft palate child (Pruzansky, 1954).

Cleft palate deficiencies in the middle third of the face have been observed by many authors (Lynch and Peil, 1966; Ross and Johnston, 1972; Blocksma et al., 1975). Absence of deficiencies have been reported in unoperated adults (Mestre et al., 1960; Sidhu et al., 1982).

It has been stated that it would be erroneous to assume that a concave profile in a cleft palate patient is always the result

of surgical interference with growth, inasmuch as a variation in the facial skeleton of non-cleft individuals also may manifest itself as a concave profile (Ricketts, 1956). Some attempts have been made to measure the various craniofacial components of cleft palate individuals exhibiting a middle third deficiency of the face by means of cephalometric radiographs and to compare these measurements with those of non-cleft individuals (Levin, 1963). An anteroposterior deficiency in the middle third of the face was observed in 29.6% of the cleft lip and palate patients studied. Bilateral clefts of the hard and soft palate yielded an anteroposterior deficiency with the largest percentage (52.4%). The exact percentage of midface deficiencies in the non-cleft group, however, was never stated.

The collapse of the alveolar arch in the maxilla has also been thought to be a result of the palatal surgery. Dunn (1952) claimed that certain types of operations such as the Von Langenbeck procedure were responsible for collapse of the arch. He preferred to repair the palate in two stages separated by six months of healing. The results were said to create normal palatal growth but a sample size of two people was not sufficient to draw any conclusions.

Henry (1955) felt that the operation on the palate should be delayed until all the deciduous teeth were present and then the teeth should be splinted so that collapse could not occur. As early as 1952, Hyslop and Wynn blamed the mucoperiosteal flap repair of the palate for producing a deformed palate and dental arch, and believed in using a flap of bone from the existing hard

palate to fill the palate defect and prevent distortion of the dental arches.

Often the blame for upper alveolar arch constriction fell on the tightness of the upper lip following lip repair. Pruzansky (1955) showed how, following lip repair in certain patients, the ends of the alveolar processes at the margins of the cleft became drawn together under the influence of the tight upper lip; and how the lesser segment of the maxillary arch may be overlapped by the greater segment. Pierce et al. (1955) suggested that this collapse happens at about the age of two years. Hagerty (1957) and Swanson (1958) both believed that collapse of the upper arch occurred after lip operation and before palate operation.

The cleft malformation itself may bring about the maxillary deformities and this could be quite separate from the effects of the operation. Walker (1959) suggested that agenesis associated with the cleft malformation may be an important factor in reducing maxillary growth. Hyslop and Wynn (1952) believed that in patients with cleft palate the growth of the maxilla is somewhat predestined, and in support of this they quoted cases where in unoperated cleft palates there are certain deformities which would readily have been attributed to the operation had one been performed. However, some researchers (de Jesus, 1959; Mestre et al., 1960; Sidhu et al., 1982) have shown that without surgery, maxillary dimensions and position of the maxilla within the craniofacial complex are normal on adult cleft lip and cleft palate individuals. Skeletal relationships of the unoperated cleft individuals did not differ significantly when compared to normal

adults.

Foster (1962) studied different types of maxillary arch collapse in different types of cleft lip and palate. He found arch collapse occurred in 58.1% of the patients. In surgically repaired patients with isolated cleft palate, it was found that "buckling" collapse occurred. This was where the premolar regions were distored medially. The fact that it was seen in patients with no lip or alveolar process cleft leads one to speculate on the role that might be played by tension from palatal scar tissue and not from repaired lip tension.

Kaplan (1978) studied 112 patients with unilateral complete clefts of the lip both pre- and post-surgically. His data indicated that a cleft lip has 10% to 20% greater growth in the transverse direction parallel to the orbicularis oris muscle than in the vertical direction perpendicular to the muscle. He concluded that surgical procedures which transpose tissue from transverse to vertical will lead to excessive vertical growth. Lip growth was found to be slightly decreased along the cleft margins. Lip shortness was observed to occur soon after repair because of scar contracture but tended to resolve with the passage of time. On the side of the cleft, transverse growth was found to be slightly less than normal (82% of normal), whereas vertical growth was signficantly reduced (67% of normal at the philtrum and 50% at the ala). As stated by the author, this study did not determine the reason for differential growth of lip skin. He hypothesized that abnormal skin tension and muscle force are the most important factors controlling lip growth aside from genetic influences.

Enemark and Jorgensen (1978) observed that unilateral-cleft-lip-and-palate patients operated on by the Tennison-Veau procedure exhibited a rather high percentage of maxillary asymmetry. Their tomographic study of 20 children who had had Tennison repairs at two months of age and Wardill palatoplasties at twenty-four months of age demonstrated bony development in the hard palate as well as fusion between the nasal septum and the maxilla on the cleft side in all subjects studied. In most patients, this bone formation was combined with an irregular thickening of the base of the bony septum with marked asymmetry of the hard palate.

(3) Results Indicating No Effect on the Midface

Some authors believe that surgery does not have a disturbing growth effect on the bimaxillary and bituberosity width, even when surgery is performed prior to eighteen months of age (Mazaheri et al., 1967).

Many outstanding surgeons insist that the underdevelopment of the maxilla existed as part of the cleft palate deformity before the operation or was a result of the type of surgery employed (Peer, 1947; Hyslop, 1952).

A great difficulty is that surgical procedures cannot be evaluated until many years after the operation, because of the continuing downward and forward growth of the palate. A palate which seems to have been successfully closed at two years of age may be badly mutilated ten or fifteen years later.

The causes of the differences in craniofacial measurements

between cleft individuals who have undergone surgery in childhood as compared with non-cleft individuals are still controversial (Bishara, 1976).

A number of investigators have attempted to determine whether craniofacial relationship differences result from surgery or exist independently of the effects of surgery on subsequent growth and development of the craniofacial complex.

Bishara (1973) studied facial growth cephalometrically in 32 normal children, in 12 subjects with surgically repaired palates, and in 8 subjects with unrepaired isolated clefts of the palate. He found significant differences in the relative size and position of the maxilla and mandible and reduced maxillary depth in the cleft group. His comparisons of the repaired cleft group with the unrepaired obturated cleft group revealed no significant differences. These data suggest that, although differences between the cephalometric morphologic characteristics of the cleft face and the normal face do exist, these differences are not necessarily a result of palatal surgery. Bishara has stated that his cephalometric data can be considered the "normal" morphological pattern for the cleft-palate-only face. He has concluded that, when these individuals are compared to normal individuals, the latter should be used primarily as a reference or baseline, and not to detect differences, since the cleft and normal samples are representative of essentially two different populations with different craniofacial characteristics.

In 1974 Mapes et al. studied photocopies of maxillary casts from 40 patients with complete unilateral clefts of the lip and

palate and noted that the growth rate of the maxilla in the cleft group lagged temporarily during the interval immediately following surgery, but then accelerated temporarily beyond the normal rate to a point where normal length was achieved. Wada and Miyazaki (1975) also studied growth and changes in maxillary arch form following surgery. Their data, based on 62 normal children and 87 children with complete unilateral clefts of the lip and palate, suggest more permanent changes as a result of surgery. In the cleft subjects who had had lip repair at six months and palatal closure at two years, the depths of all alveolar dimensions were found to be decreased at the age of two years. At four years, the latter group exhibited alveolar height and depth which were significantly smaller than in the normal group. These differences could, at least in part, be the result of surgical intervention or, as pointed out by Bishara in 1973, could simply demonstrate further the existence of two different populations with two different arrays of craniofacial characteristics.

Bishara et al. (1976) suggested that the resolution of this question might lie in the study of unrepaired cleft individuals at later stages of development. Such a study might offer some insights into which types of growth inhibition are inherited, which are biomechanically compensatory, and which are induced. They studied 12 individuals with unoperated clefts of the lip and palate ranging in age from seven to twenty-nine years and compared these subjects with 12 normal individuals matched in age, sex and ethnic background. Their cephalometric comparisons indicated no significant differences in the maxillary and cranial base parame-

ters between the cleft and normal groups. However, there was a tendency for the mandibular plane to be steeper than normal and for the total face height to be greater in the cleft group. There were as well, distinct differences in dentoalveolar and skeletal relations between both cleft groups and normals and between the two cleft groups.

Crabb and Foster (1977) have pointed out that certain of the growth deficiencies associated with repaired clefts of the lip and palate may be present in the absence of surgery. Most particularly, this includes deficiencies in vertical and lateral growth in the maxillary cuspid region immediately adjacent to the alveolar cleft. Their data clearly suggest the presence of localized growth defects as stated, which cannot be accounted for by operative trauma.

In 1977, Mazaheri et al. conducted a longitudinal analysis of growth of the soft palate and nasopharynx from six months to six years in 20 children with unilateral complete clefts of the lip and palate, 10 children with bilateral clefts of the lip and palate, 13 children with clefts of the palate only, and 15 normal children (controls). As pointed out by the authors, it has been postulated that shortness of the soft palate may result from prenatal velar underdevelopment, palatal bony deficiency or nonfunctional velar atrophy.

These authors felt that intrauterine underdevelopment and palatal bony deficiency may contribute to velar deficiency at birth. At six months of age, prior to palatal surgery, the mean velar length for all cleft types was significantly shorter than

the controls. Also it was found that velar length was affected more in the unilateral and bilateral cleft groups than in the cleft palate only group. Data collected by the authors, from birth to six years, did not support the concept of more superior positioning of the posterior border of the hard palate or of deficiency in vertical maxillary growth. Oral-nasal resonance balance in the cleft groups was noted to decrease from the age of three to six years without intervention of secondary surgery. This showed that cleft groups did not have better voice quality at an earlier age than at a later age because of an increase in size of the nasopharynx.

One other study designed to assess the results of a specific surgical procedure was conducted by Subtelny and Nieto in 1978. The authors conducted a longitudinal study of maxillary growth following pharyngeal flap surgery carried out prior to the approximate time of the pubertal growth spurt. Data from the 24 subjects in this group were compared with data from a group of 18 cleft palate individuals who had had palatal surgery but not pharyngeal flaps. Both cleft groups showed some post-surgical reduction in maxillary growth. However, the pharyngeal-flap group showed significantly greater reduction in maxillary forward growth. Interestingly, the pharyngeal-flap group showed some normal vertical maxillary dimensions with growth, whereas the non-flap group showed some reduction in vertical growth on the maxillary complex. This study may reflect surgically induced abnormalities in maxillary growth, or it may simply reflect pre-existent differences in the morphology and growth potential of the

two cleft groups.

It is clear from the disagreements on craniofacial dimensions and proportions associated with cleft palate that further study is urgently needed in this area. Some of the disparity in the data found in the literature may reflect methodologic differences. Samples should be shown from one group of cleft palate patients and would represent a baseline for future comparisons. For example, there are no cephalometric standards for six year old boys with a repaired unilateral cleft lip and palate. Surgical methods vary from surgeon to surgeon, and if there were better defined methods of surgical repair, results could be compared more meaningfully. Possibly the standardization of techniques and a more precise definition of morphological landmarks could be beneficial to different researchers in the future.

(4) Is Midfacial Retrusion a Problem in the Repaired Cleft Lip and Palate Individual?

One of the most common and difficult problems to understand in the patient with a cleft lip and cleft palate is the definite lack of development of middle third of the face. The mandibular prognathism may be only relative because of an underdevelopment in the middle third of the bony face. Prevention of this "dished-in face", which is difficult to correct, should be the goal of the Cleft Palate Team. In order to compensate for the deficiency of tissue, particularly in the anterior maxillary area, surgical and prosthetic means are employed. The ultimate objective is facial esthetics and functional harmony.

The effects of various surgical procedures on the cleft palate in regard to facial development have been given considerable attention. Generally, the consensus is that the severity of the growth arrest of the middle third of the face may be dependent upon both the particular type of cleft palate and the time, type and frequency of surgical operations.

In addition to the trauma sustained at the time of surgical closure of the cleft palate, in certain procedures there may be subsequent formation of scar tissue which has been ascribed as another cause of growth arrest. Massive scar tissue across the palatal arch could have a restraining effect upon palatal and maxillary growth, although there is no direct evidence. Which growth sites are affected is still unknown.

Some have suggested that surgical trauma incident to the raising of mucoperiosteal palatal flaps is not the cause of lack of maxillary and facial growth by the production of either a sutural growth arrest or decreased blood supply to the palate (Sarnat, 1958).

Lynch and Peil (1966) studied a group of dogs who had mobilization of the mucoperiosteal flaps for closure of the palate. A diffuse layer of scar tissue formed in the transverse dimension across the palate. Growth studies of these animals showed a significant decrease in transverse maxillary growth when compared to normal control animals or animals subjected to the same operative procedure but without the addition of the transverse band or scar tissue. They concluded that the decrease in transverse palatal growth was due to the mechanical binding effect of the inelastic

fibrous tissue produced in the transverse dimension of the palate.

Growth of the face is not completely normal and is sometimes grossly abnormal in children who have had surgical repair of a cleft lip and palate. Initially the problem of speech and the establishment of an esthetically repaired lip received the greatest attention. Midfacial retrusion was usually considered an inevitable result of the cleft anomaly. Some popular surgical procedures (e.g. the Brophy method) were recognized as causing severe inhibition to maxillary growth, and these procedures gradually disappeared from use, but the terrible results of these operations continued to plague the surgeon and prosthodontist (Ross and Johnston, 1972).

Graber's (1955) study seemed to place the blame for the observed deformity of cleft palate children on the surgery itself. This stimulated a great deal of controversy and has produced since that time tremendous interest and numerous studies in the field.

The face of the average child with a cleft maxilla fails to achieve normal forward development (Mazaheri et al., 1977; Ross and Johnston, 1972). The anterior region of the maxilla is, at all ages, retruded relative to the cranial base in an isolated cleft palate and in unilateral cleft lip and palate and it becomes progressively more retruded during growth (Foster, 1962; Hagerty and Hill, 1963; Shibisaki and Ross, 1969; Tong, 1961). This effect may be due to a decreased length of the maxilla (Ross and Coupe, 1965), although normal lengths have been reported (Harvold, 1960). The maxillary tuberosity (pterygomaxillary fissure in cephalometric studies) is in a normal anteroposterior position in

isolated cleft palate, is retruded in unilateral cleft lip and palate, and is severely retruded in younger bilateral cleft lip and palate (Ross and Johnston, 1972).

The examination of the evidence leads one to the conclusion that facial growth in children with cleft lip and palate is abnormal and one of the main reasons is due to the surgery to the palate. Ross and Johnston (1972) feel that it has a long-term effect on growth.

G. Intraoral Scar Tissue

Craniofacial growth and development in individuals with clefts of the lip and palate are influenced by irregularities in embryonic development, which cause tissue deficiencies, and by lip and palate surgery, which results in scar tissue and contractures (Vargervik, 1981).

The inhibiting effects of scar tissue and of irregularities in direction of tooth eruption on the forward development of the maxillary alveolar process can be counteracted by applying protrusive and extrusive forces on the alveolar process and teeth (Vargervik, 1981). Orthodontic treatment can indeed counteract the factors which inhibit development of the maxillary alveolar process.

The inhibiting effect that scarring and a tight lip may have on the forward growth of the maxilla can be partially counteracted (Vargervik, 1981). The formation of scar tissue intraorally is almost unique to cleft palate closure sites. As early as 1954,

Jolleys observed this fibrous tissue and felt that it exerted a growth restriction on the maxilla. Noting the inward rotation of the maxilla after surgery, and the alveolar movement behind the premaxilla, he felt that it was most easily explained by the concept of restriction of growth due to fibrosis. It was felt at this time that the contraction capability of the fibrous tissue was tremendous.

Surgical correction of cleft lip and palate always results, to a greater or lesser degree, in residual scarring and deformity. Recent studies have indicated that these residual sequelae may be much less if healing occurs in utero (Robinson and Goss, 1981; Sopher, 1975). Common features of fetal wound healing are the rapid progression of epithelial changes; the absence of scab formation and scarring; and cellular inflammatory reaction. The absence of these normal components of postnatal healing can be ascribed either to the immaturity of the tissues, in particular of the reticuloindothelial system, or to the sterile environment rendering a defense mechanism unnecessary. The protective environment and thus the lack of irritation or infection, would account for the lack of scarring. An important role of the scab in postnatal wounds is to prevent continued loss of body fluids through the breached epithelium. As fetal wounds are in a sterile environment, there is no need for a scab (Robinson and Goss, 1981).

In the Iowa Maxillofacial Growth Laboratory many experiments have been performed over the years with the long-range goal of elucidation of control mechanisms that influence growth in the maxillofacial complex. A more immediate goal was identification

and control of surgical variables used in cleft palate repairs that might interfere with post-surgical growth in the face. This group of researchers has been looking for ways to avoid or compensate for growth-inhibiting surgical variables. As stated by Kremenak and Searls (1971) "achievement of that goal would mean that palate repair surgery could be done in humans as soon after birth as was medically feasible, and that delay of palate surgery until the second or third postnatal year would no longer be necessary". Most of their work on palate surgery has been done using beagle dogs, but their findings have suggested that rats could be used in some of the work that involved manipulation of the surgical wound healing process.

It was found in their studies that the surgical variable which most interfered with post-surgical jaw growth was mucoperiosteal denudation of palatal bone adjacent to deciduous teeth, and that neither interruption of blood supply, nor extensive mobilization of mucoperiosteal flaps were variables (Kremenak et al., 1970; Kremenak and Kremenak, 1970). A basic assumption made was that assessment of growth effects of discrete surgical variables in animals without clefts was a sensible way to develop clinically useful information.

Mucoperiosteal surgery performed on one side of the hard palate of normal weanling beagle pups has been evaluated (Kremenak et al., 1967). Application of immediate post-surgical intraoral bandage produced no beneficial effect. However, it was found that the growth inhibition process could be blocked successfully by the adaptation of a laterally advanced bipedicle flap of mucoperios-

teum from midpalate to a surgically denuded region adjacent to deciduous teeth (Kremenak et al., 1970; Kremenak et al., 1971). This technique had no direct clinical application but it demonstrated the principle that initiation of the growth inhibition process in their model could be avoided by transplantation of a similar tissue to the tooth-adjacent wound region.

It was noticed as well that deciduous teeth adjacent to areas of bared palate bone moved medially toward the surgical wound during the first ten to fourteen days after surgery. The dentoalveolar complex in the wound area never again assumed its normal spatial position and the result was a maxillary deformity that involved the entire basal superstructure of the upper jaw. It was hypothesized that contraction in the healing surgical wound was the primary event that initiated the ensuing growth-inhibition process, and that techniques to prevent wound healing contraction should prevent the undesirable growth sequelae (Kremenak et al., 1971).

Later studies involved the use of topical applications of Trocinate (thiphenamil) to rabbit skin wounds and to palatal mucoperiosteal wounds in beagle pups. They showed that drug concentrations high enough to inhibit contraction also prevented healing while concentrations low enough to allow healing failed to inhibit contraction. Some success was met with a few agents in inhibition of wound contraction but growth in the healed areas was never as rapid as in unwounded tissue (Kremenak et al., 1976).

One of the events long recognized in the healing of many skin wounds is that of wound contraction. Depending upon the nature,

extent and site of the wound, contraction may give rise to marked scarring and disfigurement. Most studies show that maximum contraction occurs during that phase of healing when there is proliferation of fibroblasts and the production of collagen, i.e. when graulation tissue is formed (Abercrombie et al., 1954; Madden et al., 1974; Dunphy, 1974; Peacock and Van Winkle, 1976). Experimental studies on granulation tissues by Gabbiani and coworkers have suggested that it is the fibroblasts within this tissue that are responsible for contraction (Gabbiani et al., 1973; Ryan et al., 1974; Montandon et al., 1977). These studies have shown immunochemically that fibroblasts contain the contractile protein, actin (Gabbiani et al., 1973). These cells, termed contractile fibroblasts or myofibroblasts have been characterized ultrastructurally by the presence of bundles of oriented filaments, presumably representing actin, a convoluted nucleus and specialized junctions connecting them to other fibroblasts, and stroma so that contractile movements by individual cells result in a pulling together of adjacent cells and stroma.

Contraction of surgical wounds of the palatal mucoperiosteum has been demonstrated in young animals (Kremenak and Searls, 1971). As this phase of healing was seen to coincide with the onset of skeletal growth aberrations of the maxilla, it was proposed that contraction in the soft tissue wounds was causally related to the ensuing developmental deformities of the adjacent facial skeleton (Kremenak et al., 1967; Olin et al., 1974). Surgical repairs of palatal clefts in infants often involve the creation of wounds that subsequently contract so it is particu-

larly important to know more about the process of oral mucosal wound contraction which might allow the development of techniques to minimize this contraction and thus permit more normal growth of the maxillofacial skeleton.

Defects or fistulas have been difficult to close at times due to the limited quantity of suitable donor tissue. The tongue has been used as a donor site because of its abundant vascularity and has met with some success (Smith et al., 1982). These attempts have been made in order to circumvent the effects of intraoral scar.

A method of treatment was developed where the hard palate was closed after eruption of the permanent dentition (Friede et al., 1980). This treatment resulted in significantly less teeth in crossbite occlusion due to a wider maxillary dental arch. Poor speech development was a disadvantage in this type of treatment.

Primary veloplasty during the first year of life is a widely accepted procedure in cleft lip and palate patients. However, some surgeons have deferred closure of the residual cleft of the hard palate until the age of twelve to fourteen years, when the normal growth of the jaw is virtually complete (Schweckendiek, 1978). This prevents the palatal contraction as seen in earlier surgical results. If for any reason the residual cleft has to be closed at an earlier age, the jaw is kept under constant orthodontic supervision in order to prevent subsequent contraction. The values for width of the palatine arch and length of the maxilla and base of the skull were comparable to those of normal adults. However, as far as speech was concerned, it was found that during

the first few years (age two years to five years) children with residual clefts had greater difficulties and parents had to spend more time with them than was normally necessary. Therefore, social and psychological variables which were not assessed might have an important role. Palatal results were very good in that scarring and resulting constriction was avoided. However, the disadvantages to other developmental traits such as speech must be properly assessed if this technique is to gain worldwide acceptance.

H. Wound Healing and Scar Tissue Formation

Wound formation and repair are phenomena which can be traced far back in human history (Majno, 1975). They have been one of the major concerns in medicine in the past and still have an important place in the practice of general surgery and the management of traumatic and vascular diseases.

The body's reaction to the formation of a wound is now well known. The damaged area is invaded by plasma components (e.g. fibrin) and circulating blood cells (e.g. neutrophils, monocytes) which constitute a passive and active barrier against invasion of foreign material. When an efficient defensive reaction has been organized, the reparative phenomena starts with the synthesis of new connective tissue (Gabbiani and Montandon, 1977; Ross, 1968). The morphologic expression of reparative phenomena is the formation of granulation tissue. The new and temporary functions of granulation tissue are: (1) synthesis of a new type of connective

tissue, and (2) contraction of the wound. The modifications of connective tissue synthesis, when compared for example to normal dermal tissue, affect collagen (increase in synthesis of Type III collagen) as well as proteoglycans (Gabbiani and Montandon, 1977).

In general, the connective tissue reaction to injury may be adequately characterized as an inflammatory-reparative response. The time course of events in a typical response is plotted in Fig. II-2. The inflammatory component of the response includes the formation of a clot as well as the appearance of phagocytes. The reparative component of the response begins by the appearance of connective tissue cells which deposit a fine reticular network of fibrils derived from Type III collagen molecules. Later, the appearance of increasing numbers of fibroblasts leads to the accumulation of numerous larger fibers derived from Type I collagen molecules. At this time, the repair tissue contains predominantly fibers derived from Type I collagen molecules. This general sequence of events with respect to collagen synthesis and deposition during wound healing has been derived largely from studies on skin wounds, but seems to be similar in many connective tissue reparative responses (Gay and Miller, 1978).

