In Vitro Fusion of Cleft Palate Shelves

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Cleft palate is among the most common congenital malformations of man. One of the problems of the study of cleft palate etiology, and indeed of embryology in general is that many events are occurring simultaneously. It is thus often very difficult or impossible to determine whether events are dependent or independent of each other. According to Fraser (*1*) the process of palatal closure can fail because the head may be too wide to allow the shelves to meet in the midline; the shelves may be too narrow to meet in the midline; the tongue may interfere with closure; the shelves may fail to rotate or come into contact and fail to fuse.

In recent years it has been possible with experimental animals to study the process of palatal fusion alone under *in vitro* conditions. Hence it is possible to determine whether the failure of fusion of shelves was a primary or secondary factor in some types of cleft formation. Moriaty *et al* (*2*) and Konegni *et al* (*3*) showed that the *in vitro* process of rat palatal shelf fusion was sufficiently similar to the *in vivo* process for the *in vitro* technique to be of value in experimental studies. Pourtois (*4, 5*) further extended these studies to describe in detail the properties and stages of rat palatal shelf fusion. He found that the *in vitro* capability to fuse was acquired about thirty hours prior to the normal *in vivo* time of fusion. Shelves cultured prior to this time failed to fuse. Similar results were found in mice by Vargas (*6*).

It has subsequently been shown by Pourtois (*7*) that palatal shelves from cleft lip and palate A/Jax mice have the potentiality to fuse at the normal *in vivo* time of palatal fusion. He postulated that the cleft palate occurred with the cleft lip as the result of either a systemic alteration of the capability to fuse or as a mechanical consequence of cleft lip. He found that in all cases the palatal shelves fused *in vitro*. Hence the shelves had the capability to fuse but failed to do so from the increased width of the face or underdevelopment of shelves associated with cleft lip.

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The purpose of this investigation is to determine whether palatal shelves from cleft animals retain this *in vitro* potentiality to fuse after the normal *in vivo* time of fusion and to investigate the factors influencing this potentiality. Clefts were induced in the experimental animals by amniotic sac puncture. This method of cleft induction was chosen as the cleft results from mechanical interference by the tongue with shelf rotation and fusion (*8, 9*). Hence there was no genetic or teratogenic interference with the shelves themselves so the shelves were essentially normal.

Method and Materials

Experimental Animals. Mature Sprague-Dawley rats were kept on a diet of stock pellets ad libitum under conditions of controlled light and temperature. Each breeding night pairs of female rats were placed in a cage with one male rat at midnight and separated again at 8 A.M. the next day. The assumed time of conception was taken as half way through the breeding period, consequently the conception age of each litter was known to within a maximum of ±4 hours. The day of separation was called day zero. On day fifteen all rats were tested for pregnancy by abdominal palpation.

Amniotic Sac Puncture. Amniotic sac puncture was performed on all rat embryos, excepting the control series, at age fifteen days sixteen hours, 15/16. The technique used was similar to that described by Poswillo (*9*). The pregnant rat was anaesthetized with ether, the abdomen shaved, swabbed and opened under sterile conditions with a single midline incision and the uterus exposed. The uterus and sac were punctured in an avascular site away from the embryo with a 23 gauge needle and amniotic fluid was seen to escape from the sac. The abdomen and skin were closed in separate layers with silk sutures and the pregnant rat was returned to the animal room.

Palatal Shelf Dissections. The palatal shelves were dissected from the embryonic rats at age fifteen days sixteen hours, 15/16, seventeen days sixteen hours, 17/16, and twenty days sixteen hours, 20/16. Those aged 17/16 and 20/16 had been subjected to amniotic puncture at age 15/16, whereas those age 15/16 at time of dissection had not. The same dissection procedure was used on all embryos.

The abdomen was shaved, swabbed with alcohol and the peritoneal cavity opened by a sterile technique. The uterus was removed from the abdomen and placed in a large sterile petri dish. Individual embryos, each in their amniotic sac, were removed from the uterus and placed in separate small sterile petri dishes containing 0.5 ml of sterile Hank's Buffered Salt Solution. The dissection was performed using a dissecting microscope under strict sterile conditions. The head was removed from the body and then the tongue and mandible were removed from the remainder of the face. The brain and cranial vault were then excised

at the level of the eyes and the excess pharyngeal tissue posterior to the shelves removed.

