ROOT PREPARATION WITH CITRIC ACID
AN HISTOLOGICAL STUDY

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TABLE OF CONTENTS

DECLARATION

This thesis is submitted in partial fulfilment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide. Candidature for the Degree was satisfied by a Qualifying Examination in 1980.

This thesis contains no material which, except where due mention is made, has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

The results of the present investigation have been presented in part to the meeting of the International Association for Dental Research (Australian Section) in November 1981.

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STEPHEN YEUNG

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INTRODUCTION

It is now established that following periodontal disease and subsequent attachment loss, gingival reattachment can be achieved provided that sufficient preparation of the diseased root surface is carried out. It has been generally accepted that reattachment was achieved by long Junctional epithelial attachment.

Recent research revived interest in an old treatment method advocated at the turn of the 20th century. It was recommended that diseased root surfaces should be treated with acid. Recently, 20% Citric Acid was suggested as an appropriate therapeutic agent and, after acid demineralization, gingival reattachment was found to favour connective tissue reattachment.

The present study was designed to examine the result of acid-treated cementum and its effect upon reattachment. The topical use of 20% citric acid applied to the root surface has the potential to damage the dental pulp. This concern was also investigated in the study.

The animal model adopted was the beagle dog, relatively healthy periodontal tissue was used and the experimental design ensured that surgical trauma and its repair under the influence of citric acid or saline could be compared. The establishment of periodontal disease in

the dogs would have been desirable but time constraint precluded that approach.

The results confirmed that a difference existed in the healing patterns between surfaces treated with acid and saline. It was also found that the time periods chosen for the experiment gave an adequate insight into the healing potential. The effects of citric acid used in the experimental method failed to cause detectable pulpal changes.

CHAPTER I

LITERATURE REVIEW

1.1 Gingival Reattachment

Ever since periodontal disease (pyorrhea alveolaris*) was first described by Hunter in 1771, the objective of reattachment of connective tissue and epithelium upon the tooth surface has remained a challenge. The reattachment of connective tissue is achieved by embedding collagen fibres (principal fibres) into the surface layer of cementum to form Sharpey's fibres. (ORBAN, 1944 Oral Histology). Controversy surrounded the nature of epithelial attachment, as opinions ranged from "no attachment" to chemical and physical bonding. A protracted dispute resulted among dental researchers to decide whether or not reattachment of both connective tissue and epithelium could occur in the healing phase of periodontal disease.

1.1.1 Possibility of gingival reattachment

YOUNGER (1893, 1905) a pioneer of tooth re-implantation claimed that reattachment of soft tissue to implanted teeth was not only a possibility - but a

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*The term "pyorrhea alveolaris" meaning flowing of pus from the alveolar sockets, describes the symptoms rather than the disease. This led to the adoption of the term "periodontoclasia" which was thought to be more appropriate. Today, "periodontal disease" is universally accepted as the official terminology for this condition.

reality. He advocated complete removal of the cementum and sterilization of the root-filled tooth (soaking for 1 week in 2.5% solution of carbolic acid and then treated in a bath of 1-1000 solution of bichlorid of mercury) prior to implantation.

STEWART (1899) cited methods of treatment for pyorrhea alveolaris by a London dentist in 1760. It was believed that periodontal disease was caused by a specific infection of an oral micro-organism, and that calculus formation was a sequelae rather than a cause of the condition (Clement 1893). As a result of periodontal disease, cementum becomes hypercalcified. Stewart, thus based his treatment rationale on this knowledge. He wrote:

> Our treatment is based on the supposition that the organic matter of the cementum is replaced by calcific matter, the whole structure becoming very dense and shutting off vital connection between the tooth and the organism at large. The peri-dental membrane being no longer able to perform its functions, dies at that point.

Using eucain as local anaesthetics he surgically separated gum from teeth down to the level of alveolar bone. Then he removed most of the cementum by scraping with a sharp excavator and cut grooves into the root surface "to afford a better place for deposit and attach-ment of new tissue." This was followed by lacerating the gum with a lancet and re-adapting the tissue for healing.

Stewart also suggested that reattachment was enhanced if root surfaces were "roughened" by topically applying acid (sulphuric or hydrochloric acid) to decalcify the

surface, Stewart's work did not receive much attention, and very little progress was made for the next few decades.

The first attempt to support "reattachment" claims by histologic evidence was made by BOX (1924). He prepared sections of a tooth treated by McCall in 1912 and extracted in 1920. Later, BOX (1928) described a short-term study (1 to 9 days) of 3 cases using the same technique as McCall.

Three different schools of thought evolved; the first group, led by BLACK (1937), COOLIDGE (1938), BLANQUIE (1950) and ADLER (1951) suggested that reattachment was impossible and that it had never been proved in practice.

The second group, supported GOTTLIEB (1933), ORBAN (1948) and NOYES (1912) and believed that reattachment was theoretically possible, but technical difficulties made it impossible. GOTTLIEB (1946) proposed his "New Concept of Periodontoclasia", although most of his assertions were subsequently proved to be incorrect, his concepts dominated dental thinking for many years.

The third group were researchers who claimed that reattachment occurred clinically and in experimental studies. Early workers included STILLMAN (1917), MERRITT (1918) and BUNTING (1928), followed by LEONARD (1931, 1935), JAMES (1933), SMITH (1935) and BJORNDAHL (1948).

BECKWITH et al. (1927, 1928) were the first to investigate this problem using laboratory animals. They reported regeneration following the surgical destruction

of a section of the supporting structure in rodents, but in an area remote from and not involving the gingival crevice. Later, they reported a similar result with cats. Since then, conflicting findings have been reported by a number of investigators, using various models.

LEONARD (1943) claimed that epithelial pocket lining is capable of attaching itself to tooth surface which has been rendered free of debris, providing the surrounding connective tissue binds it tightly against the tooth surface. Following Gottlieb's concept of epithelial down-growth, he contended that reattachment after pocket elimination was epithelial in nature; since evidence of connective tissue reattachment was lacking. His assertion drew criticism from other workers and stimulated studies on the exact nature and type of reattachment. Histological study with light microscopy was the main mode of investigation.

BEUBE (1947, 1952, 1960) published data on healing and reattachment after surgical wounding in dogs. He showed that new cementum was formed in 27 days, which continued to grow in thickness and density. New bone formation was also found with fibroblasts and connective tissue filling in the space between bone and cementum.

In an effort to reproduce the condition of perio-dontal disease in an experimental animal BJÖRN (1961) using dogs, removed labial soft and hard tissue and exposed about 1/3 of the root length to the oral environ-ment. Six months later, a labial flap was mobilized to

cover this defect in an attempt to achieve reattachment. He found that new cementum formed over the exposed cementum in the healing phase. Another important aspect of his finding was that by excluding epithelium from the healing process in the periodontium, it was possible to obtain complete mesenchymal reattachment with reorganization of root cementum, periodontal membrane, and alveolar bone. Up to the end of the 1950s, it was generally believed that new cementum cannot be formed on necrotic cementum, and that consequently, in order to facilitate reattachment, the surface layer of the denuded cementum ought to be removed during a reattachment operation. Björn's findings cast doubt on such a concept.

FRIEDMAN (1958) stated that there were two types of reattachment: connective tissue and epithelial. The general consensus was that connective reattachment was more desirable since it formed a new attachment apparatus together with new cementum and new bone. On the other hand, epithelial reattachment was considered temporary and unstable. Friedman also discussed at length various methods of assessing the extent of reattachment which he considered grossly inadequate. Both clinical probing and radiographic assessment were deemed unreliable, whilst histologic (histometric) assessment was the only true guide.

LINGHORNE (1964, 1950-1957) published histologic studies of regeneration and reattachment. He reaffirmed the possibility of connective tissue reattachment and

suggested that downgrowth of the epithelium over the detached gingivae was the result, not the cause of the failure of the reattachment. His findings led MORRIS (1949) and CARRANZA (1954) to advocate a surgical technique whereby both the sulcular epithelium and necrotic cementum were removed.

Most investigators were convinced of the possibility of reattachment and massive data were available to support the concept. These included SKILLEN & LUNDQUIST (1937), McCALL (1951), MORRIS (1953, 1954), RAMFJORD (1951, 1952), ROCKOFF (1958), SHAPIRO (1953, 1957), STAHL (1962) and many others.

1.1.2 Nature of epithelial attachment

Much of the confusion regarding reattachment stemmed from a poor understanding of the nature of gingival attachment. It was unclear whether epithelium was actually attached to the tooth surface, or whether it was closely approximated to cementum.

WAERHAUG (1952) maintained that there was no epithelial attachment. He claimed that the bottom of the clinical pocket was situated at the deepest line of epithelial attachment, thus the "epithelial attachment" belongs to the lining of the pocket. Most researchers subsequently disagreed with this view.

MACAPANPAN (1954) cited cases where a strong bond existed between the gingivae and the tooth surface even though the gingivae had been torn away. This suggested

that the strength of epithelial-tooth bonding was greater than that of the intercellular adhesiveness in the epithelium. Zander (1955) pointed out the pitfall of accepting evidences such as presented by Macapanpan. He suggested that histological sections, especially when artifact was present, should not be drawn on as reliable evidence.

ORBAN et al. (1956) studied epithelial attachment in 6 dogs and 4 monkeys, using light microscopy. They concluded that gingival epithelium did attach to the enamel surface, although the actual mechanism was unclear. Such attachment was of some "firmness" because it was evidently stronger than the intercellular connections between epithelium and underlying connective tissue.

GARGIULO et al. (1961) followed on Orban's study using human materials. They felt that epithelial attachment to the tooth was relatively weak in spite of the fact that it was stronger than individual cohesiveness of the epithelial cells. Their histometric measurements found that the connective tissue attachment (from the base of epithelial attachment to the crest of alveolar bone) was constant, averaging 1.07mm., whereas the length of the epithelial attachment was variable.

WEINREB (1960) used a new histologic method to obtain ground sections of both soft and hard tissues. He concluded that there was a definite union between the gingival epithelium and the tooth surface.

His conclusions were based on:

1) Filling gingival pocket with Cavit or self-curing acrylic demonstrated continued adherence of epithelial cells to the hard tooth substance.
2) The introduction of thin strips into the gingival sulcus did not detach the epithelial attachment from its union with the hard tooth surface.
3) It was impossible to detach the gingiva from the hard tooth surface by flaps without causing tears in the epithelium, part of which continued to adhere to the tooth.

WERTHEIMER & FULLMER (1960) attempted to identify and characterize the biochemical nature of the epithelial attachment cuticle. They found that the cuticle possessed a peculiar type of carbohydrate, however, the presence of a residual lipid was not excluded. SCHULTZHAUDT et al. (1963) investigated the nature of contact between gingival epithelium and the tooth enamel surface. They believed it was a mucopolysaccharide although full identification was not achieved.

In vitro studies were also made on cellular attachment onto tooth surface. POWELL (1968) used mono-layer cultures of Hela (calf) cells and showed adhesion to the enamel of bovine incisors, although such adhesion was not as effective and firm as that to glass. MILNEK & POWELL (1970) used Syrian baby hamster kidney cells (BHK_{21}) and demonstrated adhesion to bovine incisors. They also found acidic and neutral glycoproteins and acid glycosaminoglycans secreted by the attached cells. They went on to postulate that these substances were necessary elements in the process of attachment of cells to enamel.

1.1.3 Electron microscopic studies

The advent of the electron microscope brought a renewed interest in reattachment - both of connective and epithelial tissues.

LISTGARTEN (1966) demonstrated the ultrastructure of gingival attachment. He showed that:

1) An attachment is present between the epithelial cuff and enamel or cementum.
2) The attachment apparatus (epithelial) consists of hemidesmosomes and cementing layers.
3) There are two types of cuticles.

   Cuticle A is the layer on the tooth surface, it consists of an electron dense, granular layer. (This corresponds to the primary enamel cuticle of Gottlieb and Orban.)

   Cuticle B has a less granular but mottled appearance. It could be lying directly over enamel or cementum or cuticle A.
4) Connective tissue attachment consists of collagen fibrils extending from the gingivae into the cementum, and embedded in a rather sparse, granular matrix.

Listgarten (1967) extended his study to attachment after gingival surgery using monkeys. He concluded:

> All showed formation of a new epithelial reattachment consisting of hemidesmosomes and a basement lamina. In some areas, this attachment apparatus connected the cells directly to the tooth surface. In other areas, cuticular structures were interposed between the attachment apparatus and the tooth surface. Although the layers intervening between the attachment apparatus and the tooth surface may represent acquired pellicles, they may also be due to the denaturation of superficial layers of cementum. Any factor affecting the integrity of the epithelial tissue, the attachment apparatus or these cuticles could result in breakdown of the epithelial attachment.

LISTGARTEN (1970) summarized his findings in his proposed changing concepts about the dento-epithelial

junction. He suggested that:

1) The junctional epithelium is mediated by hemidesmosomes and a cellular basement membrane.
2) The cells resist separation from the tooth surface but are capable of gliding along it.

HODSON (1966, 1967) presented quite a contrasting view regarding the dental cuticle. He wrote:

> The result shows that Gottlieb's cuticle is a fortuitous formation lying between the tooth surface and the adjacent epithelium; it is foreign to the part, of hematogenous origin and composed of denatured haemoglobin formed from conglutinated erythrocytes. The dental cuticle is a pathological formation associated with haemorrhage in hyperemic and inflamed tissues. It is suggested that it be renamed, the Hematogenous Paradontal Hyaline Layer. It is considered that the theories based on this structure and formation of the gingival sulcus, the concept of the epithelial attachment and the process known as Passive Eruption, are in the light of the new findings, no longer tenable.

TAYLOR & CAMPBELL (1972) studied reattachment of gingival epithelium to the tooth microscopically and ultrastructurally. Using marmoset premolars, they separated the gingivae from the tooth surface with steel blades. On the third day, the apical 1/2 to 3/4 of the epithelium was attached to the enamel cuticle by well formed hemidesmosomes. On the fifth day, reattachment had reached the level of the best control unoperated gingivae. These findings were consistent with the concepts of a dynamic attachment in which newly proliferated epithelial cells near the cervix attach themselves to the tooth and migrate occlusally along its surface (Listgarten 1970). The epithelial seal, although very weak, can rapidly repair itself after being mechanically disrupted.

FRANK et al. (1972, 1974) studied the ultrastructure of epithelial and connective gingival reattachment in man. Their findings mainly reaffirmed other light microscopic studies.

SAGLIE et al. (1974) examined the topography of pathologic pocket area of human teeth using scanning electron microscopy (SEM). He noted the "roughness" of the exposed cementum surface.

KOBAYASHI et al. (1976, 1977) studied the ultrastructure of the dento-epithelial junction i.e., junctional épithelium, in Rhesus monkey. They found that the basal lamina was composed of 3 layers:

1) Lamina lucida
2) Lamina densa
3) Sub-lamina lucida

Fine filaments often coursed through the lamina densa, lamina lucida and hemidesmosomes and combined them into a structural unit. The existence of a sub-lamina lucida between the lamina densa and tooth surface was a newly defined layer. Kobayashi then went on to propose that this is an area of competitive electrostatic forces of repulsion and Van der Waals forces of attraction. In an attempt to elucidate the nature of the basal lamina, using various histochemical methods, they concluded that the basal lamina was an active participant in the adhesive mechanism at the dento-epithelial junction, but the dental cuticle had a transient role and substitutes, when present, for the tooth surface.

KAPLAN (1977) used SEM to study the sulcular and junctional epithelium of normal and diseased gingivae, found that the epithelial cells of periodontal pockets have "punched out" areas. This, they believed, represented areas of increased epithelial tissue permeability in the presence of inflammation.

GARNICK (1977) proposed the use of the rat as a model system in the study of the dynamics of the long epithelial attachment.

SAGLIE et al. (1979) demonstrated the functional variations in different parts of junctional epithelium using TEM. They found:

1) the apical zone showed epithelial cells with germinative characteristics;
2) the middle zone seems to be one of major adhesiveness;
3) the coronal zone is one of major permeability.

1.1.4 Gingival reattachment after periodontal therapy

SANDERSON (1966) and KON (1969) found variations in the healing response of different animal models. This prompted STAHL et al. (1971) to study the healing behaviour of soft tissue following curettage and root planing. They concluded that individual specimens from the same host were independent and reflected an unchanging inflammatory response, given normal metabolic variation. Inflammatory response at each tooth then may be primarily controlled by local factors (i.e., plaque, trauma, etc.).

BURFIELD (1971) in a study using human teeth, showed that attachment of epithelium and connective tissue can

be expected to follow operative procedures on the periodontium. Furthermore, he demonstrated reattachment occurred even if unfavourable conditions of oral hygiene existed.

Others also reported regeneration of bone and cementum following grafting procedures (ROSS & COHEN 1968, DRAGOO & SULLIVAN 1973, FROUM et al. 1975, HAWLEY & MILLER 1975, HIATT et al. 1978, LISTGARTEN & ROSENBERG 1979). However, all of these reports failed to quantify the amount of new attachment due to the lack of reference landmarks.

CATON & ZANDER (1976, 1979) claimed that after periodic root planing and soft tissue curettage, the gingival reattachment was primarily formed by long junctional epithelium. This view was shared by other researchers (HIATT et al. 1978, MOSKOW et al. 1979, LISTGARTEN & ROSENBERG 1979). It was generally conceded that connective tissue reattachment after periodontal therapy, although desirable, was a rarity.

The most significant development in periodontal therapy in the 1970s was a gradual shift of emphasis in the modality of treatment. Attention was focussed on the preparation of root surfaces, rather than surgical manipulation of the soft tissue. Such change of attitude among periodontists and researchers was sparked off by the classic demonstration by HATFIELD & BAUHAMMERS (1971) and later ALEO et al. (1974, 1975).

Hatfield and Bauhammers showed that epithelial cell culture suffered degenerative changes when exposed to "diseased cementum". Aleo demonstrated cells from fibroblast culture, which normally attach to healthy cementum, will not do so when presented with "diseased and exposed cementum". Aleo showed that by treating the diseased root surface (e.g. mechanical root planing, chemical extraction of endotoxin with phenol) that the biological viability of the root surface could be restored and again attracted fibroblasts. It seems logical, that periodontal therapy should aim to prepare root surfaces to return them to a biologically acceptable state that will enhance epithelial and connective tissue reattachment.

1.2 Root Preparation with Citric Acid

1.2.1 Animal studies

STEWART (1899) was the first to advocate the use of acids to treat periodontally exposed root surfaces in an attempt to achieve reattachment. REGISTER (1973) revived interest in this treatment method. He studied the effects of demineralization (by chemicals) on reattachment. Both *in vitro* and *in vivo* tests were made with various chemicals; he concluded that citric acid was the most satisfactory chemical available. He claimed that citric acid

1) has no undesirable side effect, - caused no pulpal injury;
2) was biologically well tolerated by soft tissue;
3) shows significant accelerated healing.

REGISTER & BURDICK (1975, 1976) recommended 20% citric acid @ pH 1 as a suitable therapeutic concentration. REGISTER (1978) also demonstrated impressive results clinically. He reported 3 cases of endo-perio lesions with advanced bone loss. After endodontic treatment and topical application of citric acid on root surfaces he achieved considerable soft tissue reattachment and some bony regeneration.

With the aid of the electron microscope GARRETT et al. (1978) studied the effect of citric acid on diseased root surfaces. Using SEM, they found that acid application had no effect on unplaned specimens. However, on the root planed surfaces, the acid produced a fibre-like surface with frequent depressions. TEM observations showed that the acid application produced a 4 micron wide demineralized zone, which was characterized by exposed collagen fibrils. These fibrils seemed to be continuous between mineralized and demineralized zones of the root. They speculated the acid causes exposure of collagen fibrils in the dentine matrix, thus providing a suitable nidus for splicing with new fibrils during the healing process.

Furcal lesions were experimentally induced in a group of labrador dogs by (CRIGGER et al. (1978). Using citric acid to supplement their surgical procedures, they achieved closure of furcation (in mesio-distal sections) in 21 out of 23 cases. In the same experimental model, NILVÉUS et al. (1980) found that repeating the surgical

procedure did not improve the reattachment status of these furca. Statistical analysis ruled out the effects of other adjunctive therapeutic measures in this experimental model, e.g., systemic antibiotics, gelfoam (as a matrix support for the blood coagulum) and coronal repositioning of the mucoperiosteal flaps. The success of reattachment in this experimental model was attributed to the topical application of citric acid. (NILVEUS & EGELBERG 1980).

RIRIE et al. (1980) used 7 labrador retrievers, surgically exposed labial aspects of roots of maxillary incisors. After root planing and citric acid application, the wounds were closed in the conventional way. They found that compared to conventional flap surgery in the same animals, application of citric acid to the instrumented root surface resulted in an improved rate of connective tissue healing as well as a rapid and consistent re-establishment of connective tissue attachment through extensive interdigitation of new and old collagen fibrils at the tooth-gingiva interface. Subsequent reinforcement of the established connective tissue attachment included recalcification of the acid-affected dentine and deposition of new cementum.

SELVIG et al. (1981) further supported Ririe's finding by repeating a similar experiment using 3 beagles with experimentally induced furcation lesions. They showed that attachment of soft connective tissue to a root planed and acid-conditioned dentine surface can be

achieved by the same mechanism irrespective of whether the root surface has been surgically denuded or has been exposed to the environment of an experimental periodontal pocket. Similar results were found in naturally occurring through-and-through furcation defects in the aged beagle dog. (BOGLE et al. 1981).

Attempts to evaluate the relative effect of flap placement and the size of the furcation defect were made by KLINGE et al. (1981). They found that adequate post-operative flap coverage of the furcations is crucial for successful healing of the furcation defects.

NYMAN, LINDHE & KARRING (1981) used adult monkeys to study the effect of root demineralization with citric acid on reattachment. Three different treatment groups were used.

1) surgical removal of buccal bone + root planing;
2) surgical flap operation + root planing on experimentally induced periodontitis;
3) same as 2) + topical citric acid.

They found little difference in the characteristics of healing following surgery regardless of the treatment modality. Cementogenesis and collagen fibres attachment were not observable features. Consistently, they also observed gingival tissue reattached via long junctional epithelium in all three situations.

1.2.2 Human studies

In human studies, contrasting results were reported. STAHL & FROUM (1977) failed to demonstrate any cementogenesis or fibrous reattachment at sites previously exposed to periodontal disease. It is possible that failure to remove diseased cementum completely by instrumentation is responsible for their finding.

On the other hand, COLE et al. (1980) using citric acid reported that healing in all specimens was characterized by connective tissue regeneration, deposition of new cementum, and more coronally, by tightly apposed soft connective tissue. The junctional epithelium ended 1.2-2.6mm coronal to the apical border of the notch in the various specimens. There was also evidence of bone regeneration but no evidence of ankylosis or root resorption. Subsequent study by STEINER et al. (1981) using conventional (non-acid conditioning) replaced flap procedure, failed to demonstrate any soft connective tissue adhering to the tooth or evidence of new cementum coronal to the notch (a landmark of the bottom of the periodontal pocket). A thin junctional epithelium had proliferated to the level of, or beyond the notch. In spite of all reservations about possible differences between the studies, the clear-cut discrepancy in results suggests that citric acid conditioning of the root surfaces did facilitate new attachment and that corresponding results may not be possible without acid treatment.