In general, a wound is present in skin when there is a lesion of epithelium, basement membrane, and adjacent connective tissue. The wound may take the form of an incisional space or a cavity and this depends on the method of wound infliction. This damaged tissue undergoes necrosis. Over the years, a plethora of terms have been used to describe the phases of wound healing. These terms have been derived from different experimental models and by inves-

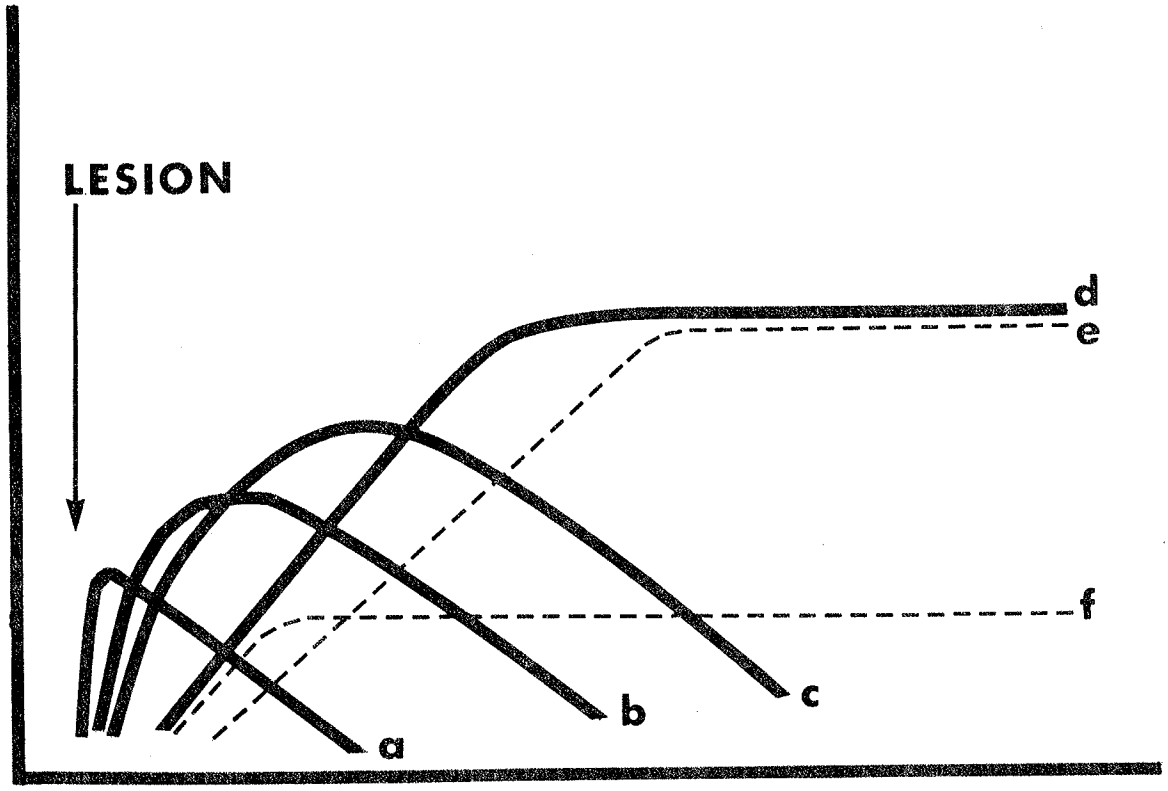


FIGURE II-2 A plot of the events in an inflammatory-reparative process as a function of time: a) blood clot; b) phagocytes; c) proliferation of capillary angioblasts; d) fibroblasts; e) fibers derived from Type I collagen molecules; f) fibers derived from Type III collagen molecules. From Gay, S. and Miller, E.J. Collagen in the Physiology and Pathology of Connective Tissue, Gustav Fischer Verlag, New York, 1978. p. 75.

tigators employing a variety of histological and biochemical techniques to assess the healing process. For the purposes of the present discussion we will use the generally accepted terminology descriptive of the exudative, resorptive, proliferative and reparative processes of normal wound healing.

Immediately following a dermal wound formation (in healing by primary intention), a wound cover is obtained by filling the lesion with blood which eventually clots (Fig. II-3A). The fibrin clot including entrapped blood cells forms a scab and provides a preliminary seal. In the first 24 hours after wounding, invading phagocytes signal the initiation of an acute inflammatory response in the margins of the lesion (Gay and Miller, 1978). The phagocytes resorb necrotic tissue and most of the clot (Fig. II-3B). At the same time, mitotic activity on the part of basal cells is observed in the epidermis, a process which will eventually reestablish continuity of the epidermal layer. Within 48 hours, wound repair and regeneration begins with granulation tissue formation. Capillaries can be seen in this stage to begin to form. As well, angioblasts and endothelial cells can be seen (Fig. II-3C). These cells are the first cells of the healing wound which have the potential to synthesize collagen. At this time there is a small reactivity of the granulation tissue with antibodies to Type III collagen. Subsequently, the granulation tissue becomes highly vascularized (Fig. II-3D) at which time one observes an increase in the reactivity of the tissue with antibodies to Type III collagen. After the capillaries within the granulation tissue have joined to create a continuous vasculature, there occurs a prolif-

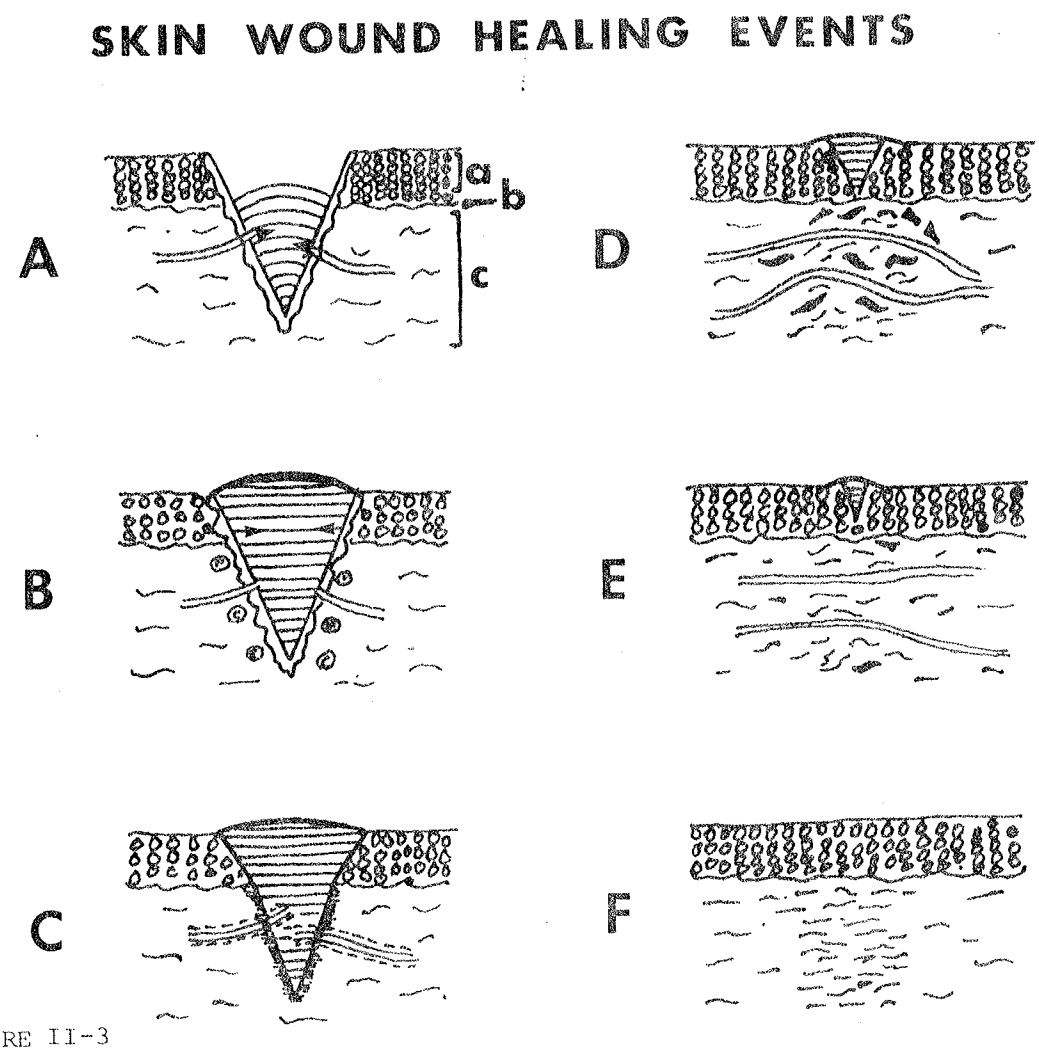


FIGURE II-3

A schematic representation of events in a healing skin wound: a) epidermis; b) basement membrane; c) dermis. A) Clot formation is initiated in the wound cavity. B) Phagocytes resorb necrotic tissue while epithelium advances from the margins of the lesion. C) The progressive budding of capillary cells and deposition of fine fibrils derived from Type III collagen molecules. D) The vascularization of granulation tissue associated with continued deposition of fibrils derived from Type III molecules as well as some larger fibers derived from Type I molecules. E) The continued deposition of fibers leads to compression of the capillaries. F) The final scar is covered by a restored epidermis. It is comprised largely of dense collagen fiber bundles. From Gay, S. and Miller, E.J. Collagen in the Physiology and Pathology of Connective Tissue, Gustav Fischer Verlag, New York, 1978. p. 77.

eration of fibroblast-like cells. At this time large cross-striated collagen fibers can be seen. Immunohistochemical studies indicate that these fibers are comprised largely of Type I collagen molecules (Gabbiani et al., 1976; Peacock et al., 1976; Gay et al., 1978). As fibroblast proliferation and synthesis of collagen proceeds, the resulting fibers occupy an even greater proportion of the lesion volume and compress the thin-walled capillaries leading to the formation of blanched scar tissue (Fig. II-3E) which exhibits a considerable degree of tensile strength. The final scar, covered by a restored epidermis, appears largely acellular, avascular, and rich in collagen fibers derived from Type I molecules between which occur a few smaller fibers derived from Type III molecules (Fig 3F). As noted above, the occurrence and relative proportions of the fibers derived from Type III and Type I molecules in various phases of wound healing have been determined by immunohistochemical techniques (Bailey, 1978; Gay et al., 1978).

It has been known for several years that healing skin wounds contract during the repair process (Bazin et al., 1974). Collagen fibers of the healing wound cannot of themselves provide the required contractile forces. It is therefore possible that these forces are provided by the proliferating fibroblasts which may be termed myofibroblasts. Even though scar formation in the skin is a remarkable biological process, tissue repair is achieved without complete regeneration of the dermal structures. For instance, the scar tissue does not contain hair follicles, glands, and melanocytes.

The principle components of dermal connective tissue are the fibrous proteins collagen and elastin. In addition there is a ground substance composed of glycosaminoglycans and glycoproteins (Peacock and Van Winkle, 1976). Physically the mechanical properties of this strong replacement tissue are due primarily to collagen, the principle component, comprising over 50% of the scar (Alvarez and Gilbreath, 1982).

Fibrinogenesis and maturation of collagen from the first 48 hours after wounding are of great significance. The fragile fibrils will begin to change into strong, insoluble cross-linked fibers. These, in turn, become organized into an oriented structure providing mechanical strength to the repaired tissue. Experimental studies have shown that connective tissue maturation changes the bulk, form, and tensile strength at a relatively constant rate from periods of two months to over five years (Madden and Peacock, 1971). Levenson et al. (1965) in their evaluation of breaking strength and tensile strength of rat skin wounds, observed that breaking strength of wounds increases rapidly from approximately the second to the fortieth postoperative day.

When tissues are being repaired, more amino acids, carbohydrates, lipids, minerals, water and oxygen are consumed than during catabolism and anabolism of normal mature tissue (Pollack, 1979).

The large amounts of granulation tissue synthesized in response to skin injury are normally resorbed and the scar tissue reverts to a composition and structure similar to that of the original tissue (Bazin and Delaunay, 1964). In contrast, some individuals, particularly after burns or deep trauma, form excess

scar tissue in the middle and deep layers of the dermis; such hypertrophic scars are especially frequent in Negroes and some Caucasians who can develop these abnormal scars after any kind of wound. Biochemical composition of these hypertrophic scars reveals a similarity, based on glycosaminoglycan content, to newly formed granulation tissue even in scars ten to fifteen years old (Bazin et al., 1974).

It would be informative to determine the proportions of the types of collagens which are present in the normal palatal soft connective tissues and in scar repair tissue in surgically treated palate. Similar work involving normal skin, normal scar, and keloid (hypertrophic) has suggested significant differences in collagen synthesis patterns relative to normal skin (Diegelmann et al., 1979). It was found in this study that keloid fibroblasts had an autonomous capacity to synthesize increased amounts of collagen compared to fibroblasts from normal skin or normal scar. The particular collagen phenotypes which would be expected are Type I and Type III collagen. The changes in their relative proportions at various time points during scar formation in comparison to normal palatal soft tissues would be essential data to correlate with the remodelling capacities of normal and scar tissue in the palate.

I. Collagen and its Role in Scar Tissue Formation

Barbanell et al. (1978) described collagen fibers as being composed of aggregations of smaller fibrils, which in turn are formed by cross-linking between individual collagen molecules.

Collagen is synthesized intracellularly in osteoblasts and fibroblasts and secreted as procollagen which has additional terminal peptides. Each collagen molecule is composed of three polypeptide chains wound into a right-handed supercoil. Each polypeptide chain consists of about 1052 amino acids, of which every third one is glycine. Proline is also of high concentration, some of which becomes hydroxylated to form hydroxyproline which is an amino acid characteristic of collagen. Each polypeptide chain is coiled in a left-handed helix. The molecular weight of collagen is about 300,000 and it measures 15 Angstroms by 2800 Angstroms.

There are at least five types of interstitial tissue collagens which have been identified (Prockop and Williams, 1982). Type I collagen is present in skin, tendon, bone, gingiva, and periodontal ligament. It is composed of two identical polypeptide chain subunits, the alpha 1 (I) chains and a third structurally different polypeptide subunit, the alpha 2 (I) chain. Therefore, the subunit structure of Type I collagen is alpha 1 (I) alpha 2 (I). Type II collagen alpha 1 (II), is found in cartilage. Type III collagen alpha 1 (III), is found in young dermis, foetal tissues, blood vessels and periodontal ligament. Type IV collagen alpha 1 (IV) occurs in basement membrane. Type V collagen or A B collagen has been shown to have two distinct chains, designated as alpha A and alpha B or A and B (Chung et al., 1976). Another chain alpha C has been discovered and the alpha A, alpha B, alpha C chains of Type V collagen is designated as alpha 1 (V), alpha 2 (V), and alpha 3 (V), respectively (Bornstein and Sage, 1980). Type V collagen has been found in whole placenta, the

amion, chorion, cornea, and in human gingiva (Narayanan and Page, 1983).

The different collagen types can be separated through the use of chromatography or electrophoresis.

The collagen content in normal scars of skin is initially higher than normal skin but reverts in the same level as normal skin in old scars. For hypertrophic skin scars the collagen content is not significantly different from normal skin (Bailey et al., 1975).

Hypertrophic scars are characterized by a marked increase in proteoglycans, especially chondroitin-4-sulphate (Shetlar et al., 1978). It is therefore possible that alterations in collagen-proteoglycan interaction may contribute to the development of this abnormal healing. Recent experiments suggest a significantly increased collagen-proteoglycan interaction in human hypertrophic scars as compared with non-hypertrophic scars, normal skin, and granulation tissue (Linares, 1983).

In normal scar the major reduced cross-link in early wounds is dihydroxylysinonorleucine Lys (OH)2 - Nle but after a few months the major reduced components are hydroxylysinonorleucine Lys (OH)2 - Nle and histidinohydroxymerodesmosine His - Mdes (OH) .

In hypertrophic scars the major reduced cross-links present are Lys (OH)2 - Nle and Lys (OH) - Nle. Initially the proportion of the Lys (OH)2 - Nle is higher than Lys (OH) - Nle (about 3:1) but rapidly plateaus to a ratio of about 1:1.3.

Collagen synthesis and maturation is a prerequisite of wound

healing (Gay et al., 1978) and development of wound tensile strength (Viljanto, 1964). Current information suggests that normal mammalian skin contains 80-90% of Type I collagen and 10-20% of Type III collagen (Miller, 1976).

A high proportion of Type III collagen, in contrast to the small amount normally present in mature skin, has been demonstrated in rat dermal granulation tissue (Bailey et al., 1975b). During the wound healing process collagen synthesis was high and it was therefore suggested that the ratio of Type I and Type III collagen may change. It was found that the proportions of Type III collagen in the acute and chronic inflammations were higher (40 and 30% respectively) than the normal mature rat skin (about 10%).

Studies in children in Finland have shown in early phases of healing (24-72 hrs), Type III collagen production (Viljanto, 1976). Type III collagen has been demonstrated in pepsin-solubilized collagen of rat dermal granulation tissue (Bailey et al., 1975b; Gabbiani et al., 1976), human hypertrophic scar, and in guinea pig dermal scars (Barnes et al., 1976; Bailey et al., 1975a). Type I collagen increases in the later stage of human wound healing may indicate that the original cells of the wound area, or their descendants, can synthesize both collagen types, as has been shown for isolated human skin fibroblasts (Gay et al., 1976c).

The mechanical strength of the wound increases in proportion to the increase in its collagen content (Gay et al., 1978).

Type III collagen is formed in guinea pig scar tissue ini-

tially, followed by the appearance of Type I collagen. If, however, contraction is prevented, only Type I is formed. Preventing contraction prevents epithelialization (Jackson, 1978).

It has been shown that fibers of the periodontal ligament (PDL) contain collagens with the chain structures alpha 1 (I) alpha 2 and alpha 1 (III) (Butler et al., 1975). The PDL is one of several soft connective tissues shown to contain both Type I and Type III collagens. Other examples are skin (Epstein, 1974; Chung and Miller, 1974), aorta (Trelstad, 1974), uterine leiomyoma (Chung and Miller, 1974), cardiac muscle (McLain, 1974), and gingiva (Ballard and Butler, 1974).

J. Summary

Orofacial clefts are the most common of all facial malformations occurring in all major racial and ethnic groups. The psychological impact of an abnormal facial appearance can be quite distressing to the parent and later to the child. The major functional disturbances involve the mechanism of speech and the development of the midface and dentition.

Much has been researched and reported about the growth and development of the midface of the cleft lip and cleft palate child. In my opinion most cleft palate children do indeed have a midface retrusion which seems to be a result of the surgical trauma (Ross and Johnston, 1972; Mazaheri et al., 1977; Sidhu et al., 1982). The formation of scar tissue intraorally is almost unique to cleft palate closure sites. Little research has been

done in the area of palatal scar tissue but there have been a number of studies undertaken in the field of dermal scar.

Thus it would be desirable to determine the proportions of the types of collagens present in normal palatal soft connective tissues and in scar repair tissue in surgically treated palate. The proportions of types of collagen present in normal palatal soft connective tissues would provide a baseline to which palatal scar tissue could be compared. Changes in proportion of collagen types could indicate the cells which are active in palatal healing and this could eventually lead to understanding the mechanism involved in scar formation. Understanding the mechanism could eventually result in our ability to control it and thus avoid scar tissue formation and its undesirable sequelae. Similar work in dermal scar has suggested significant differences in collagen synthesis patterns relative to normal skin (Diegelmann et al., 1979). As stated earlier, the particular collagen phenotypes which would be expected are Types I and III, since these collagen types have been isolated from other gingival sites (Ballard and Butler, 1974). Changes in the ratio of Type I collagen to Type III collagen at various time points during the formation of palatal scar would be important data to compare with normal palatal soft tissue. This might suggest the mechanism of scar formation and how it differs from the normal tissue. The study of palatal scar tissue and normal palatal tissue will also elucidate their remodelling capacities and might lead us to the control and thus reduction of unfavorable scar tissue in the palate. Palate surgery could then be done in humans soon after birth and delay until

two to three years of age would no longer be necessary.

CHAPTER III

STATEMENT OF THE PROBLEM

The factors involved in regulating the remodelling of oral connective tissue during normal growth and during orthopaedic or orthodontic therapy are as yet unknown. The collagen response in surgically manipulated palatal mucosa has not been studied. The pattern of synthesis of different collagen types could be instrumental in the identification of the important cell populations involved in palatal scar formation. After their identification one could then proceed to examine the regulatory mechanisms involved and thus the prevention of scar tissue in the palate.

Since our primary objective is to study palatal scar formation and its effect on facial growth and development of the occlusion, it is necessary to develop an experimental model system with a cleft and a resulting scar.

The first step would be to determine the optimal age of the animal in which a surgical cleft will be created, in order to assure the potential for growth and developmental changes. The effect of the cleft creation and the subsequent results of scar tissue formation can be measured if there is a reasonable amount of growth remaining.

Once this age has been determined, the second step would be to investigate histologically the tissue response on a time-course basis, to determine the sites of non-collagenous protein and collagen synthesis.

The next step would be to determine biochemically the types of collagen synthesized at various time points in different sites in normal and healing palatal tissues. In future studies these collagen synthesis patterns would aid in identification of those

cell populations involved in palatal scar remodelling. Ultimately, these isolated populations could be studied for the control mechanisms in palatal tissue remodelling.

CHAPTER IV

THE DEVELOPMENT OF A MODEL

INTRODUCTION

The development of a model which can mimic the effects of cleft palate surgery has been attempted in the dog (Kremenak et al., 1971; Lynch and Peil, 1966; Jonsson and Hallmans, 1980), monkey (Sarnat, 1958) and pig (Atherton et al., 1974). Since the maintenance of these large animals is costly, it would be desirable to select an animal which is both smaller and readily available. Findings suggest that rats can be used in some of the work that involves manipulation of the surgical wound healing process (Fejerskov, 1973; Searls and Biggs, 1974).

In general it has been found that palatal surgery interferes with maxillary growth (Jonsson and Hallmans, 1980; Kremenak et al., 1971; Lynch and Peil, 1966). However, in many studies no effort has been made to separate the effects of the many surgical and growth variables involved and it is therefore very difficult to draw conclusions concerning the mechanisms underlying this growth interference.

Creation of a surgical cleft in the rat would allow one to observe the histological processes of wound healing in the palatal mucosa. This healing response could be further monitored by pulse labelling with (^{3}H)-proline for radioautography following surgical injury and thus assess the rates and magnitudes of the proliferative response of various cellular elements of the palate of the rat. It has been shown that proline is utilized in the synthesis of a wide variety of intracellular proteins in the non-hydroxylated form (Tristram, 1953). Hydroxyproline is a unique

amino acid in protein structures. It is essentially found only in collagen and its presence is generally taken as an indicator of collagenous metabolism. Hydroxyproline is not incorporated directly into the polypeptide chains of collagen, but rather is produced by the specific hydroxylation of pre-existing proline molecules incorporated into the alpha-chains during collagen biosynthesis. Therefore, labelled proline is commonly used to measure collagen synthesis. Glutamic acid, ornithine, and arginine are important components of collagen and can also be derived from proline (Stetten, 1955).

MATERIALS AND METHODS

The Experimental Animal

When considering a mucous membrane area for studies of the cytological changes in the surface epithelium and underlying tissues during wound healing, a number of conditions must be fulfilled (Philipsen, 1971):

(a) The stratified squamous epithelium of the mucous membrane must be thick, i.e. it must be composed of a large number of cell layers enabling a detailed study of the reaction of the single strata to experimental wounding.

(b) The experimental area must be easily accessible for experimental procedures, e.g. the production of standardized excisional wounds.

(c) The experimental area must be well-defined making it easy to

trace even after a lesion is completely healed.

After a pilot investigation (see Appendix 1), the palatal region of the Sprague-Dawley male rat aged eighteen days was chosen for experimental use. The area of the midpalatal suture between the erupting first molars was found to fulfill all of these criteria.

The mucous membrane in this area is tightly bound to the underlying bony tissue. The epithelium is thick, keratinized, and of stratified squamous type with tall, slender connective tissue papillae. The midpalatal suture is a reliable histological landmark and the first molars are relatively stable throughout the palatal growth period in the rat (Hughes et al., 1967).

Histologic Study and Autoradiography

Young male Sprague-Dawley rats, eighteen days of age, outbred in this facility (Faculty of Dentistry Animal House), were maintained on Wayne Laboratory, Blox-F6 specialty feed, given water ad libitum, and kept at a temperature of 20 ± 1.5°C. Rats were randomly selected for experimental and control groups for each of eight post-surgical time periods: 0, 1, 4, 7, 10, 14, 21, 28 days post surgery.