Using microdissection instruments the remaining mid-third of the face is divided at the midline taking care not to damage the edges of either shelf which at this age are close to the midline (Figure 1A). The individual segments then lie with the cheek surface resting on the base of the petri dish with the medial edge of the palatal shelf facing toward the operator. The microdissection knives are then gently placed nasal to the shelves and the tissue cranial to this is removed (Figure 1B). The

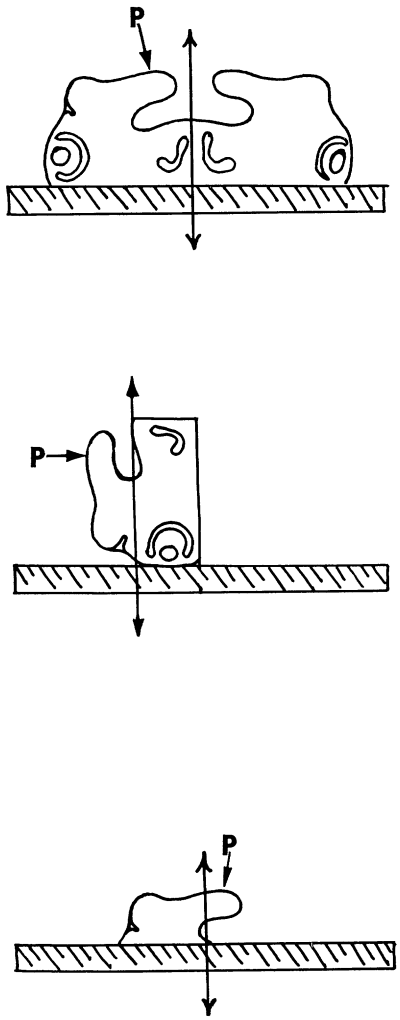

FIGURE 1. A. Cross-section of mid-third of face, arrows showing plane of dissection. P indicates palate. B. Cross-section remaining portion of tissue, arrows showing plane of dissection. P indicates palate. C. Arrows show plane of dissection used to obtain palate, P.

palatal shelf and attached tissue then rests with the nasal surface of the shelf in contact with the base of the petri dish. In this position the shelf is clearly distinguishable and the extraneous tissue is dissected away (Figure 1C).

In vitro Culture. After dissection the palatal shelves were cultured under the same standardized conditions. The technique used NCTC 109 media in an environment of 95% air and 5% CO_2. The dissected shelves were placed with their medial edges in contact on a millipore filter, which in turn rested on a metal grid in a plastic organ culture dish.

The cultures were maintained at 37° ± 0.5°C for 72 hours. After incubation, the preparation still resting on the millipore filter was placed in 4% Peters Buffered formalin for 24 hours, and the 20/16 tissues subsequently decalcified in E.D.T.A. The tissues were then embedded in paraffin, sectioned serially in the frontal plane at 8μ and stained with haematoxylin and eosin. The only departure from standard histologic technique was that all tissues were placed in eosin for two minutes between the 70% and 80% alcohols during dehydration following fixation. This superficial staining greatly facilitated the subsequent orientation of the tissues for sectioning.

Results

In vitro fusion with penetration occurred between all ten pairs of palatal shelves dissected and cultured from normal rat embryos age fifteen days sixteen hours (Figure 2). In this experiment "fused" described the situation where an epithelial barrier remained intact between the mesenchymal tissues or where this barrier had been penetrated by mesenchymal tissue. Hence the term fused is subclassified into laminated and penetrated groups. Each preparation was classified according to the most advanced stage of fusion attained.

Preparations were included in this study when it was evident that they had been placed in correct orientation with the medial edges of the palatal shelves together. In some preparations, which were not used in this study, it was seen that the shelves had been placed with the medial edge of one shelf in contact with the cut lateral edge of the other shelf. This error was easy to make as the shelves are homogeneous and small, each measuring about 2 × 1 × 0.5 millimeters. It is of interest to note that in these instances although there was only a single layer of epithelium between the two masses of mesenchyme, no breakdown of the epithelium with fusion occurred (Figure 3). These tissues were healthy and were cultured under exactly the same conditions as those tissues which were correctly oriented in which fusion occurred.

Embryos which had been subjected to amniotic sac puncture at age fifteen days sixteen hours, 15/16, were obtained at age seventeen days sixteen hours, 17/16. Some of these embryos were non-viable and resorbed, some apparently normal and some had cleft palates. It was

