10. DISCUSSION

10.1 Methodology

10.1.1 Anaesthesia

The Hypnorm®/Hypnovel® anaesthetic combination was found to produce the best results for the animal procedures, with both rapid onset and profound anaesthesia produced. However, supplies of Hypnorm® are no longer available, and the results achieved with Nembutal® were only practically useful for animal sacrifice, given the long time required for onset and the varying dosage requirements. Should further studies be carried out in the future, alternate anaesthetic agents will need to be trialled.

10.1.2 Thermal insult

The inconsistent appearance of ankylosis in the experimental teeth in this study was in contrast to that found in previous studies 76, 95. The thermal insult procedure used in the current study, a single 20 minute application of dry ice, was adapted from that developed by Dreyer 75, 95. In his study, the 20 minute thermal insult reliably produced ankylosis within the interradicular areas of the teeth in all time periods from 7 days onwards. The study by Shaboodien 76, which used the same experimental method, found ankylosis in the interradicular region of all experimental teeth from the 14 days observation period onwards. In the current study, none of the animal groups exhibited ankylosis in every experimental tooth. In fact, in four of the six groups, ankylosis was seen in only one out of the five animals. In one case, ankylosic union was observed in only a single section from one animal (Table 1).

In experimental teeth which had not developed ankylosis, changes were still observed, particularly within the pulp tissue. These changes included tissue disorganisation and decreased cellularity compared with the control tooth in the corresponding section. Often, the surface of the alveolar bone abutting the periodontal ligament also appeared irregular compared to the control side, and
occasionally the periodontal ligament itself seemed to be narrower. These observations suggest that the thermal insult of the experimental intervention was sufficient to cause tissue damage but perhaps not severe enough to initiate the processes leading to ankylosis.

The experimental protocol of the current study was designed to standardise the thermal insult intervention over each observation time group in an attempt to produce consistent results in each of the animals. To this end, all of the animals from each observation group underwent the thermal insult procedure during the same session, which meant that the interval between the first and last animals treated in each group was generally no longer than two hours. Therefore, the distribution of ankylosis development was unusual, since all five animals from each group underwent the same procedure at the same time and under the same conditions, yet the results observed at the end of the time period were not consistent across the group. It would be expected that most, if not all animals from each group should either develop ankylosis or otherwise remain unaffected.

One of the factors which may have influenced the effect of the thermal insult intervention is the good thermal insulating of tooth structure, particularly the dentine. For this reason, Dreyer suggested that a sufficient length of time is required for a thermal stimulus, when applied to a tooth, to produce changes in the surrounding tissues. In that study, a 10 minute application of dry ice was considered long enough to reliably produce resorptive changes, whereas a single 20 minute application or multiple freezing episodes were suggested for the production of ankylosis. Although the freezing time used in the current study fulfilled these guidelines, other factors may have been involved, including variations in the thickness of the dentine and enamel.

Other potential influences may be the pressure with which the dry ice pellet is held onto the tooth surface, as well as the size of the pellet. In the current study, the ice pellets were held down onto the tooth with gentle but firm pressure, and
the size of the pellet generally approximated the size of the crown of the first molar tooth to which it was being applied, in order to minimise soft tissue injury. However, as the CO$_2$ sublimated from the solid to gaseous state, the size of the pellet decreased before it was eventually replaced with a new pellet. It may be possible that the cold stimulus from the smaller ice pellet prior to its replacement was insufficient to maintain freezing of the underlying dental tissues. Interestingly, Dreyer noted that ankylosis was only sporadically seen in animals which were used for the study of root resorption, and those animals had been subjected to a shorter freezing time$^{95}$. A similar pattern was noted in the current study, suggesting that some of the animals in this study may not have experienced sufficient freezing time for the development of ankylosis.

In future ankylosis studies using thermal insult as an experimental intervention, thought should be given to undertaking a trial prior to the main experiment, in which different freezing times are used. A freezing time longer than 20 minutes or multiple freezing episodes should be considered, although the latter option may not be practical. Additionally the dry ice pellets should be replaced more frequently, and the pellet size should be kept as large as possible without causing unnecessary damage to the soft tissues of the buccal mucosa and tongue. It may also be useful to ensure that the ice pellet is held firmly against the occlusal surface of the tooth at all times. The occlusal surface has been suggested as the best site for application of the cold stimulus, as better thermal conductivity occurs when the stimulus is applied parallel to the dentinal tubules$^{98}$.