Each rat was anaesthetized by using ether anaesthesia for approximately three minutes. The rat was then removed from the ether and numbered with an ear punch. Control rats were then placed in the recovery cage. Experimental rats were placed on an operating table and the mouth was propped open using cotton pliers. The tongue was held under the cotton pliers so as not to

infringe upon the surgical area. The first molars were then sighted and a surgical burr (number 4, friction grip) was placed between them in the region of the midpalatal suture. The palatal mucosa was penetrated as was the underlying palatine bone into the nasopharyngeal cavity. Upon withdrawal of the burr, the palatal mucosa only was then lacerated in an anterior direction up to the region of the premaxillary bone, but not quite to the incisive foramen. The width of the lesion created was 3 mm and the length was 8-10 mm (see Figure IV-1).

Each rat was weighed before and after surgery and the intramolar width was measured using precision calipers (Staedtler, Germany) and a boley gauge (see Fig. IV-2). The intramolar width was taken as the shortest distance of palatal epithelium between the first molars. This caliper measurement was transferred to a straight line and marked. Later the distance was measured using a boley gauge, accurate to 0.1 mm.

Following surgery each animal was placed in a recovery cage and a dish of minced feed (Wayne Laboratory, Blox-F6 specialty feed) was introduced for one week. Two rats (one experimental and one control) were selected randomly from each age group and injected intraperitoneally with L-proline-2, 3-H (New England Nuclear, Lot 1468-128; 24.9 ci/millimole specific activity) with a dosage of 5 uCi/g. body weight, two hours before sacrifice. In view of the possible circadian periodicity of cell division, as demonstrated by Roberts et al. (1979) in the rat molar periodontal ligament, the injection of radioisotopes and sacrifice were performed after 10:00 a.m. because 8:00 a.m. to 10:00 a.m. was the

BASAL ASPECT OF THE SKULL OF THE RAT

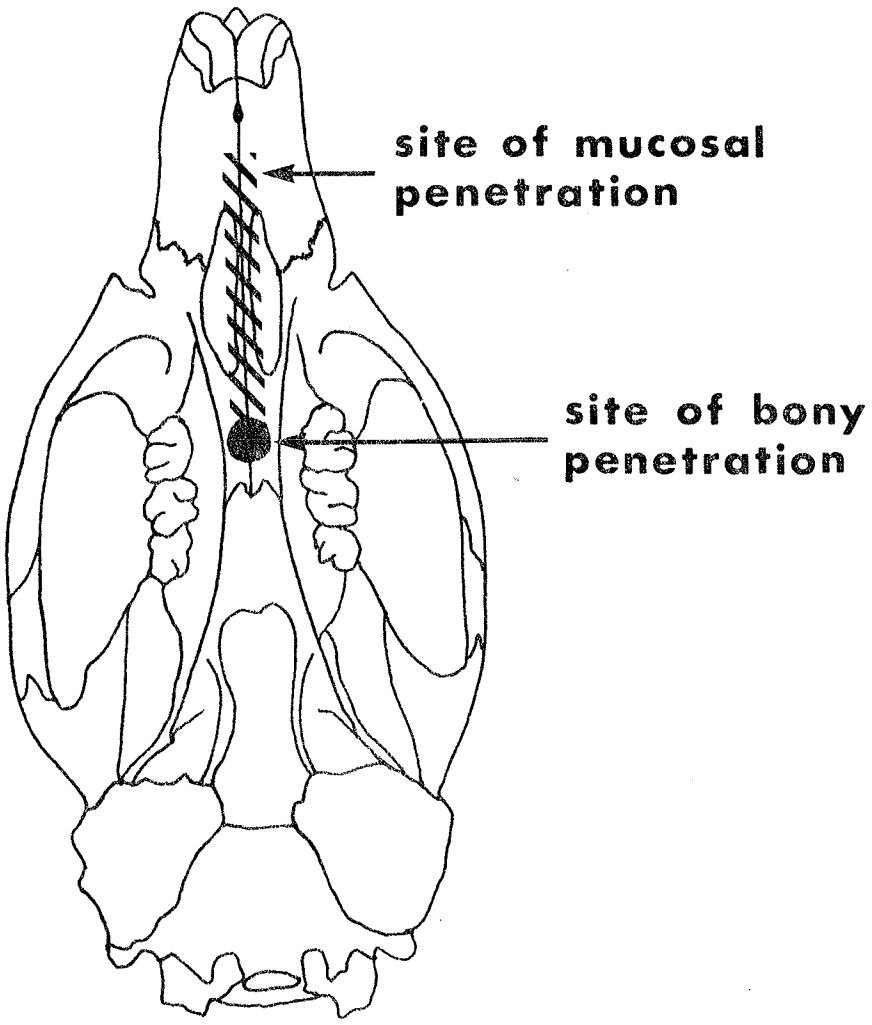


FIGURE IV-1 Basal aspect of the skull of the rat showing where surgery was performed with a surgical burr mounted in a high speed dental handpiece. The actual width of the lesion created was 3 mm. and the actual length of the lesion created was 8 to 10 mm.

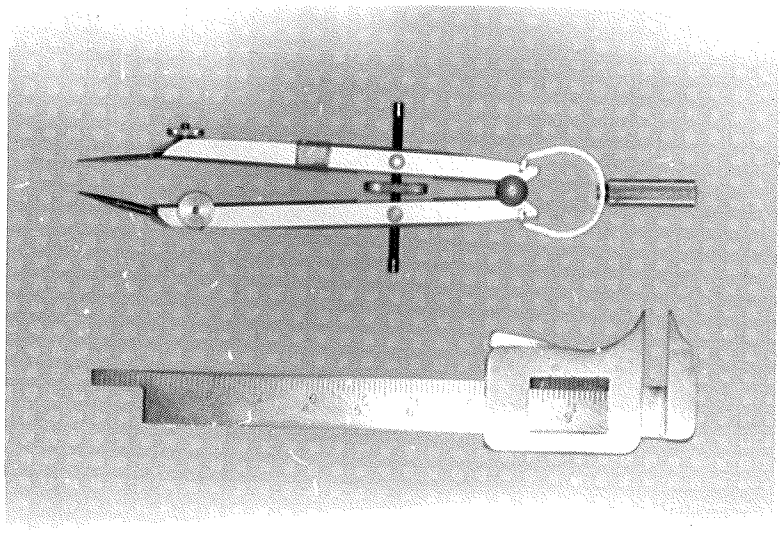


FIGURE IV-2 Instruments used to measure intramolar width of first molars in rat palates.

Above - calipers

Below - boley gauge

peak period of mitoses (Roberts et al., 1979). By avoiding this period of cell proliferation, the likelihood of protein synthesis would be maximized. After sacrifice, the maxilla was dissected immediately. The mandible was cut along both rami and reflected back along with the tongue. The section of palate removed included the anterior rugae from the incisive foramen to a point 3.0 mm behind the third molar. The zygomatic arches were cut and the section included all three molars. This was then fixed in Bouin's solution for 24 hours and decalcified in Cal-Ex (Fisher Scientific Company, New Jersey, U.S.A.) for 12 hours. After embedding in paraffin, 5 um serial sections were cut and mounted. Every fourth slide was stained with haematoxylin and eosin.

The intervening serial sections of control and experimental specimens were processed together for radioautography using the dipping technique of Kopriwa and Leblond (1962). The slides were dipped in Kodak NTB2 emulsion (Eastman-Kodak, Rochester, New York). Exposure was made in the dark at 4°C. for 10 days. The radioautographs were developed with Dektol, fixed with Kodak Rapid Fixer and stained through the emulsion with haematoxylin and eosin.

Photomicrographs of the palatal wound site and of a comparable site in the control animals were made of each radioautograph as transparencies which were projected on a screen with 1 X 1 cm grids using a photographic enlarger. Grain counting was performed over unit areas of the grid.

Five areas were counted for each zone using ten different slides. Zones were divided into epithelial, connective tissue and

bony regions (Fig. IV-3). This was repeated for every photomicrographic transparency. The average was calculated for each zone. The background control slides were also counted. All counting was undertaken by the same person to eliminate inter-operator difference. The consistency of the operator's counting has been confirmed with an error study (see Appendix 2). The data was tabulated, corrected for background and subjected to statistical analysis.

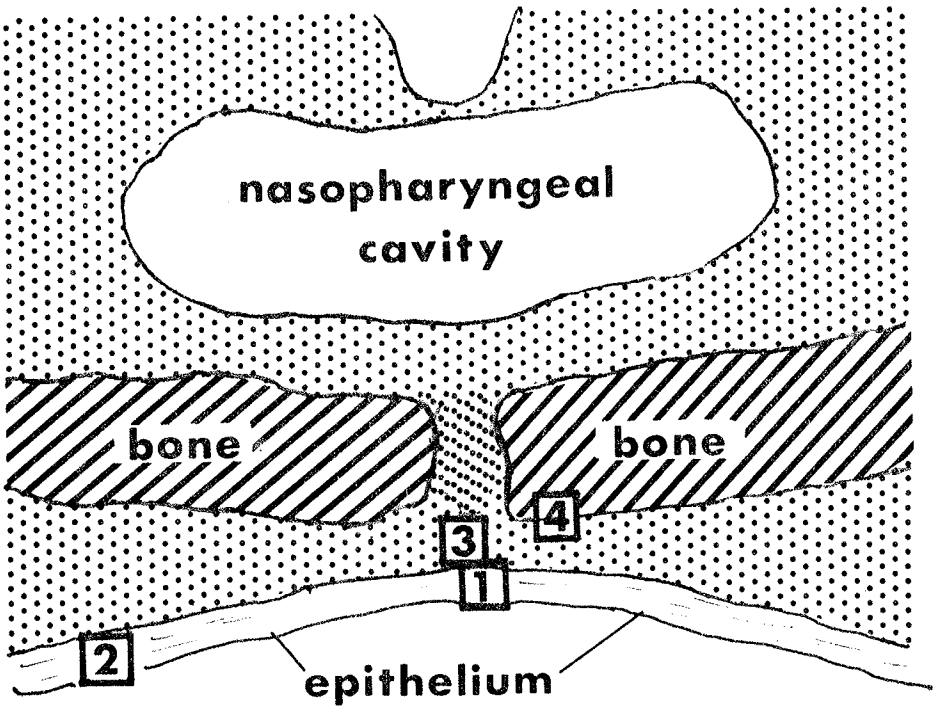
OBSERVATIONS AND RESULTS

Visual Results of Palatal Surgery

Observations of the healing process revealed that the epithelial wound edges were fused together four days after surgery. The scar which was formed was located in the middle of the palate and a scarred palate was easily distinguishable from a control palate (Fig. IV-4). The scarred palate contained posterior rugae which were asymmetrical in nature and did not meet in the midline. The anterior rugae of the scarred palate had gaps in the midline where surgery had been performed. The middle of the palate appeared somewhat shallower in the wounded palate compared to the control. It appeared narrow (contracted) in the first molar region and V-shaped compared to the control which was wider and parallel. Intramolar measurements revealed that the first molars were closer to each other than succeeding teeth in the arch. This first molar region was the area of actual bony penetration. The tissue in the

FIGURE IV-3

PALATAL GRAIN COUNT SITES



AREAS (ZONES) COUNTED:

1 Epithelium close to wounding site

2 Epithelium peripheral to wounding site

3 Connective tissue in wounded region

4 Bony junction (with connective tissue)

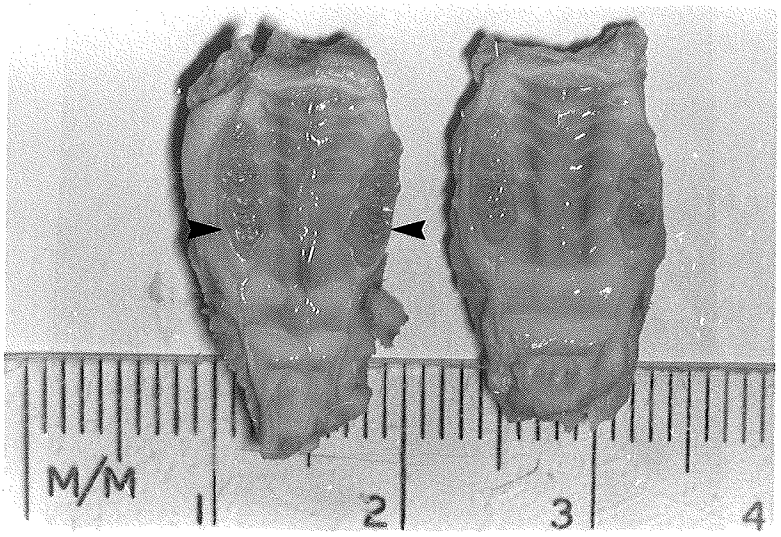


FIGURE IV-4 Enlarged photo (X 2.5) of experimental rat palate (left) and control rat palate (right) 14 months after experimental procedure. Note the contracted intramolar width especially in the first molar region (arrow). The posterior portion of the palate is at the top of the photo. The rugae pattern is symmetrical in the control palate (right) and asymmetrical in the experimental palate (left).

middle of the experimental palate was the scar tissue which was studied in further experiments.

Light Microscopic Characteristics of Normal Palatal Epithelium

The oral epithelium consisted of several cellular layers and rested on a basement membrane (Fig. IV-5). The first layer was the stratum basale (germinativum). It was composed of a single layer of dividing cuboidal cells. The region above this single-layered structure was the multilayered stratum spinosum. The cells here were polyhedral and appeared connected to one another. Above this layer was the stratum granulosum in which the cells appear somewhat flattened with their long axis parallel to the surface of the epithelium. The final layer of cells was the stratum corneum and it contained cells which were closely stacked with no visible nuclei.

Deeper to the epithelium was a layer of loose connective tissue containing blood vessels, nerves and fibers of collagen, reticulin and elastin. Deeper to this layer was a layer of palatal bone which is divided in the midline to form the midpalatal suture. Above this region was the loose connective tissue of the nasal mucosa and the single layer of columnar cells lining the nasal cavity (Fig. IV-6). The light microscopic results of normal palatal epithelium can be seen from Figure IV-6 to IV-13.

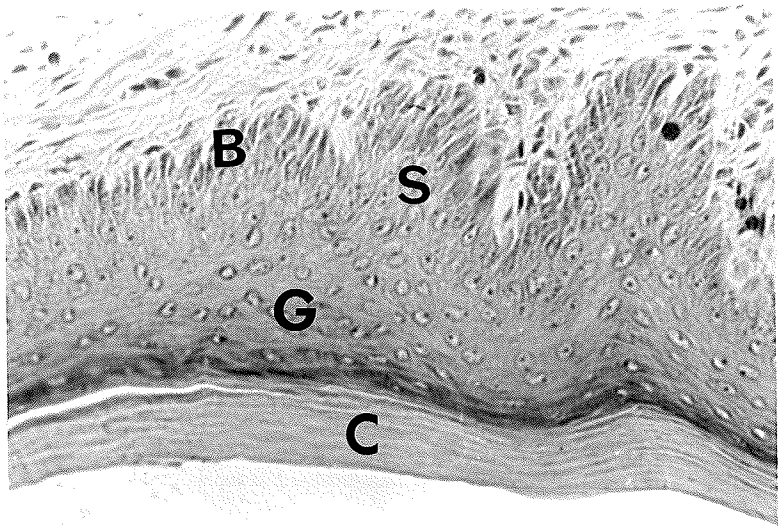


FIGURE IV-5 Photomicrograph of the layers of oral epithelium in the palate of the rat (X 2,500)

B - stratum basale

S - stratum spinosum

G - stratum granulosum

C - stratum corneum

Legend for Figures IV-6 to IV-25

NS = Nasal Septum

M = Maxillary Sinus

NP = Nasopharynx

B = Palatal Bone

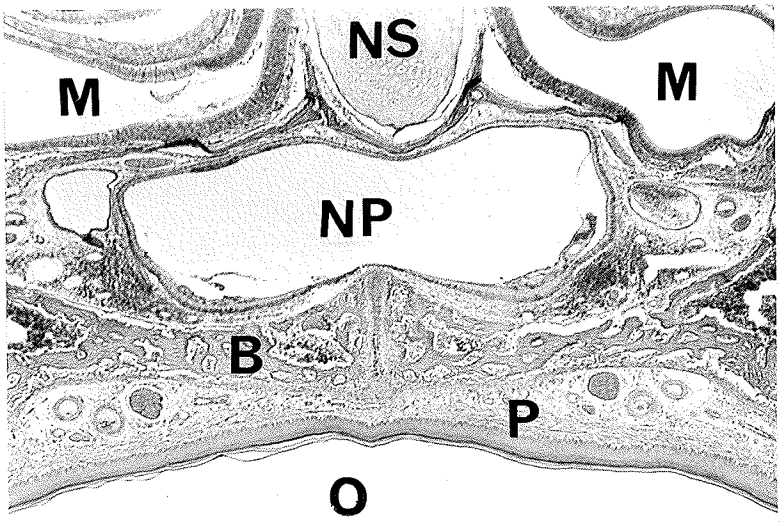
P = Palatal Soft Tissue

O = Oral Pharynx

F = Oro-nasal Fistula

FIGURE IV-6 Coronal cross-section of a control rat palate, 0 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-7 Coronal cross-section of a control rat palate, 1 day into the post-operative period. Haematoxylin and eosin. X740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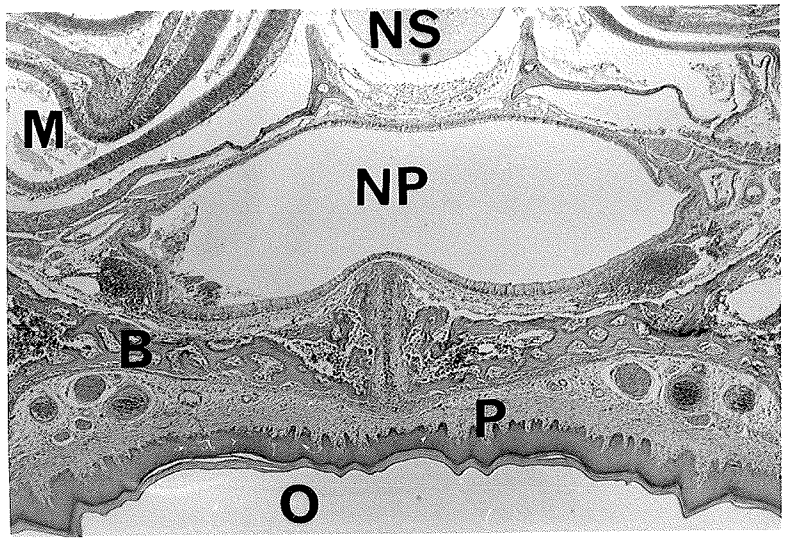
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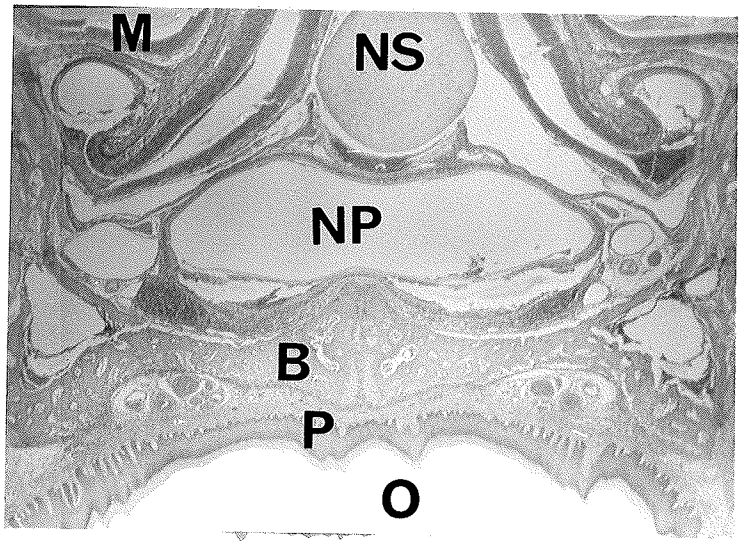
FIGURE IV-8 Coronal cross-section of a control rat palate, 4 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-9 Coronal cross-section of a control rat palate, 7 days into the post-operative period. Haematoxylin and eosin. X740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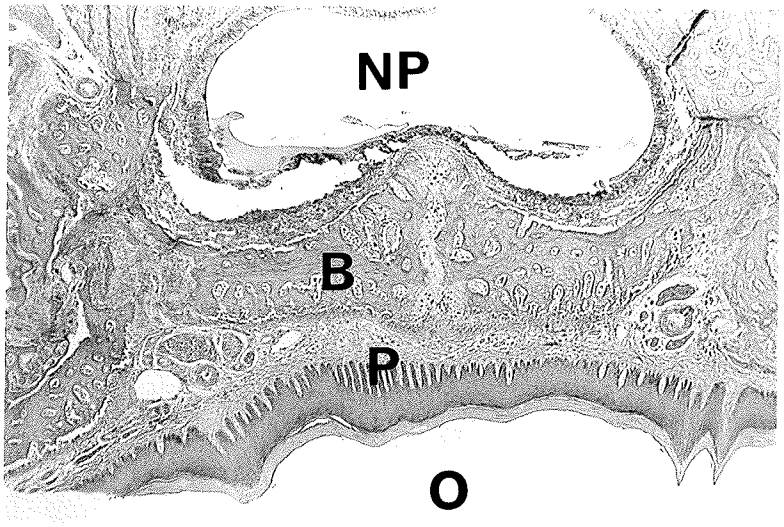
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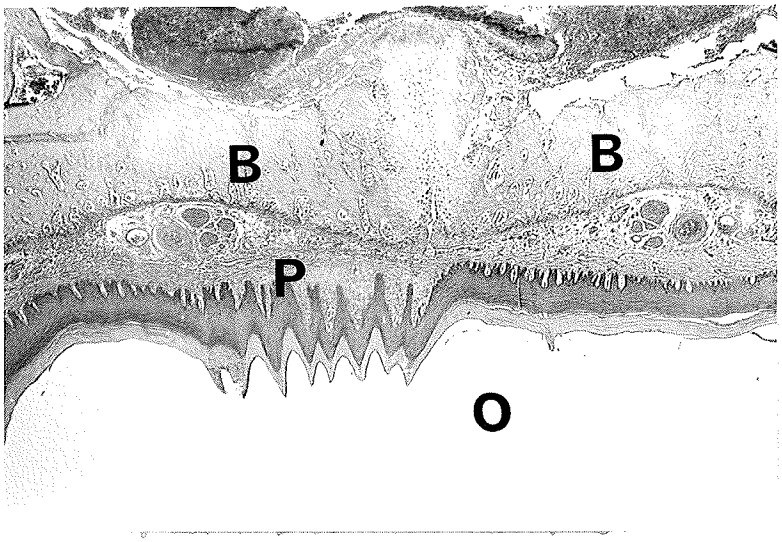
FIGURE IV-10 Coronal cross-section of a control rat palate, 10 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-11 Coronal cross-section of a control rat palate, 14 days into the post-operative period. Haematoxylin and eosin. X1460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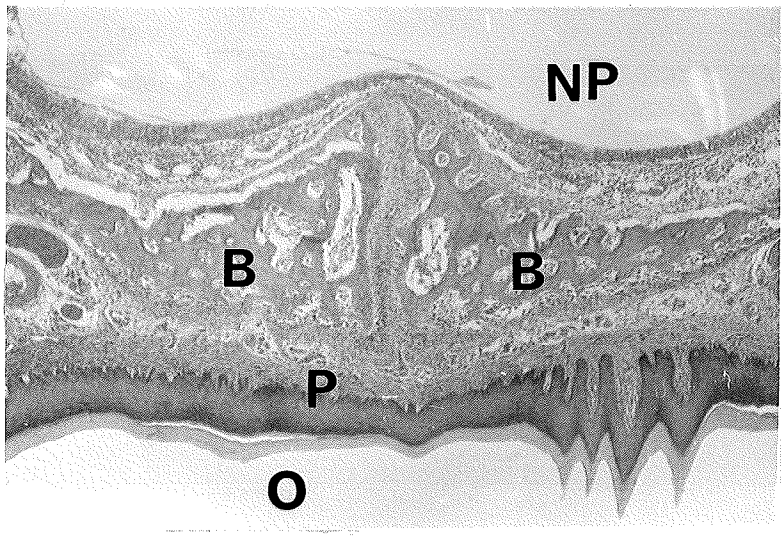
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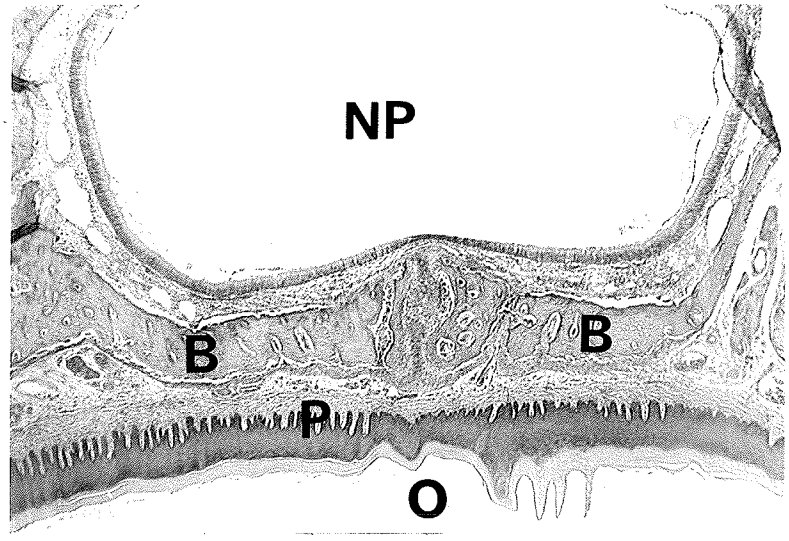
FIGURE IV-12 Coronal cross-section of a control rat palate, 21 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-13 Coronal cross-section of a control rat palate, 28 days into the post-operative period. Haematoxylin and eosin. X1460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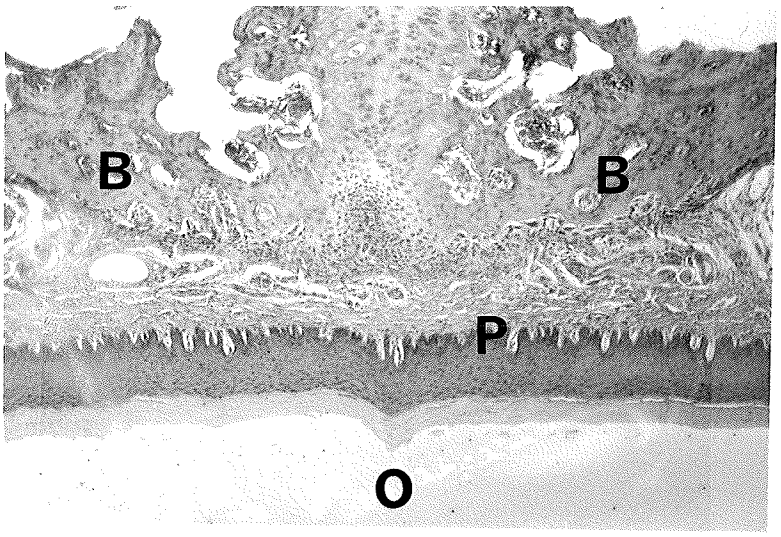
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The Reaction of Palatal Epithelium during Wound Healing under Light Microscopy

The results of the light microscopical investigations into the reaction of the palatal epithelium and underlying connective tissue and bone to standardized surgical wounding can be summarized according to the days following the surgery.