Other clinical human studies were reported by LIU & SOLT (1980) and SHILOAH (1980). Liu used citric acid conditioning as an adjunct to surgical treatment of localized gingival recession. Shiloah, on the other hand, used topical citric acid during laterally positioned pedicle grafting. Both reported more predictable successful results.

Controlled clinical studies by COLE et al. (1981) showed that there was only a 2.1mm gain in probing attachment level for pooled acid treated surfaces, as compared to 1.5mm for the non-acid treated control surfaces. This result suggested that the use of citric acid application might only provide a small improvement in probing attachment levels.

1.2.3 Other related studies

Other aspects of effects of citric acid were also investigated. FINE (1980) compared the efficiency of different solutions to elute potentially toxic materials from root surfaces. He found that trichloro-acetic and citric acid removed more calcium and more toxic material from the root surface and subsurface than did either water or phenol-water.

BOYKO (1980) studied cell adherence to the root surface. Using cultured fibroblast-like cells derived from periodontal ligament, he found significantly more cells attached to demineralized roots than to non-demineralized roots. The method of demineralization did

not influence the result. The results suggested that one of the ways in which citric acid demineralization of root surfaces could contribute to the reported enhancement of healing of periodontal defects was by improving the attractiveness of the root surface as a substrate to which cells can adhere.

TVEIT & SELVIG (1981) studied the in vivo remineralization of root surfaces after citric acid treatment. They found the citric acid had produced a completely demineralized surface zone in the root dentine and the subjacent hard tissue showed a zone of partially reduced mineral content. Following exposure to the oral cavity, the demineralized surface layer appeared unchanged in width. The subjacent zone, however, had been reduced in width in all instances when compared with control specimens from the same teeth. They concluded that the clinical implication is that the completely demineralized surface layer of root surfaces which remain exposed following periodontal surgery with citric acid application, should be considered condemned. It must be pointed out that during this study, demineralization was achieved using 2.6M citric acid for 10 minutes under constant agitation. Such procedures are unlikely to be used clinically.

1.3 Pulpal Reactions to Periodontal Therapy

Current dental therapy is directed toward the maintenance of the viability of the dental pulp. All new forms of dental therapy need to be evaluated for their

direct or indirect effects on the pulp, before new methods can be accepted for routine use.

When a tooth is exposed to periodontal disease for a period of time, it often results in loss of attachment and pocket formation. Conventional periodontal therapy consists of instrumentation of the periodontal pocket, and more importantly, planing of the diseased root surfaces to achieve reattachment. It is necessary to consider what effects citric acid have beyond that of conventional periodontal therapy.

1.3.1 Effects of periodontal disease on pulpal tissue

It is generally agreed that pulpal disease could initiate or perpetuate (or both) periodontal disease (Czarnecki et al., 1979; Seltzer, 1971). The presumed routes of transmission of pathologic changes are:

1. apical canal
2. accessory canals
3. permeability of chamber floor or root surfaces (Sinai, 1973)

However, there seems to be some dispute as to whether the reverse situation does exist (i.e., periodontal condition inducing pulpal pathology), and if so, to what extent.

Lang and McConnell (1920) attributed intrapulpal calcification as a result of tooth exposure to periodontal disease.

Craney (1925) made some attempts to grade the various degrees of periodontal destruction in the specimens of his

study. He postulated a cause-effect relationship between periodontal disease and pulpal pathology although he observed some normal pulps as well.

Brammer (1927) reported atrophic changes in pulps of teeth with periodontal disease. Cahn (1927) observed accessory canals communicating between the periodontal ligament and the pulp, and hence concluded disease in one region will lead to disease of the other.

Seltzer et al. (1963) proposed the concept of "retrograde pulpitis". They observed that periodontal lesions produce a degenerative effect on the dental pulps of the involved teeth. They blamed interference with the nutritional supply of the pulp as the cause of such change (e.g. atrophy, cell death, calcification etc.); whereas bacteria and their toxic products are thought to be responsible for pulpal inflammation and necrosis.

Rubeck and Mitchell (1965) claimed that "pulpitis and or pulpal necrosis can and do occur as a result of periodontal inflammation involving an accessory or apical canal." They also concurred with Simring and Golberg's (1964) theory of "retrograde Periodontitis".

Bender and Seltzer (1972) introduced the term pulpodontic-periodontic syndrome. They attributed this pulpal periodontal relationship to the high incidence of accessory canals.

Sinai and Soltanoff (1973) induced periodontal lesions in 75 white rats and studied the pulpal effects

of periodontal diseases in these animals. They claimed that a relationship exists between diseases of these two tissues even though this only happened in 8 out of 44 specimens. However, they pointed out the pulpal response was never inflammatory, but rather reparative, resulting in deposition of secondary dentine, or resorptive, followed by a reparative response. Their experimental model drew some criticisms. The periodontal lesions in rats were induced within a period of 8 weeks. It is doubtful if data from the experiment can be extrapolated to human conditions.

Langeland et al. (1974) found bacteria present in the pulp as well as on root surface of periodontally involved teeth. They concluded: "The cumulative effect of periodontal disease, as indicated by the factors of calcifications, apposition of calcified tissue, resorption, or inflammation from root caries or from involved lateral canals, will be damaged pulp tissue, but total disintegration is a certainty only when all main apical foramina are involved by bacterial plaque." Since their samples were collected by conventional extraction technique, it is doubtful if their observations and conclusions were valid.

However, opposing views were held by other workers. Fish and MacLean (1936) had conclusively shown that bacteria inside the dental pulp of periodontally involved teeth is not a regular feature. Previous studies by Henrici (1921), Collins (1919) and Colyer (1924) where

micro-organisms were reported to be inside the dental pulps, were probably due to the pumping action of luxation and extraction, and therefore unrelated to pulpal infection.

Sauerwein (1956) studied 104 teeth and reported no regressive change in 15% of the teeth, and changes in the remaining teeth were within normal limits.

Mazar and Massler (1964) in a two-part study involving 106 teeth plus 22 in paired controls, failed to establish any direct relationship between pulpal changes and periodontal disease status. This was in agreement with Sauerwein's results. Mazar and Massler found the pulps of the same patient showed similar appearance regardless of the amount of periodontal involvement. They also disputed age, *per se*, as the causative factor in pulpal changes observed.

Hattler *et al*. (1977) using rice rats as an experimental model, found no detectable change in the pulpal tissue of periodontally involved teeth, as compared to the pulps of normal teeth. Perhaps the scarcity of accessory canals in rice rats did influence the outcome of their study.

Czarnecki and Schilder (1979) studied 46 human teeth with carefully documented concurrent periodontal involvement. Controlled teeth were obtained from the same patients where possible. Again, they failed to identify periodontal disease as a causative agent in pulpal pathosis.

It is not surprising to find such conflicting results in the literature since the parameters of study were never defined or standardized. For example, the effect of restorative dental procedures, presence or absence of accessory canals, age and other systemic influences were never eliminated in these studies. The earlier investigations lacked controlled samples and therefore were unscientific in their approach. More recent studies using animal models again failed to duplicate or simulate human condition. Finally, difficulties in tissue fixation and processing and subsequent error in interpreting artifacts, all contribute to very doubtful results.

Hence, the question of pulpal reaction to periodontal disease remains unresolved. It is generally agreed that:

1. pulpal pathosis can cause and/or maintain periodontal inflammation,
2. the presence of accessory and apical canals provide communication channels whereby disease from one tissue can be transmitted to the other.

However, it is also the known fact that in the majority of cases, periodontal disease involves only the cervical third or even half of the root surfaces. In this region, accessory canal is not a common feature. If disease is to be transmitted from one tissue to the other as postulated, the question of transmissibility of disease across dentinal barrier needs to be investigated.

The permeability of dental hard tissues was studied by Bevelander and Amler (1945) using radioactive isotope P_{32}. All morphological varieties of dentine were found to be permeable to P_{32} and absorb it in a measurable quantity. Other radioactive isotopes were used by Bartelstone (1954) to study the permeability of enamel, dentine and cementum. More recently, Pashley et al. (1981) compared permeation of ^{131}I through dog's dentine in both in vitro and in vivo conditions, and found the rate of permeation to be very similar.

BERGENHOLTZ & LINDHE (1975) applied soluble factors from human plaque to cavities prepared in monkey's teeth for 8 continuous hours (every 5 minutes). They found inflammatory response in the pulpal tissue characterized by vascular exudation and infiltration of neutrophils and monocytes. In the same experimental model, BERGENHOLTZ (1977) used cultured filtrates and disintegrated cells of plaque bacteria (human) and applied them to the dentinal cavities. Again, inflammatory reponse was induced in the dental pulps. Hence, it was established that factors of periodontal disease (e.g., plaque, bacterial products etc.) can induce pulpal inflammation across dentinal barrier. Furthermore, BERGENHOLTZ et al. (1977) also demonstrated that when challenged with Bovine Serum Albumin (BSA) by placing it on freshly exposed dentine, immunized monkeys will develop severe pulpal inflammation. This suggests antigenic materials can be transmitted across dentinal tubules and set up an antigen-antibody reaction with the formation of immune-complexes.

Despite the above findings, BERGENHOLTZ & LINDHE (1978) reported (in monkeys) that periodontal destruction and plaque accumulation on exposed root dentine did not cause severe alteration in the pulp of roots involved.

From the present evidence, it seems some pulpal response is elicited by exposure of root surface to periodontal disease, although the extent of such reaction as related to severity and duration of periodontal disease, is still not clear. Furthermore, whether such pulpal reactions and changes are reversible or irreversible remain to be determined.

1.3.2 The effects of periodontal therapy on pulpal tissues

Routine scaling and root planing of periodontally diseased teeth is believed to cause some pulpal reaction. During these procedures cementum and dentine may be removed. It is claimed the effects are similar to cavity preparation procedures in restorative dentistry. (Seltzer, 1971).

Stahl (1963) studied the pulpal response to gingival injury by surgically stripping gingivae from molars of 69 rats. The presence of irregular dentine was used as a criterion of pulpal response. He found 21 out of 69 showed irregular dentine in their pulps, some of them as early as 5 days following gingival injury. He postulated that irregular dentine formation at these pulpal sites was associated with irritation of odontoblastic processes which occurred after gingival wounding.

Brazda & Jiricka (1978) repeated and extended Stahl's experiment, using 73 molars from 49 Wistar-Dejvice rats. In one group of rats, gingival mucosa mesial to left first upper molar was removed. The animals were then sacrificed at different time intervals. In another group of rats, the surgical trauma was repeated 4 and 6 days after initial surgery. The animals were then sacrificed at different time intervals. They found that:

1. Pathological changes in the periodontium provoke a response in the dental pulp.

2. The intensity of the pulpal reaction depends on the degree and frequency of traumatization of the periodontium.

3. Pulpal response ranges from acute inflammation to irregular dentine formation. They wrote: "We found that the pulp responded to a single traumatization of the gingivodental attachment by hyperaemia, which was joined relatively soon by oedema of the pulpal fibrillar connective tissue and by moderate infiltration. An increase in the intensity and frequency of traumatization was accompanied by an increase in the extent of these changes in the pulp, together with haemorrhage from the hyperaemic blood vessels and necrotic changes in the odontoblast layer. In the zone where the ondotoblasts were destroyed, strips of tissue characterized by pronounced fuchsinophilia, a PAS-positive reaction and PTAH staining were very soon formed. We later observed in these places the formation of new odontoblasts, multiplication and concentration of the collagen fibres, followed by hyalinization and osteoid transformation, and lastly the formation of irregularly organized reparative dentine."

It is evident that periodontal injury and root preparation procedures can induce various pulpal reactions, although it is not possible to quantify such reactions in relation to the "insult" received by the tooth. Also, it is not known if these reactions are reversible. Seltzer (1971) speculated that in deep periodontal instrumentation, blood vessels through accessory canals and in the furcation regions of molars could be severed, causing infarction to portions of the pulpal tissue, and possibly to eventual partial or total pulpal necrosis.

1.3.3 Effect of root preparation with topical citric acid on pulpal tissue

Register revived the interest of using acid to periodontally diseased root surfaces with the aim to achieve better reattachment. In his early reports (1973) he claimed there was no detectable changes noted in the pulps of the animal teeth he treated with 0.6N hydrochloric acid for 15 minutes.

Subsequent studies (1975) using citric acid up to 0.5 pH for 5 minutes, also revealed lack of marked pulpal response (i.e., appearance of secondary dentine by 6 weeks). However, there was no controlled experimentation to specifically examine the pulpal response as a result of root surface conditioning with citric acid.

Ririe et al. (1980) reported only 2 minor pulpal changes in their series of dog experimentation, and suggested any pulp effect will only be of minor consequence. However, since no controlled study had been carried out, the question remains unanswered.

Other histological study had been carried out on pulpal reaction when citric acid was used as a cavity cleanser following cavity preparation in Restorative Dentistry. Lee et al. (1971, 1973) examined the pulpal response after using a 50% aqueous solution of citric acid pH 1.5* on freshly cut dentine. Time periods examined were 2,4,6 and 8 weeks. They found no gross or irreversible pulpal irritation to the citric acid solution.

This was later confirmed by Cotton and Siegel (1978) using human volunteers. They found that citric acid cavity cleanser initially caused a significantly higher incidence and intensity of deep inflammatory response, which decreased with time. However, they did not notice any irreversible pulpal reaction after application of citric acid cavity cleanser.

Other studies on acid etchants by Gotto and Jordan (1973), Retief et al. (1974), Stanley et al. (1975) and Eriksen & Leidal (1979), using mainly 50% phosphoric acid or citric acid, generally agreed that pulpal responses of various degrees were elicited, although the severity and reversibility had not been established. Most of these studies also looked at other variables such as microleakage, irritation due to composite filling materials and mechanical trauma from cavity preparation. It is

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*Epoxylite 9060 Cavity Cleanser (Lee Pharmaceuticals) contains 45.6 wt% anhydrous citric acid $C_6H_8O_7$ at pH 0.8-1.

important to note that 50% aqueous solution of citric acid is a much more concentrated solution than the 20% citric acid at pH 1 used in periodontal therapy. Caution should be exercised for any direct comparison.

Other usage of citric acid solution was proposed by Loel (1975) in endodontics. Epoxylite 9060 was suggested as a root canal debriding agent. It was also found that direct pulpal exposure to citric acid cleanser for 45 minutes achieved very good tissue destruction of the pulp. Once again such mode of application is unlikely to be encountered in periodontal therapy.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Five young beagle dogs, one male and four females, were used in this series of experiments. The average age and weight of the dogs was 18 months and 11 kilograms respectively. These beagle dogs came from the IMVS field station, where a colony of beagles are continuously bred for experimental purposes. Beagles are docile and have excellent handling characteristics for laboratory conditions. They are also of constant weight and were free of disease.

The periodontal surgery used in this series of experiments was conservative and produced no stress to the recovered dog if the usual test of appetite was applied. All in all, they make excellent experimental animals for periodontal research.

The dogs exhibited a moderate amount of calculus, especially on the buccal aspects of premolars and molars of both jaws. Mild gingivitis was present adjacent to the calculus deposits but no periodontal pockets exceeding 3mm were detected on probing.

Citric Acid, $C(OH)(COOH)(CH_2 \cdot COOH)_2 \cdot H_2O$ (analytical grade, manufactured by B.D.H. Chemicals Aust. Pty. Ltd.) was used to prepare 20% aqueous solution at room temper-

ature. The solution was then buffered with hydrochloric acid to pH 1. A fresh solution was prepared for each session. Sterile normal saline was used in the control sites.

2.2 Methods

2.2.1 Outline of methods and experimental design

Surgical procedures (2.2.2) were performed on all the dogs. Opposing jaws were treated similarly to act as controls. At various time intervals, the dogs were sacrificed, specimens were retrieved by block dissection.

2.2.1.1 : Schedule

Dog No	Experimental Period
1	8 weeks
2	4 weeks
3	2 weeks
4	1 week
5	1 day

2.2.1.2 : Site allocation

Mandibular third and fourth premolars were treated with citric acid, the maxillary second and third premolars were used as controls and treated with saline.

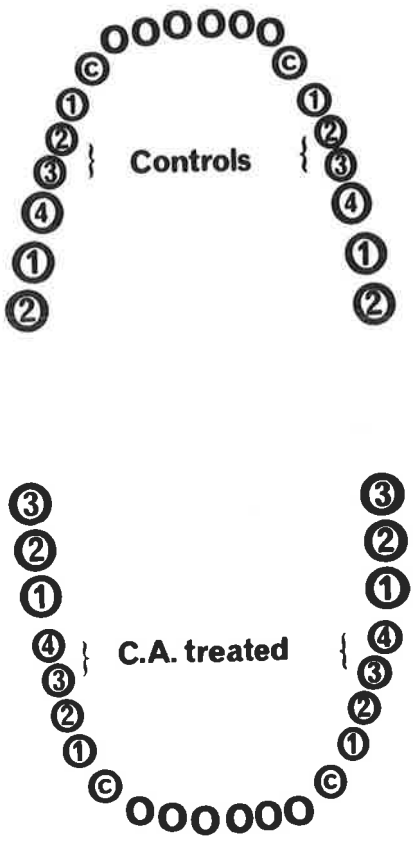


Fig. 2.1: Teeth used in this experimental study.

2.2.2 Induction Procedure

2.2.2.1 : Anaesthetics and Preparation[8]

Premedication

Each animal was premedicated with Innovar-Vet 2ml subcutaneously 1 hour prior to operation.

General Anaesthesia

General anaesthesia was achieved with 2ml of intravenous Nembutal.

Haemostatis

The sulcus related to the premolar teeth was infiltrated with lignocaine 2% (Adrenalin 1:80,000).

Antibiotic Cover

Streptopen 4ml I.M. was given routinely.

Swabbing

The oral cavity and face of the dog were routinely swabbed with sterile aqueous solution (containing chlorhexidine 0.05% and Cetramide 0.5%).

2.2.2.2 : Surgical Procedure

Incision and Flap Design

The incision was made along the gingival sulci extending from the distal of the canine to the mesial of the first molar. No relief incision was used and only a buccal flap was involved.

2.2.2.3 : Bone removal

Buccal bone was removed over both roots and the cemental surface was notched at the most apical extent of the exposed surface, approximately 7 mm from the original crestal height.

2.2.2.4 : Root planing

Only the mesial root was root planed thoroughly (i.e. only on the buccal aspect). The mesial root was thoroughly planed cervical to the notch, the distal root was not touched.

2.2.2.5 : Citric acid application

After mechanical preparation of the root surfaces topical citric acid was applied for 2 minutes. The area was then thoroughly irrigated with saline.

2.2.2.6 : Suturing

After re-adapting the surgical flap, cat-gut sutures were used to close the wound.

2.2.2.7 : Control sites

The procedures were then repeated on the control site - using saline in place of citric acid.

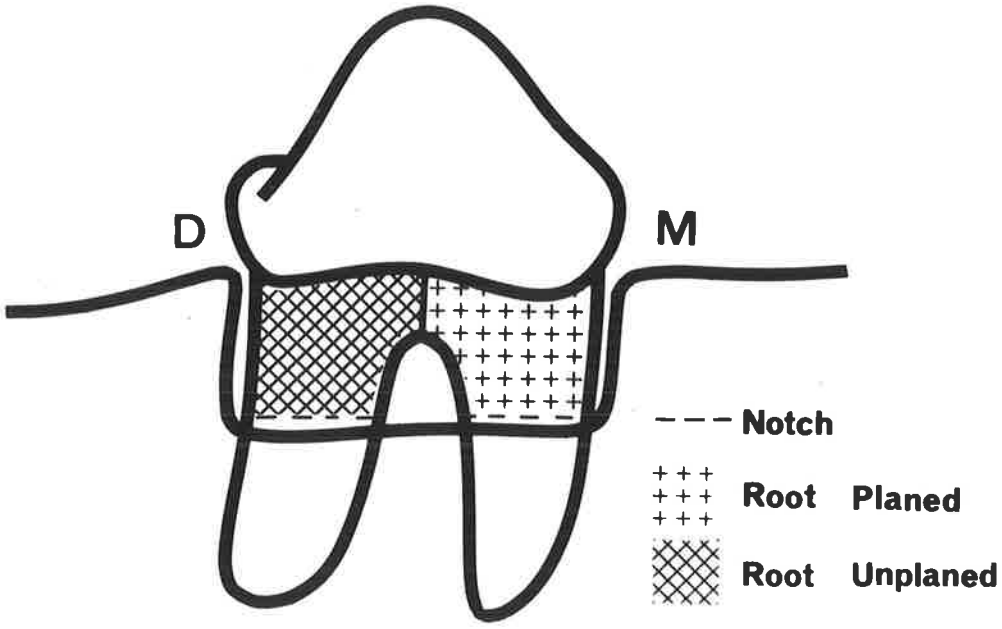


Fig. 2.2: Diagrammatic representation of a Beagle's mandibular premolar showing exposed root surface and indicating position of notch.

2.2.3 Retrieval operation

2.2.3.1 : Profusion

At the appropriate time period, the dogs were sacrificed using a profusing technique (Appendix I).

2.2.3.2 : Biopsy procedure

Specimens were obtained by block dissection (Appendix II).

2.2.4 Histological preparation

Routine histological techniques were used in preparing and processing the specimens for light microscopy (Appendix III).

Bucco-lingual serial sections were cut at 6 (Appendix IV).

Haematoxylin and eosin were used as routine stains, while Polychromes and Van Gieson's stains were used to differentiate between old and new collagen, and to demonstrate collagen fibres (Appendix V).

2.2.5 Microscopic evaluation

Initially, one representative block from each time period, was serially sectioned and stained with H&E (Appendix IV & V). It was found that the histological picture was consistent within each specimen from the mesial to the distal. The mid-root sections were examined for both periodontal and pulpal results.

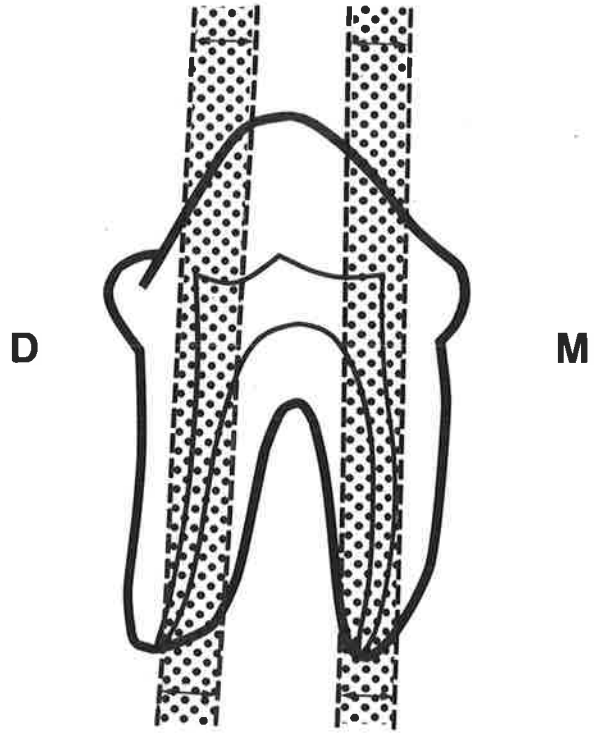


Fig. 2.3: Diagrammatic representation of buccal view of dog's premolar. The shaded area represents the region where serial sections were taken and evaluated.