10.1.3 Immunostaining
10.1.3.1 Cytokeratin AE1/AE3
This antibody produced reliable and consistent staining, with negligible background stain. The use of this antibody was also relatively simple, and good results were possible with a generic multi-species detection kit. Identification and counting of epithelial cell clusters was quite easy, due to the contrast attained
between positively stained and unstained structures. Prior to the current study, this antibody had been successfully used in an earlier study of epithelial cells in rat periodontal ligament. For these reasons, cytokeratin AE1/AE3 can be recommended as a reliable choice for immunohistochemical study of epithelial structures within the periodontal ligament.

The only unexpected staining reaction noted with this antibody was the appearance of positively stained particles within the periodontal ligament and sometimes the alveolar bone. These objects appeared to be small cells, although it was not possible to precisely identify them or even to confirm that they were not artefactual. Although they exhibited a staining reaction with the cytokeratin antibody, these elements did not appear to be epithelial cells due to the fact that they were scattered throughout the periodontal ligament and surrounding tissues, whereas the epithelial cells are normally situated close to the root surface. It is speculated that the structures observed in this study may represent phagocytic cells which have recently ingested material containing cytokeratin. This is a possibility on the experimental side, as macrophages within the periodontal tissue may be responsible for removing remnants of the epithelial cells following their lysis after the experimental intervention.

10.1.3.2 PGP 9.5

This antibody stain was considerably more technique sensitive compared with cytokeratin AE1/AE3. Initial use with the multi-species detection kit yielded substantial background staining which, coupled with the small size of the structures being investigated, complicated the identification of nerve fibres. Background staining was reduced when fresh normal horse serum was used in place of the blocking agent supplied with the multi-species kit, but results approaching that seen with the cytokeratin stain were only achieved when staining was carried out in the IMVS immunocytochemistry laboratory. In that laboratory, blocking sera, secondary antibodies and streptavidin peroxidase were freshly made for each staining run. If PGP 9.5 is to be used in future
investigations of periodontal ligament innervation, freshly made antibody and blocking solutions should be employed rather than a generic multi-species detection kit.

10.1.4 Method error
The results of the weighted kappa statistics for epithelial cell and nerve fibre count indicated good agreement between the initial and recounted data sets. Weighted kappa statistics results usually range from zero to one, with values near zero indicating poor agreement, while a value of one indicates perfect agreement. The values obtained in this study, 0.6105 for epithelial cells and 0.7351 for nerve fibres, suggest that the counting method used was reliable.

10.2 Ankylosis
Two morphologic types of ankylosis have been described. One type is preceded by resorption of cementum and dentine, with ankylosis occurring in the old resorption lacunae. The other type involves the replacement of periodontal ligament with bone without prior resorption of cementum. In this case, the alveolar bone appears to be in contact with an intact cementum surface.

The ankylosis observed in the current study was of the latter type described above. The ankylotic tissue which was fused to the affected tooth appeared to be separated from the dentine by an intact cementum layer. Resorption did not usually directly affect the area of ankylotic union, although it was often seen in adjacent sites. This is in agreement with the findings of Dreyer.

The ankylotic material in this study appeared to become more solid with time, progressing from fine bony trabeculae with connective tissue interspersed in the 7 days observation group to a solid bone mass in the 18, 21 and 28 day groups. The 18 days observation group was particularly notable for the fact that the entire furcation was filled with dense woven-bone type material and the only visible periodontal ligament was along the buccal and palatal roots. This appearance is
very similar to that reported by Dreyer et al \textsuperscript{75} in rats treated with multiple freezing episodes, where widespread solid ankylosis was observed after 14 days.

This pattern of ankylosis development over the observation time periods suggests that the condition may be progressive in nature, and this may have potential clinical implications. Luxation has been recommended as a possible treatment for ankylosed teeth \textsuperscript{55, 66}, and it would seem logical that it would have a greater chance of success if the area of ankylosis was quite small and consisted of fine contacts rather than solid bone-like material. The results from the current study indicate that luxation may only be a realistic treatment option for ankylosis if it is carried out within the first two weeks after initial development of the condition. It is of course impossible to determine this time point in the clinical setting.