Immediately Following Surgery (Time Zero)

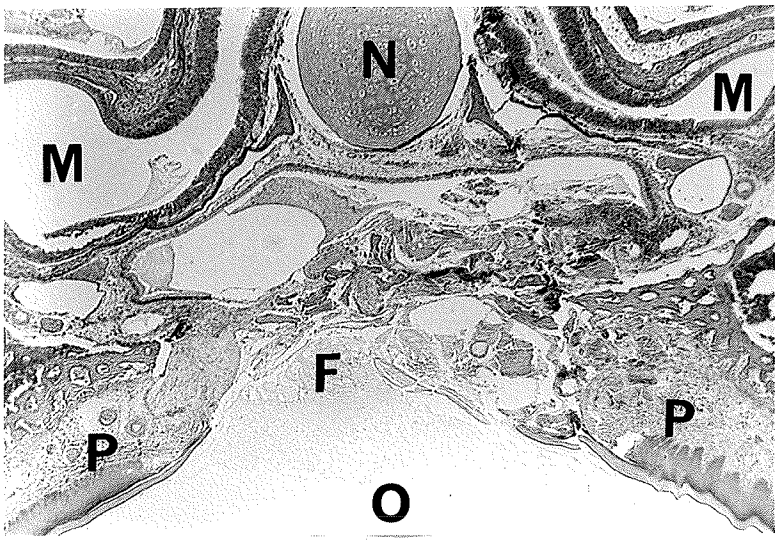
The cut surface through the epithelium connective tissue and bone could be clearly seen (Fig. IV-14). Polymorphonuclear granulocytes were adjacent to the wound, whereas the wound cavity itself was filled with blood coagulum. Along the wound margin in the stratum granulosum and the upper cell layers of the stratum spinosum, the cells were stained similar to the cytoplasm in the stratum corneum or lighter. Pyknosis could be seen in the area adjacent to the wound site. The cells bordering the incision have been flattened in the stratum spinosum and stratum basale (Fig. IV-15).

One Day Following Surgery

The cells bordering the wound seemed to be more organized from the standpoint of less cellular infiltrate in the cleft when compared to time zero. The wound margin was still well-defined. There were many more leukocytes present in the coagulum of the wound area. The nasal cavity was still filled with blood coagulum

FIGURE IV-14 Coronal cross-section of an experimental rat palate, 0 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-15 Coronal cross-section of an experimental rat palate, 0 days into the post-operative period. Cells bordering the incision can be seen to be flattened and pyknotic. Haematoxylin and eosin. X3300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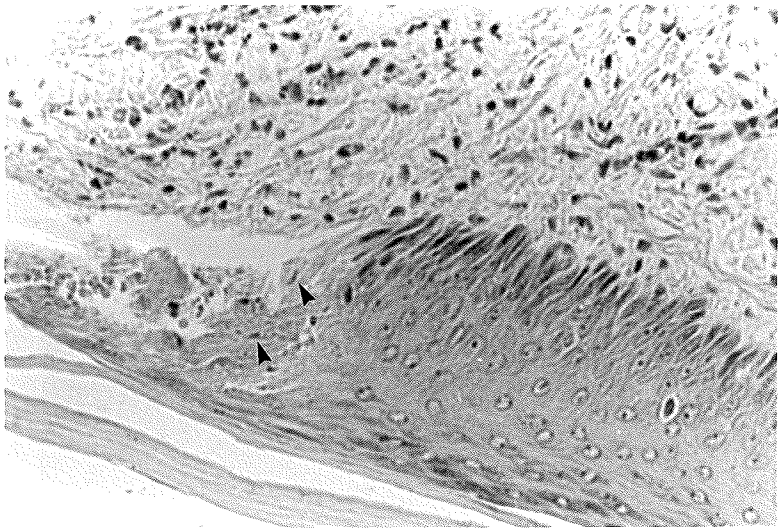


and there was massive cellular infiltrate present. It could be seen that the wound had a definite clot at this time and the center of the cavity contained fibrin (Fig. IV-16).

Four Days Following Surgery

The layers of the epithelium were once again clearly demarcated (Fig. IV-17). The epithelial wound edges had migrated and were fused in most cases. There were a few examples where the cleft had remained open, creating an oronasal fistula (Fig. IV-18). The epithelium over the wounded region was still thin compared to the non-wounded epithelium.

Bony recontouring had begun to take place and the bone wound edge did not appear as sharp and pointed in nature as previously described (Fig. IV-19).

The nasal cavity had reorganized and the columnar cells were facing the center of the nasal cavity. The nasal cavity had an irregular outline, but was still cylindrical in shape like the control sample.

There appeared to be a lack of vascularization in the loose connective tissue under the wound region.

The midpalatal suture was not present and there were no cartilagenous cells.

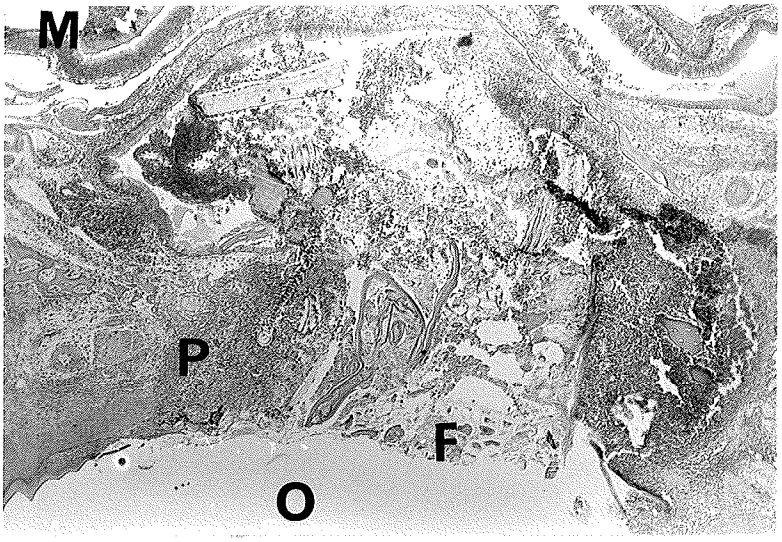
Seven Days Following Surgery

The epithelial layers were joined completely and appeared normal with notable exceptions. There was a lack of rete peg for-

FIGURE IV-16 Coronal cross-section of an experimental rat palate, 1 day into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-17 Coronal cross-section of an experimental rat palate, 4 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-18 Coronal cross-section of an experimental rat palate, 4 days into the post-operative period. Residual cleft is present. Haematoxylin and eosin. X740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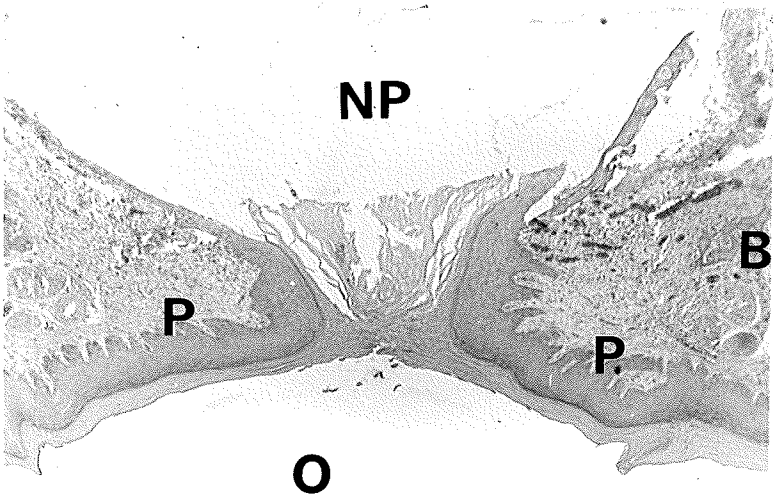
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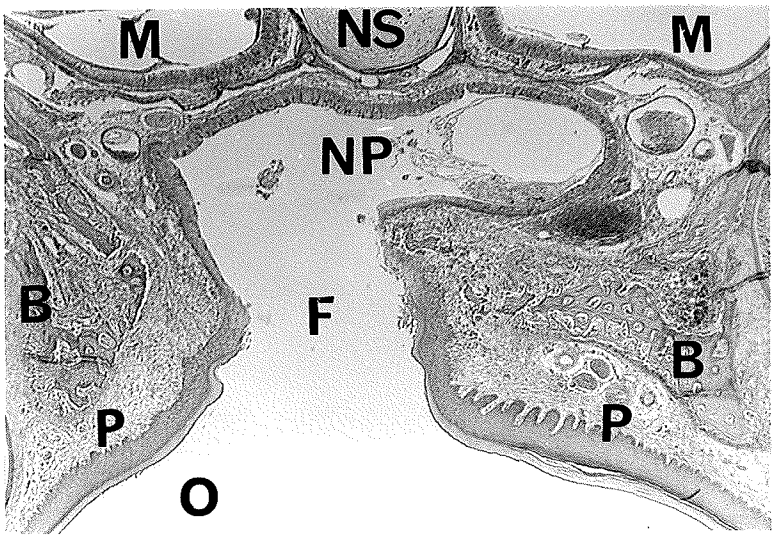
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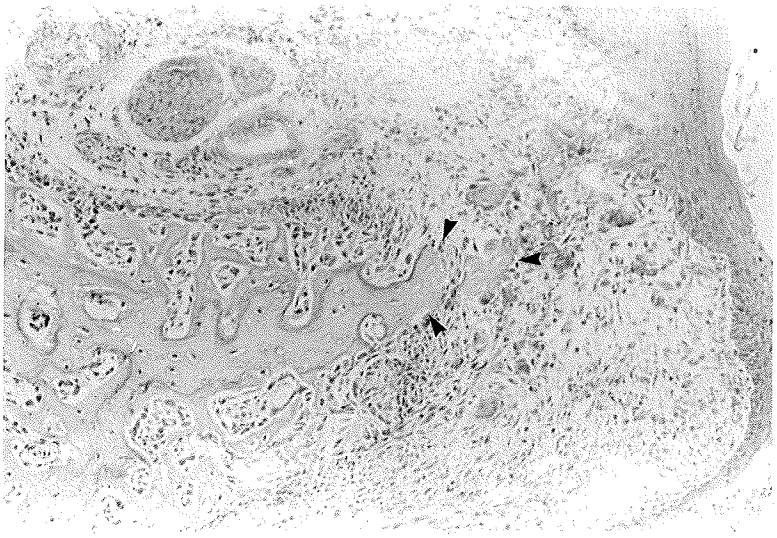
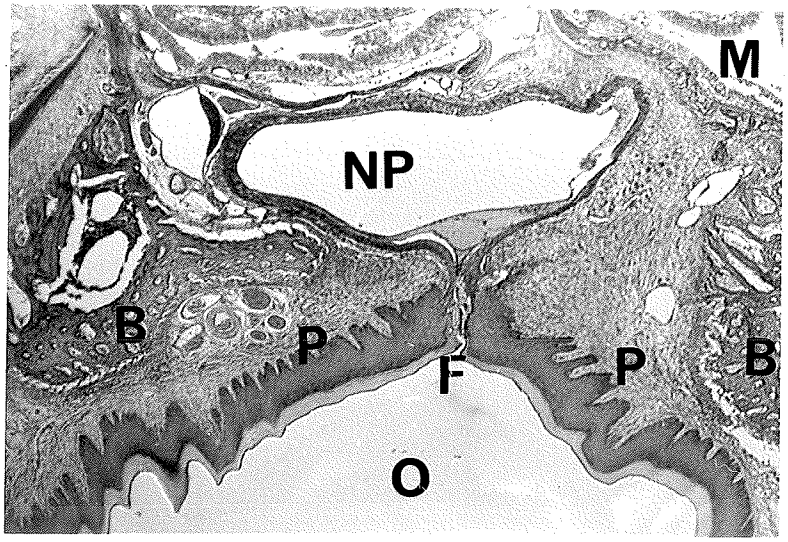
FIGURE IV-19 Coronal cross-section of an experimental rat palate, 4 days into the post-operative period. Haematoxylin and eosin. Note area of bony recontouring. X3300

FIGURE IV-20 Coronal cross-section of an experimental rat palate, 7 days into the post-operative period. Haematoxylin and eosin. X740

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mation and the stratum basale was smooth in appearance (Fig. IV-20).

The underlying connective tissue was avascular compared to the control palate and compared to the adjacent non-wounded regions. Bone was not present and had been replaced by soft connective tissue which extended to the nasal cavity. There was no sign of cartilaginous cells.

Bony spicules were no longer present and the bone from the palatal shelf which bordered the cleft appeared rounded in nature.

The nasal cavity was cylindrical but was not symmetrical as seen in the seven day control palate.

Ten Days Following Surgery

The epithelium in the wounded region had remained slightly thinner than seen on the periphery, but had increased in rete peg prevalence compared to seven days following surgery. The epithelial convolutions were fewer in this region compared to the peripheral regions (Fig. IV-21).

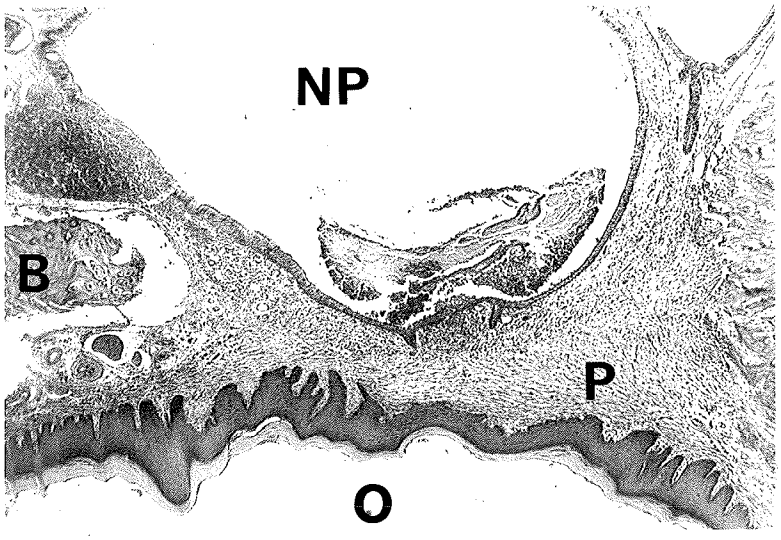
The palatal bone had been rounded in shape, towards the nasal cavity and appeared somewhat thickened at the edges.

The connective tissue in the wounded area was still relatively avascular and lacked organization in its appearance when compared to the peripheral unwounded regions.

Not much change had occurred in the nasal cavity compared to the seven day palate.

FIGURE IV-21 Coronal cross-section of an experimental rat palate, 10 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-22 Coronal cross-section of an experimental rat palate, 14 days into the post-operative period. Haematoxylin and eosin. X740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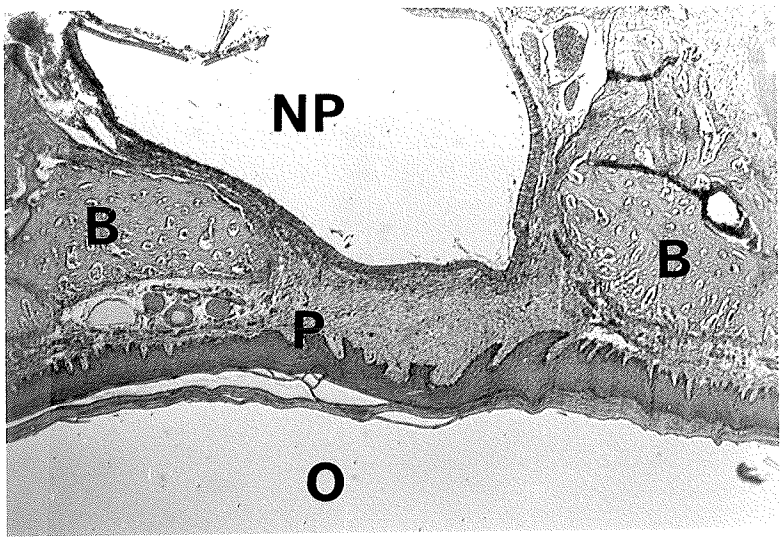
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Fourteen Days Following Surgery

The epithelium appeared to be normal except for the lack of rete pegs in the wounded region. Connective tissue was still relatively avascular. Bony changes were similar to those described for ten days following surgery and the nasal cavity remained unchanged (Fig. IV-22).

Twenty-one Days Following Surgery

The example of a completely healed palate and an exmaple of a residual fistula in the palate are shown here which represent the two possibilities of this wounding procedure (Fig. IV-23 and Fig. IV-24).

Oronasal Fistula Example

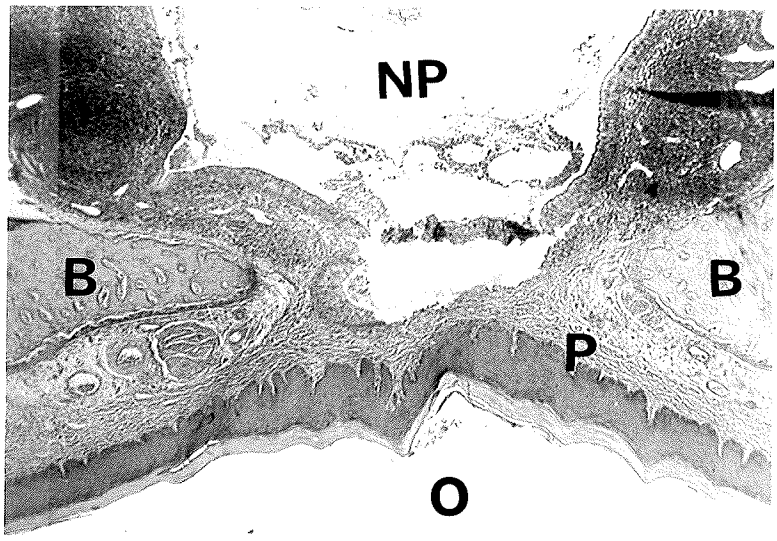
In the first example there was an oro-nasal fistula and it was seen that the epithelium layer of stratum corneum began to thin out until it fused with the nasal epithelium (Fig. IV-24). The underlying layers of stratum granulosum and stratum basale became thin rather abruptly. The stratum basale remained unchanged and then disappeared as it met with nasal epithelium.

Connective tissue adjacent to the fistula was avascular and dense in nature compared to a loose stroma beneath the non-surgical site.

The bone appeared rounded in nature and the nasal epithelium appeared somewhat thickened as it approached the oral cavity.

FIGURE IV-23 Coronal cross-section of an experimental rat palate, 21 days into the post-operative period. Haematoxylin and eosin. X740

FIGURE IV-24 Coronal cross-section of an experimental rat palate, 21 days into the post-operative period showing open fistula formation. Haematoxylin and eosin. X740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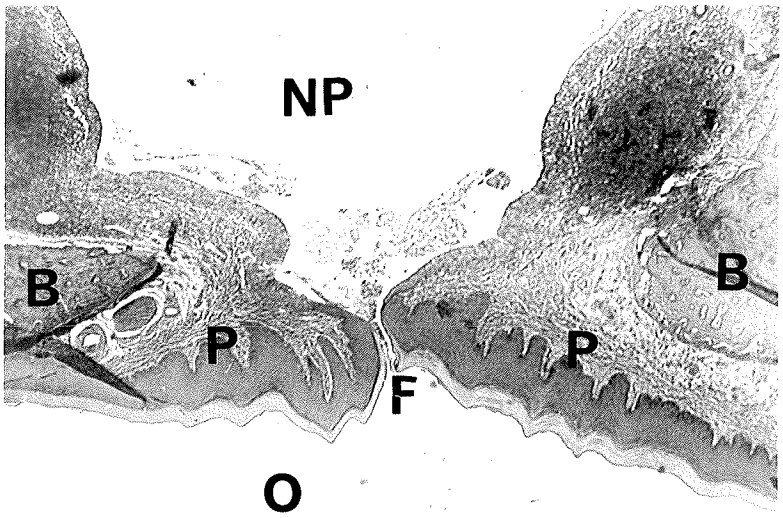
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Healed Palate

In the case of the twenty-one day post surgical healed palate, the epithelium lacked rete pegs. The connective tissue was avascular and slightly dense with cells when compared to the peripheral non-wounded regions (Fig. IV-24).

The bone was rounded in the region adjacent to the cleft and the nasal cavity appeared normal.

Twenty-eight Days Following Surgery

After twenty-eight days of healing there was rete peg formation beginning in the scarred region. The epithelial layers appeared normal in content and number on this day (Fig. IV-25).

The connective tissue was relatively avascular in appearance and was more dense than the adjacent unwounded region. The bone was rounded in appearance and the nasal cavity was normal.

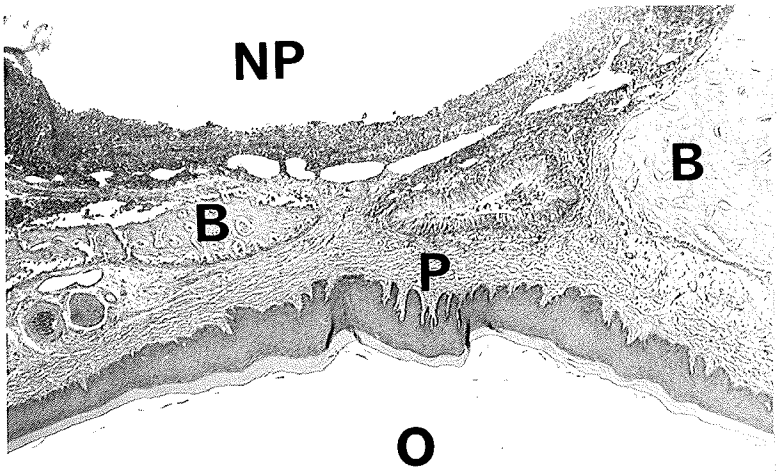
Intra-Molar Width Results

Intra-molar width was found to increase with time in the control animal from a mean of 4.08 mm at day zero to 5.21 mm at day twenty-eight (see Fig. IV-26 and Table IV-1).