The criteria for evaluating pulpal response were based on the F.D.I. recommendation (1978). In the classification of dental materials for biological evaluation according to F.D.I. recommendations, topical citric acid usage is classified under Type IV Class 2a (periodontal materials, etchants). The suggested tests include:

1. Acute Systemic Toxicity Test; oral route
2. Ame's Test (mutagenicity test)
3. Styles' Test (cell transformation test)
4. Dominant lethal Test
5. Cytotoxicity Test
6. Sensitization

but excludes any pulpal evaluation. Since citric acid is a normal body metabolite, some of the above tests may be redundant.

Similarity exists between the topical application of 20% citric acid on root planed surfaces and that of acid etching freshly cut dentinal surfaces after cavity preparation. It is necessary to determine whether topical application of citric acid to root surfaces will evoke a pulpal reaction.

The criteria used for pulpal evaluation in this study are:

1. Inflammation - based on the degree or density of round cell infiltration
   - 0 None
   - 1 Mild
   - 2 Moderate
   - 3 Severe
2. Degenerative changes - based on changes in odontoblasts including loss of cellular details and vacuoles formation
3. Calcification - based on demonstratable calcified materials in the pulpal tissues e.g., secondary dentine, pulp stones and dentinal bridges.

The criteria for assessing gingival healing and reattachment are:

1. Inflammation - based on the degree or density of round cell infiltration
   - 0 None
   - 1 Mild
   - 2 Moderate
   - 3 Severe
2. Granulation tissue formation
3. Changes on root surface, including the presence of
   - resorption bays
   - secondary cementum
   - Sharpey's fibres

4. Connective tissue healing including collagen maturity, organization and orientation of collagen bundles

5. Epithelial healing - signs of cuticle formation and epithelial reattachment

6. Type of gingival reattachment

    a) long junctional epithelium

    b) connective tissue + normal junctional epithelium

CHAPTER III

RESULTS

Of the 40 teeth used in this experiment 15 experimental and 15 controls were double embedded in celloidin and paraffin wax (Appendix III). The remaining (5 experimental and 5 control) teeth were embedded in Araldite. The paraffin sections were cut at 6 microns using a rotary microtome. The Araldite sections were cut at the same thickness using a Jung's Sledge Microtome. This method of cutting was unsatisfactory. Consequently, 10 teeth were excluded from the study. Imperfection in pulp tissue fixation and accidents during cutting resulted in a further 6 half-teeth being excluded from histological evaluation (i.e., 3 were excluded from pulpal evaluation and 3 were excluded from reattachment evaluation).

The normal histology of the gingival attachment in the young Beagle is demonstrated in Figs 3.1 and 3.2. These photomicrographs were taken from an unoperated site.

Photomicrographs of dental pulps of unoperated teeth are shown in Figs 3.3 and 3.4. They showed a normal and intact odontoblastic layer, relatively accellular zone and fibrous central core, rich in blood supply.

Symbols used in photomicrographs

D = Dentine

N = Notch

C = Cementum

NC = New Cementum

F = Cemental Fragment

A.C. = Alveolar Crest

B = New Bone

E = Epithelium

J.E. = Junctional Epithelium

R.B. = Resorption Bay

C.C. = Clastic Cells

P.L. = Periodontal Ligament

H&E = Haematoxylin & Eosin Stains

P.P.C. = Picropolychrome Stain

U.G. = Van Gieson Stain

B.V. = Blood Vessels

Od = Odontoblast

G = Gingiva

P = Pulp

Col = Collagen

Y.Col = Young Collagen

Cal = Calculus

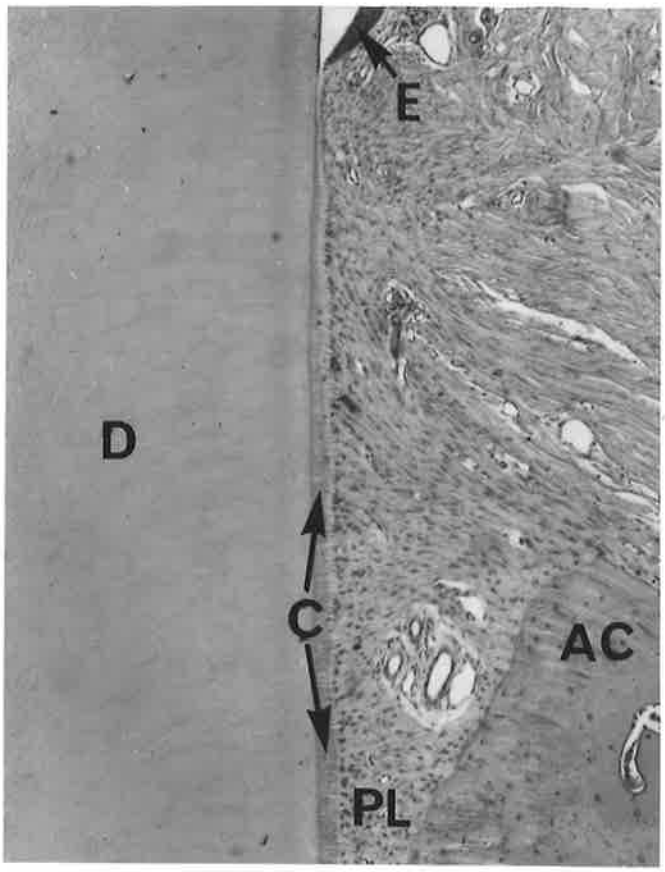


Fig. 3.1: Gingival attachment of unoperated premolar. H&E x 75.

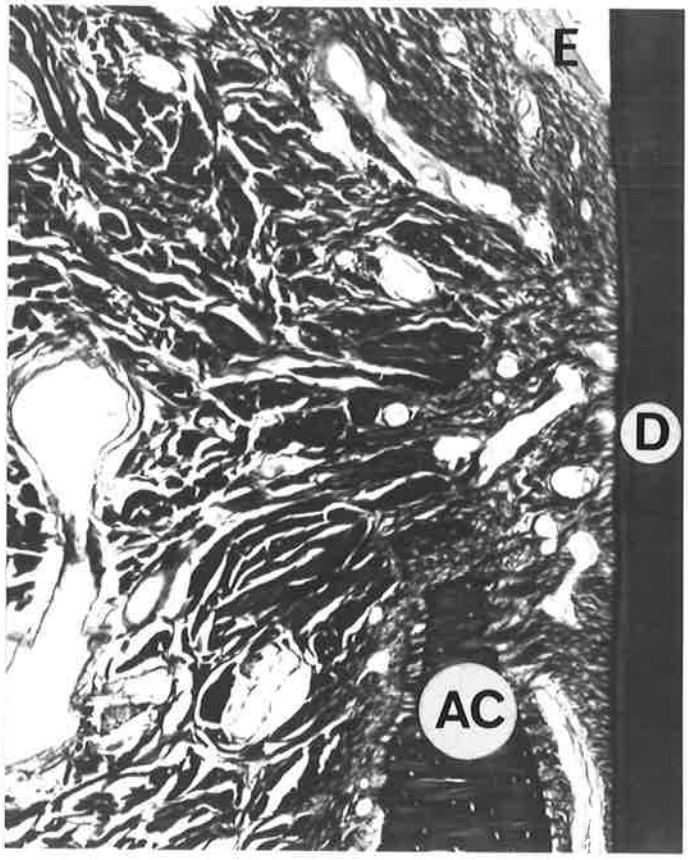


Fig. 3.2: Gingival attachment of unoperated premolar. P.P.C. x 100.

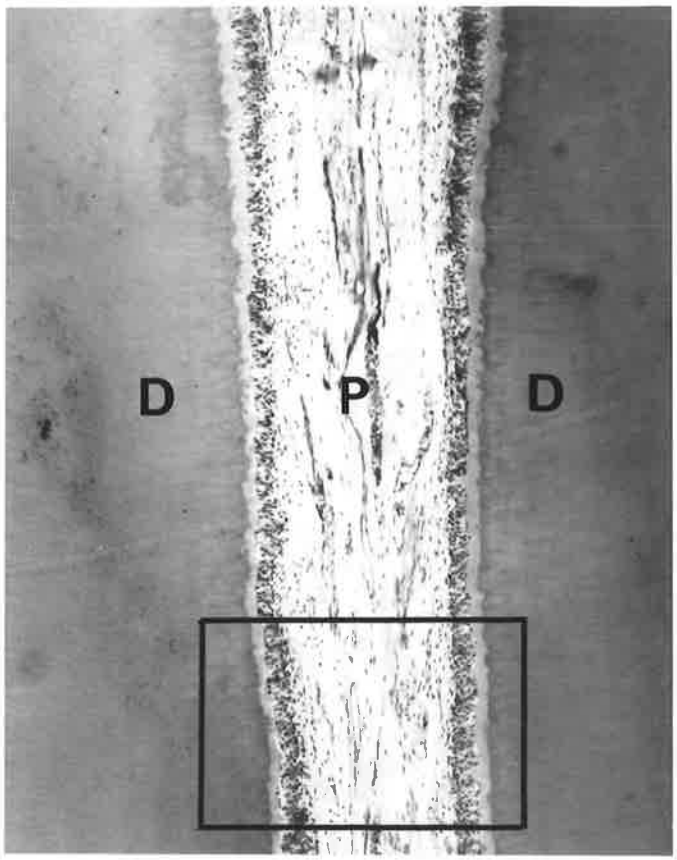


Fig. 3.3: Pulp of unoperated tooth. H&E x 62.5.

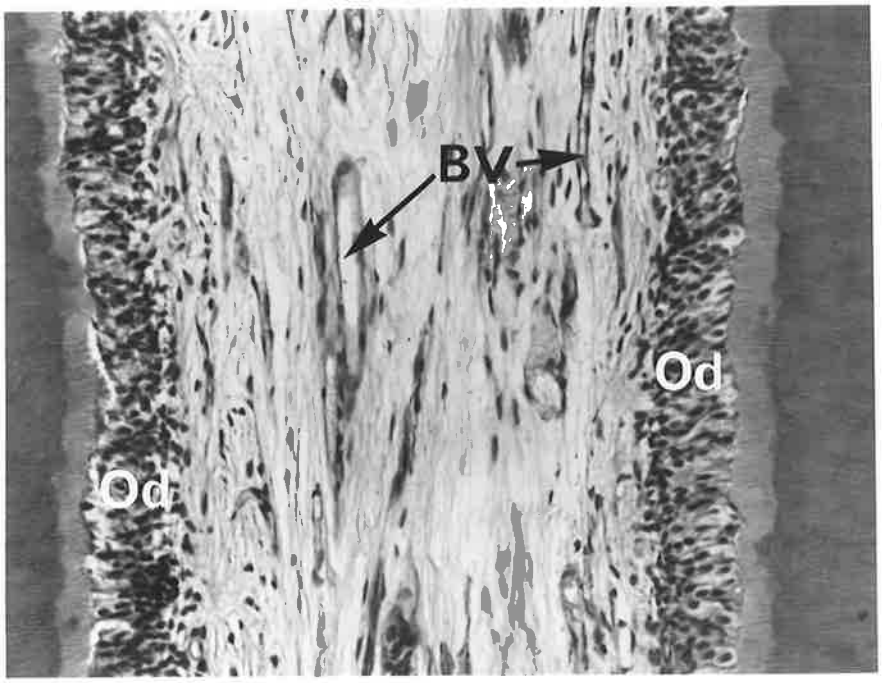


Fig. 3.4: High power photomicrograph of area indicated in Fig. 3.3. H&E x 200.

PART A: REATTACHMENT

ONE DAY

There was a general histopathological similarity among the various specimens of this time period. Citric acid treatment and root planing did not influence the outcome. All specimens showed a lack of soft tissue attachment to the tooth surface. Typically, the soft tissue had a well demarcated wound edge within which there were features of acute inflammation (Figs. 3.5 - 3.8).

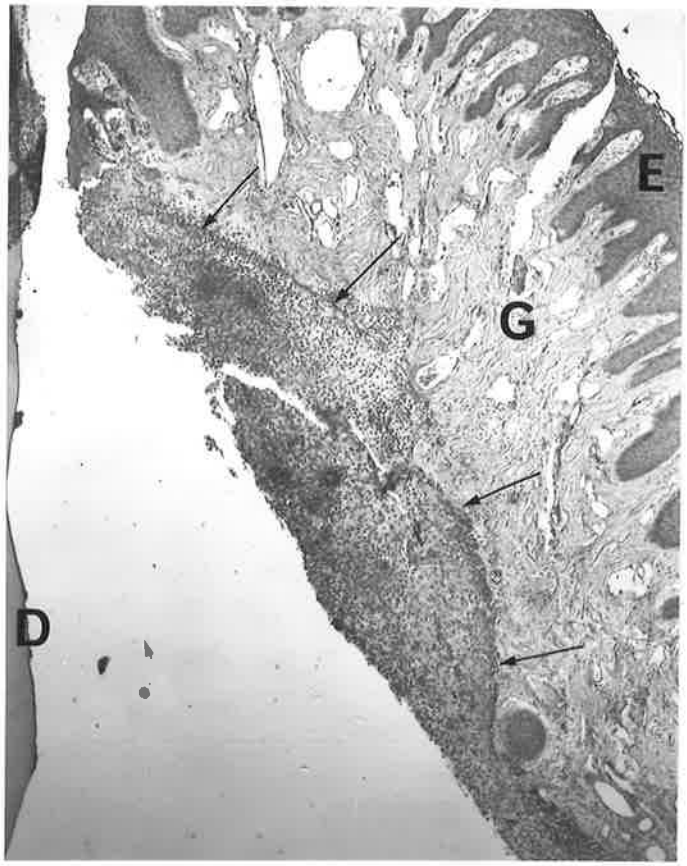


Fig. 3.5: 1 Day specimen, root planed and saline-treated. H&E x 50. Arrows indicate demarcation of inflammation at wound edge.

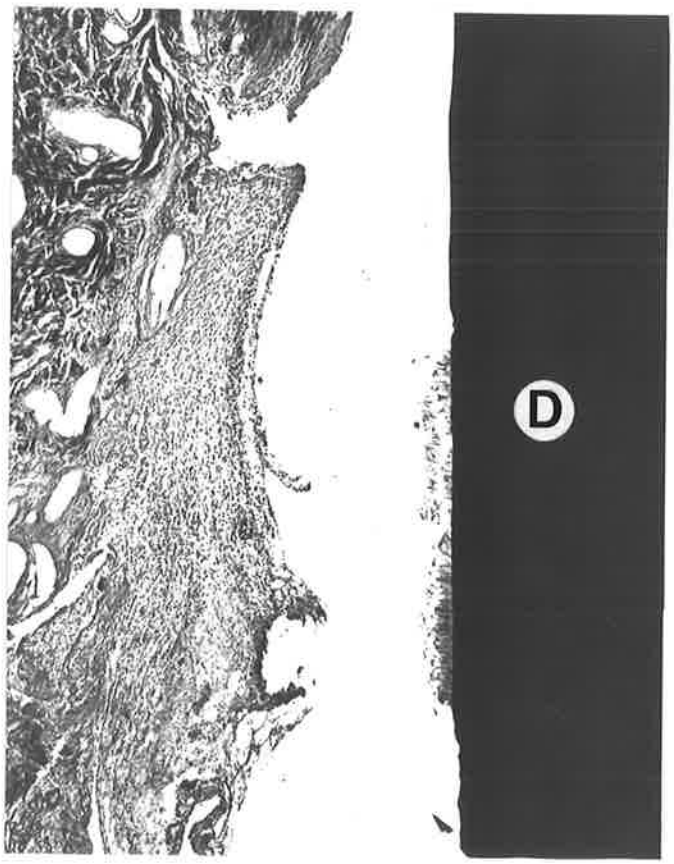


Fig. 3.6: 1 Day specimen, unplaned and saline-treated. P.P.C. x 50.

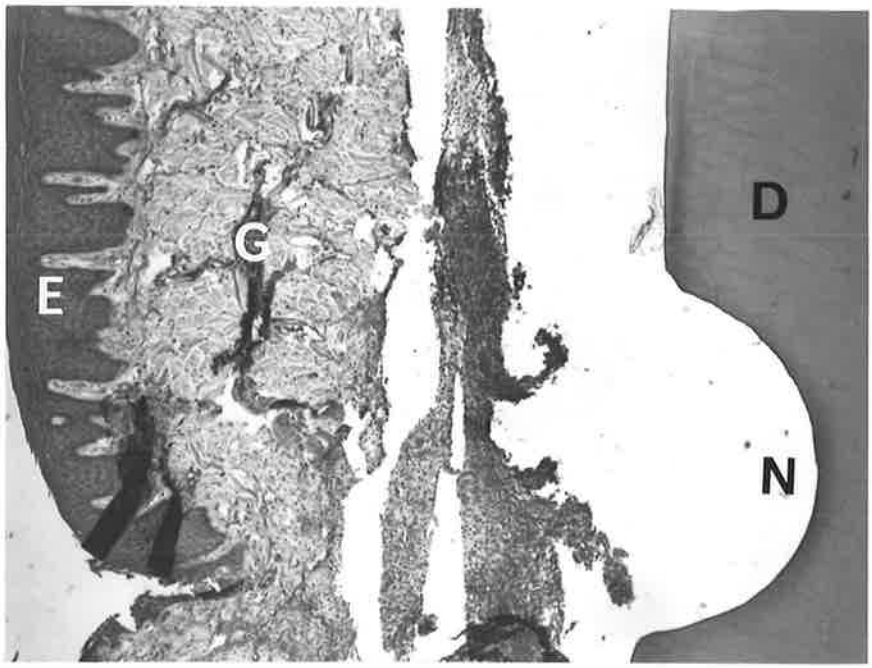


Fig. 3.7: 1 Day specimen, root planed and citric acid-treated. H&E x 50.

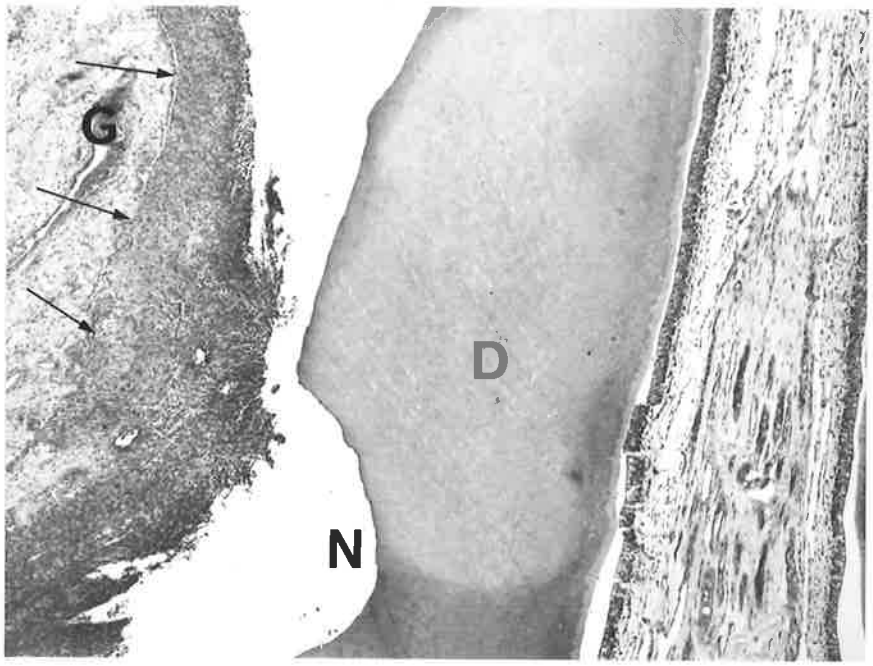


Fig. 3.8: 1 Day specimen, unplaned and citric acid-treated. H&E x 50. Arrows indicate demarcation of inflammation at wound edge.

ONE WEEK

Saline

At one week, the group showed a noticeable decrease in inflammatory cell infiltration compared with the one day specimens. The unplaned root surfaces demonstrated advanced reattachment of gingival tissue (Fig. 3.9). In the region subjacent to the junctional epithelium, where cementum was left intact, reattachment was at an advanced stage. However, more apical to this region adjacent to the notch where cementum had been lost, separation of soft tissue away from the root surfaces had occurred. This pattern was similar to the sites that were root planed.

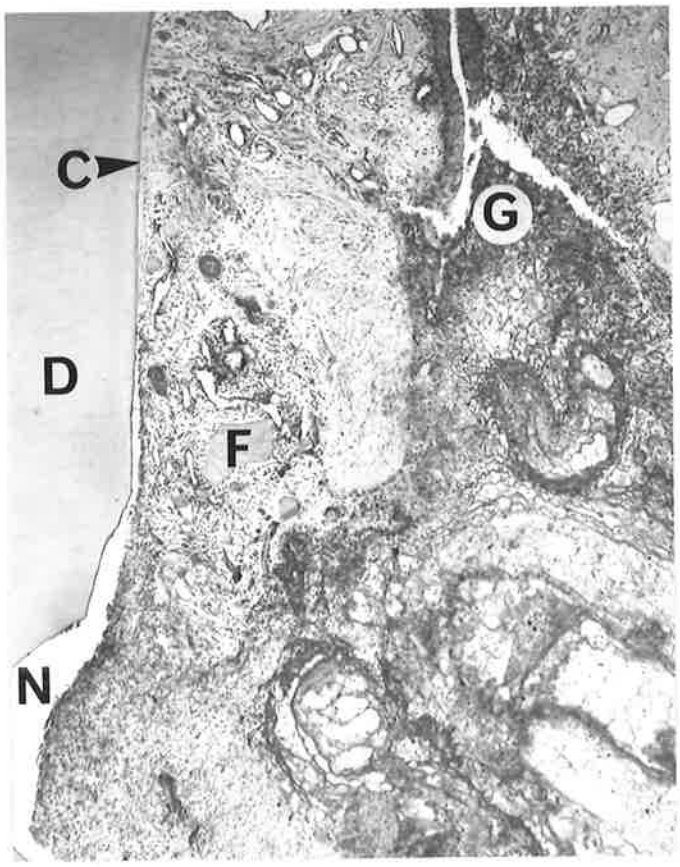
On the root planed surfaces, there was some separation of soft tissue from the root surfaces during tissue processing. Epithelial cells also migrated some distance along the root surfaces. It was considered that the potential for long junctional epithelium existed (Fig. 3.10).

Citric acid

The specimens showed a marked reduction of inflammatory cell infiltration, when compared with the one day specimens. This is similar to the one week saline controls.

There was close adaptation of soft tissue to the unplaned root surfaces. Some separation of soft tissue from root surfaces occurred near the gingival margin, possibly due to the processing, but there was no sign of apical migration of epithelial cells (Figs. 3.11, 3.12).