The progressive nature of ankylosis has been examined in a study conducted by Hammarstrom et al \textsuperscript{101}, in which ankylosis was investigated following the replantation of extracted teeth in monkeys. They found that with more severe periodontal injuries such as those associated with an extended time delay prior to replantation, the subsequent ankylotic area which developed increased in size until most of the periodontal ligament was replaced by bony tissue. However, with a shorter time period between extraction and replantation, a transient ankylosis was observed, with regression of the newly formed hard tissue within the periodontal ligament after several weeks.

It is interesting to note in the current study that the complete obliteration of the furcation in the 18 days group was not seen in the 21 and 28 days group. Here the ankylosic material was quite solid, without connective tissue cells interspersed, but the contact area between it and the root surface was usually small. In some cases, especially in the 21 days observation group, the ankylosic area consisted of fine trabeculae similar to that noted at 7 days. Although this apparent reduction in the degree of ankylosis may be related to the same factors,
discussed earlier, which may have caused the irregular development of ankylosis seen throughout the observation groups, it may also represent a regression of the ankylotic union similar to that noted by Hammarstrom et al. Shaboodien reported ankylosis at 14 days, persisting up to 86 days. However, it was not clear in that study whether the appearance of the ankylotic areas changed over time. Future studies may be required to more thoroughly investigate changes in ankylosis morphology over a longer time period.

10.3 Epithelial cells

10.3.1 Distribution
The distribution and appearance of the epithelial cell clusters observed in this study are similar to previous reports. These clusters appeared either as circular aggregates or strands of cells. This variation in appearance is probably related to the plane of section, as the epithelial cell rests are usually described in the literature as forming a network within the periodontal ligament.

Epithelial cell clusters were observed in the furcations of all control teeth, although the cell counts from individual teeth ranged between one and 20 cells/clusters. This finding is in contrast to reports from an early study of periodontal ligament epithelial cells in the rat, in which these structures were identified in the molars of only 50 per cent of the animals examined. This discrepancy may be explained, in part, by methodological differences as only H&E stained sections were used in the earlier study, whereas immunostaining, with its associated greater accuracy in cell identification, was employed in the current study.

10.3.2 Experimental intervention
The results of the current study indicate that the thermal insult had an effect on the number of epithelial cells and clusters within the furcation region, with significantly fewer cells noted in the experimental teeth compared to the control teeth. Epithelial cells were often completely absent from the furcations of experimental teeth. The aseptic tissue necrosis caused by the thermal insult...
applied to the experimental teeth would appear to be the reason for the decrease in epithelial cell count observed. Time was not found to have a significant effect on the count on either control or experimental side. This suggested that the number of epithelial cells remains stable in the normal periodontal ligament over the short term (up to 28 days), and that the cells do not regenerate following hypothermic necrosis, at least not within the first 4 weeks following the experimental intervention. However, repopulation of the periodontal ligament by the other cellular components of the tissue within 14 days after thermal insult has been reported 74.

Similar findings to those of the present study have been reported by Reitan 21 in an investigation of the behaviour of epithelial cell rests during orthodontic tooth movement. He found that when hyalinization of the periodontal ligament occurred during tooth movement, atrophy of the cellular elements of the ligament, including epithelial cells, occurred. As soon as the adjacent bone was removed by undermining resorption, cellular proliferation and regeneration of the tissue took place, but the epithelial cells did not reappear. This finding may not be unexpected given that the epithelial cells within the periodontal ligament are remnants of a developmental structure, Hertwig’s root sheath.

A more recent ultrastructural investigation of teeth previously subjected to orthodontic forces during rapid maxillary expansion has suggested that the epithelial cells may in fact regrow 39. Sismanidou et al 103 have also reported regeneration of epithelial cell clusters in the healing periodontium following orthodontic expansion. However, it appeared that not all of the epithelial cells regenerated, with the authors noting that single epithelial cells which were present earlier did not reappear. Fujiyama et al 37 described regeneration of the epithelial cells after 10 weeks. In that case, denervation was used as the experimental intervention. As for epithelial cell behaviour in the normal periodontal ligament, previous investigations have found that they decrease in number with age in both the human 21, 104 and rat 102. The observation period of
28 days in the current study appears to be too short to demonstrate an observable time-related decrease in epithelial cell count.