The experimental group, however, showed an initial increase in intra-molar width followed by a decrease to 3.39 mm at day twenty-eight (Table IV-1).

The results were subjected to the statistical mixed analysis of variance and there were significant differences found in

FIGURE IV-25 Coronal cross-section of an experimental rat palate, 28 days into the post-operative period. Haematoxylin and eosin. X740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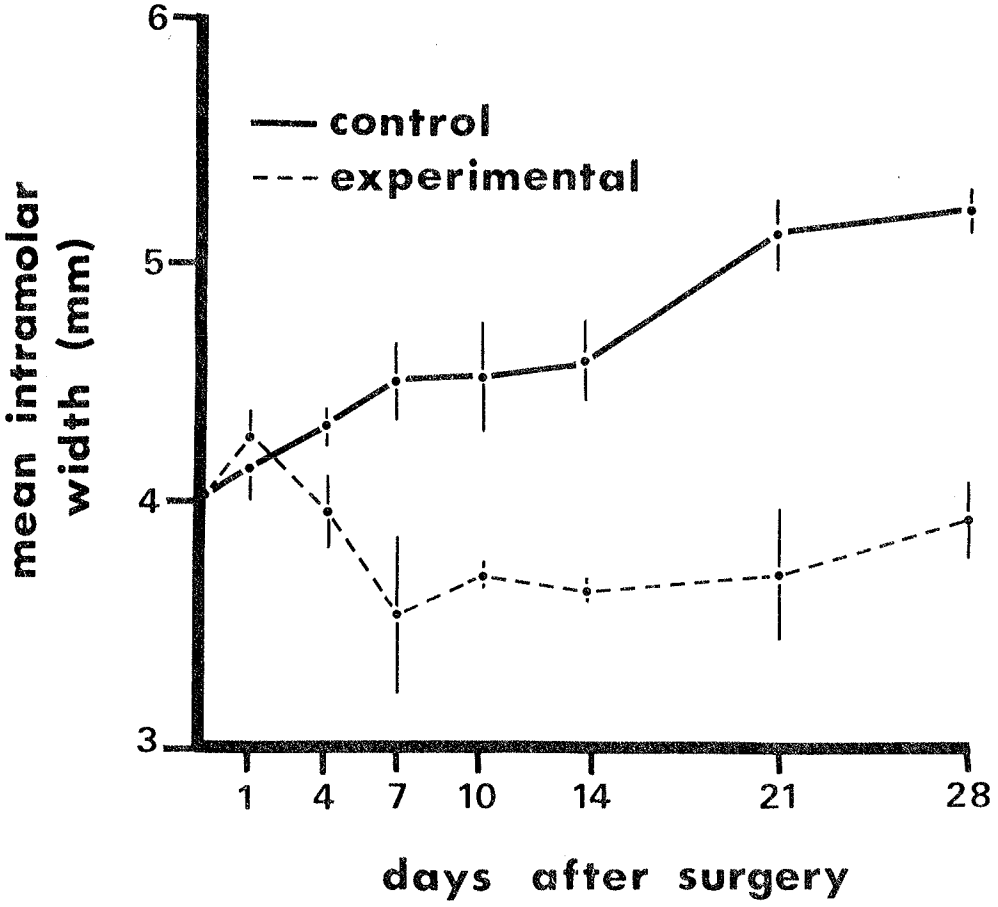


FIGURE IV-26 Mean intramolar widths of control and experimental rats from 0 to 28 days following palatal wounding.

TABLE IV-1

MEAN INTRAMOLAR WIDTHS OF CONTROL AND EXPERIMENTAL RATS FROM 0 TO 28 DAYS AFTER PALATAL WOUNDING

TIME FOLLOWING SURGERY (DAYS)	MEAN INTRAMOLAR (MM.)			
	CONTROL		EXPERIMENTAL	
	$\bar{X}$	S.D.	$\bar{X}$	S.D.
0	4.083	0.076	4.083	0.075
1	4.150	0.138	4.267	0.104
4	4.367	0.082	3.967	0.175
7	4.500	0.179	3.567	0.329
10	4.517	0.233	3.700	0.090
14	4.600	0.155	3.650	0.055
21	5.167	0.121	3.733	0.234
28	5.217	0.099	3.967	0.163

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

intra-molar width between experimental and control groups (p less than 0.01) over the time period of twenty-eight days (Table IV-2). The interaction between control and experimental animals with time was also found to be significant at the level of less than 0.05 (Table IV-2).

Weight Results

When weight differences were plotted (see Fig. IV-27), it was found that the control group showed a steady increase in weight which seemed to level off at day twenty-eight (Fig. IV-27).

The experimental group, however, initially lost weight in the first and fourth days and began to recover by post-operative day seven. On this day the mean weight was found to be the same as the animal weighed on the day of surgery. Weight gain was then slow and most animals did not reach the weight attained by the non-surgerized animals (Table IV-3). There was some overlap between control and experimental weights on the twenty-eighth day following surgery. The differences in weight between experimental and controls was subjected to a mixed analysis of variance (Table IV-4). There were statistically significant differences in weight gain between the experimental and control groups (p less than 0.01). This would suggest that palatal surgery is an important variable in growth of the rat.

There were statistically significant differences in weight gain in the time periods studied as expected (p less than 0.01). There were also statistically significant differences in the slopes and general pattern of weight gain between the two groups

TABLE IV-2

MIXED ANALYSIS OF VARIANCE TEST FOR INTRAMOLAR WIDTH IN CONTROL AND EXPERIMENTAL RATS FROM 0 TO 28 DAYS FOLLOWING PALATAL WOUNDING

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F
ANIMAL (CONTROL OR EXPERIMENTAL)	1	0.006	1.34
TIME	7	0.502	119.30**
INTERACTION	7	0.019	4.60*

* SIGNIFICANT AT $P < 0.05$

** SIGNIFICANT AT $P < 0.01$

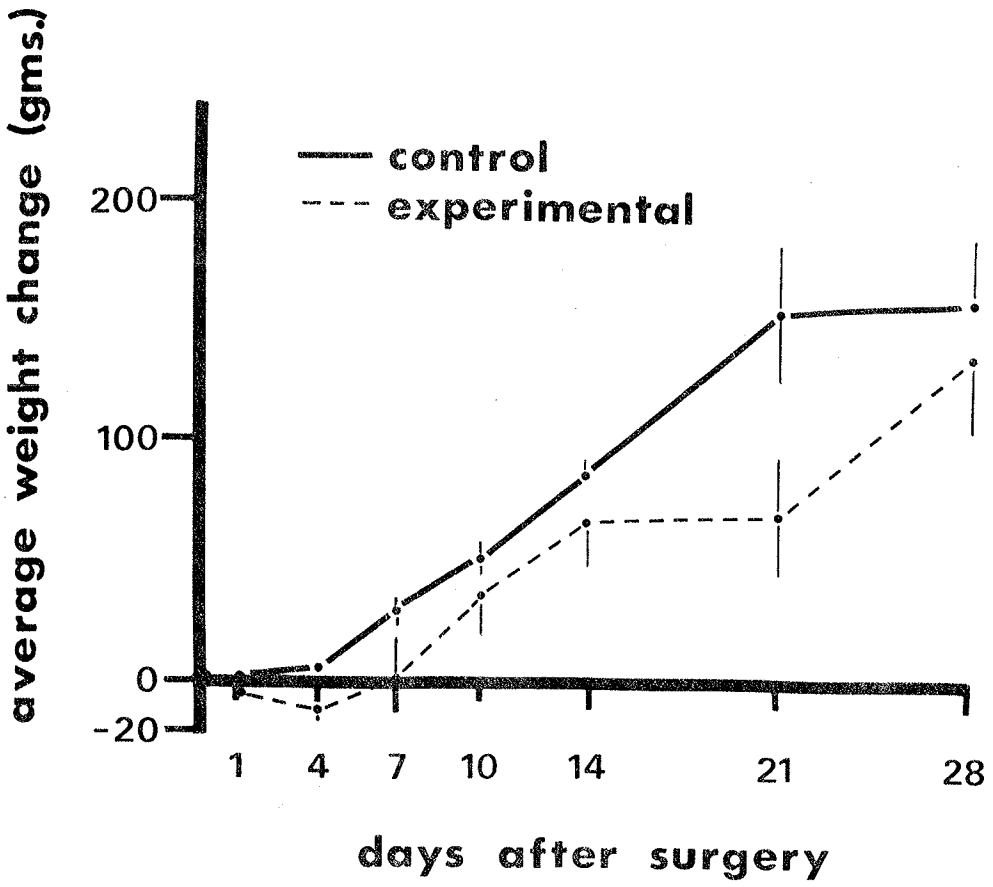


FIGURE IV-27 Average weight change of control and experimental rats from 0 to 28 days following palatal wounding.

TABLE IV-3

MEAN WEIGHT CHANGE IN CONTROL AND EXPERIMENTAL RATS FROM 0 TO 28 DAYS FOLLOWING PALATAL WOUNDING

TIME FOLLOWING SURGERY (DAYS)	AVERAGE WEIGHT CHANGE (GMS)			
	CONTROL		EXPERIMENTAL	
	$\bar{X}$	S.D.	$\bar{X}$	S.D.
0	0	0.0	0	0.0
1	0	0.408	-1	0.645
4	6	2.309	-7	4.596
7	29	2.887	0	18.248
10	52	6.702	36	16.250
14	87	6.934	67	17.448
21	153	28.346	67	24.498
28	158	26.440	137	30.341

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

TABLE IV-4

MIXED ANALYSIS OF VARIANCE TEST FOR WEIGHT DIFFERENCES ($W_2 - W_1$) IN CONTROL AND EXPERIMENTAL RATS FROM 0 TO 28 DAYS FOLLOWING PALATAL WOUNDING

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F
ANIMAL (CONTROL OR EXPERIMENTAL)	1	2505.426	31.93
TIME	7	7129.836	90.86
INTERACTION	7	531.535	6.77

ALL VALUES SIGNIFICANT AT $P < 0.01$

with time (p less than 0.01). Experimental animals were visibly smaller in most groups studied. However, general daily activity, fur colour and texture were observed to be similar to that of the control animals.

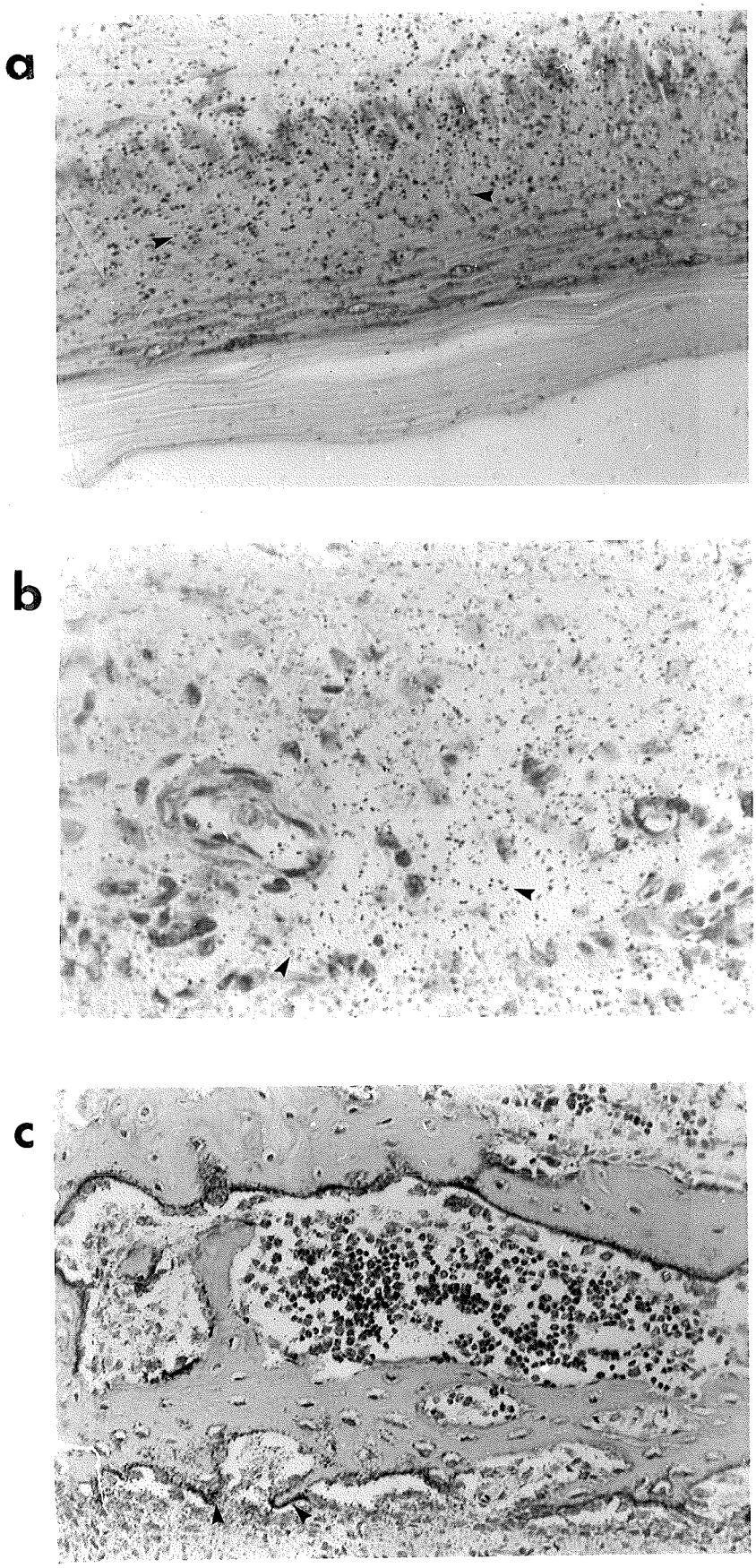
Autoradiography Results (Grain Counting)

Photographic examples of grains in (a) epithelium, (b) connective tissue, and (c) bone can be seen in Figure IV-28.

Epithelial Tissue Near Wound Site

Grain counting in the epithelial layers of stratum granulosum and stratum spinosum near the wound site are summarized in Table IV-5. The control rats had a low count of 20 grains on day zero. The day one grain count of almost 40 grains was the highest level recorded for any day. A graph showed two peaks which occurred on day one and day twenty-one (Fig. IV-29). The curve began to drop again on the final day (twenty-eight). This cyclical nature of proline uptake in epithelial tissue near the midpalatal suture indicated rapid collagen turnover in that region.

Similar results were obtained in the experimental animal with a few exceptions. The initial grain count was very low (10 grains) on day zero (Table IV-5). There was a cyclical nature to the curve but the second rise was not as high as seen in the control group (Fig. IV-29). The grain count was very uniform from the fourteenth day to the twenty-eighth day and showed no sign of further cyclical action during the test period under study.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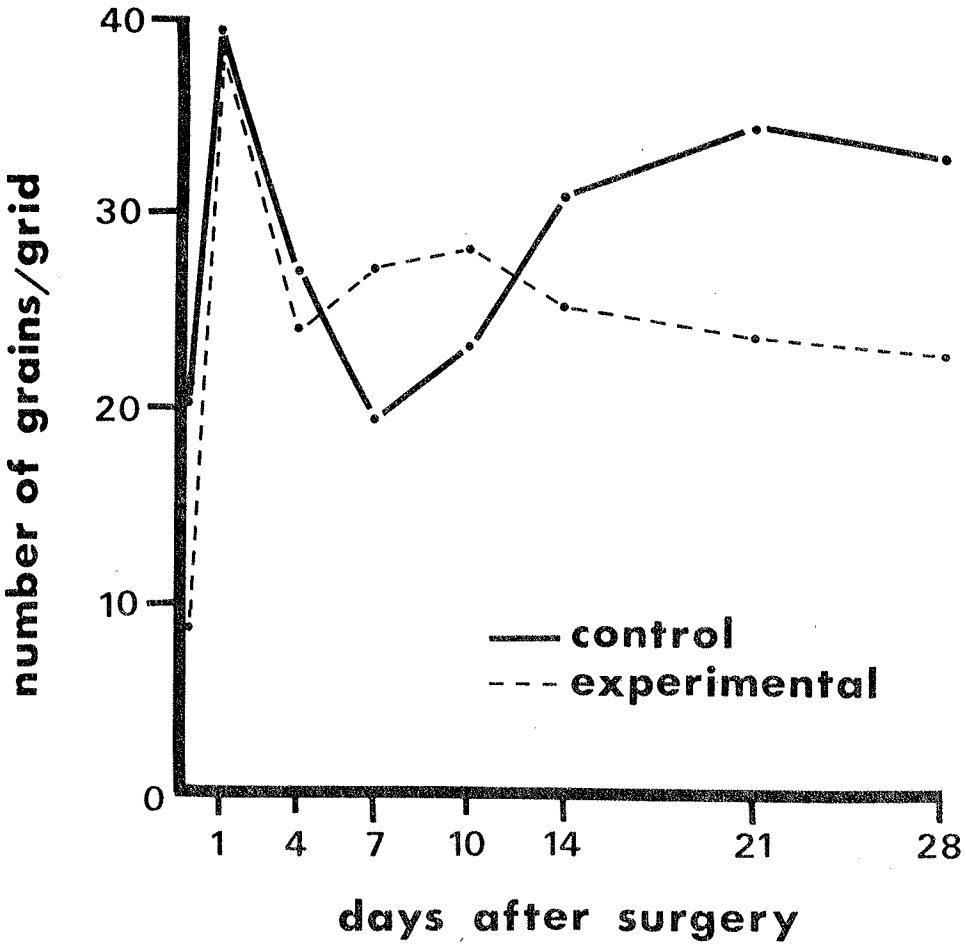

FIGURE IV-29 Grain count of epithelium (close to wound site: Zone 1) in control and experimental rats from 0 to 28 days following palatal wounding.

AGE (DAYS)	EPITHELIUM (MIDDLE) ZONE 1		EPITHELIUM (PERIPHERAL) ZONE 2		CONNECTIVE TISSUE ZONE 3		BONE ZONE 4	
	C	E	C	E	C	E	C	E
0	20.0	8.67	14.0	11.0	4.0	3.2	30.0	45.0
1	39.67	39.33	37.67	28.33	3.9	5.6	25.0	13.0
4	27.0	24.0	20.33	23.67	5.3	6.0	80.0	17.8
7	19.67	27.33	18.67	20.0	4.4	7.8	60.0	80.0
10	23.33	28.33	20.33	19.0	5.8	5.4	35.0	85.0
14	31.0	25.67	19.67	19.0	5.8	5.8	55.0	70.0
21	34.0	23.33	26.0	27.67	6.8	5.8	30.0	45.0
28	30.33	22.67	27.0	27.67	3.6	3.8	40.0	50.0

TABLE IV-5

GRAIN COUNTS (PER UNIT AREA) AT DIFFERENT AGES IN EPITHELIUM, CONNECTIVE TISSUE, AND BONE OF RAT PALATE

C: CONTROL; E: EXPERIMENTAL

ALL VALUES ARE EXPRESSED AS AVERAGES

Epithelial Tissue Peripheral to the Wound Site

Both control and experimental animals showed almost identical curves when grains were counted near the periphery of the palatal mucosa (Fig. IV-30). The results are summarized in Table IV-5. There was an initial rise at day one, followed by a drop on day four, and a period of no change to the fourteenth day. Thereafter, the curve shows a slight increase which was similar on day twenty-one and twenty-eight. This could be interpreted as a cyclical rise but much more gradual than in the midpalatal epithelial region.

Results of Connective Tissue Grain Count

The results of the grain count of the palatal connective tissue are summarized in Table IV-5. The control data showed grain count values varying from 3.6 to 6.8 throughout the experimental time period.

The experimental data showed grain count values varying from 3.2 to 7.8 throughout the time period tested. There was a peak incidence on day seven and a general decreasing trend thereafter (Fig. IV-31). Both control and experimental plots followed the same general shape, except on day seven which had a grain count difference of 3.4.

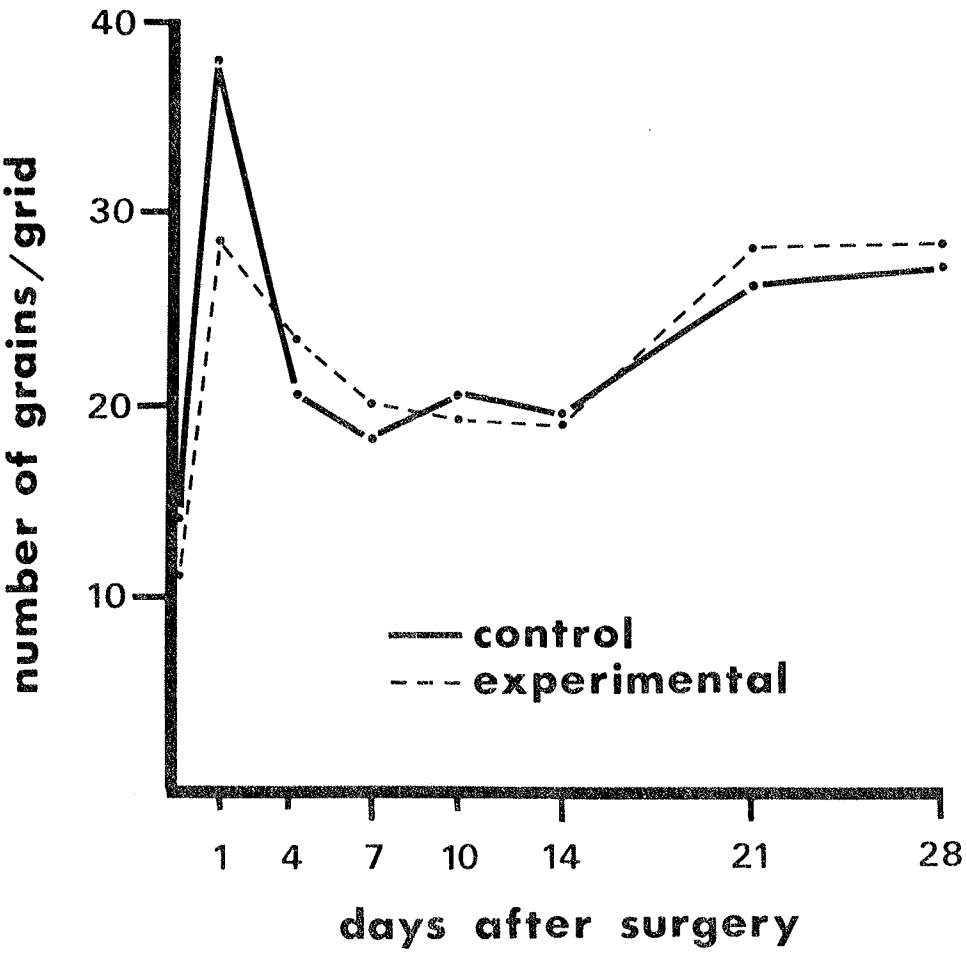

FIGURE IV-30 Grain count of epithelium (peripheral to wound site: Zone 2) in control and experimental rats from 0 to 28 days following palatal wounding.