Fig. 3.9: 1 week specimen, unplaned and treated with saline. H&E x 50. Separation of soft tissue from dentinal surface became evident near and at the notch.



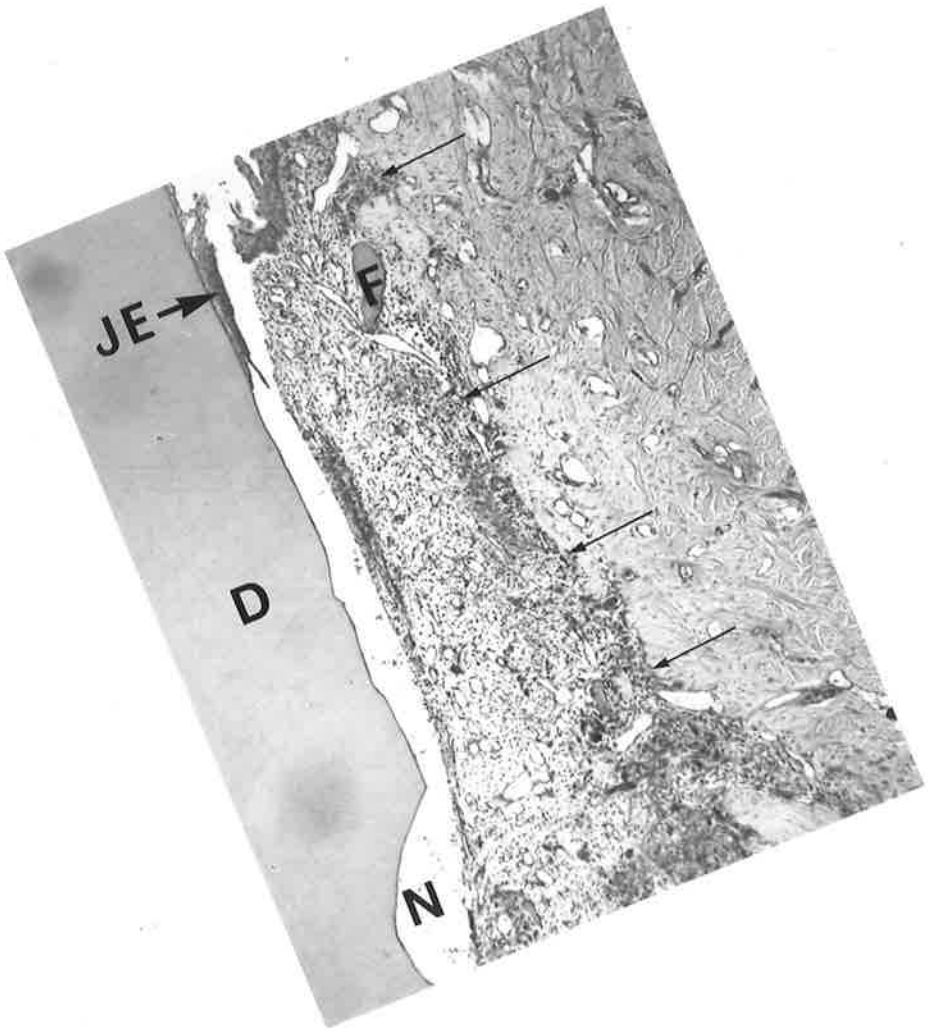


Fig. 3.10: 1 week specimen, root planed and saline-treated. H&E x 50. The separation of gingival tissue from the root surface is apparent. Although the junctional epithelium attached very well, the break in the epithelial layer is an artifact. The demarcation between inflamed wound edge and normal gingiva is indicated by arrows.

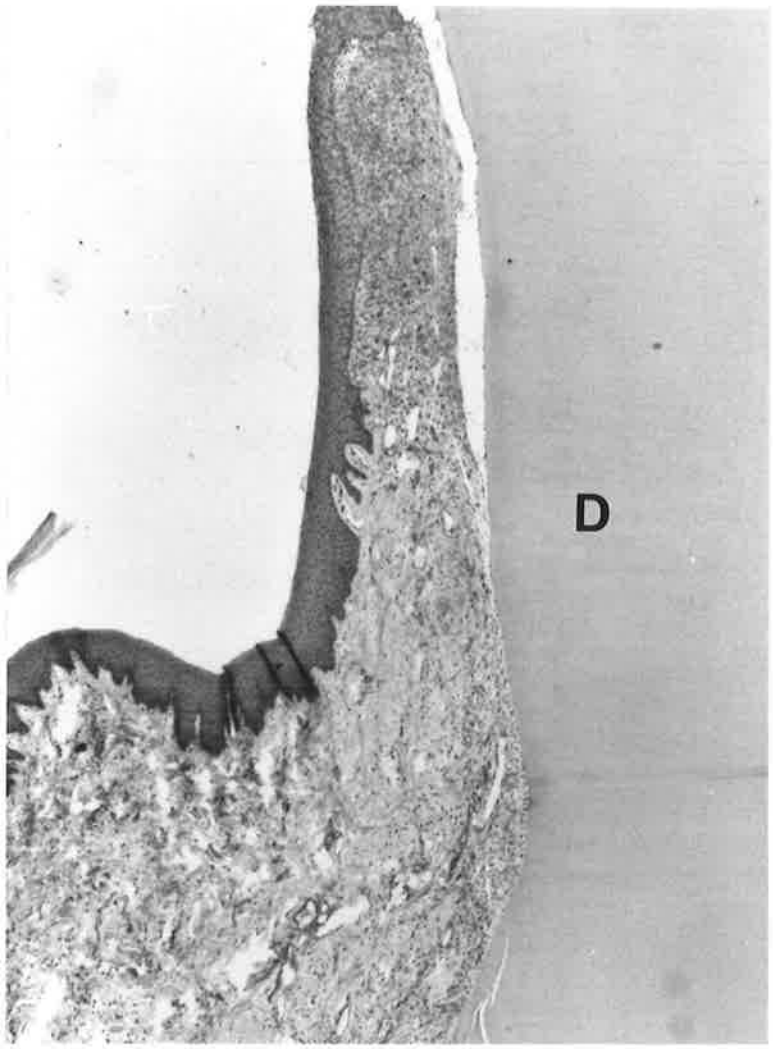


Fig. 3.11: 1 week specimen unplaned but citric acid-treated. H&E x 180.

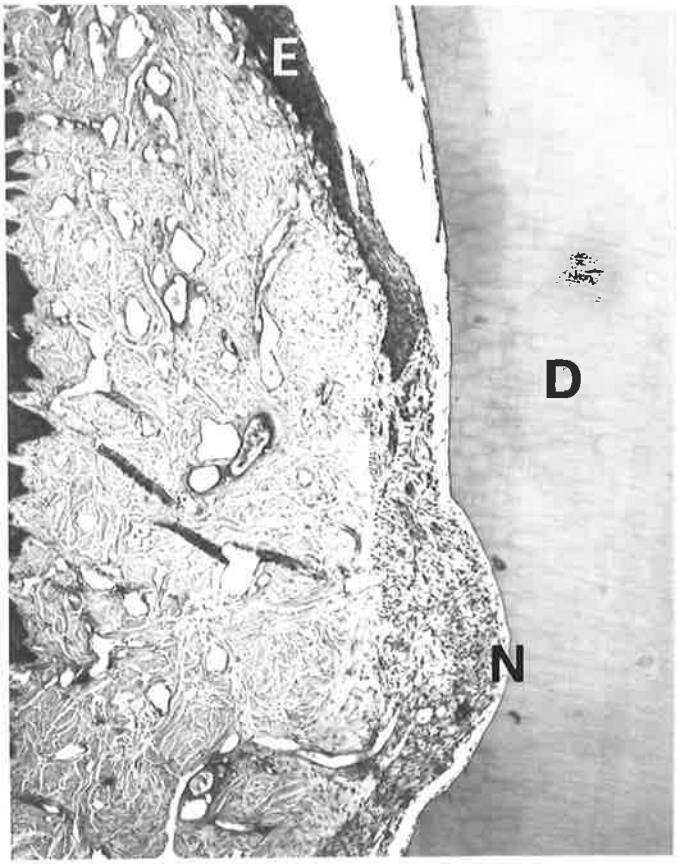


Fig. 3.12: 1 week specimen, root planed and citric acid-treated. H&E x 50.

TWO WEEKS

Saline

At two weeks, the group treated with saline alone showed varying degrees of inflammation. Overall, it was rated marginally more severe than the experimental group, but the difference was not significant. All the root surfaces in this group showed resorption bays associated with clastic cell activity. There was also evidence of bony regeneration with the alveolar crest extending beyond the apical border of the notch.

Of the unplaned root surfaces in this group, two out of three showed connective tissue reattachment, whereas the remaining specimen had long junctional epithelium extending to the coronal border of the notch. On the root planed surfaces - two out of three had long junctional epithelium, with the third specimen showing connective tissue reattachment (Figs. 3.13, 3.14).

Citric acid

In the citric acid treated group, there was a general reduction in inflammatory cell infiltration. The cell types were predominantly lymphocytes and plasma cells, together with some young fibroblasts. Occasional giant cells and macrophages were present, especially around spicules of bone-like substance. There was a general organization of the intercellular fibrous network. Collagen fibres were forming into bundles, although they were not properly orientated. Two of the three teeth

in this group showed evidence of clastic cells in resorption bays. The remaining tooth showed fairly advanced connective tissue reattachment without any evidence of root resorption.

All citric acid-treated root surfaces, both root planed and unplaned, had a connective tissue reattachment. The junctional epithelium appeared similar to that of a normal unoperated site, there was no evidence of epithelial downgrowth. Bony regeneration was also noted at the alveolar crest, extended beyond the apical border of the notch in some instances (Figs. 3.15, 3.16).

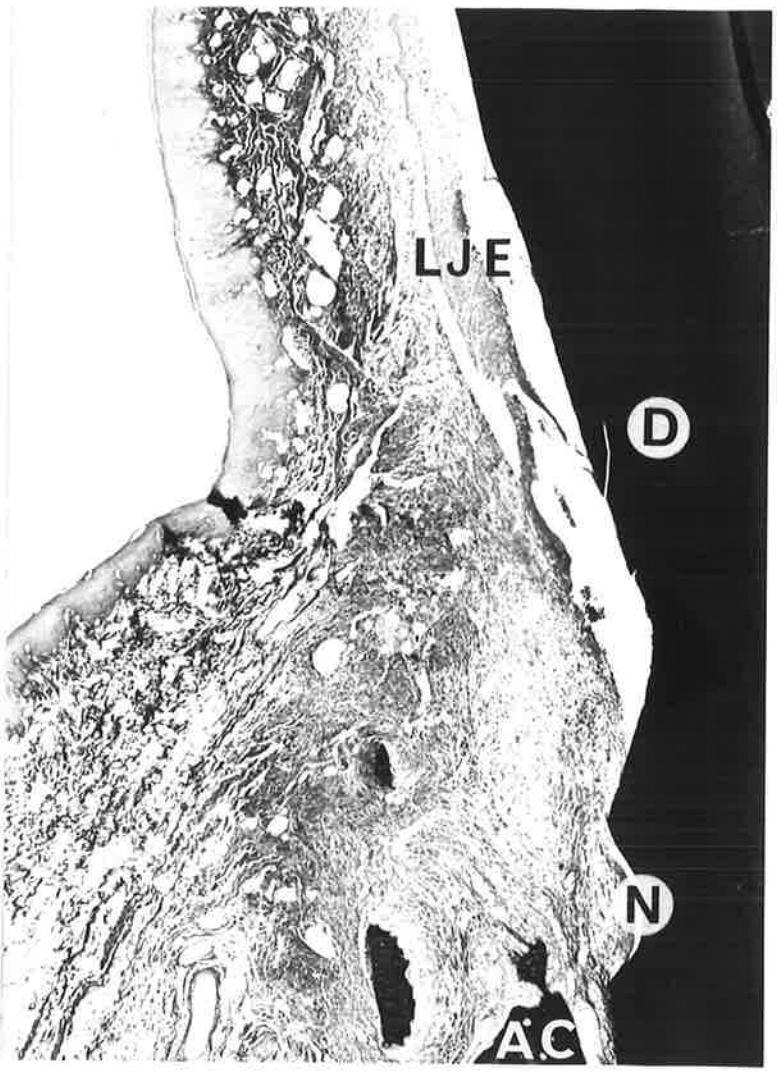


Fig. 3.13: 2 week specimen, saline-treated and unplaned. P.P.C. x 72.

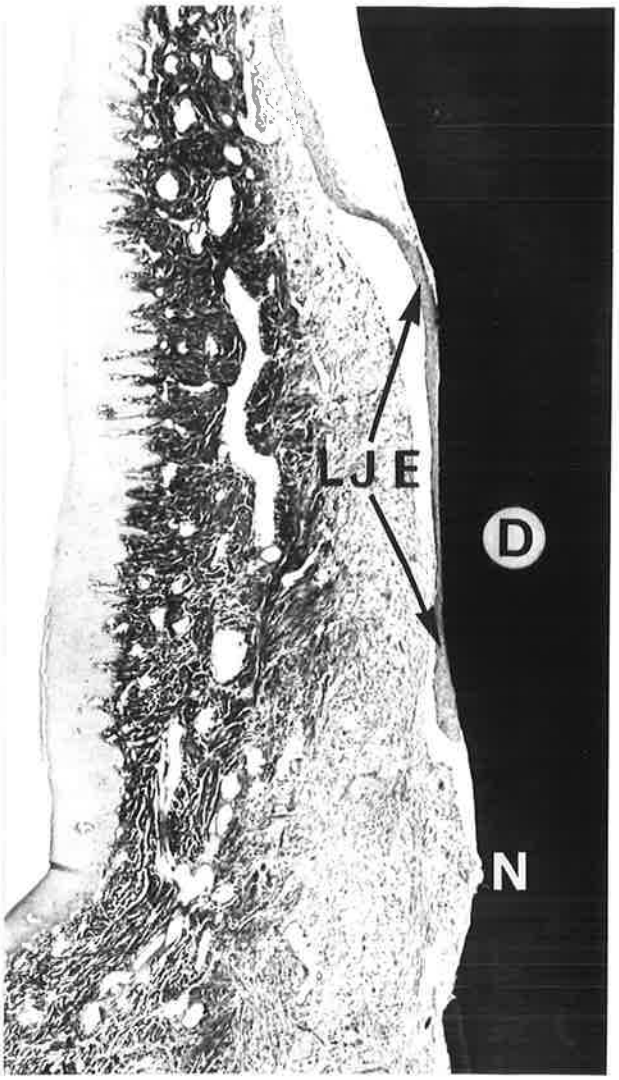


Fig. 3.14: 2 week specimen, saline-treated and root planed. P.P.C. stain x 80.

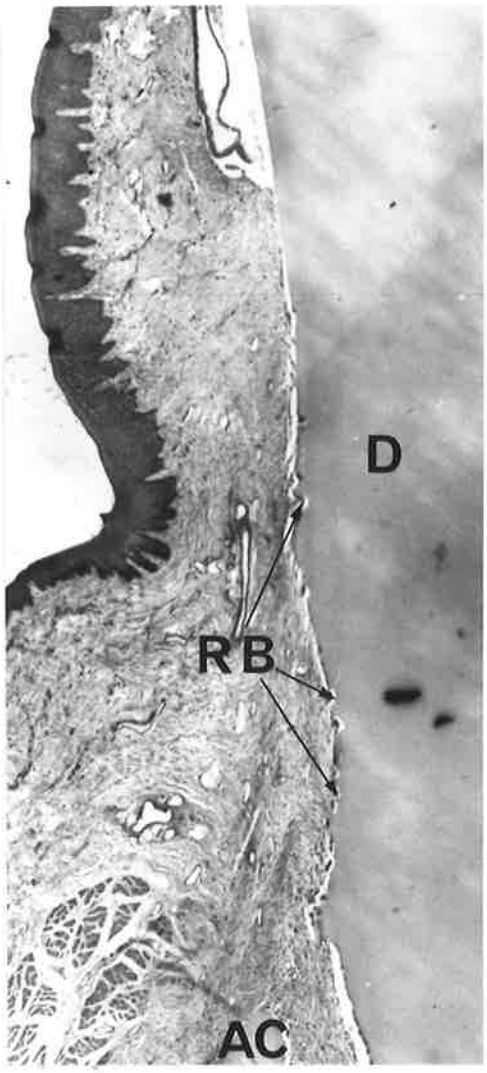


Fig. 3.15: 2 week specimen, unplaned and citric acid-treated. H&E x 80. Multiple resorption bays as marked R.B.

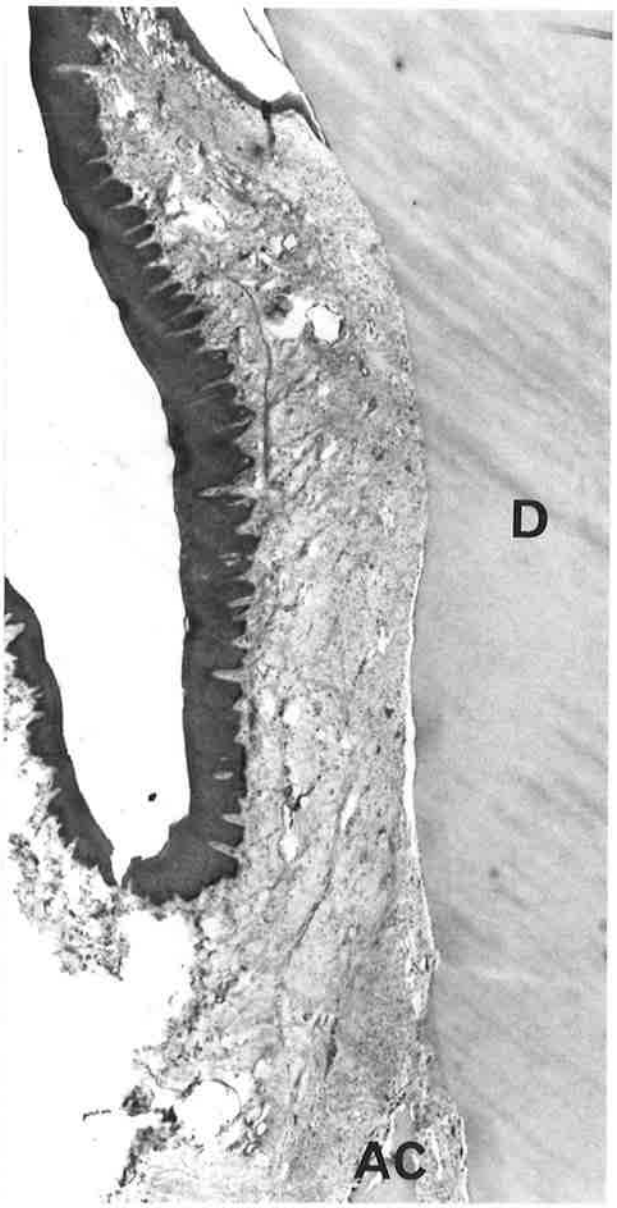


Fig. 3.16: 2 week specimen, root planed and citric acid-treated. H&E x 100.

FOUR WEEKS

Saline

In the control group, the healing of gingival tissue had reached an advanced stage with resolution of the inflammatory cells and orientation of the collagen tissue. Using the picropolychrome stains, it was possible to differentiate between mature and young collagen (Fig. 3.17).

Resorption bays on root surfaces were a consistent finding, but not all of these areas showed new cementum formation. On the root planed surface of one specimen, new cementum formation was associated with ankylosis (Fig. 3.18).

On the unplaned root surfaces, one out of three showed downgrowth of epithelium, whereas in the root planed surfaces, two out of three showed this phenomenon (Figs. 3.19, 3.20).

Citric acid

All the root surfaces examined showed resorption bays with new cementum lining these areas (Fig. 3.24). There was also new cementum formations of various thickness on all root planed surfaces. The gingival attachment was primarily fibrous in nature. There was a close adaption of collagen fibres against the root surfaces, running in a direction parallel to the root surface.

The unplaned root surfaces generally showed a more advanced fibrous organization. There were areas where Sharpey's fibres were present, stained red with picropolychrome stains, suggesting that they were old fibres from the unplaned cementum. These were seen to be intimately associated with younger collagen fibres which stained blue (Fig. 3.21).

In both the experimental and control groups, bony regeneration was seen in all specimens extended beyond the notch. However, no notable difference in the extent of bony regeneration was detected between the two groups.

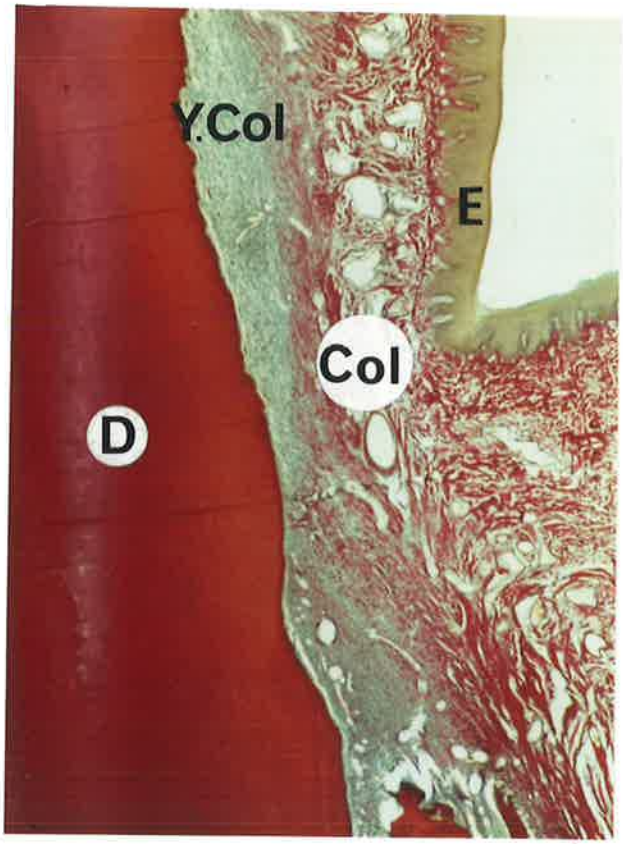


Fig. 3.17: 4 week specimen, citric acid-treated and root planed. P.P.C. x 120. Demonstrates differentiation between mature and young collagen. Mature collagen stains red, and young collagen stains blue.