The epithelial cell clusters seen on the experimental side in the current study were usually smaller in size compared to the control side, sometimes consisting of only a few cells. When large epithelial cell clusters were observed on the experimental side, they often exhibited less intense staining with cytokeratin AE1/AE3 and so had a faded appearance. This finding suggests that the epithelial cells may be undergoing degeneration following the thermal insult. Whether this process was continuous and would thus lead to a further decrease in epithelial cell count over the long term is unclear from the data collected in this study. It is also possible that this appearance may actually represent newly formed cells containing fewer cytokeratin fibrils. Further investigations with longer observation periods would be required to assess these possibilities.

10.3.3 Epithelial cells and ankylosis

A relationship between epithelial cell count and ankylosis was evident in the current study, with the epithelial cell count being higher in experimental teeth which were not affected by ankylotic union. However, the number of epithelial cells around non-ankylosed experimental teeth was still lower than the control teeth. When this relationship was assessed directly, it was found that the probability of ankylosis development in a particular region decreased by 75.5% for every one unit increase in the epithelial cell count. These findings suggest that the epithelial cells may, in fact, provide protection against the development of ankylosis. This function was first suggested by Löe and Waerhaug \(^{35}\), when they found that replanted teeth did not become ankylosed in areas where vital periodontal ligament containing epithelial cells remained. The study by Lindskog et al \(^{36}\) examined this relationship further, finding that the alveolar bone showed local resorption adjacent to tooth root areas where odontogenic epithelium had been implanted. The current study suggests that the presence of epithelial cells may have a protective effect which is proportional to the number of cells, where
the probability of ankylosis is decreased as epithelial cell count increases. On the other hand, it is interesting to note that many experimental teeth registered epithelial cell counts of zero within the furcation, yet did not show signs of ankylosic union. It may be that the relationship between epithelial cells within the periodontal ligament and ankylosis development is more complex, and may involve additional factors other than a simple protective function from the root sheath remnants alone.

Any protective effect of the epithelial cells against ankylosis would appear to be a local one, on the evidence from previous studies as well as the current one. In the study by Lindskog et al. resorption of the alveolar bone away from the implanted odontogenic epithelium occurred in a well-defined bay-like area immediately adjacent to the implanted tissue, thus recreating the normal space between the alveolar bone and epithelial cells. In the current study, epithelial cell clusters were sometimes observed within the periodontal ligament of teeth which were affected by ankylosis. These clusters were located some distance from the region of ankylosic union, in areas where the periodontal ligament retained its normal width and morphology (Figure 27, Figure 28). This was generally outside of the furcation area.

10.4 Nerve fibres

10.4.1 Distribution

The nerve fibre distribution observed in this study is similar to that described in earlier investigations. The majority of the nerve fibres identified in the current study were seen in the outer part of the periodontal ligament, near the alveolar bone, as well as within the middle part of the ligament. This general pattern of nerve fibre distribution was noted in both control and experimental teeth. Nerve fibres were rarely seen in the vicinity of the root surface, and when they were present they consisted of small, fine nerve endings. These fibres are probably involved in nociception, particularly as it is thought that the smaller nerve fibres in the periodontal ligament perform this function.
Maeda et al.\textsuperscript{48} suggested that periodontal sensory nerves (i.e., mechanoreceptors) would be most abundant where the tooth is most movable, thus explaining their finding that the apical part of the ligament was richly supplied with nerve fibres. The current findings supported this in that the periodontal ligament of the furcation region of the rat molar tooth was found to be sparsely innervated. This was particularly noticeable when comparisons were made with the gingival tissue which was richly innervated throughout. Although the current study did not examine the apical area of the periodontal ligament in detail, there did appear to be more nerve fibres along the alveolar bone adjacent to the buccal and palatal roots than within the furcation.

The common finding of nerve and blood vessel associations in this study suggests a functional interrelationship between these structures. This would likely be a regulation of blood flow via signals transmitted to the blood vessels by the associated nerve fibres. Sympathetic innervation is known to be present in the periodontal ligament\textsuperscript{10}, and the function of this innervation is thought to be the control of regional blood flow\textsuperscript{47} as well as regulating cell proliferation during wound healing\textsuperscript{51}. Sensory nerves may also have a function in the control of blood flow within the periodontal ligament\textsuperscript{10}. These interactions between nerves and blood vessels would be particularly important following the tissue damage associated with the experimental intervention used in this study. As discussed earlier, cellular repopulation of the ligament occurs after the initial necrosis, and it would be expected that changes in blood supply would be necessary for this to occur.