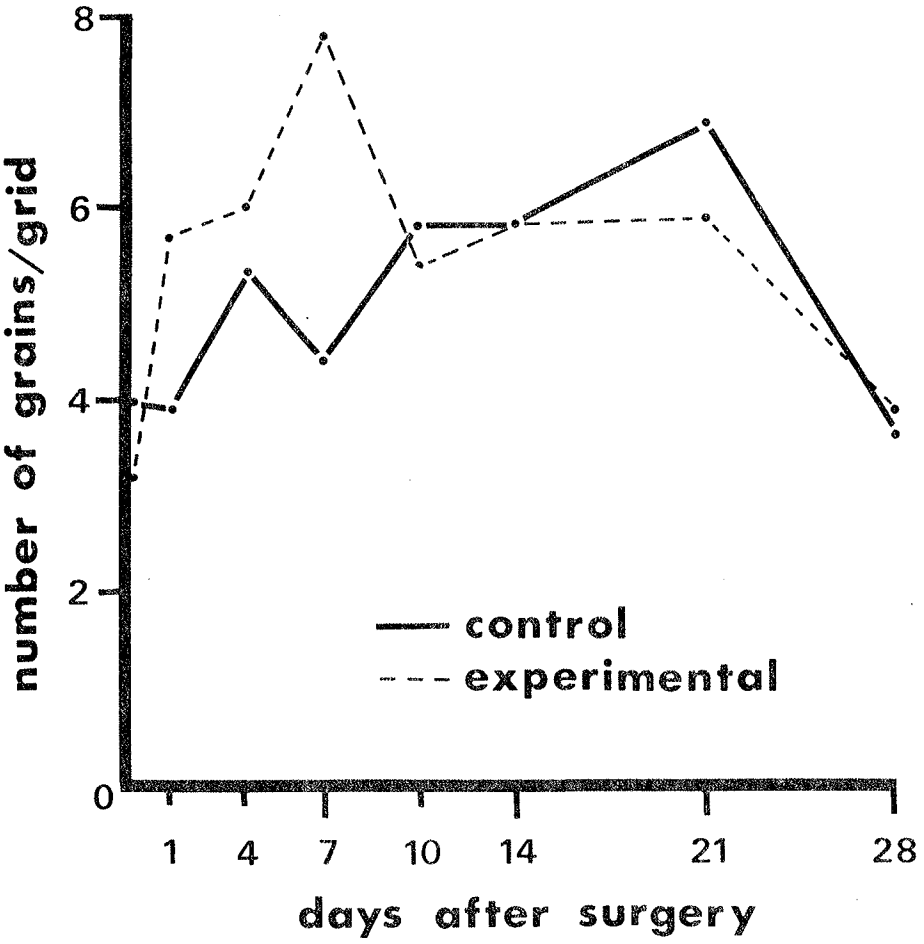

FIGURE IV-31 Grain count of connective tissue (in wounded region: Zone 3) in control and experimental rats from 0 to 28 days following palatal wounding.

Results of the Bone Grain Count

The results of the grain count of palatal bone tissue are summarized in Table IV-5. Heavy labelling occurred on the bony interface adjoining connective tissue. High grain counts were noted on day four of control rats and days 7, 10 and 14 of the experimental rats. Control and experimental grain counts were plotted in Figure IV-32. General curves of experimental and control animals were found to be similar in shape with slightly higher activity in the experimental animals.

DISCUSSION

The microscopical observations showed distinct changes in epithelium, connective tissue and bone a few hours postoperatively, especially in the cell layers adjacent to the wound margin. In this type of wound a tissue mass was replaced by coagulum and thin fibrin and inflammatory cells. Part of the wound margins was in direct contact with saliva and microorganisms.

The number of altered cells was largest in the nucleus-containing layers which had the highest degree of differentiation, namely the stratum granulosum.

The degenerative nuclear changes which were observed in the stratum granulosum have been interpreted as pyknosis.

The initial signs of active reparative reactions in the epithelium were observed after 24 hours of wounding. In oral wound healing studies Engler *et al.* (1966) found migrating

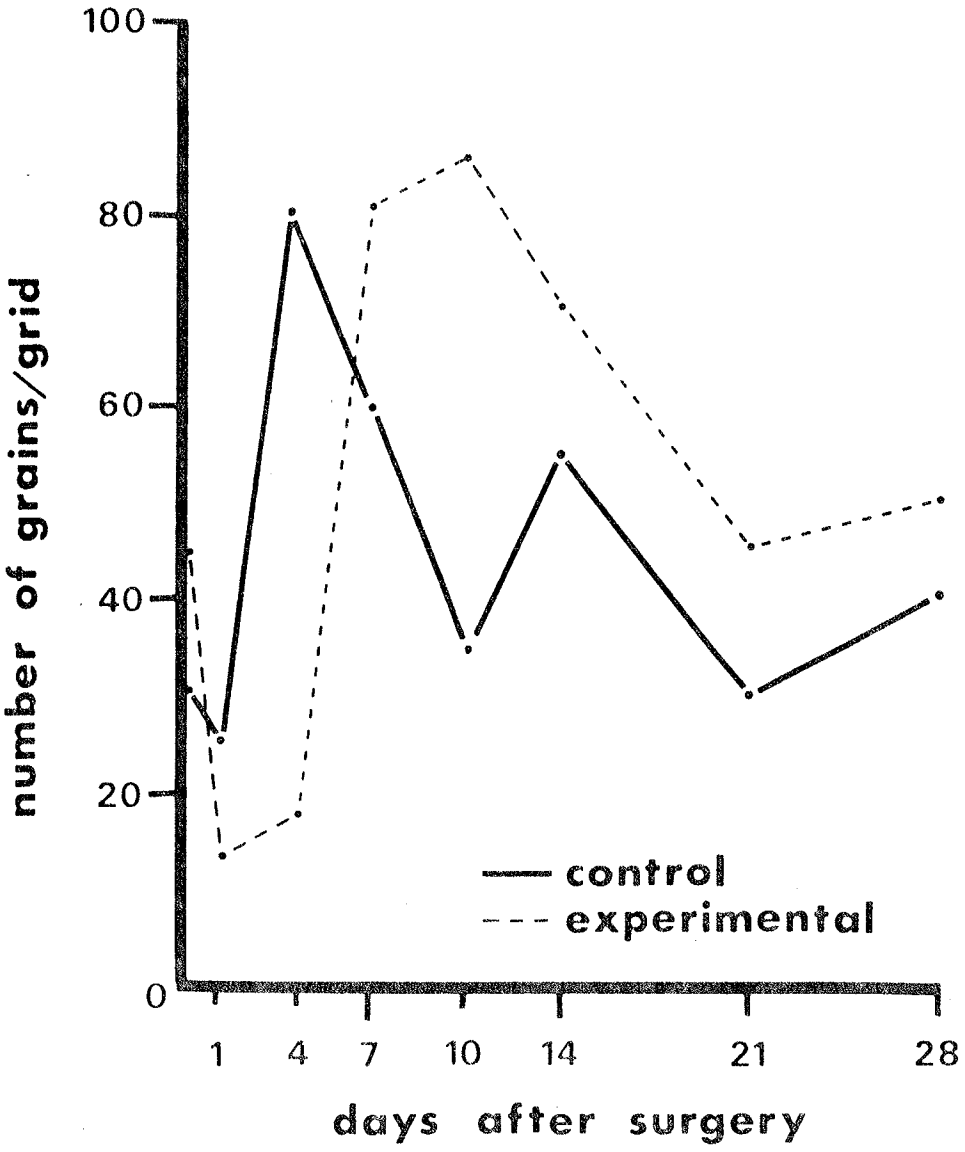

FIGURE IV-32 Grain count of bone (Zone 4) in control and experimental rats from 0 to 28 days following palatal wounding.

epithelium 13 to 24 hours after gingivectomy and Mittelman et al. (1964) observed migration after 18 hours in gingival incisional wounds. The starting point for the epidermal migration is extremely variable and can not be accurately measured in this study. The migration of epithelial cells was in the direction of the opposite wound margin. It appeared that the migration occurred on top of the underlying normal connective tissue base. There was an intact dermal surface of fibrin created before epithelialization. The inflammatory cells appear possibly as a defence mechanism against bacteria as well as to create a more favorable environment for new growth. Vizium et al. (1964) found animals (rabbits) which lacked a normal functioning haematopoietic system, had no epithelial migration after dermal wounds were inflicted.

Some animals failed to achieve palatal continuity and this resulted in the creation of an oronasal fistula. This result can be observed in some cleft palate patients after a surgical repair (Ross and Johnston, 1972). The reason for only a few experimental animals with fistula formation could be due to a slightly more aggressive wound inflicted by the operator. It is possible that this wound was unable to fuse due to its large size and subsequent lack of sufficient epithelial tissue or underlying connective tissue.

The excellent tracer qualities of (^{3}H)-proline for the autoradiographic study of collagen matrix synthesis at the tissue, cell and ultrastructural levels have been well documented (Ross and Benditt, 1962a; Tonna, 1971; Tonna and Pavelec, 1971). Analysis of data represented by plots of the average grain counts,

per time period must also take into account the fact that labelled circulation matrix precursors of secondary origin exist (Tonna et al., 1980). Reutilization from secondary sources produces further labelling of newly synthesized collagen and a tendency to keep the plots elevated at later time periods. However, this is balanced against the rate of turnover of collagen which acts to reduce the grain counts (Tonna et al., 1980). All of these parameters must be taken into account in interpreting the data. In midpalatal regions the slopes of the peak decline were similar in control and experimental animals up to day ten. Following injury, cell turnover was very marked. The discovery that matrix turnover was great in non-injured tissue could be attributed to forces of mastication which were new to this rat of eighteen days of age. This is in agreement with studies by Claycomb et al. (1967) who found that oral tissue, which is under profound functional stress, should be regarded as a repair tissue undergoing continuous wound healing. Their results also found a multi-peak incorporation of (^{3}H)-proline into oral collagen (Claycomb and Summers, 1965).

CONCLUSIONS

1). Throughout the wound healing period of the palate there appeared to be less vascularization in the loose connective tissue directly under the wounded region.

2). Creation of a palatal cleft with a high speed handpiece and a round burr resulted in scar tissue along the midpalatal suture region. In a very few cases it resulted in an orona-

sal fistula.

3). Light microscopy revealed a lack of rete peg formation into the dermis until twenty-eight days following wound affliction.

4). Intramolar width was found to be a significant variable when experimental and control animals were compared over time. Arch width was consistently less in the experimental animals at all ages.

5). Cleft created animals weighed consistently less than control animals but began to 'catch up' near the end of the time period studied.

6). Autoradiographic results of epithelial grain counting revealed a multi-peak proline uptake curve with similar curves for experimental and control animals.

7). Autoradiographic results of connective tissue and bone grain counting revealed much less labelling occurring in the connective tissue and heavy labelling occurring at the connective tissue bony interface.

CHAPTER V

DEVELOPMENT OF AN ORGAN CULTURE SYSTEM TO STUDY COLLAGEN SYNTHESIS IN THE HEALING RAT PALATE *IN VITRO*

INTRODUCTION

The development of an organ culture system which can maintain a whole mature palate undergoing wound healing in response to mechanical injury will provide a model *in vitro* in which conditions comparable to cleft palate repair can be imitated. By isolating the tissue *in vitro* one can eliminate those factors *in vivo* which would modulate and mask the presence of possible regulatory mechanisms and thus facilitate the measurement of such mechanisms.

Other tissues such as periodontal tissues have been cultured and they involved either a single tooth and surrounding alveolar bone explants from mouse (Melcher *et al.*, 1973; Turnbull and Melcher, 1974; Melcher and Turnbull, 1976), or three teeth and surrounding bone from mouse in a continuous flow system (Yen and Melcher, 1978). The latter system is of limited value for the investigation of palatal wound healing because of the prohibitive cost of any experimentation involving additions of radioactive materials to the large volumes of required medium.

The culture of mature organs in a synthetic medium was accomplished using the four week old rat (Trowell, 1959). In his study skin was cultured with the raw side down in quite large sheets. After 3 days the tissue appeared healthy histologically and the cultures performed just as well in air as in oxygen.

Organ culture systems which involve a fibrous joint have been developed by Meikle *et al.* (1979) and Yen *et al.* (1980). Meikle *et al.* (1979) have described the culture of coronal and sagittal cranial suture and adjacent bone from new-born rabbits. Viability

was demonstrated by radioautography of (^{3}H)-proline and ^{3}H-leucine incorporation into sutural protein and (^{3}H)-proline incorporation into collagen. Mechanical forces were applied across these sutures resulting in increase in both protein and collagen synthesis.

Yen et al. (1980) have developed an in vitro system which utilized the cranial suture of adult mice. Radioautography of (^{3}H)-proline labelled explants and the presence of collagenase-digestible radiolabelled proteins have demonstrated the viability and protein synthetic capacity of this system.

Establishment of a culture technique which will allow palatal organ explants to survive will introduce the possibility of investigating mechanisms regulating wound healing in palatal mucosa. Because collagen is the predominant protein in both hard and soft connective tissues (Bornstein and Traub, 1979), it represents the parameter of choice to examine the effects of possible cellular regulators upon connective tissue remodelling.

Within the palatal mucosa there are three main collagen chains, the alpha 1 and alpha 2 chains of Type I collagen and the alpha 1 chains of Type III collagen (Butler et al., 1975; Limeback et al., 1978; Sodek and Limeback, 1979).

The factors which regulate the remodelling of the oral connective tissue during normal growth and during orthopaedic or orthodontic therapy are unknown. Because collagen is the predominant component in palatal connective tissue, it is necessary to determine the rate of collagen synthesis in normal and surgically scarred palatal tissues at various time intervals after palate

surgery. This will provide data on the remodelling capacity of scar tissue relative to normal palatal tissue. Mechanical stress has been shown to have a temporary stimulative effect on collagen synthesis in craniofacial sutures (Yen et al., 1980; Meikle et al., 1982), but little is known of the long-term synthetic pattern of a palatal scar, despite the significance of the long-term effect on a growing child.

A supposed role for Type III collagen has been proposed as a structurally essential component in rapidly remodelling tissues such as wound sites (Bailey et al., 1975; Kent et al., 1976; Gay et al., 1978), induced osteogenic sites (Reddi et al., 1977), and in normal oral tissues (Butler et al., 1975; Yen and Melcher, 1978).

The objectives of this study were two-fold. Firstly, to establish an organ culture system for the rat palatal mucosa. Secondly, to determine the proportions of the types of collagens present in normal palate soft connective tissues and in scar repair tissue in surgically treated rat palates.

MATERIALS AND METHODS

Palatal Cleft Preparation

Eighteen day old male Sprague-Dawley rats, bred in our facility at the Faculty of Dentistry Animal House, were anaesthetized with ether. A surgically-created palatal defect (8 mm in length) was created using a large round burr in a high speed handpiece.

The defect was located in the palatal midline between the right and left molar segments. Four animals were treated as above and sacrificed by ether anaesthesia and decapitation at each of the following time points after surgery: 1, 2, 3, 4 weeks. The same number of animals of equivalent age were sacrificed at each of the above times to serve as untreated controls.

Dissection and Culture System

Dissection of the palate from the anterior premaxillary region to behind the third molar with the surrounding alveolar bone was performed. This palate with part of the nasal cartilage was placed in culture medium under a dissecting microscope. Cuts were made to remove the three molars on each side of the palate and the underlying cartilage of the nasal cavity. The strip of epithelium had underlying dermis and bone attached and was trimmed to lie flat in the organ culture system (Fig. V-1). The average explant was 8 mm long and 4 mm wide and 2 mm thick.

The explants were cultured in Trowell-type (Trowell, 1959) organ culture dishes (60 x 15 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) in which tissue rested on stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California), which were suspended over a center well in the dish (Fig. V-1). Sufficient medium (800 ul) was introduced to reach the platform of each grid so that the explants stood at the gas-medium interface. The center well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator main-

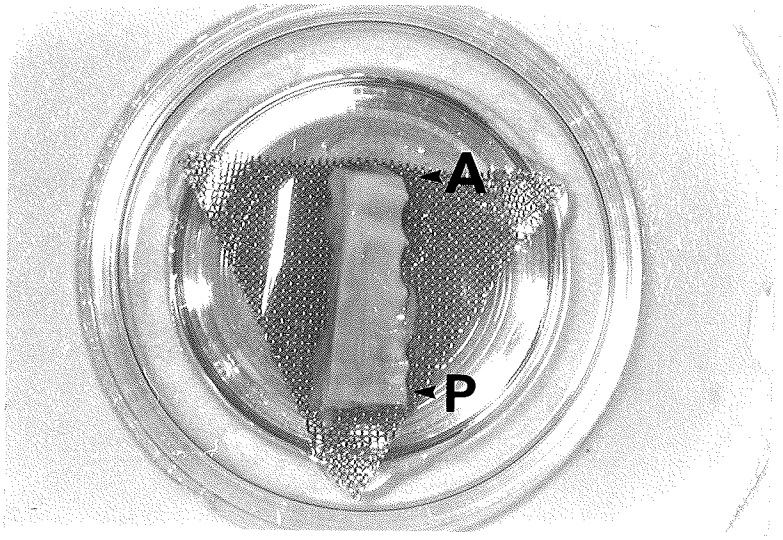

FIGURE V-1 Palatal tissue explant resting on a grid in a Trowell-type organ culture dish (magnified X 2).

A = anterior palate

P = posterior palate

tained at 37°C. Explants were cultured for 1.5 hours.

Medium, Gas, Isotope

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York) (Waymouth, 1959), supplemented with 300 ug/ml ascorbic acid (Fisher Scientific, Fair Lawn, New Jersey) and an antibiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomycin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a mixture of 95% oxygen and 5% carbon dioxide, humidified by bubbling through water.

Eighty ul of 20 uCi/ml (^{14}C)-glycine (CFA 30, 1-(^{14}C)-glycine, Amersham Corporation, Oakville, Ontario) with a specific activity of 51.2 uCi/mmol were added to the medium of each experimental and control culture dish 1.5 hours prior to culture termination.

Collagen Extraction

At the end of the culture period the palates were dissected and palatal tissue was removed from the anterior and posterior portions of the explant as shown in Figure V-1. These samples were placed in separate 1.5 ml plastic centrifuge tubes which were subsequently frozen for storage.

Following thawing each sample was cut into fine pieces and

placed back into a 1.5 ml plastic centrifuge tube. The addition of 250 ul of 0.1 mg/ml pepsin (2 X crystallized, Sigma Chemical Co., St. Louis, Missouri), in 0.5 N acetic acid to each tube was completed. The tubes were then placed in a cooler at 16°C for 15 hours and the procedure was then repeated for an additional 6 hours. The samples were then centrifuged for one minute and the supernatant was removed and pooled with the previous supernatant. This was dialyzed against four changes of 1% acetic acid at 4°C for 24 hours. The samples were then freeze dried in preparation for gel electrophoresis.

Radioautography

Two separate samples were tested for radioautography at each time point. At the end of the culture period one control and one experimental explant for the time period of two weeks after surgery, were fixed in Bouin's solution for 48 hours. After extensive washing in 70% alcohol, the explants were demineralized for 14 hours in Cal-Ex (Fisher Scientific, Fair Lawn, New Jersey). After embedding in paraffin, serial sections were cut at 5 u intervals. Every fourth slide was stained with haemotoxylin and eosin. Suitable slides thereafter were prepared for radioautography by dipping in Kodak NTB-2 nuclear tracking emulsion (Eastman Kodak Co., Rochester, New York), stored in the dark at 4°C for 10 days, subsequently developed in Dektol developer and fixer (Eastman Kodak Co.) and stained through the emulsion with haemotoxylin and eosin.

Collagen Separation and Quantification

Collagen alpha-chains and procollagens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The procedure followed for the separation of those collagen components was described by Laemmli (1970) and utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel and Tris/glycine buffers (Bio-Rad Laboratories, Richmond, California). Type I and Type III collagen alpha-chains were separated using the interrupted electrophoresis method of Sykes et al. (1976).

Freeze dried samples were dissolved in 70 ul of reservoir buffer containing 2 M urea, 2% sodium dodecyl sulphate and 0.1% bromophenol blue and heated at 60°C for 30 minutes to denature the collagen. Electrophoresis was performed for one hour at 125 V. The samples were then reduced by the addition of 20% mercaptoethanol to the sample wells. After standing for 45 minutes, the electrophoresis was continued until the tracking dye reached the base of the gel.

For fluorographic visualization of separated radiolabelled collagen bonds, gels were washed twice in dimethyl sulfoxide (Fisher Scientific, Fair Lawn, New Jersey) and impregnated with 2,5-diphenyloxazole (New England Nuclear, Boston, Mass.) as described by Bonner and Laskey (1974). The gels were placed on filter paper and dried on a slab drier (Bio-Rad, Richmond, California) and exposed to Kodak XRP-1 x-ray film (Kodak Canada Inc., Toronto, Ontario) at -70°C for 2 weeks.

Individual sample tracks were then scanned at 550 nm and pro-

portions of Type III collagen relative to Type I were quantified on a spectrophotometer (Beckman DU-8, Toronto, Ontario).

OBSERVATIONS AND RESULTS

Anterior Palate

The summary values of the percentage of type III collagen found in the anterior palate, for both control and experimental animals are found in Figure V-2 and Table V-1. The values for the control group ranged from 2.87% to 4.02%. The experimental animals showed the highest value at week one which was 8.50% type III collagen. The percent of type III collagen production decreased steadily to the lowest value at 4 weeks after surgery. These values were subjected to a mixed analysis of variance as discussed below.

Posterior Palate

The summary values of the percentage of type III collagen found in the posterior palate, for both control and experimental animals are found in Figure V-3 and Table V-2. The control value was initially high at one week after surgery (6.14% type III collagen) and decreased to below 3% on weeks two, three and four.

The experimental values were higher than the controls on all the weeks tested and showed a steady decrease from 7.52% (type III collagen) at one week post surgery to 2.20% at week four. These values were subjected to a mixed analyses of variance as discussed

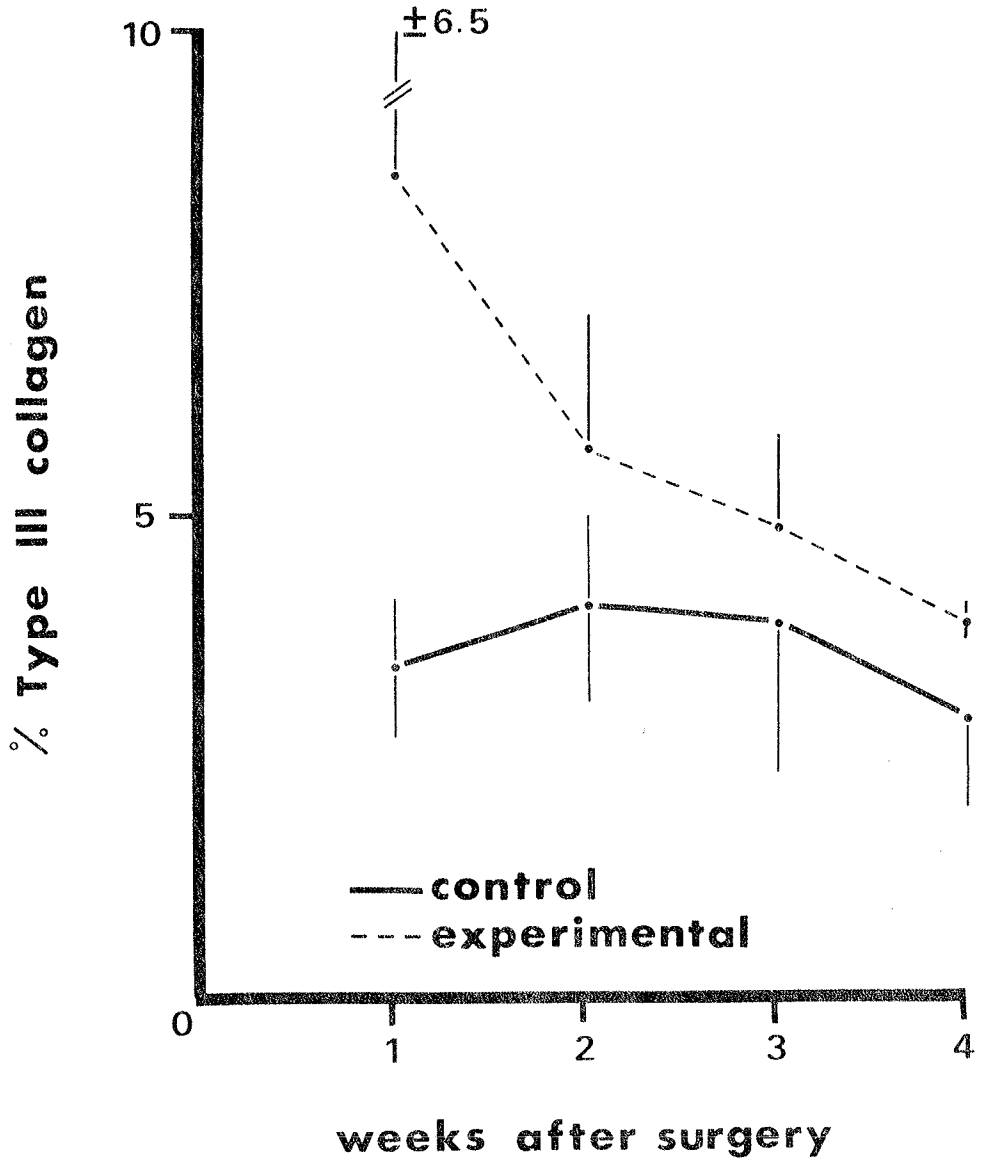

FIGURE V-2 Percentage of type III collagen present in anterior portion of the rat palate, 1 to 4 weeks following palatal surgery. In vitro results using (^{14}C)-glycine.