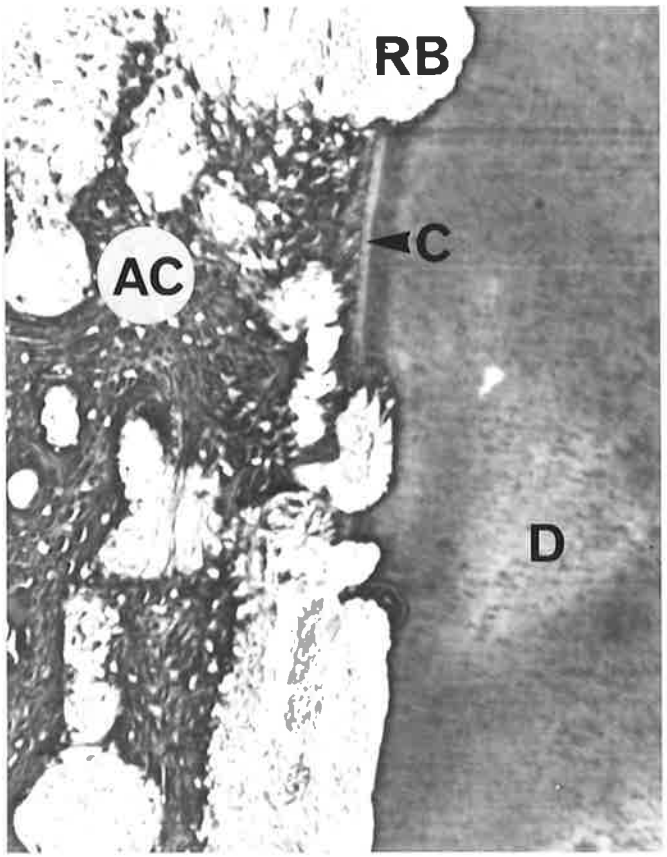


Fig. 3.18: 4 week specimen, showing ankylosis. Root planed and saline-treated. P.P.C. x 200. There was some residual cementum not removed by root planing. Ankylosis occurred at this site.

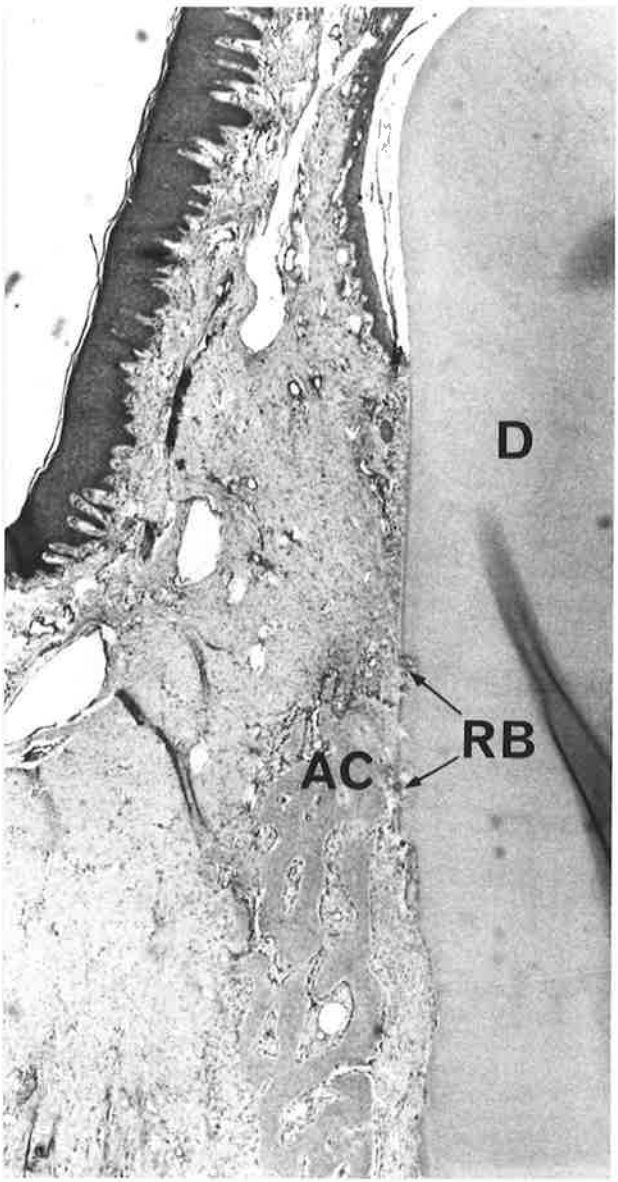


Fig. 3.19: 4 week specimen, unplaned and saline-treated only. H&E x 55.

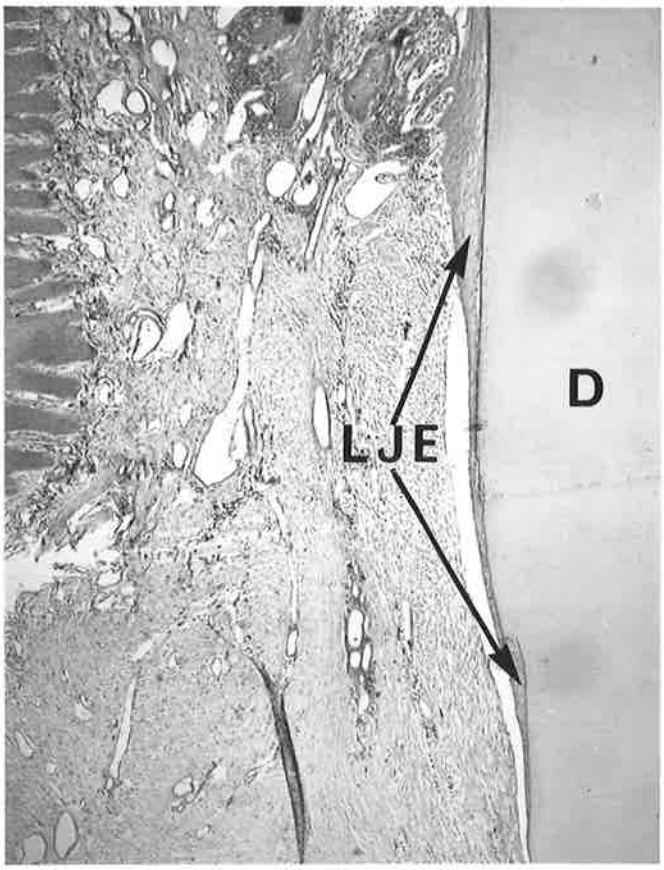


Fig. 3.20: 4 week specimen, root planed and saline only. H&E x 40.

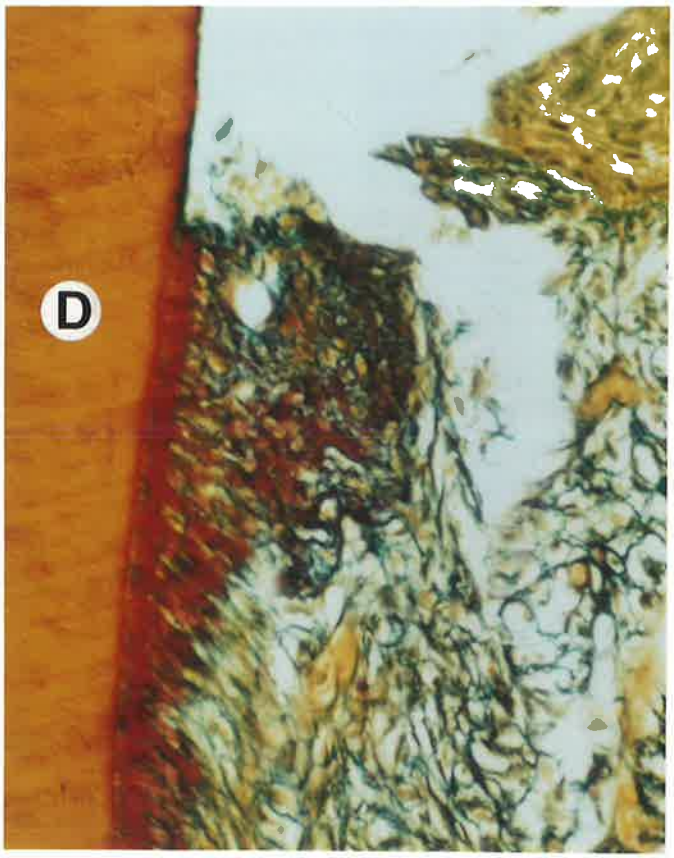


Fig. 3.21: 4 week unplaned specimen. P.P.C. x 250. Young collagen fibres interdigitate with mature collagen fibres (Sharpey's).

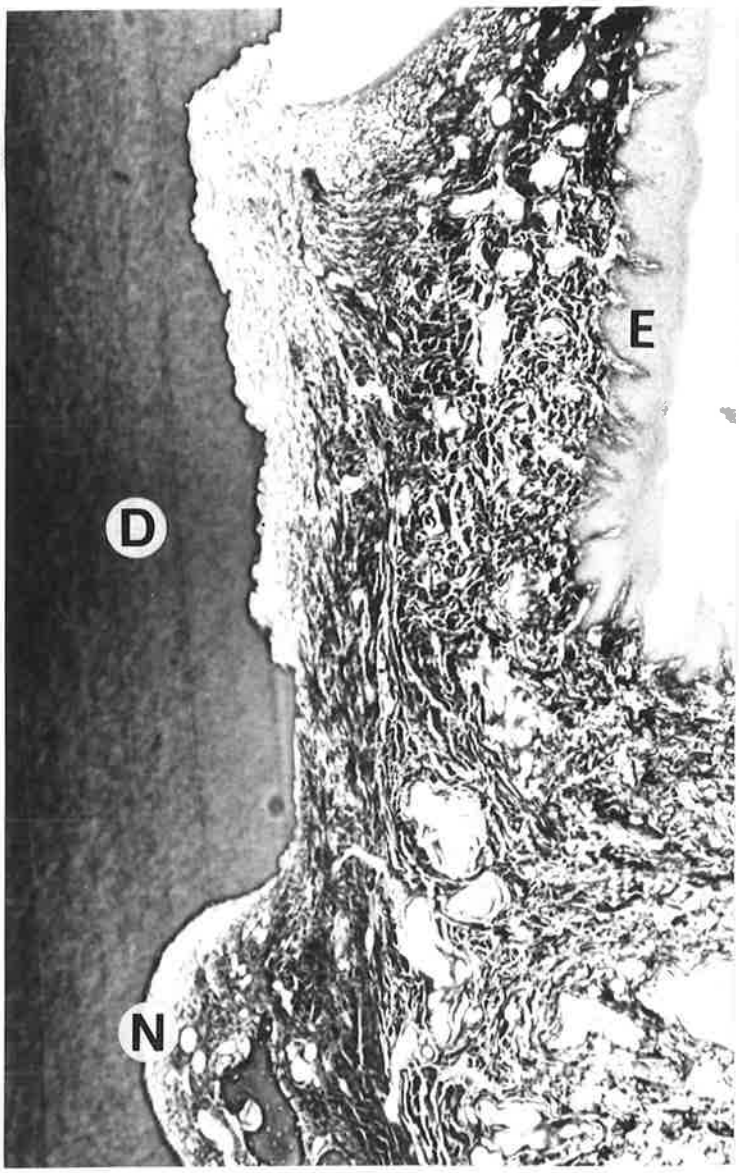


Fig. 3.22: 4 week specimen unplaned but citric acid-treated. P.P.C. x 100.

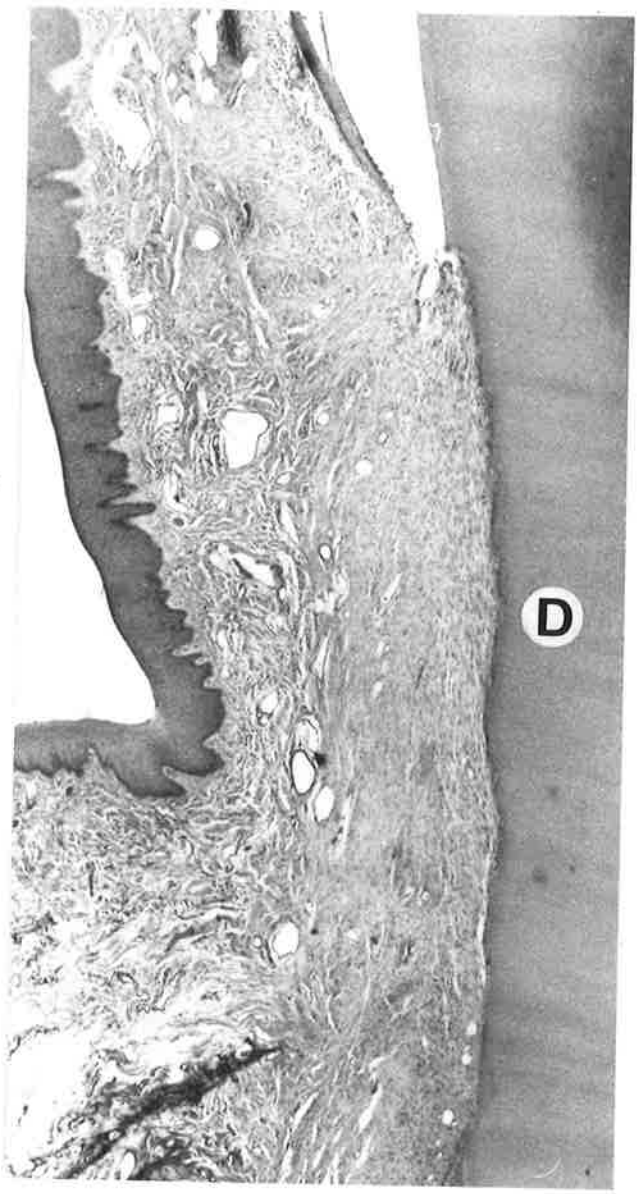


Fig. 3.23: 4 week specimen, root planed and citric acid-treated. H&E x 100.

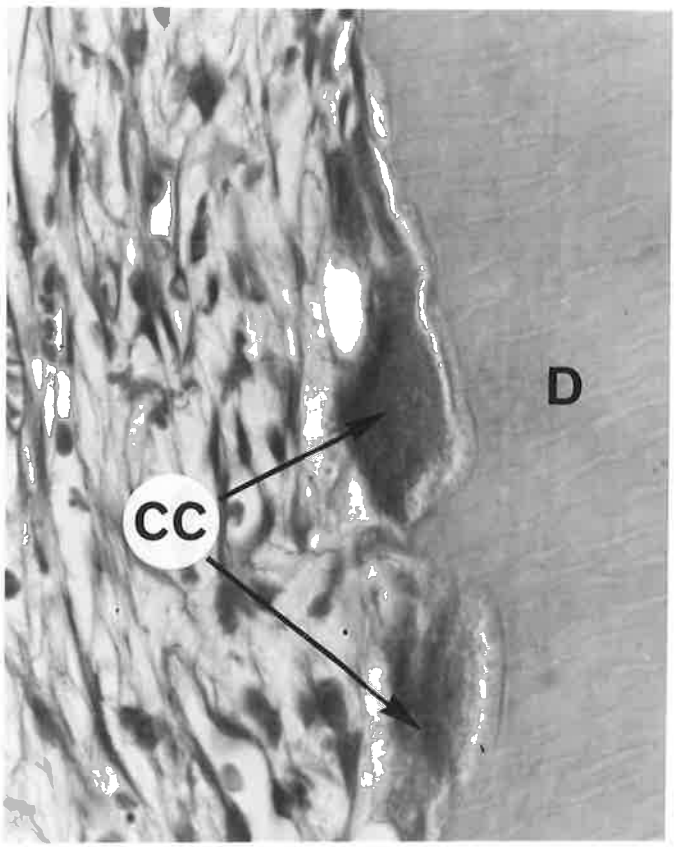


Fig. 3.24: 4 week specimen, unplaned and treated with citric acid. H&E x 575. Clastic cells situated in resorption bays.

EIGHT WEEKS

Saline

Gingival repair had reached an advanced stage, there was no sign of inflammation at the old wound site. Collagen fibres showed a high degree of organization, but a difference was demonstrated between the fibres of the healed tissue and those of the unoperated site.

In the control group, two specimens showed mild to moderate inflammation of the gingivae. This gingivitis is related to the accumulation of plaque and calculus (Fig. 3.26). Numerous resorption bays were present along root surfaces but new cementum formation was inconsistent. On the unplaned root surfaces, one specimen had connective tisue reattachment, while two had a long junctional epithelium. On the root planed controls, all specimens showed long junctional epithelium.

Citric acid

Soft tissue repair was similar to that of the control group, root resorption had occurred on both root planed and unplaned surfaces. Most of the resorption bays had new cementum depositions and all specimens in this group had connective tissue reattachment. Bony regeneration was common among the specimens of this time period, passing cervical to the notch in some instances. One case of ankylosis was noted in the citric acid-treated root surface (Fig. 3.29).

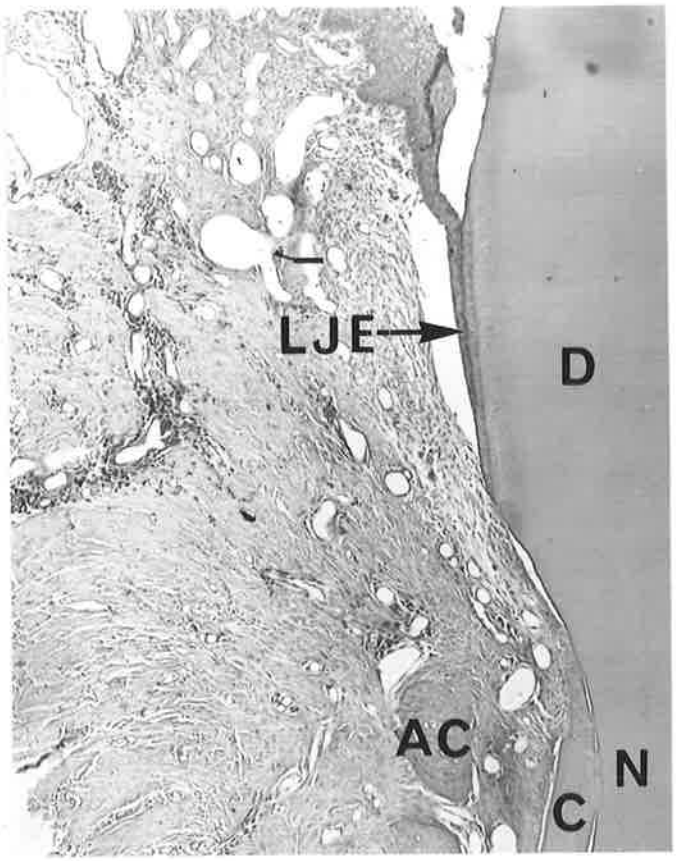


Fig. 3.25: 8 week specimen, unplaned and saline-treated). H&E x 50.

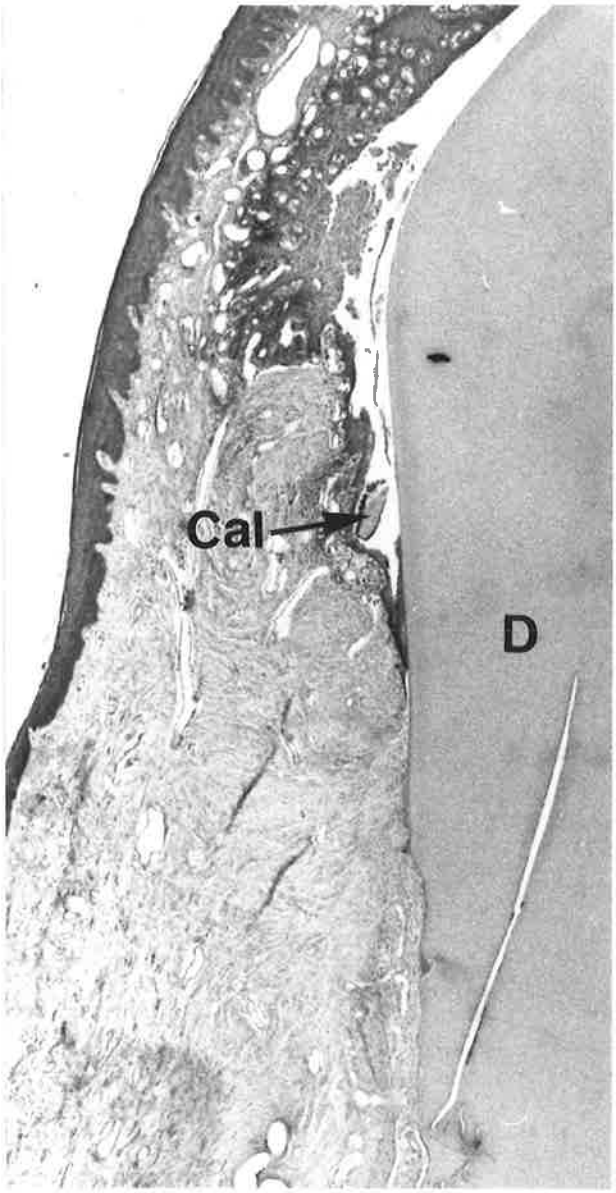


Fig. 3.26: 8 week specimen, root planed and saline-treated only. H&E x 50.

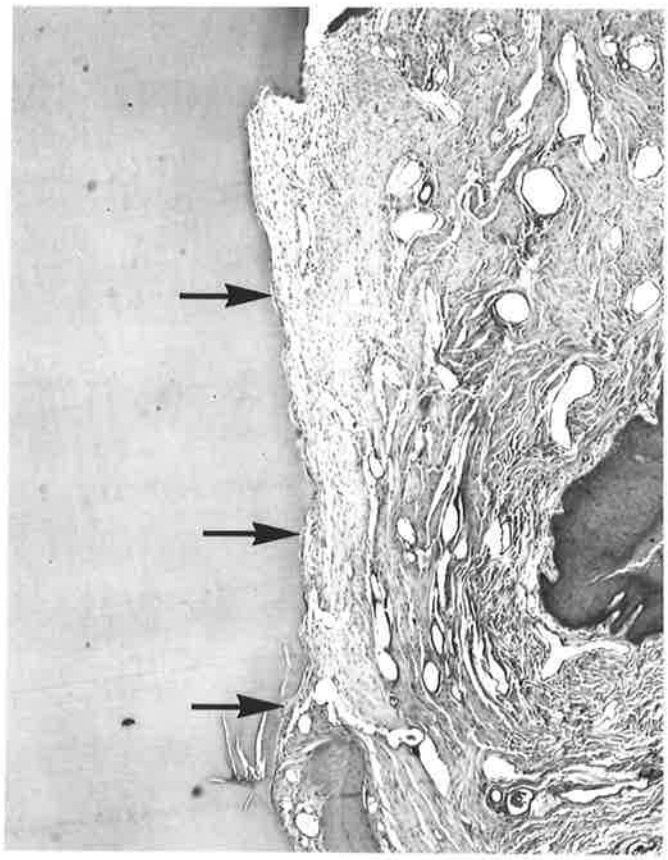


Fig. 3.27: 8 week specimen, unplaned and citric acid-treated. H&E x 60. New cementum deposition indicated by arrows.