\textbf{10.4.2 Experimental intervention}

\textbf{10.4.2.1 Nerve fibre count}

Statistical analysis of the nerve counts revealed that there were significantly more fibres within the furcations of control teeth compared to the experimental teeth. This finding was not surprising in the earlier observation groups, given that there would be decreased cellularity in the periodontal tissue initially as it undergoes
necrosis after the thermal insult. An increase in the nerve fibre counts would then be expected over the longer term during the cellular repopulation of the ligament. However, statistical analysis did not demonstrate a significant relationship between nerve counts and time, which suggests that the expected increase in nerve fibres over time does not in fact occur.

Explanations for this finding include the possibility that the nerve fibres take longer to regenerate following the initial tissue necrosis, so that the longest observation period in this study (28 days) was not sufficient to observe any increase in nerve count back to the normal level. Another possibility is that the nerves within the furcation region of the periodontal ligament do not regenerate to a significant degree following aseptic necrosis, although this would seem improbable. It has been suggested, however, that the epithelial rests may be involved in the development of the innervation of the periodontal ligament. As discussed earlier, the epithelial cells do not appear to regenerate following the experimental intervention, and this may have an influence on the subsequent re-innervation of the periodontal ligament during repair of the experimentally induced tissue necrosis. Whether this proposed effect leads only to a delay in the re-innervation process, or is long term in nature, is unclear from the current data.

10.4.2.2 Distribution of nerve fibres
Comparisons between control and experimental teeth in the current study found that there were significant differences between the sides with regard to nerve fibre count in the middle and root zones of the periodontal ligament. These differences in counts were especially evident in the region of the ligament closest to the root surface. An explanation for these findings may be that the cellular necrosis caused by the thermal insult would be expected to be most severe nearest to the site of application of the cold stimulus. Thus, the nerve fibres near the underside of the crown would have been more affected than those in the middle part of the ligament. By the time the cold stimulus reached the nerves
situated near the bone, it may have been sufficiently dissipated and absorbed within the periodontal ligament tissue so as to prevent extensive damage.

10.5 Root resorption
Significantly more root resorption was observed in experimental teeth compared to control teeth in the present study. This was an expected finding due to the fact that the experimental protocol was adapted from one first developed as a model for the study of resorption. In that study, a 10 minute application of dry ice was found to reliably produce root resorption. Longer and multiple freezing episodes were reported to have had little effect on the resulting area of resorption, although ankylosis was found to develop subsequently in such cases.

Previous authors have described the chronology of root resorption following periodontal ligament hyalinisation associated with orthodontic forces. Hyalinisation was seen one or two days before the onset of resorption, which was initiated between two and seven days after the application of orthodontic force. Dreyer used the hypothermic stimulus to evoke the same tissue reactions as orthodontic forces, and reported a similar time pattern with regard to resorption. In addition, active resorption appeared to reduce or even cease after 14 days when repair of the resorption bays was initiated. Repair of the resorbed areas was complete by 28 days after the thermal insult, regardless of whether a single 10 minute, 20 minute or multiple freezing episodes were used.

In the current study, no time-related interaction was noted with regard to the incidence of resorption, indicating that it did not increase or decrease significantly between 7 and 28 days following thermal insult. However, there did appear to be a greater extent of root surface affected by resorption in the later observation groups compared to the earlier time periods. Additionally, active resorption was observed even in the 28 days observation group, in contrast to the findings of Dreyer. In the present study, repaired resorption was not commonly seen in
the experimental teeth, but was noted in one section taken from the 18 days observation group.

10.5.1 Resorption and epithelial cell count
A statistically significant relationship was found between the epithelial cell count and resorption, which was similar in nature to that described between epithelial cell count and ankylosis. The findings of the present study indicated that the probability of resorption in a particular section decreased by 55% for every one unit increase in epithelial cell count. This suggests that the epithelial cells within the periodontal ligament may have a protective function against root resorption, similar to that proposed against ankylosis. Several other authors have previously proposed such a function. Brice et al.\textsuperscript{39} found evidence of migration of epithelial cells into resorption bays and proposed that they may be involved in the mediation of cementogenesis and subsequent repair of the root surface. This was based on the consistent finding of epithelial cells within repairing resorption bays but not in areas where active resorption was occurring. Also, the ultrastructural appearance of the cells was very similar to the proliferating epithelial cell rests of Malassez described by Yamasaki and Pinero\textsuperscript{28}.