TABLE V-1

PROPORTION OF TYPE III COLLAGEN IN ANTERIOR RAT PALATAL TISSUE AT VARIOUS TIME INTERVALS FOLLOWING SURGERY

TIME OF SACRIFICE (WEEKS AFTER SURGERY)	EXPERIMENTAL %		CONTROL %	
	MEAN	S.D.	MEAN	S.D.
1	8.50	6.5	3.46	0.8
2	5.57	1.5	4.02	0.9
3	4.85	0.9	3.95	1.7
4	3.92	0.2	2.87	1.0

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

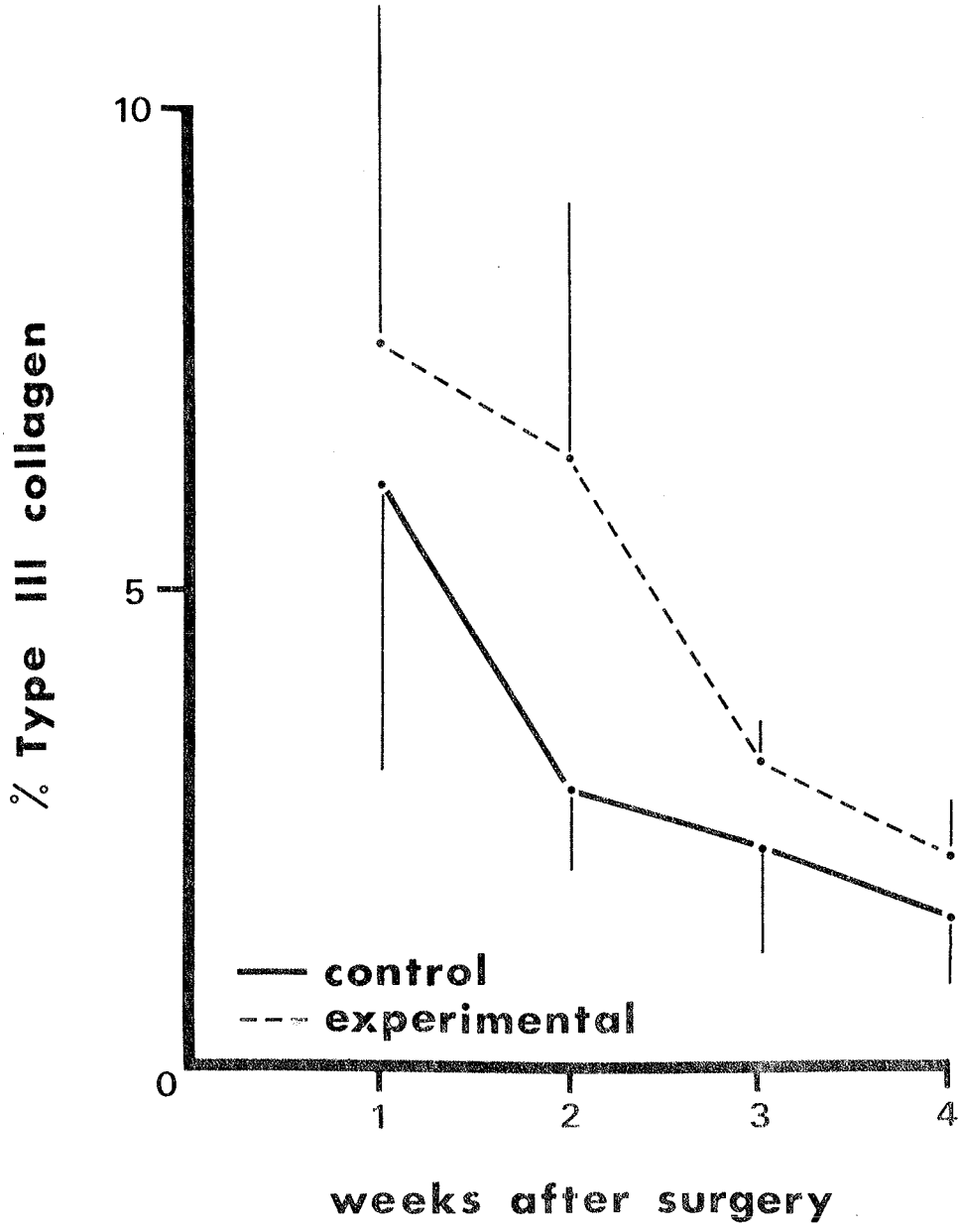

FIGURE V-3 Percentage of type III collagen present in the posterior portion of the rat palate, 1 to 4 weeks following palatal surgery. In vitro results using (^{14}C)-glycine.

TABLE V-2

PROPORTION OF TYPE III COLLAGEN IN POSTERIOR RAT PALATAL TISSUE AT VARIOUS TIME INTERVALS FOLLOWING SURGERY

TIME OF SACRIFICE (WEEKS AFTER SURGERY)	EXPERIMENTAL %		CONTROL %	
	MEAN	S.D.	MEAN	S.D.
1	7.52	3.8	6.14	3.0
2	6.45	2.7	2.90	0.9
3	3.17	1.0	2.27	0.4
4	2.20	0.7	1.52	0.6

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

below.

Statistical Significance of the Data

The results of the mixed analysis of variance have been summarized in Table V-3. Control and experimental values were shown to be significantly different when compared to each other at the 99% level of confidence. The average value for percentage of type III collagen production for experimental and control animals was found to be 5.27% and 3.39% respectively. Analyses between anterior and posterior palate showed no statistical significance.

Time was shown to be an important variable and differences in type III collagen production from week one to week four were significant at the 99% level of confidence. This analysis proved to be significant when experimental and control values were pooled. The values were averaged and a steady decline was seen to occur from week one to week four. Mean values were 6.40, 4.73, 3.56 and 2.63 for weeks one to four respectively.

Analyses between other variables showed no statistical significance.

DISCUSSION

The finding of increased type III collagen production in experimental rat palate compared to controls was in agreement with studies on rat dermal granulation tissue by Bailey et al. (1975b). It also agreed with the findings of Bailey et al.

TABLE V-3

ANALYSIS OF VARIANCE TEST FOR VALUES IN TABLES V-1 AND V-2

VARIABLE	DEGREES OF FREEDOM	MEAN SQUARE	F VALUE
CONTROL VS EXPERIMENTAL	1	60.12	7.52 *
WEEKS	3	44.61	5.58 *
INTERACTION	26	7.98	

* $P < 0.01$

(1975a) who found increased quantities of type III collagen in human hypertrophic scar tissue. The possibility that after palatal wounding there was a partial reversion to the synthesis of "embryonic" collagen in the scar tissue was one advanced by Miller (1976), and this was a possible explanation for the findings of this study. The scar tissue found in the rat palate was only one to four weeks old, and that could be a reason for the presence of type III collagen production. With the passage of time, this percentage of newly formed type III collagen could decrease. Developing palatal scar tissue may be similar to fetal dermal tissue development which shows a decrease in proportion of type III collagen with the passage of time. That trend was seen in this study where even experimental palates decreased their type III collagen production with time. Normal, long-standing scars in human dermal tissue have been shown to have a very low proportion of type III collagen after long time periods (Miller, 1976).

Time was shown to play an important role in both control and experimental palates. These rats were very young and had not yet experienced the forces of mastication before the experiment was undertaken. This could partially explain the results of a high type III collagen production in the early weeks after litter separation (Figure V-3).

The findings that the anterior of the palate did not differ significantly in its proportion of type III collagen production from the posterior palate (both control and experimental) was interesting. The texture and thickness of the anterior palates was quite different than the posterior palates and a difference in

their proportion of type III collagen production would not have been unexpected. However, the lack of statistically significant differences indicates that the anterior and posterior palates might differ only in the quantity of collagen present and not in actual collagen proportion (type III to type I) (see Chapter VI).

The relative percentage of type III collagen in the palate of the control rats was 3.39 over the four week time period studied. This is significantly lower than the 15% in the rat periodontal ligament (Sodek and Limeback, 1979) or 20% in the bovine periodontal ligament (Butler et al., 1975). This could be a reflection in the more severe as well as constant physical and bacterial stresses placed on the periodontium. The in vitro model used in this study might also account for such differences when compared to the in vivo system.

This study used the technique of gel electrophoresis and fluorographic visualization to quantitate the changes in proportions of type III and type I collagen at various points after surgical wounding. There is no information regarding which cells make which type of collagen and where this is done. The collagen types may be synthesized by different cell populations, in which case, the collagen type may be used as a marker to identify certain cell populations, using immunohistochemical techniques. Studies (Gay et al., 1976; Engel et al., 1980; and Kao et al., 1980) suggest the same cells could synthesize both type I and type III collagens. During wound healing, the precedence of type III collagen in the earliest phases over type I collagen, and the later predominance of type I collagen, may be due to a shift in

cell function. This would imply a regulatory control at the transcription step of protein synthesis. However, the possibility remains that control at both levels may occur. Collagen types may serve as markers for cell populations or cell function. When reasons for shifts in cell populations or cell functions are identified, then the process of tisssue remodelling and scar formation will be better understood.

CONCLUSIONS

1). After palatal surgery there was a significant difference between the proportion of type III collagen production (to type I collagen production), when compared to non-operated control animals. This was seen to be significant in the anterior as well as the posterior palates using the *in vitro* model previously described.

2). Time was shown to be a significant factor in proportion of type III collagen production in both control and experimental palates. There was a decreasing trend of type III collagen proportion in both groups.

3). The *in vitro* model system was shown to be a good system for the measurement of new collagen production using the radioactive tracer (^{14}C)-glycine.

CHAPTER VI

THE EFFECT OF SCARRING ON COLLAGEN SYNTHESIS IN THE RAT PALATE IN ORGAN CULTURE

INTRODUCTION

In order to study the effect that scarring the rat palate had on collagen production, the incorporation of labelled glycine into collagen was measured biochemically. It was thought that this study would measure the rate of new collagen synthesis in the anterior and posterior portions of the palate of control and experimental animals. Differences between the metabolism of palatal scar tissue and normal palatal tissue would explain the behavioural differences as seen clinically in repaired cleft palate patients and normal patients. One possibility for this difference is that the rate of remodelling is different in repaired clefts as compared to normal patients. Remodelling, however, involves a combination of synthesis and degradation of cellular components. It is the purpose of this study to look at the synthesis of collagen in order to determine if it plays a role in scar formation.

MATERIALS AND METHODS

Dissection, Culture System, Medium, Gas and Isotope

Wound infliction was carried out in a similar manner as described in Chapter IV. Four animals were surgerized and sacrificed by ether anaesthesia and decapitation at each of the following time points after surgery: 1, 2, 3, 4 and 5 weeks. The same number of animals of equivalent age were sacrificed at each of the above times to serve as untreated controls. Dissection of the

palate from the rats was done similarly to that described previously as well as the culture techniques (Chapter IV).

The explants were cultured in Trowell-type (Trowell, 1959) organ culture dishes (60 X 16 mm, Falcon #3037, Becton, Dickenson and Co., Cockeysville, Maryland), in which tissue rested on stainless steel grids (Falcon #3014, Becton, Dickenson and Co., Oxnard, California), which were suspended over a center well in the dish. Sufficient media (800 ul) was introduced to reach the platform of each grid so that the explants stood at the gas-medium interface. The center well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator maintained at 37°C. Explants were cultured for 1.5 hours.

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York) (Waymouth, 1959), supplemented with 300 ug/ml ascorbic acid (Fisher Scientific, Fair Lawn, New Jersey) and an antibiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomycin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a mixture of 95% O2 and 5% CO2, humidified by bubbling through water. Eighty ul of 20 uCi/ml (^{14}C)-glycine (CFAA 30, 1-(^{14}C)-glycine), (Amersham Corporation, Oakville, Ontario) with a specific activity of 51.2 uCi/mmol were added to the medium of each experimental and control culture dish 1.5 hours prior to culture termination.

Collagen Extraction

At the end of the culture period, the palates were dissected and palatal tissue was removed from the anterior and posterior portions of the explant in the area of scar formation and in the control palates. The samples were weighed and placed in separate 1.5 ml plastic centrifuging tubes, which were subsequently frozen for storage.

Following thawing, each sample was washed in isotope free medium and placed in prepared dialysis bags with 500 ul medium. This was dialyzed against cold medium for 6 hours at 4°C. Dialysis was then repeated against two changes of 50 millimolar tris/HCl buffer, pH 7.6, containing 5 millimolar CaCl2. Palatal samples were then placed in microtubes and 200 microlitres of tris buffer, 25 ul of bovine serum albumin, 6.5 ul of N-ethyl-maleimide (Sigma), 25 ul of bacterial collagenase (1 mg/ml in tris) (Advance Biofactures Corp., Linbrook, New York) was added. This was incubated for one hour in a shaking water bath at 37°C. It was then centrifuged at 10,000 X g. for 2 minutes and the supernatants were transferred to plastic microtubes. The palatal tissue was saved and incubated overnight in 100 ul 60% formic acid at 37°C. Then 42 ulitres of 50% trichloracetic acid was added to each supernatant and centrifuged (10,000 X g) for 2 minutes. The supernatant (A) was removed and saved. 300 ulitres of 7% trichloracetic acid was added to the pellet (precipitated sample) and recentrifuged for 2 minutes. The supernatant was removed and pooled with A (collagenase digestible protein). Afterwards 50 ul of 60% formic

acid was added to the remaining pellet and incubated overnight at 37°C (non-collagenous protein). From the collagenous digestible protein (C.D.P.) and the non-collagenous digestible protein (N.C.P.), aliquots were removed and counted on a Searle Mark III scintillation counter (Chicago Nuclear) for (^{14}C)-glycine.

OBSERVATIONS AND RESULTS

Anterior Palatal Samples

The summary values of the anterior palate for both control and experimental animals are found in Figure VI-1 and Table VI-1. The anterior palates which were scarred showed the heaviest glycine uptake around the second week of healing. This value of 204.70 disintegrations per minute (DPM)/mg tissue was the highest value found of all the samples studied. Thereafter, the readings for experimental and control were quite similar. The control samples had an initially high reading of 145.53 DPM/mg tissue but gradually decreased to a level of 88.26 DPM/mg tissue after three weeks (Table VI-1). Values of experimental palates were found to be different from values for control palates over the period of time studied.

Posterior Palatal Samples

The summary values of the posterior palate for both control and experimental animals are found in Figure VI-2 and Table VI-2.

The posterior palatal samples, which were scarred, had a

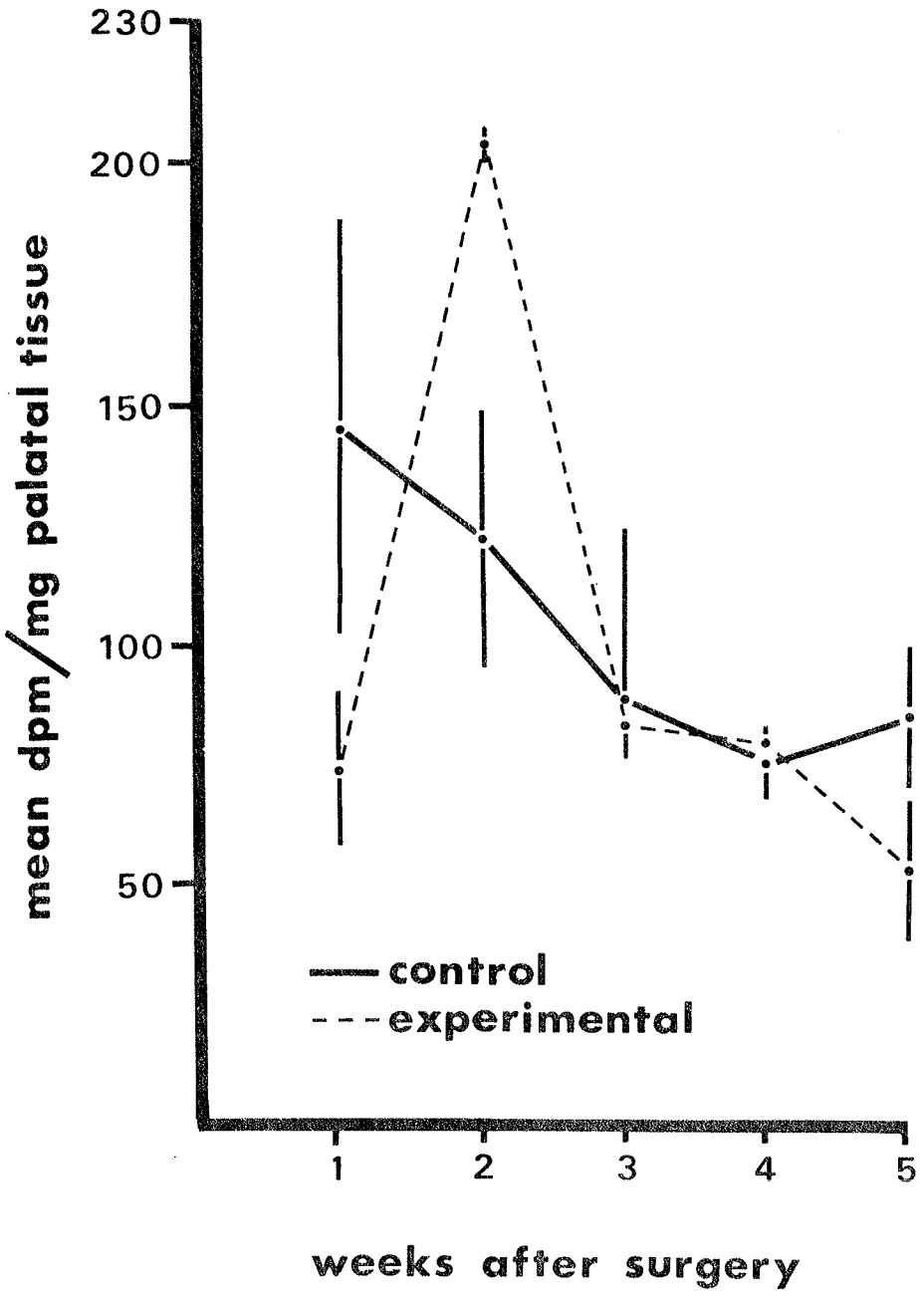

FIGURE VI-1 Collagenase digestible protein as measured in the anterior of the palate of the rat. Palates were cultured (in vitro) for 1.5 hours in ^{14}C-glycine. dpm = disintegrations per minute

TABLE VI-1

COLLAGENASE DIGESTIBLE PROTEIN AS MEASURED IN THE ANTERIOR OF THE PALATE OF THE RAT USING ^{14}C-GLYCINE

TIME FOLLOWING SURGERY (WEEKS)	MEAN DISINTEGRATIONS PER MINUTE (DPM) PER MILLIGRAM OF TISSUE			
	CONTROL	S.D.	EXPERIMENTAL	S.D.
1	145	44.45	73	15.49
2	122	28.85	204	5.50
3	88	35.35	82	8.45
4	76	8.45	80	4.35
5	84	15.38	52	14.32

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

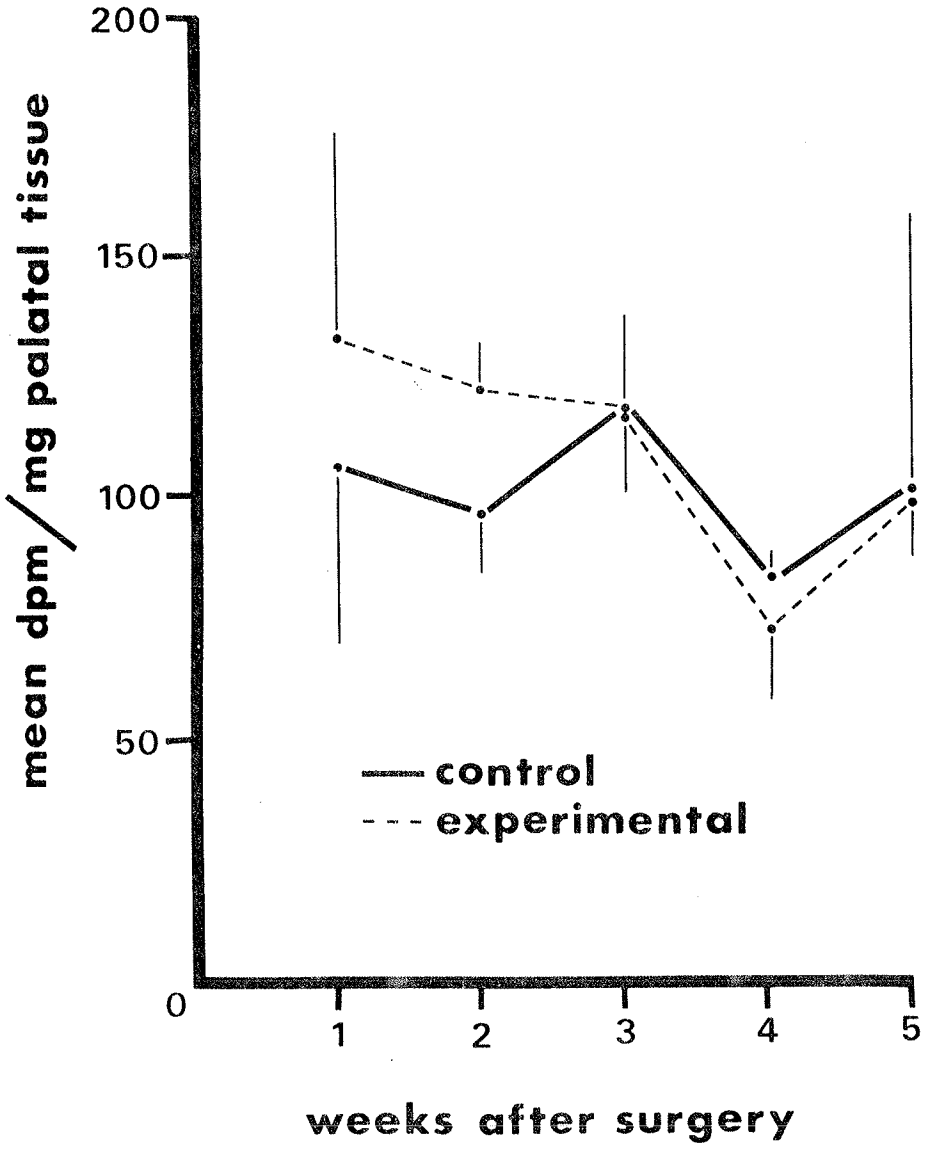

FIGURE VI-2 Collagenase digestible protein as measured in the posterior of the palate of the rat. Palates were cultured (in vitro) for 1.5 hours in ^{14}C-glycine. dpm = disintegrations per minute

TABLE VI-2

COLLAGENASE DIGESTIBLE PROTEIN AS MEASURED IN THE POSTERIOR OF THE PALATE OF THE RAT USING ^{14}C-GLYCINE

TIME FOLLOWING SURGERY (WEEKS)	MEAN DISINTEGRATIONS PER MINUTE (DPM) PER MILLIGRAM OF TISSUE			
	CONTROL	S.D.	EXPERIMENTAL	S.D.
1	107	39.35	133	42.43
2	96	15.33	123	9.42
3	119	18.18	111	18.18
4	80	1.88	73	13.90
5	102	57.17	102	14.39

EACH VALUE CALCULATED FROM SAMPLES FROM 4 OR MORE RATS.

higher value of disintegrations per minute/mg tissue at week one and week two, but thereafter were found to be slightly less than the control groups at weeks three and four. At the final week (Week 5), both values were approximately equal.

Statistical Significance of the Data

The data was subjected to a mixed analysis of variance and time was found to be a significant variable when control and experimental results were pooled. The results of this comparison are shown in Table VI-3. This was significant at the level of $P<0.01$. The values of the experimental palates were found to be different than the values of the control palates when expressed over time. The significance of this finding was at the 95% level of confidence (Table VI-4).