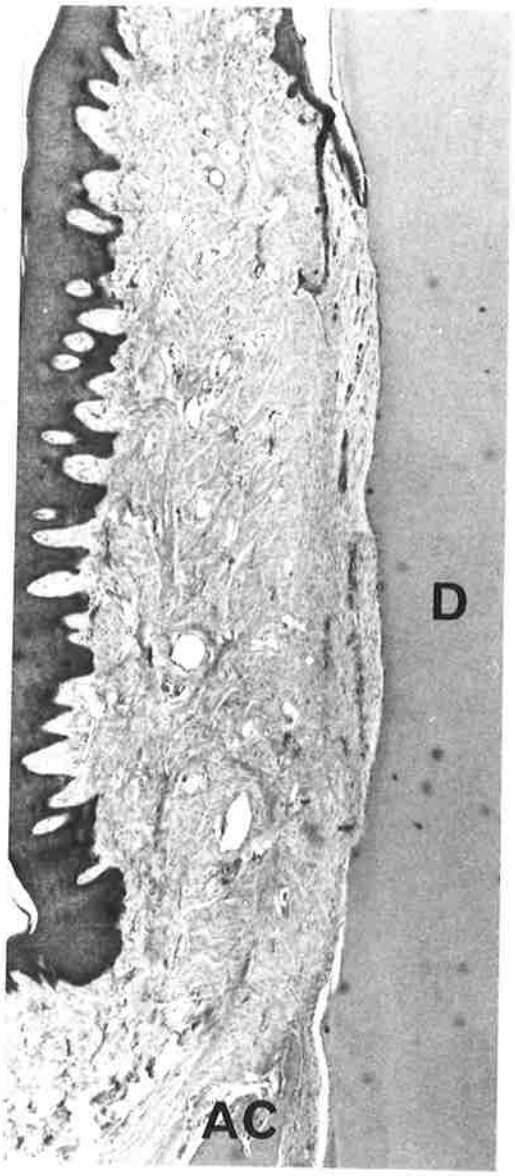


Fig. 3.28: 8 week specimen, root planed and citric acid-treated. H&E x 100.

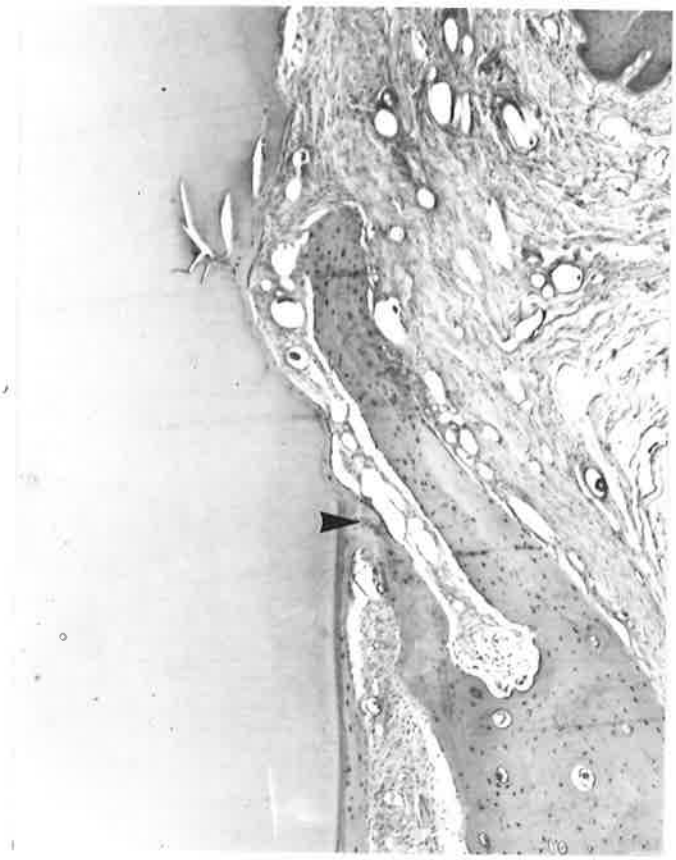


Fig. 3.29: 8 week specimen, citric acid-treated and unplaned, showing ankylosis at one site. H&E x 150.

TYPES OF GINGIVAL REATTACHMENT

Unplaned
(Distal Roots)

TIME PERIOD	TOOTH NO.	CITRIC ACID TREATED	TOOTH NO.	CONTROL
8 Weeks	1	C.T.	3	C.T.
	2	C.T.	4	L.J.E.
	32	C.T.	31	L.J.E.
4 Weeks	5	C.T.	7	C.T.
	6	-	8	C.T.
	34	C.T.	33	L.J.E.
2 Weeks	9	C.T.	11	C.T.
	10	-	12	C.T.
	36	C.T.	35	L.J.E.
1 Week	13	C.T.	15	-
	14	C.T.	16	C.T.
	38	C.T.	37	-
1 Day	17	-	19	-
	18	-	20	-
	40	-	39	-

L.J.E. : Long Junctional Epithelium
C.T. : Connective Tissue Type

TYPES OF GINGIVAL REATTACHMENT

Root Planed

(Mesial Roots)

TIME PERIOD	TOOTH NO.	CITRIC ACID TREATED	TOOTH NO.	CONTROL
8 Weeks	1	C.T.	3	L.J.E.
	2	C.T.	4	L.J.E.
	32	C.T.	31	L.J.E.
4 Weeks	5	C.T.	7	C.T.
	6	C.T.	8	L.J.E.
	34	C.T.	33	L.J.E.
2 Weeks	9	C.T.	11	C.T.
	10	C.T.	12	L.J.E.
	36	C.T.	35	L.J.E.
1 Week	13	C.T.	15	L.J.E.
	14	C.T.	16	-
	38	C.T.	37	-
1 Day	17	-	19	-
	18	-	20	-
	40	-	39	-

L.J.E. : Long Junctional Epithelium
C.T. : Connective Tissue Type

PART B: PULPAL RESPONSE

Of the 30 specimens used in this study, 3 specimens were excluded from histological evaluation due to gross tissue deterioration as a result of inadequate fixation and processing. Of the remaining 27 specimens assessed, all showed a consistent healthy and normal pulpal picture throughout the various time periods. Inflammation was not present either as a generalized event covering the entire pulp, or locally opposing the sites of surgery and notching. Larger blood vessels were regularly congested with erythrocytes.

Degenerative changes in odontoblasts were not seen in any of the specimens. All sections showed intact odontoblast layers with all cellular integrity preserved.

Intra-pulpal calcification in the form of pulpal stones or secondary dentine was not seen in any of the specimens. Some specimens showed abundance of fibrous tissue centrally, running longitudinally along the main axis of the teeth. This feature was considered as a variation of the normal pulpal picture (Figs. 3.44, 3.45).

Within each time period, the pulpal pictures remained the same for both experimental and control teeth. Furthermore, serial sections revealed lack of variation or change from one location of a pulp to the other locations of the same pulp. In other words, root planing did not induce any noticeable pulpal changes.

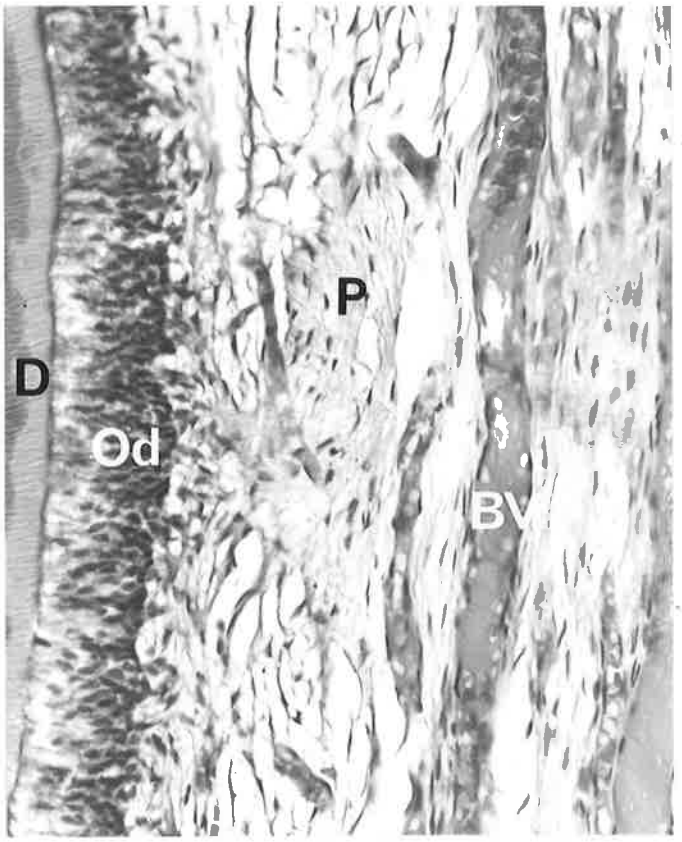


Fig. 3.30: 1 day specimen, citric acid-treated and unplaned. H&E x 250.

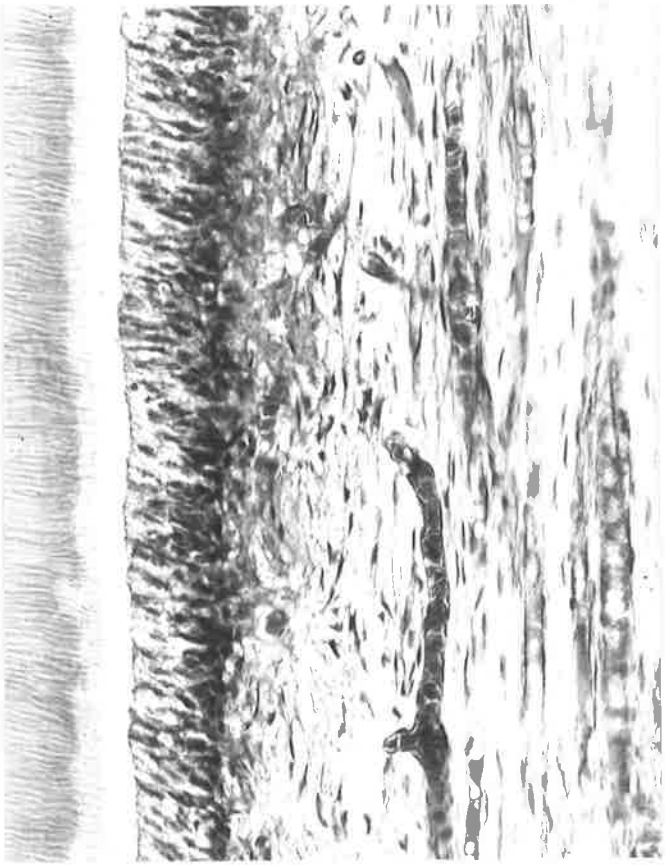


Fig. 3.31: 1 day specimen, citric acid-treated and root planed. H&E x 250.

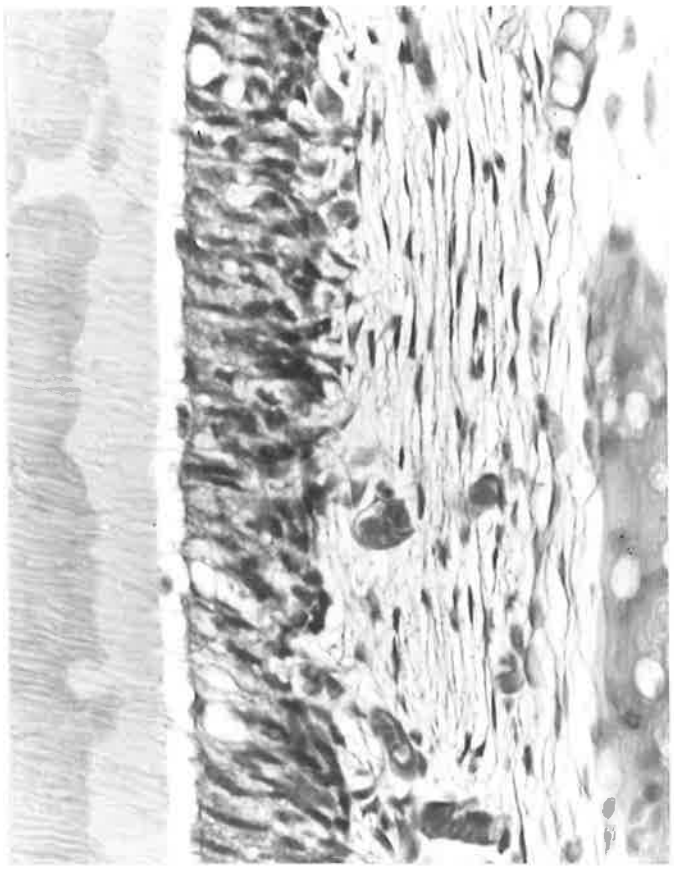


Fig. 3.32: 1 day specimen, unplaned and saline-treated. H&E x 280.

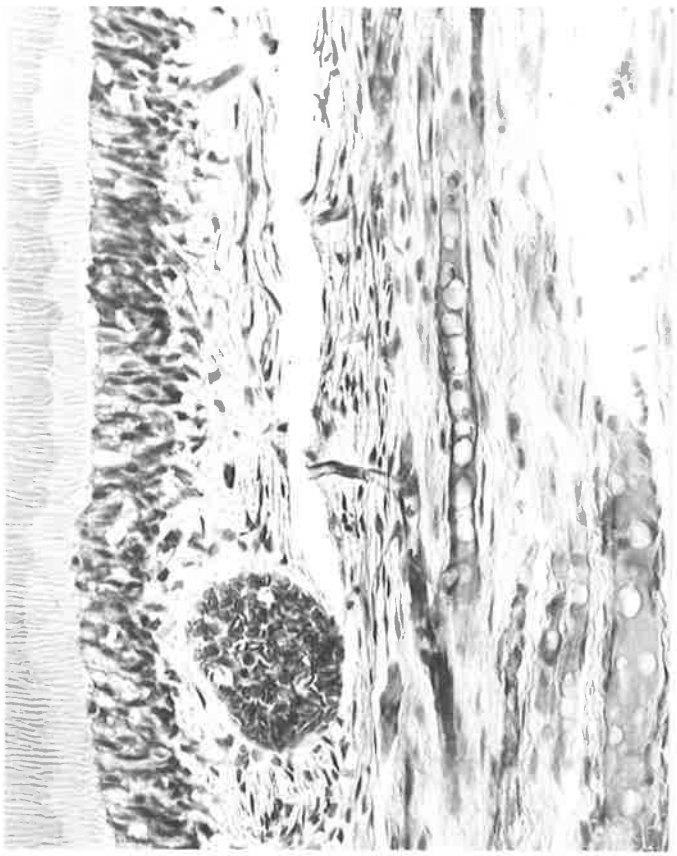


Fig. 3.33: 1 day specimen, root planed and saline-treated. H&E x 200.

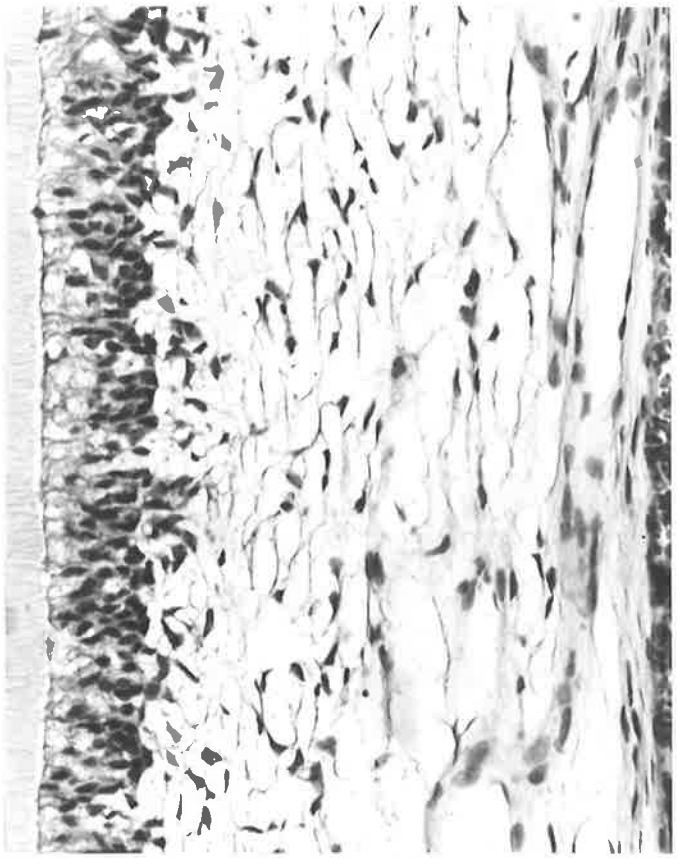


Fig. 3.34: 1 week specimen, unplaned and citric acid-treated. H&E x 250.

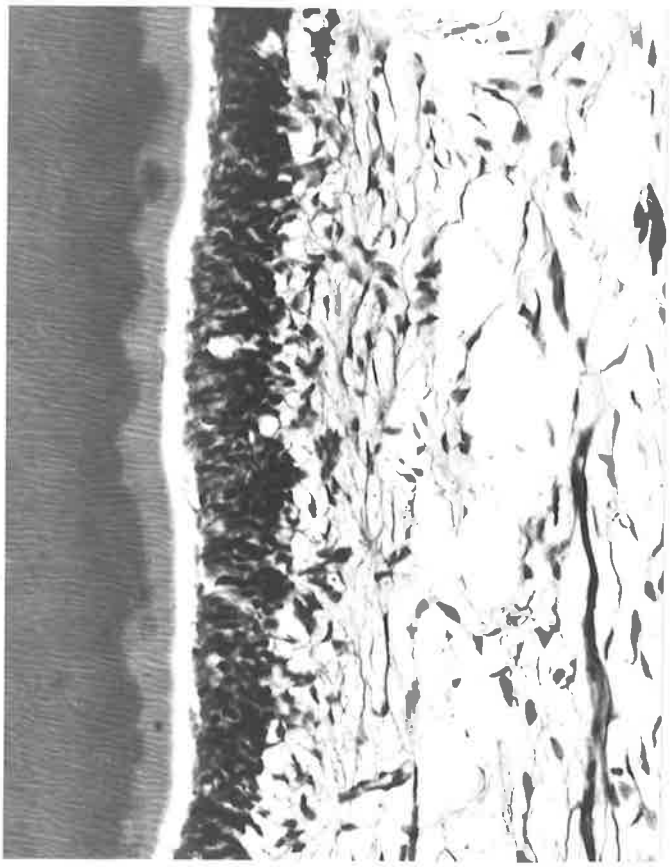


Fig. 3.35: 1 week specimen, root planed and citric acid-treated. H&E x 220.

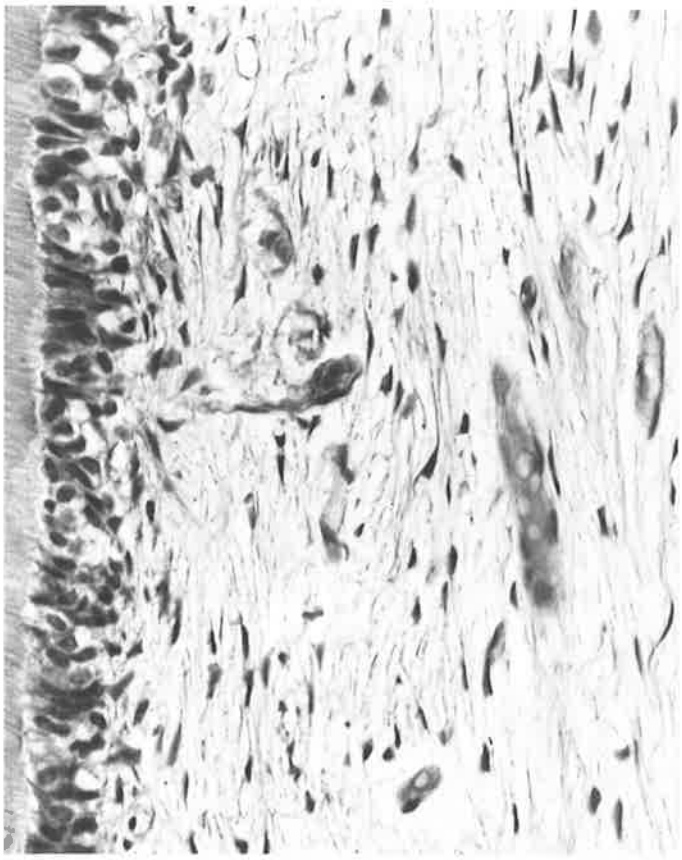


Fig. 3.36: 1 week specimen, unplaned and saline-treated. H&E x 250.

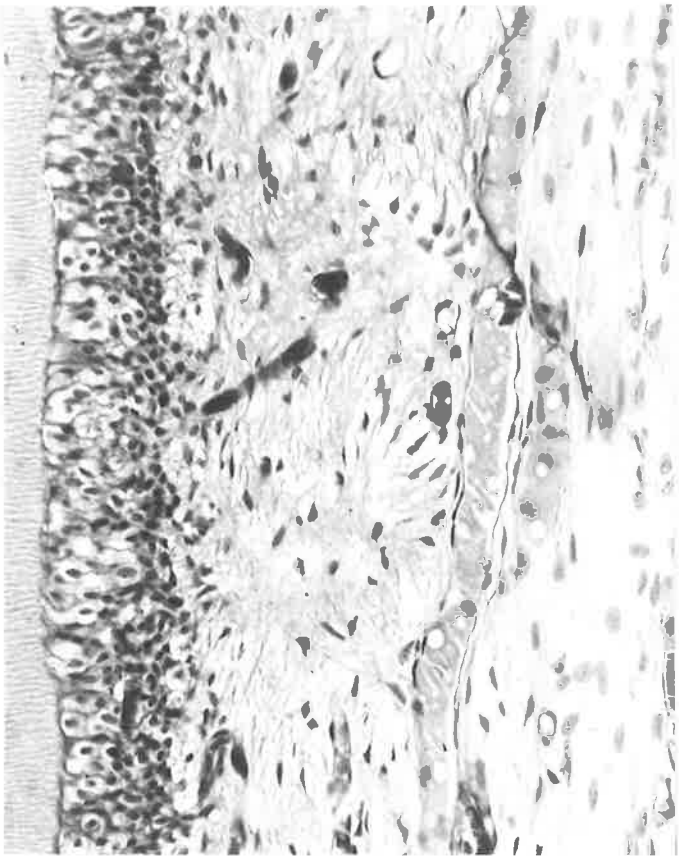


Fig. 3.37: 1 week specimen, root planed and saline-treated. H&E x 250.

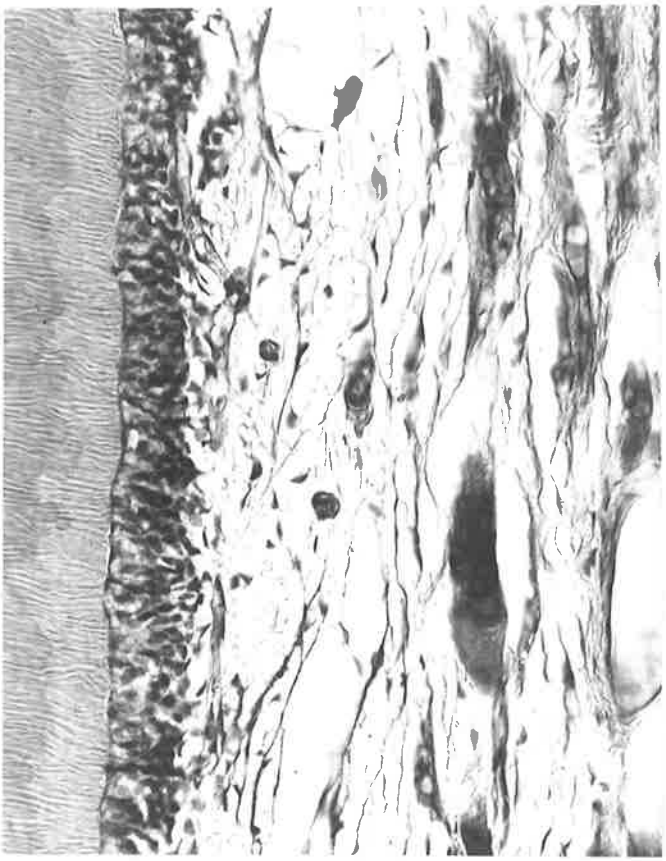


Fig. 3.38: 2 week specimen, unplaned and citric acid-treated. H&E x 250.