In their study investigating the effects of denervation on the periodontium, Fujiyama et al.\textsuperscript{37} found that along with the reduction in distribution of epithelial rests of Malassez and subsequent development of dentoalveolar ankylosis, root resorption was also activated. These areas of resorption showed signs of active cementum formation and repair approximately 10 weeks later, which corresponded with regeneration of the epithelial cells within the ligament. The authors suggested that these cells may be involved in the promotion of cementogenesis and that they may negatively regulate root resorption.

10.6 Relationship between resorption and ankylosis
Resorption within the furcation region of ankylosed experimental teeth was often seen adjacent to the area of ankylosic union in the present study. This
observation raises the question of whether there is a relationship between the two conditions. Early studies of ankylosis development in replanted teeth reported that root resorption often preceded ankylosis, although it was not a prerequisite stage. The fact that root resorption in the experimental teeth of the present study was usually more widespread than the areas of ankylosis suggests that the thermal insult applied to these teeth may have been sufficient only in causing resorption, but not enough to lead to more widespread ankylosis. Alternatively, the observed resorption may represent an early stage in the process which will eventually culminate in the development of ankylosis. However, the timing of ankylosis development following thermal insult reported in previous studies suggests that this may be unlikely, although areas of ankylosis have been reported after five to seven weeks and ankylosis has been noted to become more widespread between 56 and 86 days.

Dreyer et al suggested that ankylosis following hypothermic insult may occur as a result of an effusive reparative response by cells associated with the surfaces of the bone and tooth. Raghoebar et al proposed that ankylosis may develop as a result of a disturbance in the normal repair process of root resorption. Instead of the appearance of cementoblasts and the formation of cementum, osteoblasts may take over and form osteoid material, leading to ankylosis. However, the ankylosic union observed in the current study was usually associated with an intact cementum surface, indicating that local root resorption had not occurred prior to the ankylosic union. It may be possible that the effusive reparative response described by Dreyer et al could have been occurring as part of the healing of the periodontal ligament tissue following freezing, and that this was independent of any concomitant resorptive processes.

10.7 Relationship between nerve fibres and epithelial cells
Several studies have explored the relationship between nerves and epithelial cell clusters in the periodontal ligament, from both an anatomical and physiologic aspect. Intimate anatomical relationships between nerve fibres and epithelial
clusters have been demonstrated\textsuperscript{27}, and physiologic interdependence has been inferred with regard to epithelial cell maintenance\textsuperscript{38} and development of innervation\textsuperscript{27}. Statistical analysis of the data from the present study, however, did not identify a significant relationship between nerve fibre count and epithelial cell count. This was the case when several associated variables were accounted for in the analysis and also when the counts were assessed directly without adjusting for factors such as side and location within the ligament. This finding was surprising given the evidence suggesting a close relationship between these periodontal ligament components. It may be that the finding was influenced by the statistical power of the current study; therefore examination of a larger number of sections may yield different results. It is also possible that an interrelationship between nerves and epithelial cells may exist over the longer term and as such was not detected within the timeframe of the current study.

10.8 Pulpal changes in experimental teeth

Following application of the cold stimulus, clearly discernable changes were observed within the coronal pulp chambers of the experimental teeth. In the earliest observation group the pulp tissue appeared necrotic, with absence of the odontoblastic layer, significantly decreased cellularity and generalised tissue disorganisation. With time after the experimental intervention, the cellularity of the tissue increased to the point where it approached the level of the corresponding control tooth, although the odontoblastic layer did not reappear at any of the observation periods. This finding indicates that the thermal stimulus used in the current study did not cause necrosis of all cells within the pulps of the experimental teeth and that these remaining viable cells were capable of proliferating. For this to occur, a blood supply must also remain at some point within the pulp system. Precise identification of the cell types in the repopulated pulp chambers was beyond the scope of this study, but it would be interesting to determine whether they were derived from mesenchymal cells or from more specialised survivors of the original pulp tissue.
Together with the cellular changes in the pulp, hard tissue alterations, which became more pronounced over time, were also observed in the experimental teeth. In the earlier observation groups, an amorphous layer was noted on the inner walls of the pulp chamber. This layer was seen to thicken with time, and by the 18 days observation group it was noted that cells had become trapped within the newly formed material, producing an appearance similar to cellular cementum. This reaction was not found in any of the control teeth, and its development suggests some form of reparative or protective response such as that provided by secondary dentine formation. However, the newly formed material seen in this study did not have the structural characteristics of secondary dentine.