The values obtained for the anterior portion of the palate were significantly different than those values found in the posterior of the palate when expressed over time. This test of significance pooled experimental with control palates and only distinguished anterior from posterior. The level of confidence found was 95% (Table VI-4).

Experimental values differed from control values in both anterior and posterior regions of the palate, but the trends were not significant according to the statistical analysis.

Results comparing the anterior experimental palate to the posterior experimental palate showed no statistical significance.

TABLE VI-3

MEAN DISINTEGRATIONS PER MINUTE (DPM) PER MG IN EXPERIMENTAL AND CONTROL RATS FROM ONE TO FIVE WEEKS (AFTER PALATAL WOUNDING).

TIME (WEEKS)	N	MEAN EXPERIMENTAL AND CONTROL VALUES
1	14	114 ± 10
2	10	136 ± 11
3	12	100 ± 10
4	16	73 ± 9
5	16	85 ± 9

F VALUE = 5.645

SIGNIFICANT AT $P < 0.01$

TABLE VI-4

ANALYSIS OF VARIANCE TEST FOR VALUES IN TABLES VI-1 AND VI-2.

VARIABLE	DEGREE OF FREEDOM	MEAN SQUARE	F VALUE
1. TIME	4	8019.23	5.64 **
2. TIME EXPERIMENTAL VS CONTROL	4	3124.08	2.58 *
3. TIME ANTERIOR VS POSTERIOR	4	4042.73	2.83 *

* $P < 0.05$

** $P < 0.01$

DISCUSSION

Newly synthesized collagen, as measured in disintegrations per minute/mg tissue, was found to be greater in the experimental palates than in the control palates. This was the trend that was observed, but it was not significant when subjected to statistical analysis. The reason for this finding might be explained when one examines the age of the rats used. Eighteen day old rats (experimental and control) had received primarily a liquid diet up to the time of experimentation and had not been separated from their mothers as yet. Introducing a control rat to exclusively hard food diet would create the need to chew this food in order to survive. The palates at this time are soft and not accustomed to heavy forces of mastication. The result of these masticatory forces could cause an increase in collagen production, which would mask any differences resulting from the scarring of the experimental group.

The experimental palates showed an increased collagen production, especially in the first two weeks. After two weeks of healing, the levels began to approach the values found for the control palates. The results of these values, however, were not found to be statistically significant.

The anterior portion of the palate was found to have the highest amount of collagen production two weeks following surgery. It was at this time that many of the palates were superficially healed and perhaps there might be increased collagen production due to a reorganization in the area of the anterior palatal scar.

The posterior portion of the palate had the highest level of collagen production one week following wounding. Since the bone and connective tissue was completely penetrated here as well as the epithelium, we might expect to see a difference when compared to the anterior of the palate. It appeared that this posterior region began to reorganize itself at a faster rate than the anterior region. This might be due to the underlying bone which started to recontour very early as seen histologically (Chapter IV). The epithelium as well as the underlying connective tissue had a lack of bony support and the process of repair under such adverse conditions was expected to be different from the situation of surface soft tissue wounding, as seen in the anterior portion of the palate.

Time was found to play a significant role in both the experimental and control palates. When a comparison between the experimental and control palates over time was subjected to statistical analysis, it was found that there was a significant difference at the 95% confidence level (Table VI-4). This analysis pooled experimental values in both anterior and posterior palatal samples. New collagen production was expected to be different in a palate undergoing wound healing and this finding was no surprise. Early (1 week) values for collagen production in the control palates were high and this can be explained by the effect of hard diet on the forces of mastication in the palate, as previously discussed.

When anterior palates (both control and experimental) were compared to posterior palates (both experimental and control),

there was found to be a significant difference in collagen production when subjected to a mixed analysis of variance (Table IV-4). The level of significance for this difference was at the 95% level of confidence. The anterior palate was different in its production of collagen than the posterior palate. In the anterior region the epithelial tissue was thicker and had larger rugae present. This might accommodate the harder and thicker base required for the forces of mastication. As the food is chewed, it moves into the posterior portion of the palate and this thickness in mucosa is not required. The collagen synthesis in the posterior region was not as high and that could indeed be a result of lack of functional demands.

When palatal samples were taken, a small portion of non-wounded tissue was always included. This experimental technique was unavoidable as the separation from scar tissue to normal tissue follows a gradient similar to that found in human palatal scar. This was then a source of experimental error which might explain the results of experimental palates having higher collagen production, but not at a significant level of confidence.

CONCLUSIONS

1). The *in vitro* organ culture system was successful for (^{14}C)-glycine uptake and was found to be a good system for the synthesis of collagenase digestible protein.

2). The anterior experimental palates were found to have the

highest collagen production at 2 weeks following wounding.

3). The posterior experimental palates were found to have the highest collagen production at 1 and 2 weeks after wounding.

4). Time was found to be a significant factor when experimental and control animals were compared (significant at the 95% level of confidence).

5). Time was found to be a significant factor when anterior palates were compared to posterior palates in both groups (experimental and control). This was significant at the 95% level of confidence.

6). There was a trend which showed experimental palates produced more collagen than the control palates, but it was not found to be statistically significant.

CHAPTER VII

DISCUSSION

The experimental model which was developed and used in this study, proved to be a practical and convenient model for the investigation of wound healing and scar tissue formation in the palate of the rat. The eighteen day old Sprague-Dawley male rat was young enough to alter certain growth processes such as intramolar width and general body size as seen in many cleft palate patients (Ross and Johnston, 1972). Palatal scar tissue was easily identified in the experimental animals and parameters such as irregular rugae formation and contracted palatal width were seen clinically. The small size of the animal made it more economical when radioisotopes were used in vivo. The size of the palatal tissue used under culture conditions (in vitro) was also small and very easy to maintain for the time period required. There were no specific reasons for choosing male rats over females. The region of the midpalatal suture between the erupting first molars was selected because of its reliability of location and its convenience for the surgical procedure.

Microscopically palatal scar tissue has been shown to exhibit a disorganized fiber arrangement (Madden and Peacock, 1971), as well as a lack of vascularity in the connective tissue (Linares, 1983). The tissue examined in my study exhibited many of the features associated with the palatal scar tissue of repaired cleft patients (Figs. IV-14 to IV-24). The radioautographic study was carried out using (^{3}H)-proline and following the technique of Kopriwa and Leblond (1962) and Tonna (1974). Implicit in the interpretation of the grain labelling, in correlation with the biochemical data, was the assumption that (^{3}H)-proline was largely

incorporated into collagenous proteins. This was disputed by Orlowski (1976) and Rossman et al. (1975). However, Sodek et al., (1977) showed (^{3}H)-proline was a reliable label for collagen in vivo, for biochemical analysis of (^{3}H)-hydroxyproline, a characteristic amino acid for collagen, in the periodontal ligament.

The grain counting procedure was done only to obtain an indication of the differences in proline uptake or collagen synthetic activity by the epithelium, connective tissue and bone in the palatal region. There are limitations in grain counting for quantitative analysis, because variations in temperature, humidity, dipping technique, drying conditions and radiation all affect grain development. Rogers (1979) felt that radioautography was semi-quantitative at best. In this study all the radioautographs were prepared and processed by the same operator, and all the conditions were kept as uniform as possible. The grain counting procedure was done by the same operator and an error study (Appendix II) showed acceptable accuracy in the counting procedure. In order that the data on grain counting be statistically significant, three more rats would have to be labelled at each time period and prepared for histology. Since this was not the primary interest in this study, that procedure was not undertaken.

The light microscopy revealed a number of interesting characteristics in wound healing in the palatal region. Bone was shown to be sharp after surgical penetration and after four days (post surgery) it was shown to remodel and become rounded with time. The epithelial and connective tissue layers in the area of the wound (scar tissue) showed a lack of vascularity with the passage

of time. This was in agreement with Longacre et al. (1976) where with time, the scars showed signs of contracture and reduced vascularity. Rete peg formation was visibly absent until 28 days after surgery when they began to appear in the region of the scar. Some of the palates did not repair by closing off the oral and nasal cavities from each other. These palates repaired but had an oronasal fistula present and they occurred at a rate of about one in ten surgeries. Reasons for the presence of a fistula in some of the palates could be due to excessive removal of palatal mucosa and connective tissue by the operator. There was obviously a limit to the amount of tissue which could be removed and still undergo repair by sealing off the oral and nasal cavities. There were not enough of these rats with oronasal fistulae present to do any meaningful study with them. It would be interesting to see how intramolar widths and general body weight would compare to the normal and fully repaired cleft samples in this study. Oronasal fistulae are present in 10 to 20% of repaired cleft palate patients according to Peer et al. (1954). In addition, scar tissue contraction during healing tends to pull the soft tissue away from the bone because of the shape of the palatal vault. Healing results in callus-like bone formation under the flaps which causes the irregularities in the surface of the bony palate. This clinical impression was supported by work on dogs by Hugg and Kremenak (1968). The rats which were surgerized in this study had significantly smaller body weights when compared to the non-surgerized control animals. This is in agreement with Ross and Johnston (1972) who reported that physical retardation of children with

clefts was greater than children without clefts. Facial structures in humans exhibit a difference in growth timing with the prepubertal growth spurt occurring later in children with clefts (Shibisaki and Ross, 1969). It has not been established whether growth continues later in children with clefts to enable them to "catch up" eventually, but evidence which is in agreement with this study suggests that it does not (Birch et al., 1972).

The decrease in intramolar width in the surgerized animals in this study is in agreement with studies by other authors (Jolleys, 1954; Lynch and Peil, 1966; Kremenak et al., 1967). In humans there are many different techniques of cleft palate repair. One basic problem in most clefts is an inadequate amount of tissue in the region of the cleft. The surgeon must either borrow tissue from a region close to the cleft or perform the repair under tension. Repair under tension creates an environment unfavorable for soft palate function and speech, and the usual procedure is to mobilize palatal mucoperiosteal flaps, using releasing incisions close to the alveolar process. When the flaps are joined together in the midline, areas of denuded bone remain in the palate close to the alveolus. These areas subsequently epithelialize and become areas of scar tissue which contract during healing and create a constricting force on the maxilla (Jolleys, 1954; Lynch and Peil, 1966; Kremenak et al., 1967). The midline flaps which are under tension also become scar tissue.

The finding that intramolar width decreased with time in experimental rats and then began to increase at a very slow rate was interesting. The question arises as to whether there was

actual contraction due to the action of myofibroblasts or whether there was relative contraction due to a lack of remodelling around the midpalatal suture. A dense network of connective tissue extends from the buccal mucosa to the palatal mucosa forming a continuum. Studies using tattooing (Edwards, 1968; Hugg and Kremenak, 1968) have shown the non-elastic nature of the tissues even after years of orthodontic repositioning of teeth. It was therefore not surprising to see a decreased intramolar width when a relatively small hole was placed in the palatal midline, quite distant from the molars themselves. The most probable explanation for the lack of increase in intramolar width is a lack of remodelling in the midpalatal suture region of the wound site. After the period of one week, intramolar width was seen to increase in experimental animals (data not shown), but the finding never completely regained normal dimensions. This would lead one to speculate that the relatively contracted maxillae of repaired cleft palates are due to limited or interrupted remodelling patterns. The possibility of altered physiological and functional oral environment was not evaluated in this experimental model. An altered environment such as an airway obstruction could lead to dental arch collapse and lowered tongue and mandibular position. The possibility of palatal soreness with a lowered tongue position and subsequent molar collapse may contribute to the lack of increase of intramolar width. However, no animals were observed to have changed mandibular position as well as drooling or other signs of pain or soreness. This is not a problem in repaired cleft patients who do not exhibit a higher incidence of mouth breathing.

In humans the effect of repeated palatal repairs is hard to measure and it could introduce a new contracting force after each surgical repair. According to Ross and Johnston (1972), the scar tissue in the palate causes a mild contraction of the maxillary segments during the initial healing period and this is in agreement with the intramolar width study conducted in this experiment.

Whether the tissue examined in my study was representative of that stage scar tissue which would be most significant in affecting facial growth is unknown. The oldest animal which was examined for collagen types was eight weeks of age. The collagen content of the palatal scar was not examined after this age and further studies might show a difference in collagen production. A small number of surgically scarred rats were examined clinically at 14 months of age and still exhibited scar. However, a larger sample would be required to verify the duration of palatal scar. A major concern in this study is the limited range of ages examined post-operatively. Longer post-operative periods may reveal biochemical differences between control and palatally-scarred animals.

The significance of the proportion of type III collagen increase in palatally surgerized animals is in agreement with its appearance in early phases of wound healing in skin as demonstrated by Gay et al., 1978. Type III collagen has been found to be present in foetal skin and uterine wall (Chung and Miller, 1974); synthesized by synovial cells from patients with rheumatoid arthritis (Eyre and Muir, 1975) and associated with inflammation (Weiss et al., 1975). Type III collagen persisted in the dentin

of patients with osteogenesis imperfecta, where normally it would be replaced by type I collagen (Sauk et al., 1980). In patients with the heritable disease, Ehlers-Danlos Syndrome type IV, there was a deficient synthesis of type III collagen (Aumailley et al., 1980). It appears that type III collagen serves an important role in rapid remodelling situations, such as wound healing, as well as abnormal growth and development of connective tissue. Its association with vascularization in the form of reticular network (Gay et al., 1978) might suggest a supportive role for the blood capillaries. Type III collagen contains the amino acid, cysteine, whose sulphide bonds provide strong crosslinks between the alpha chains. This may explain the easily friable dermis in patients with Ehlers-Danlos Syndrome type IV, who lack type III collagen. Type III collagen is present in tissues such as the aorta, uterine wall, and periodontal ligament (Chung and Miller, 1974; Butler et al., 1975; Narayanan and Page, 1983) and consequently might have a stress-bearing role. This would agree with the findings of this study where the proportion of type III collagen increased after palatal wounding.

The finding that control animals had higher levels of type III collagen in the early weeks of the study and decreased with time, might be related to the masticatory stresses which were placed on the palate in the eighteen day old animals. This finding agrees with the study by Claycomb et al. (1967) who felt that oral collagen existed in a state of continuous wound healing and repair. The level of oral collagen metabolic activity in their animals was high initially and decreased with time.

As discussed in Chapter V, the change in collagen types could be a result of a shift in cell populations which make different types of collagen; or a change in cell function. Fibroblasts in culture have been shown by immunofluorescence to be able to synthesize both type I and type III collagen (Gay et al., 1976; Engel et al., 1980). In Ehlers-Danlos Syndrome type IV there is a deficient production of type III collagen (Aumailley et al., 1980). This might implicate a regulatory defect at the gene expression level and the level of transcription in protein synthesis. The possibility of the existence of different cell subpopulations might be capable of producing only type I collagen, or both type I and type III collagens. If this were the case, then the different cell types could be isolated and studied separately for regulatory effect by various biological mediators. Also, the cell types may be traced during their differentiation, so that osteoprogenitor populations could be identified. Antibodies to the collagen types would be used as histochemical markers.

The removal of a tissue from its in vivo environment must provoke biological changes (Schilling, 1978) and these changes may be further accentuated by the act of an in vitro culture. Cells may release and synthesize greatly increased amounts of lysosomal enzymes, both in response to dissection trauma and to agents present in the culture medium, and this can result in modified tissue behaviour (Weissmann et al., 1972). The media chosen for organ culture systems is designed to provide an in vitro nutritional environment which approximates, as closely as possible, to that occurring in the intact animal. Waymouth (1974b) described the

object of media design was to enhance the genotypic and phenotypic stability of cells *in vitro*. The inclusion of ascorbic acid in culture media has been shown to eliminate excessive hydration (Reynolds, 1966b), and to increase collagen production (Reynolds, 1966b). These factors serve to illustrate that the *in vitro* system could indeed be quite different than the *in vivo* system.

The effect of time on the percentage of type III collagen in palatal scar tissue was seen to decrease in both experimental and control samples. Type III collagen has been compared to fetal dermal tissue which also shows a decrease in proportion of type III collagen with time. Long-standing scars in human dermal tissue have been shown to have a very low proportion of type III collagen after long time periods (Miller, 1976). Type III collagen might play a role in providing initial support and a matrix upon which the other tissues are deposited. It is possible that after this matrix is laid down, certain cells might be signalled to increase the type I collagen production and decrease the type III collagen production. Further studies in the identification of cell subpopulations responsible for collagen type synthesis might answer this question.

Much further research is required in the search for the regulatory mechanisms of scar tissue remodelling in the palate. Identification of collagen synthesis patterns would aid in identification of those cell populations involved in palatal scar remodelling. Ultimately, these isolated populations could be studied for the control mechanisms in palatal tissue remodelling.

CHAPTER VIII

CONCLUSIONS

CONCLUSIONS

1). The experimental *in vivo* model used in this study, performing palatal surgery on eighteen day old male rats, proved to be a practical and convenient one for the studies of palatal scar tissue remodelling.

2). After palatal wounding, it was shown histologically that the period of time required for palatal re-epithelialization was approximately four days. One animal in ten developed an oronasal fistula.

3). Intramolar width in the rats studied was shown to be decreased in all experimental rats while it increased in all control rats with time.

4). Radioautographic results showed that protein synthetic activity occurred in the palatal epithelium, connective tissue, and bone throughout the palatal region.

5). The *in vitro* model used in this study was shown to be a practical and convenient one for the studies of biochemical analysis of palatal scar tissue.

6). The proportion of type III collagen production (compared to type I collagen production) was shown to be significantly higher in experimental rat palates compared to the controls.

7). The proportion of type III collagen was shown to decrease over the time period studied. This was significant in both

experimental and control palates in the age groups studied.

8). Total collagen production per mg of palatal tissue was shown to be significantly different when anterior palates were compared to posterior palates in both control and experimental animals. This difference was seen over the period of time of one to five weeks.

9). Further studies were suggested:

a). A time-course study to investigate collagen degradation in palatal scar tissue in order to compare this with collagen synthesis.

b). Identification of possible cell subpopulations which might be responsible for the synthesis of different types of collagen in palatal scar tissue.

c). To study the role of proteoglycans in scar tissue formation.

d). A time-course study to determine the relationship between masticatory function and palatal tissue collagen synthesis.

e). A time-course study of the collagen synthesis in rats of three weeks of age or older in order to determine if production of collagen in palatal mucosa was high only initially after litter separation from the mother.

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APPENDIX I

AGE STUDY

INTRODUCTION

A pilot study was undertaken in order to determine the youngest possible age at which a rat could undergo palatal surgery. It was assumed that the earlier the age that surgery could be performed, the more analogous the animal model would be to the clinical situation where facial and dental growth and development are affected.

Intramolar width is an important variable in orthodontic treatment of cleft lip and palate individuals. Contraction of the maxillary dental arch is a common feature of surgically treated cleft palate patients. Expansion applicances are often the initial procedure undertaken in correction of a malocclusion in a cleft lip and palate child. Monitoring the intramolar width while observing weight gain and general body development and scar tissue formation would lead us to a useful model in the rat.

The rat would serve as a superior model for analysis of collagen since its smaller body weight (compared to dogs and monkeys) would be more economical for radioisotope studies.

Once a desirable age of Sprague-Dawley rat is determined, it would be possible to proceed with a histological study on the effects of surgery and resultant scar tissue of the palatal tissues.

MATERIALS AND METHODS

This investigation selected the male Sprague-Dawley rats as the experimental model because of its size, which was suitable to create a palatal cleft and observe the scar formation. These animals were bred in our facility at the Faculty of Dentistry Animal House. The midpalatal suture region between the first molars was chosen as the surgical site because of its convenience and reliability as well as its accessibility for surgical manipulation.

Male Sprague-Dawley rats, outbred in this facility, were maintained on Wayne Laboratory, Blox-F6 specialty food, given water ad libitum, and kept at a temperature of 20° ± 1.5°C. Five rats were randomly selected for each of seven age groups: 15, 16, 17, 18, 19, 20 and 21 days. The usual time of separation of the litter from the mother was twenty-one days after birth.

In each age group there were three experimental and two control rats which were chosen at random. Each of the rats was weighed and received ether anaesthesia and then numbered according to a code.

The experimental group received surgery in an area between the erupting first molars in the region of the midpalatal suture. Surgery consisted of placing a high speed handpiece with a number 4 surgical burr attached into the region and penetrating through the oral epithelium and bony tissue. The burr was then moved anteriorly into the rugae

region, being careful only to surgerize the soft tissue.

Rats from each age group were placed in the same cage and observed on a daily basis. A special diet of finely minced food (Blox F-6) was placed in each cage for one week in order to permit the wounded animals easier digestion in the recovery period.

RESULTS

The number of control as well as experimental animals which survived in the early age groups of 15 and 16 days was very low (Table A-1). At 17 days of age all the controls survived, but only 40% of the surgerized animals lived for one week or more. The 18 day age group had 100% of the controls which survived and 80% of the experimental animals. The results of the remaining days tested were similar to the 18 day group.

Visible scar creation in the palatal region was seen to occur after one week of healing.

DISCUSSION

On the basis of this experiment, it was decided that the 18 day old Sprague-Dawley male rat would be the youngest age which could be surgerized without causing death before a time period of one week. General observations of health and body

TABLE A-1

RESULTS OF PILOT STUDY IN WHICH 5 EXPERIMENTAL RATS RECEIVED ETHER ANAESTHESIA AND PALATAL SURGERY AND 5 CONTROL RATS RECEIVED ETHER ANAESTHESIA ONLY.

AGE (DAYS)	NUMBER OF RATS ALIVE ONE WEEK FOLLOWING SURGERY	
	CONTROL	EXPERIMENTAL
15	2	0
16	2	0
17	4	2
18	5	4
19	5	4
20	5	5
21	5	4

weight showed the experimental rats to be of less weight than the controls, but this was expected since surgery of the palate created temporary eating difficulties. Fur of the sick rats was noted to be very sparse and the body had a bloated appearance in the intestinal region. Any rat which took on this appearance was expected to die and this occurred within the time period of one week.

The control rats which died in group 15, 16 and 17 days of age were presumed to be sensitive to the ether anaesthesia at this young age.

The formation of scar tissue intraorally was seen after approximately one week. The persistence of this scar tissue, which is almost unique to cleft palate closure sites, will be studied in further experiments of this nature.

CONCLUSIONS

The youngest possible age at which a rat could survive palatal surgery was determined to be 18 days of age. This was three to four days earlier than the usual separation of the litter from the mother. Earlier age groups had a high mortality rate and were presumed to be sensitive to ether anaesthesia, as well as not capable of withstanding the surgical procedure.

This early age of 18 days will create a model where dental and facial growth and development can be studied. Palatal scar tissue will also be studied as it was one of the

results of the wounding procedure.

APPENDIX 2

ERROR STUDY OF GRAIN COUNTING PROCEDURE

ERROR STUDY OF GRAIN COUNTING PROCEDURE

Ten transparencies of the epithelial scar tissue area radiographs were projected with a photographic enlarger at a distance of 2 feet onto a screen graduated with 3 x 3 cm grids. Five areas were counted in random order and this was done on three separate days. Each recorded count was covered so that subsequent counting would not be biased.

The data (Table Appendix 2) was treated statistically according to the method described by Chebib and Burdick (1973). The percentage error for the grain counting procedure, at the 99% confidence level, was 10% at maximum. In other words, for every 25 grains counted, the maximum error was 2 to 3 grains at the 99% confidence level.

TABLE A-2

ERROR STUDY OF CONSISTENCY OF GRAIN COUNTING PROCEDURE

SLIDE \ AREAS	DAY 1	DAY 2	DAY 3	MEAN $\bar{X}$	S.D.
1	24	24.4	25.0	24.46	0.5
2	24.2	25.2	24.4	24.60	0.5
3	24.6	25.4	24.2	24.73	0.6
4	22.6	24.0	24.8	23.80	1.1
5	25.6	23.8	24.2	24.53	0.9
6	23.8	25.8	24.2	24.60	1.0
7	24.4	24.0	24.4	24.26	0.2
8	25.4	24.8	24.0	24.73	0.7
9	25.0	22.6	23.6	23.73	1.2
10	26.0	23.8	24.2	24.66	1.1

FIVE AREAS WERE COUNTED ON EACH DAY AND AVERAGED.
TEN SLIDES WERE PROJECTED

POOLED MEAN, $\bar{X}$ = 24.41

POOLED STANDARD DEVIATION, SP = 0.868