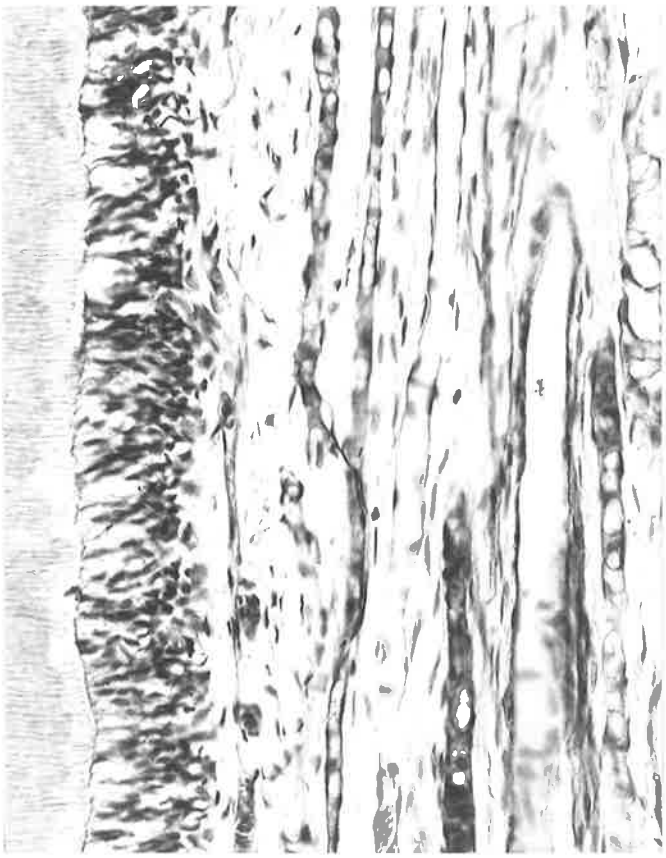


Fig. 3.39: 2 week specimen, root planed and citric acid-treated. H&E x 250.

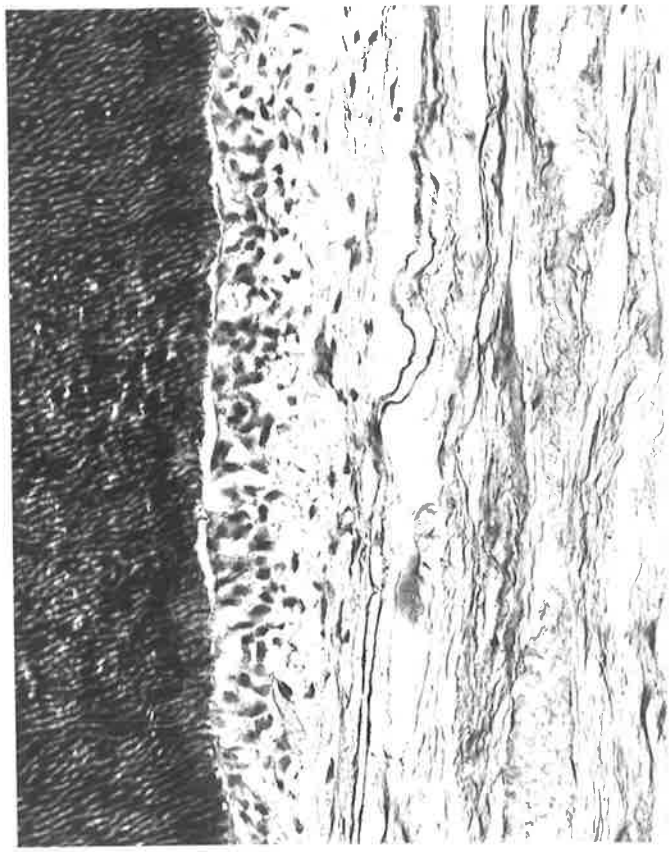


Fig. 3.40: 2 week specimen, unplaned and saline-treated. P.P.C. x 250.

Fig. 3.41: 2 week specimen, root planed and saline-treated. P.P.C. x 250.

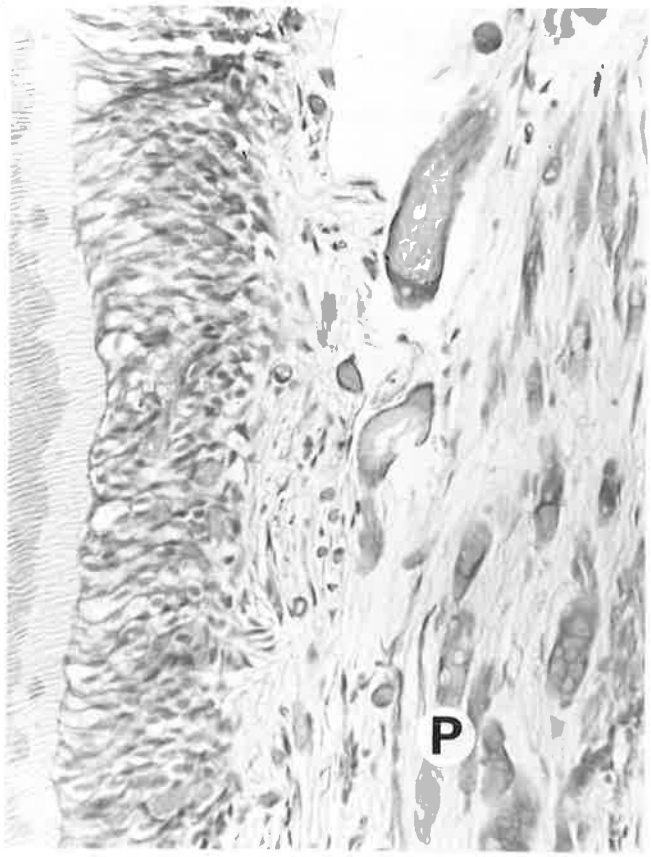


Fig. 3.42: 4 week specimen, unplaned and citric acid-treated. H&E x 250.

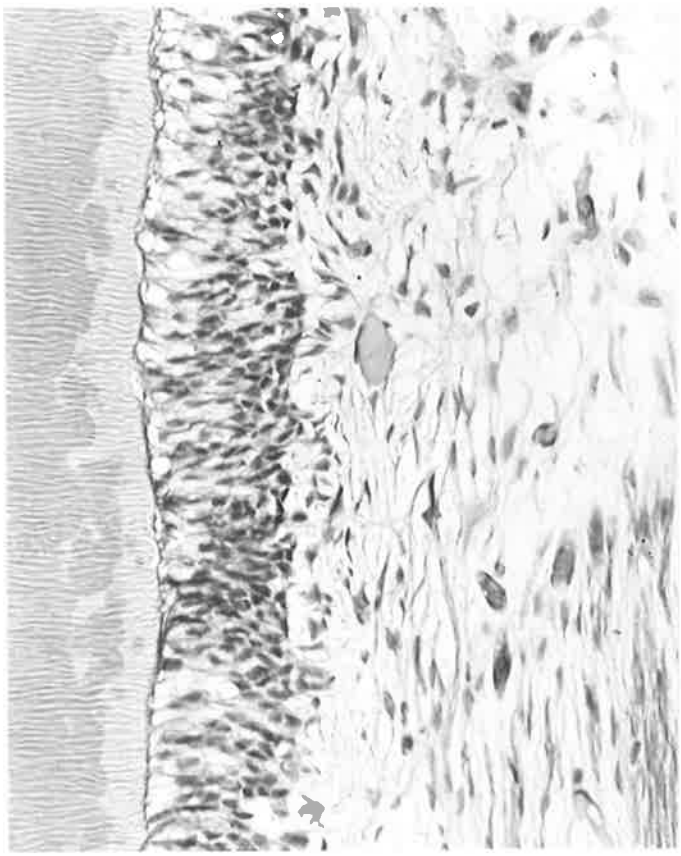


Fig. 3.43: 4 week specimen, root planed and citric acid-treated. H&E x 250.

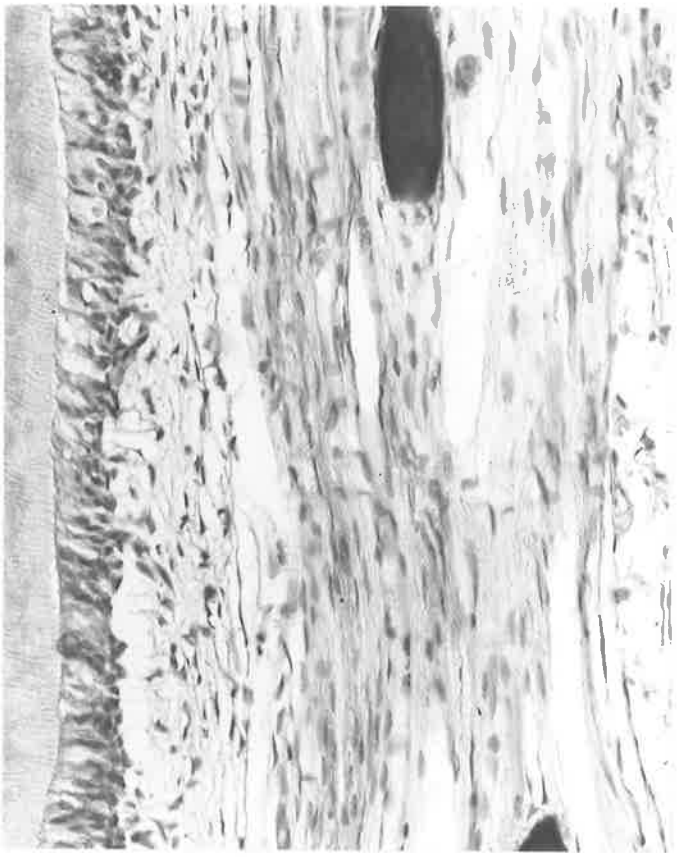


Fig. 3.44: 4 week specimen, unplaned and saline-treated. H&E x 250.

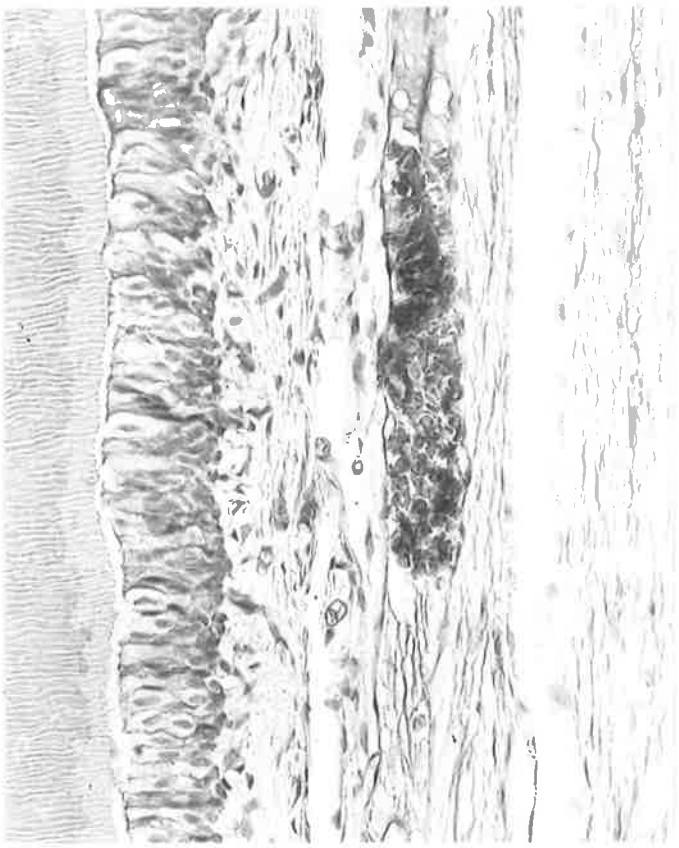


Fig. 3.45: 4 week specimen, root planed and saline-treated. H&E x 250.

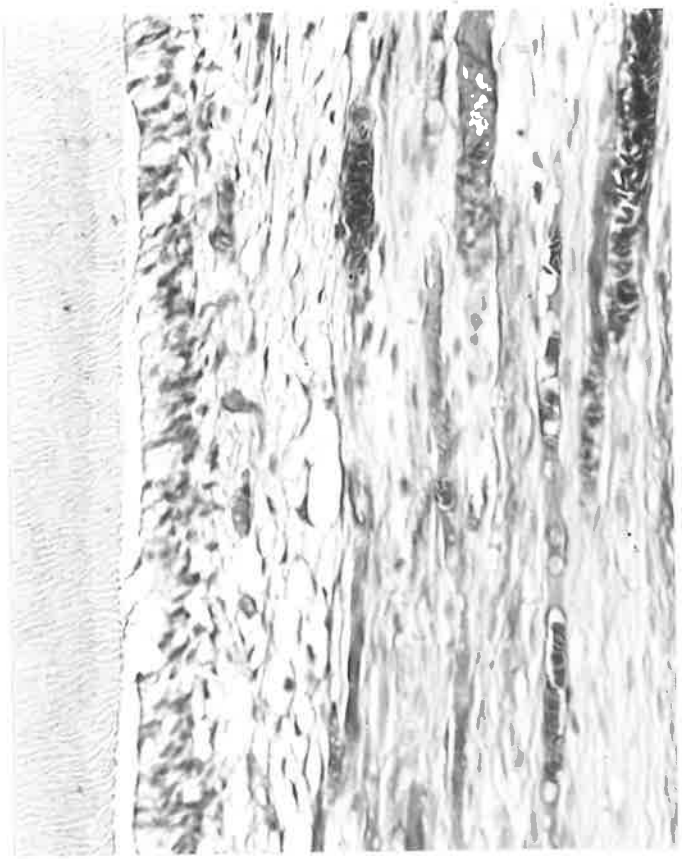


Fig. 3.46: 8 week pecimen, unplaned, citric acid-treated. H&E x 250.

Fig. 3.47: 8 week specimen, root planed and citric acid-treated. H&E x 500.

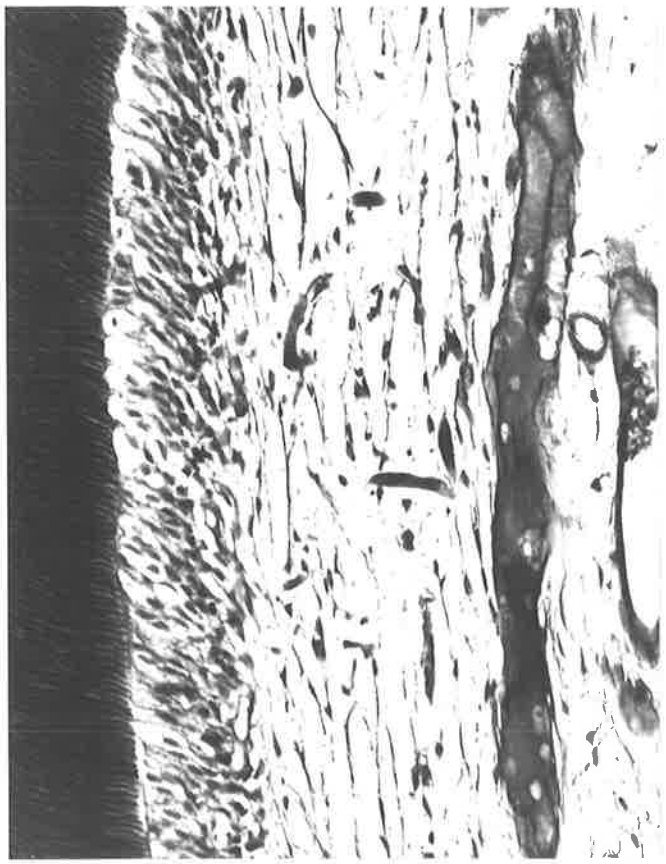


Fig. 3.48: 8 week specimen, unplaned and saline-treated. P.P.C. x 250.

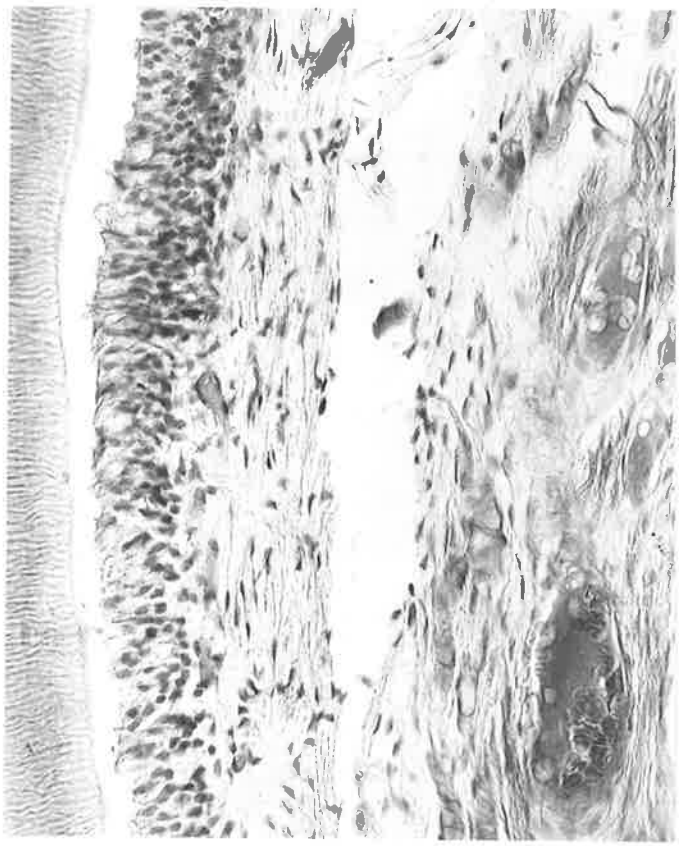


Fig. 3.49: 8 week specimen, root planed and saline-treated. H&E x 200.

CHAPTER IV

DISCUSSION

A. Reattachment

1. Wound healing

In this study, normal sequence of healing was observed in sections of early time periods (up to 2 weeks). There was a change from acute inflammation (1 day) to resolution (1 week) and repair (2 weeks). No difference was detected between specimens treated with citric acid and saline. It therefore, appeared that the use of 20% citric acid for 2 minutes did not cause tissue necrosis or delayed wound healing. This is in agreement with early findings of Register (1973), Register and Burdick (1975, 1976), Crigger et al. (1978). In a study on healing of experimental furcal defects in dogs, Crigger and co-workers observed uneventful healing of gingival tissue after surgery and topical citric acid application. The results from the present study confirmed their observation.

2. Root resorption

Root surface resorption was observed in sections from the 2 week period onward. There were varying numbers of resorption bays and a varying extent of resorption area. Root resorption appeared to be associated with connective tissue (fibrous) reattachment, and was absent in the presence of a long epithelial attachment. Experim-

ental sites (root planed and treated with citric acid) showed the least amount of resorption. This finding is in agreement with Register & Burdick (1975, 1976) and Ririe et al. (1980).

However, on unplaned root surfaces (both citric acid- and saline-treated), root resorption was observed (Figs. 3.19, 3.22). The extent of resorption varied greatly between specimens. Such findings have not been reported elsewhere in the literature.

3. Cementogenesis and relation to notching

Histologic sections from the 8 week period showed new cementum deposition at resorption sites (Fig. 3.27). This suggested that resorption had ceased and that repair by remineralization had begun. New cementum formation at the notch area was conistently observed in greater quantity than other dentinal repair sites. This finding agrees with that of Linghorne (1957), Patterson et al. (1967), Morris (1969, 1978), Frank et al. (1974) and Listgarten (1972); but is at variance with that of Garrett et al. (1981). In a study involving beagle dogs, Garrett et al. found that:

1) The majority of specimens showed reattachment of connective tissue in the full extension of the notch;
2) New cementum was consistently formed on exposed dentine in the depth of the notches as well as on the walls of the notches and the adjacent unnotched area. In unnotched areas, new cementum was also observed on old cementum not completely removed with root planing.

They concluded: " ... notches seem to have no effect on the rate or quality of new cementum formation. ... some doubt on the ability of notches *per se* to stimulate cementogenesis."

In the present study, cementogenesis began in the notches prior to other dentinal surfaces; the quantity of new cementum deposition was greater at the notch than that of other dentinal surfaces.

4. Bony regeneration

Bony regeneration occurred in sections as early as the 2 week time period. Sections from later time periods showed more extensive bony regeneration and, in some instances, the new alveolar crest had passed the coronal border of the notch. However, no difference was observed between the osteogenic capacity of the citric acid-treated sites when compared with the non-acid-treated specimens. This finding agrees with Ririe *et al*. (1980) but is at variance with Nyman *et al*. (1981) who reported no bony regeneration in monkeys. The present study does not support claims made by Crigger *et al*. (1978) and Cole *et al*. (1980). In a study on healing of experimental furcation defects in dogs, Crigger *et al*. reported the bone level was significantly higher in the acid-treated than in the contralateral, sham-operated teeth. Using human material, Cole *et al*. observed alveolar bone extended coronal to the apical extent of the notch in 9 out of 10 specimens.

It is possible that variation in results is due to lack of standardization of model systems used in the various studies.

5. Ankylosis

Ankylosis was observed in two specimens in this study, both were on unplaned root surfaces, one treated with citric acid and the other with saline (Figs. 3.18, 3.29). This occasional finding supported the observations by Register & Burdick (1976). Crigger et al. (1978) and Nilvéus et al. (1980); but is at variance with findings by Cole et al. (1980), Steiner et al. (1981) and Bogle et al. (1981). Cole et al. and Steiner et al. could not find any ankylosis in human teeth they studied, whereas Bogle et al. reported ankylosis as a common histologic finding. It is possible that the older beagle dogs used in Bogle's study could influence and promote the incidence of ankylosis.

6. Orientation of fibrous attachment

In all the root planed specimens which exhibited connective tissue type of reattachment, the collagen fibres were seen running mainly parallel and along the root surface (Figs. 3.23, 3.28). The citric acid-treated specimens, although showing a stronger and more advanced connective tissue reattachment, consistently displayed close adaptation of collagen fibres running parallel to the dentinal surface. Where new cementum had formed on dentinal surfaces, collagen fibres were observed to have been incorporated in the process of remineralization.