10.9 Future research directions
The present study did not identify any changes in epithelial cell and nerve fibre counts with time. However, although these populations are stable over the duration of the current observation period, it can only be speculated as to whether this is the case over the longer term. Further investigations of the epithelial cell and nerve fibre responses over a longer duration may shed light on whether there is some degree of regeneration of these periodontal components, and to assess any interrelationships between the two. This last point would be of particular interest given the evidence for a close association between nerves and epithelial cells in the periodontal ligament. It would also be valuable to determine the role of stem cells within the pulp and the periodontal ligament in any regenerative processes.

Prior investigations of periodontal innervation have shown that the density of nerve fibres is greatest in the apical part of the ligament. Due to this fact, study of nerve and epithelial cell interactions may be facilitated by focusing histologic examination on this region. However, a different experimental intervention would be required, as application of a cold stimulus to the crown of the tooth is unlikely to have a direct effect on the apical tissue. A different thermal insult could be
employed for such a study, including the use of a cryoprobe applied directly to the buccal alveolar bone plate. Procedures of this type have been used previously to examine periodontal healing and have been shown to cause local devitalisation of the bone and the ligament immediately beneath \(^74, 108\). Denervation of the ligament via transection of the inferior alveolar nerve has also been suggested as a means of reducing epithelial cell distribution and inducing ankylosis \(^37\). However, both of these procedures would be time consuming and technically more difficult compared to the dry ice stimulus used in the present study.

In the current study, nerve fibres and epithelial cells were stained individually on separate sections. Although consecutive sections were used to facilitate comparisons, the development of a double-labelling protocol to allow immunostaining of both epithelial cells and nerves within the same section would permit better elucidation of interactions between the two.

Although the current study set out to investigate the relationship between epithelial cells and ankylosis, an interaction was noted with resorption which suggested a protective function of the epithelial cells. To examine this further, future studies could be carried out using a 10 minute freezing time, thus reducing or eliminating the confounding factor of ankylosis.

Lastly, identification of the cytokeratin-positive objects observed in this study would be of interest to determine their significance, if any, in the functioning of the periodontal ligament. Electron microscopic examination should provide sufficient information to allow identification, and perhaps speculate on their function.
11. CONCLUSIONS

The following conclusions can be drawn from the results of the present study:

1. Epithelial rests of Malassez may have a protective function against the development of dentoalveolar ankylosis, proportional to the cell count in any given region.

2. Epithelial rests of Malassez may have a protective function against the development of external root resorption, with a similar proportional relationship to that seen with ankylosis.

3. The freezing technique employed in this study produced a long term reduction in epithelial cell and nerve fibre counts within the periodontal ligament, as well as an increase in the incidence of root resorption.

4. The epithelial cell rests of Malassez do not appear to regenerate after necrosis.

5. The morphologic appearance of dentoalveolar ankylosis produced by freezing changes with time, progressing from fine bony trabeculae to solid bone occupying the whole furcation, then diminishing towards smaller areas of ankylosic union.

6. The freezing technique does not always produce consistent results with regard to the development of ankylosis. In several observation groups fewer teeth were ankylosed than non-ankylosed.

7. No significant relationship appears to exist between epithelial cell and nerve fibre counts, but any potential relationship between these two periodontal components may not be clearly reflected in their cell counts.

8. Cytokeratin AE1/AE3 was an excellent immunostain for the detection of epithelial cell rests of Malassez, whilst PGP 9.5 was technique sensitive, requiring freshly made blocking, secondary antibody and streptavidin solutions to produce optimal results.
9. The null hypothesis that epithelial cell rests of Malassez do not provide a protective function against ankylosis and external root resorption was rejected.

10. The null hypothesis that nerve fibres and epithelial cells are not interdependent was retained.