However, the fibres generally ran in an oblique direction. This finding does not support observations made by Ririe et al. (1980) and Crigger et al. (1978), although Crigger did concede that areas where fibres were arranged more parallel to the root surface also occurred. Ririe et al., using TEM were able to demonstrate the difference between young and old collagen fibres by virtue of their cross-sectional dimension (493 ± 27Å vs.± 870 47Å). Furthermore, they were able to demonstrate the interdigitation between old and new collagen fibres at the soft-hard tissue interface. In contrast, they found among the non-acid-treated specimens, the connective tissue fibres generally coursed parallel to the root surface in a vertical direction.

The question of orientation of collagen fibres in healing gingival wounds (particularly in humans) was discussed by Linghorne & O'Connell (1950), Stahl (1966) and Morris (1969). These authors agreed that new connective tissue fibres ran parallel to the tooth surface. Stahl demonstrated that this arrangement could persist for up to a year. This was confirmed by Burfield (1971). Morris proposed that the pattern assumed by these fibres was identical to that of periosteum and concluded that the periodontal membrane in this area had reverted to the form of the less specialized periosteum.

It was not possible to demonstrate interdigitation of old and new collagen fibres in the present study since high resolution TEM is required. On unplaned root surfaces, picropolychrome staining suggested that inter-

digitation of old and new collagen fibres may have occurred (Fig. 3.21). However, any such union occurred outside the mineralized cementum and there was no suggestion that mineralization was occurring at the union site.

Citric acid treatment does promote early connective tissue reattachment but functionally orientated fibre insertion was not observed. As suggested by Stahl (1966) and Burfield (1971) given enough time, maturation of connective tissue attachment will reach a stage of consisting of functionally orientated fibres and thus become indistinguishable from the unoperated site.

7. Mode of reattachment

Controversy surrounds the mode of gingival reattachment following periodontitis and corrective surgery or simply surgical detachment. Currently, one view claims that gingival reattachment may occur after both situations, but such reattachment is mainly achieved by epithelial cells. This view is supported by Skillen & Lundquist (1937), Ramfjord (1951, 1952), Morris (1955), Yukna (1976), Caton & Nyman (1980) and Caton et al. (1980). On the other hand, Linghorne & O'Connell (1950, 1951, 1955, 1957) and Stahl & Persson (1962) reported connective tissue reattachment following surgical wounding and gingival detachment.

Recently, Register (1973), Register & Burdick (1975, 1976), Cole et al. (1980), Ririe et al. (1980), Crigger et al. (1978), Nilvéus et al. (1980) and Selvig et al.

(1981) claimed predictable connective tissue reattachment using citric acid demineralization procedure in conjunction with thorough root planing. Stahl and Froum (1977) and Nyman et al. (1981) have disagreed that connective tissue attachment can be achieved in a predictable manner. In the present study, it was observed that citric acid treatment following root planing did consistently produce connective tissue type reattachment. In contrast, the non-acid-treated sites almost invariably produced long epithelial attachment.

This finding agrees with Register, Register & Burdick, Crigger, Ririe, Cole, Nilvéus and Selvig; but is at variance with Stahl & Froum and Nyman et al. The present study is confined to surgical wounding where cementum has not been previously exposed to periodontal disease. However, most authors agree that there is no detectable difference in the speed and character of wound healing and gingival reattachment in these two situations. It is probably reasonable to extrapolate the findings from this experimental model to that of periodontal disease situation.

8. Effects of citric acid on reattachment

Electron microscopic studies by Garrett et al. (1978) revealed citric acid application (after root planing) produced a 4 micron demineralized surface zone, consisting of exposed collagen fibres. Enhanced cementogenesis was claimed subsequent to acid conditioning.

Ririe et al (1980) reported interdigitation of young collagen fibres with denuded old collagen fibres from demineralized dentinal surface. They went on to propose that:

1) acid conditioning of the root surface may have obviated the need for the initial step in this biological sequence of events, i.e., demineralization of the dentinal surface.
2) acid conditioning resulted in a mechanism of reattachment to the dentinal surface not unlike that which occurs in the healing of a soft tissue incisional wound.

The question of cytotoxicity of cementum after exposure to periodontal disease was first brought to light by Hatfield and Bauhammers (1971) and Aleo et al. (1974, 1975). Aleo found by treating the diseased cementum with phenol and water extraction, the biocompatibility of fibroblast cell culture was restored.

Shiloah (1980) suggested tht citric acid has the potential to extract endotoxin from diseased root surfaces. Such contention was supported by in vitro studies of Boyko et al. (1980). They found significantly more cells attached to demineralized roots than non-demineralized roots, the method of demineralization not influencing the result. This suggests that one of the ways in which acid-demineralization of root surfaces could contribute to the reported enhancement of healing of periodontal defects is by improving the attractiveness of the root surface as a substrate to which cells can adhere.

In another study by Fine et al. (1980) it was demonstrated that trichloroacetic acid and citric acid were more effective than water or phenol in removing calcium and toxins from the root surface and subsurface. Preliminary characterization of the material eluted indicated the presence of endotoxin.

The physical characteristics of dentinal surface after acid demineralization may contribute to the enhanced collagen attachment. Squier & Collins (1981) demonstrated that 3 μm is the minimal pore size that permits connective tissue penetration into the millepore filter and that when infiltration does occur, the resulting soft tissue attachment markedly restricts the extent of epithelial downgrowth. SEM studies by Garrett et al. (1978), Lee et al. (1973) and Brannstrom & Johnson (1974) revealed frequent depressions associated with openings of dentinal tubules. Such openings could provide the necessary "micropores" for collagen fibres to infiltrate, thus establishing early reattachment. These hypotheses lack definite supporting evidence.

B. PULPAL RESPONSE

Seltzer (1971), Stahl (1963) and Brazda & Jiricka (1978) reported pulpal reactions following surgical trauma and instrumentation of root surfaces. These pulpal reactions ranged from acute inflammation to irregular dentine formation. In the current study, pulpal histology consistently lacked pathological changes; irrespective of

surgical wounding, instrumentation or notching. This suggests that the degree of trauma inflicted during the experimentation was well tolerated by the pulpal tissue.

No difference between acid- and saline-treated specimens was noted, pulpal evaluation in both situations was negative. This finding is in agreement with Register (1973) and Ririe et al. (1980).

It would appear from the present study that 20% citric acid applied topically to exposed root surface for 2 minutes does not elicit any pulpal changes, transient or permanent.

CHAPTER V

SUMMARY AND CONCLUSIONS

An experimental study was carried out designed to investigate the effects of citric acid demineralization of root surfaces on

1) Gingival reattachment
2) Pulpal response.

Full-thickness buccal flaps were raised in the premolar regions of five beagle dogs. Alveolar bone was removed to simulate periodontal bone loss, the mesial roots were thoroughly planed to remove cementum while the distal roots were not instrumented. Saline was used as the control for 20% citric-acid treatment. Gingival flaps were repositioned and sutured. Specimens were removed by block dissection after 1 day, 1, 2, 4 and 8 weeks, and subjected to histopathological investigation.

Results:

1) Topical use of 20% citric acid did not induce tissue necrosis or delay the process of healing.

2) After root planing, demineralization with 20% citric acid for 2 minutes consistently gave a connective tissue reattachment after 2 weeks. Saline-treated surfaces healed with both long epithelial and connective tissue reattachment.

3) Root planing in one and two week specimens was found to delay healing of the surgical wound when compared with unplaned sites.

4) Reattachment with long junctional epithelium was not associated with root resorption. Regions healing with connective tissue attachment were commonly associated with areas of root resorption.

5) Cementogenesis was evident in the treated areas, especially where resorption had occurred and in association with the notch.

6) Bony regeneration occurs in all teeth but there is no significant difference between citric acid-treated and the non-acid-treated sites.

7) Ankylosis was seen in two of the 30 specimens but was unrelated to citric acid treatment.

8) Early collagen fibre alignment in the healing site was parallel to the root surface.

9) 20% citric acid applied topically to exposed root surface for 2 minutes did not elicit pulpal changes.

These results obtained from the dog are not valid for extrapolation to the human. Comparison with human studies (Cole et al., 1980) indicated that similar results were obtained in that model. However, Nyman et al. (1981) using monkeys had not confirmed these findings.

APPENDICES

APPENDIX I: PROFUSION TECHNIQUE

Apparatus set-up

10% buffered formal saline was used for profusion. This was stored in a 5-gallon container and positioned about 7 ft. above ground level.

Through a series of tubings reducing in bore (diameter), the container of formalin was connected to a catheter inserted into the left Common Carotid Artery of the dog. Another catheter was inserted into the right External Jugular Vein. This provided for drainage of blood and formalin during profusion. The operation table was tilted to a head-down position. This facilitated a better Flow (Fig. I.1).

Anaesthesia

General anaesthesia was obtained in essentially the same manner as in 2.2.2.1 except endotracheal intubation was used routinely.

Catheterization

The left Common Carotid Artery was isolated with blunt dissection. An appropriate size catheter was then inserted and tied with silk ligature. The catheter was then connected to tubings from the formalin flow tank.

Catheterization was achieved in a similar fashion with the right External Jugular Vein. This was then left draining over a bucket.

Profusion

After making sure the catheters were not clogged the profusion taps were opened, and formalin allowed to flow through the animal. Anaesthetic and airway support were withdrawn a short while later.

Profusion continued for about an hour until clear formalin appeared in the draining tube. The animal was considered properly fixed, and the taps were then turned off.

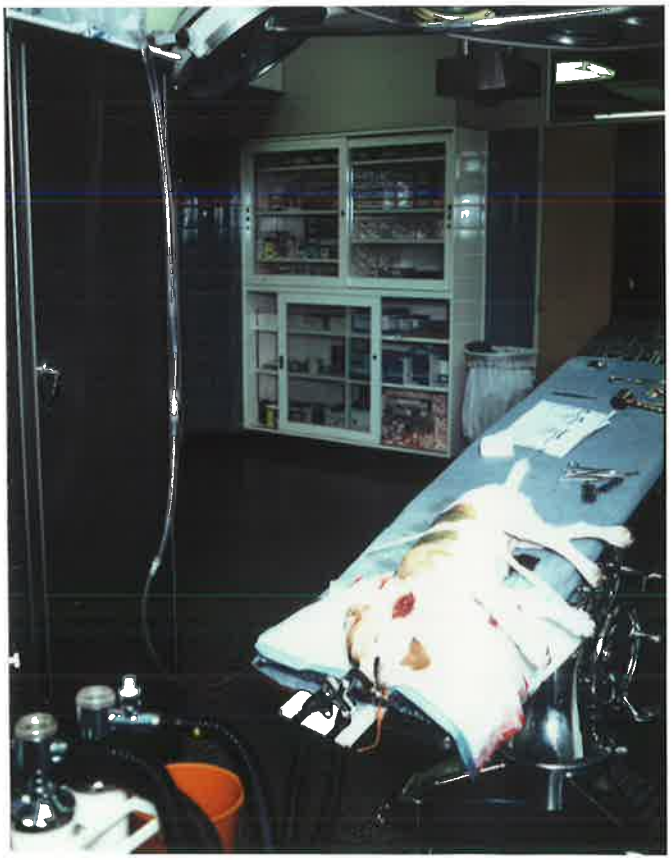


Fig. I.1: Apparatus set-up for profusion.

APPENDIX II: BIOPSY PROCEDURE

Skin and soft tissue were dissected from the jaws leaving only the attached gingivae. On the lower jaw such dissection extended to the angle of mandible. A channel was cut along the body of the mandible approximately at the distal of lower first molars. The body of the mandible was then separated from the two rami with chisel and mallet (Fig. II.1).

Again, using chisel and mallet, two maxillary sections were obtained, each section included a canine and 4 premolars (Figs. II.2, II.3).

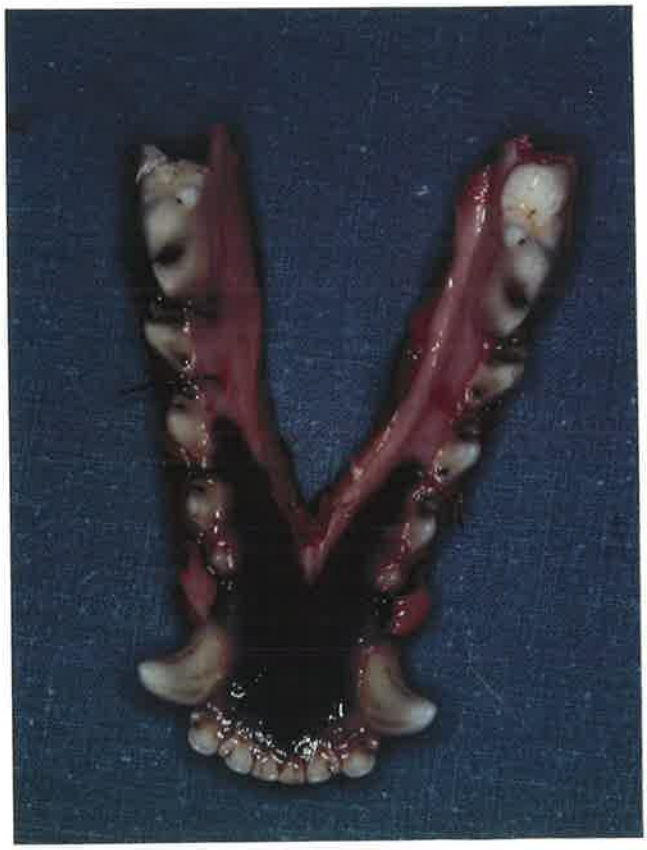


Fig. II.1: Mandibular specimen resected.

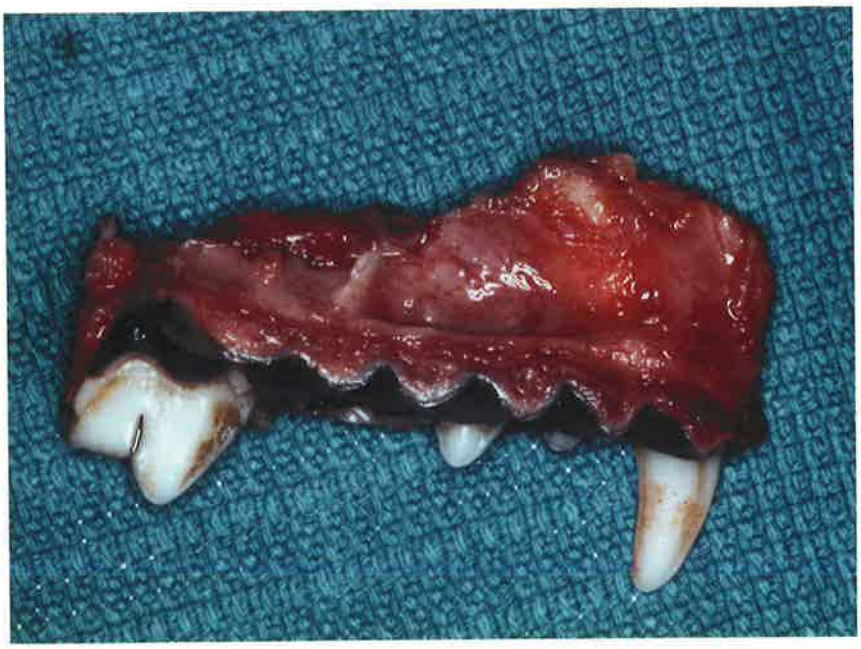


Fig. II.2 Block dissection of left maxillary specimen.

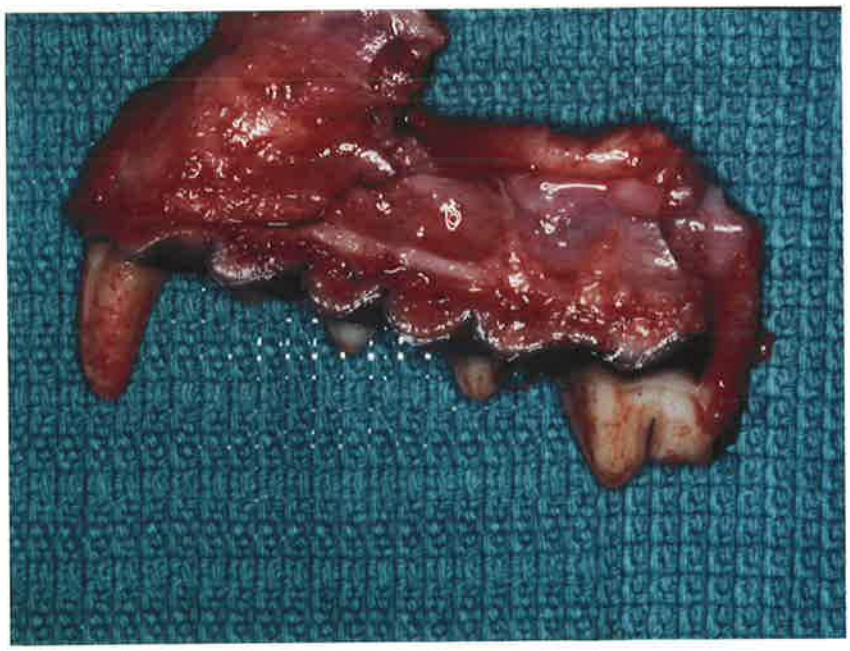


Fig. II.3: Block dissection of right maxillary specimen.

APPENDIX III: TISSUE PROCESSING

(I) Fixation – in 10% formal saline (PO_4 buffered) at least 24 hours.

(II) Trimming
- Reduce specimen to manageable size
- cutting by motorized, diamond embedded cutting disc with water coolant.

(III) Decalcification
- Place specimens into individually labelled jars of Formate formic
- change solution daily
- X-ray to determine end pt.

(IV) Neutralization
1) Running water 1 hr
2) Na_2 SO_4 5% at least 36 hr.

(V) Dehydration
- wash 1 hr in running water
- progresssive alcohol dehydration

At 37°C
70% alcohol overnight
80% alcohol 1 hr
90% alcohol 1 hr
95% alcohol 1 hr
100% Alcohol I 1 hr
100% Alcohol II 1 hr
100% Alcohol III 1 hr
Equal parts of methyl salicylate and alcohol - 1 hr.

(VI) Double embedding (under vacuum)

At 37°C
- methyl salicylate + 0.5% celloidin - 2 days
- methyl salicylate + 1.0% celloidin - 2 days

At 60°C
- 2/3 methyl salicylate + 1/3 wax - 1 hr
- 1/2 methyl salicylate + 1/2 wax - 1 hr
- 1/3 methyl salicylate + 2/3 wax - 1 hr
- wax - change I - 2 hr
- wax - change II - 2 hr
- wax - change III - overnight

- Use Tissue Embedding Machine and block specimens in moulds.

APPENDIX IV: STAINING

(1) HAEMATOXYLIN AND EOSIN

HAEMATOXYLIN	EOSIN
5 gm Haematoxylin	10 gm Eosin
50 gm Ammonium alum	5 gm Potassium dichromate
300 ml Glycerol	100 ml Sat. Picric acid
700 ml Distilled water	100 ml Absolute alcohol
1 gm Sodium iodate	800 ml Distilled water
20 ml Glacial acetic acid	

1. Dissolve the haematoxylin in about 20 ml of absolute alcohol.
2. Dissolve the alum in some of the distilled water by heating and allowing to cool.
3. Mix in other ingredients.

* * * * * *

Routine Staining.

Staining procedures

1. Place slides into xylol for 15 min.
2. Into 100% alcohol I 2 min.
3. Into 100% alcohol II 2 min.
4. Into 70% alcohol for 2 min.
5. Put into running tap water.
6. Haematoxylin for 7 min. or longer depending on the stain.
7. Take to running water for 5 min.
8. Differentiate in 0.2% acid-alcohol, HCl in 70% alcohol.
9. To water again to blue for 5 min.
10. Eosin for 3 min. or longer depending on the stain.
11. Wash in in water for 2-3 dips until most of the stain has been washed away.
12. Dehydrate in 70% then 2 x 100% alcohol.
13. Clear in xylol and mount.

* * * * * *

(2) POLYCHROME STAIN FOR DIFFERENTIATING PRECOLLAGEN FROM COLLOGEN
(Herovici, 1963; Lillie et al., 1980))

Nuclear stains:

(i) Celestine blue 0.25 g.
Iron alum 2.5 g.
Distilled water 50 ml.

Boil for three minutes, cool, add 10 ml of glycerol.

(ii) Aluminium sulphate, 5 per cent aqueous solution 100 ml.

Heat to boiling point; then add very slowly,

Haematoxylin, 1 per cent alcoholic solution 50 ml.

Allow to boil for three minutes; when cool add:

Distilled water 100 ml.
Ferric chloride, 4 per cent aqueous solution 10 ml.
Hydrochloric acid, concentrated 1 ml.

Cytoplasmic stain.

Metanil yellow 0.25 g.
Distilled water 60 ml.
Acetic acid 5 drops.

Connective tissue stain.

Picropolychrome mixture:

(i) Methyl blue 0.05 g.
Distilled water 50 ml.
(ii) Acid fuchsin 0.18 g.
Picric acid, saturated aqueous solution. 50 ml.

Mix (i) and (ii) and add,

Glycerol 10 ml.
Lithium carbonate, saturated aqueous solution 0.5 ml.

Acetic acid solution

Acetic acid 5 drops.
Distilled water 60 ml.

Lithium carbonate solution.

Lithium carbonate, saturated aqueous solution 2 drops.
Distilled water 60 ml.

(2) continued:

Procedure

1. Stain in celestine blue (five minutes).
2. Wash in running tap water (five minutes).
3. Stain in haematoxylin mixture (five minutes).
4. Wash for thirty minutes in running tap water.
5. Stain in metanil yellow (two minutes).
6. Differentiate in acetic acid solution; rinse in water.
7. Place in lithium carbonate solution (two minutes).
8. Stain in picropolychrome mixture (two minutes).
9. Transfer to 1 per cent acetic acid (two minutes).
10. Dehydrate, clear and mount in Canada balsam containing salicylic acid (0.1 g to 100 ml of balsam)

    Nuclei-black; cytoplasm-green to yellow; young collagen-blue; mature collagen-red.

* * * * *

(3) VAN GIESON'S METHOD FOR COLLAGEN FIBERS

SOLUTIONS

WEIGERT'S IRON HEMATOXYLIN SOLUTION

Solution A

Hematoxylin crystals	1.0 gm
Alcohol, 95%	100.0 ml

Solution B

Ferric Chloride, 29% aqueous	4.0 ml
Distilled water	95.0 ml
Hydrochloric acid, concentrated	1.0 ml

Working Solution

Equal parts of Solution A and Solution B.

VAN GIESON'S SOLUTION

Acid fuchsin, 1% aqueous	2.5 ml
Picric acid, saturated aqueous	97.5 ml

STAINING PROCEDURE

1. Deparaffinize and hydrate to distilled water.
2. Weigert's hematoxylin solution for 10 minutes.
3. Wash in distilled water.
4. Van Gieson's solution for 1 to 3 minutes.
5. Dehydrate in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.
6. Mount with Permount or Histoclad.

RESULTS

Collagen	- red
Muscle and Cornified epithelium	- yellow
Nuclei	- blue to black

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