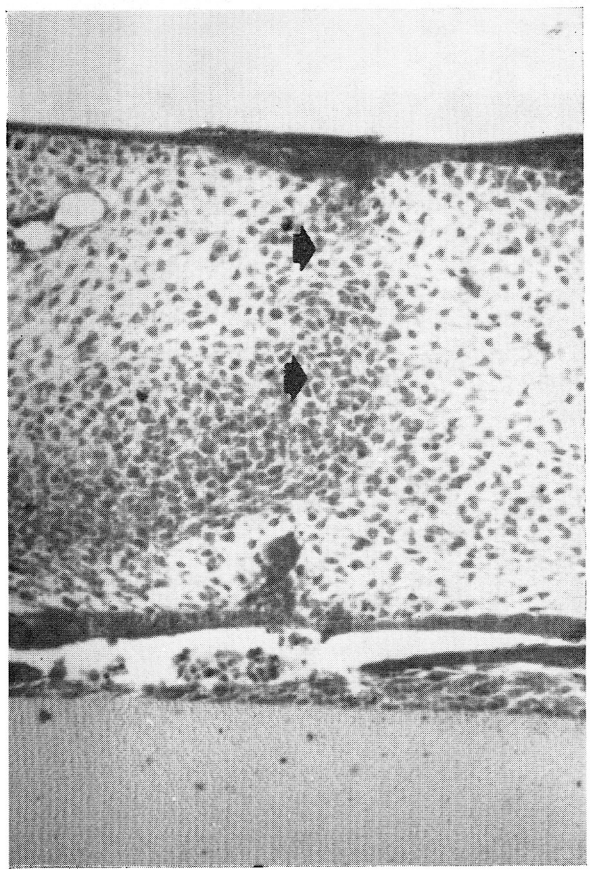

FIGURE 2. Normal 15/16 palatal shelves grown in vitro for 72 hrs. Arrows show area of midline fusion. × 80 H & E.

noted that most viable animals with an intact palate had an abnormally high palatal vault. Palatal shelves were dissected from those viable embryos with complete cleft of the secondary palate. It was found that of the ten preparations, which met the criteria of correct orientation, nine were fused and one was not fused. Of the fused group, eight were with penetration and one with lamination (Figures 4 & 5).

Embryos which had been subjected to amniotic sac puncture at age fifteen days sixteen hours, 15/16, were obtained at age twenty days and sixteen hours, 20/16. A similar range of resorptions, cleft palate and apparently normal embryos were found. Palatal shelves were dissected and cultured from the cleft embryos. These preparations were larger than those from the 17/16 embryos, although the actual shelf itself was of similar size, the palatal shelves of the cleft animals did not appear to have increased in size at a similar rate to the remainder of the embryo. None of the ten 20/16 preparations which were correctly

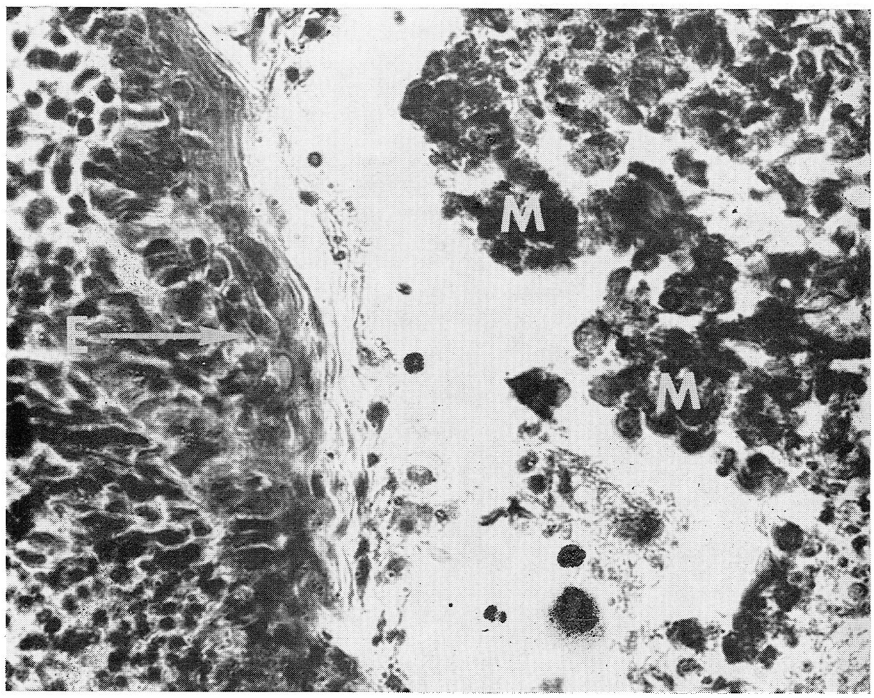

FIGURE 3. Normal rat palates aged 15/16 cultured for 72 hrs. with epithelial surface, E, in contact with cut mesenchymal surface, M. No evidence of epithelial breakdown is present. × 180.

oriented fused. To determine whether the length of time of culture had anything to do with this failure of fusion, five pairs of shelves were cultured for nine days. None of this group fused.

Microscopically palatal shelves from embryos age 15/16 are composed of an undifferentiated cellular mesenchyme covered by a thin, one-or two-cell epithelial layer. The dental lamina and tooth buds are present at the lateral edges of the shelves. Subsequent to *in vitro* culture for 72 hours there is increased differentiation and organization of the mesenchymal tissue but without bone formation. The covering epithelium remains thin and the dental structures continue to differentiate. The first stage of fusion is a lamination of the covering epithelium into an epithelial barrier between the two separate masses of mesenchyme. Subsequently the distinct lamination is lost. The epithelial barrier then appears to be disrupted and the adjacent mesenchyme intermingles with that of the opposing shelf.

At age 17/16 the palatal shelves have normally fused, the *in vivo* time of fusion being at about sixteen days twelve hours. Ossification has commenced in the lateral margins of the palate and the oral surface is covered by a thin non-keratinized epithelium. In the cleft embryos,

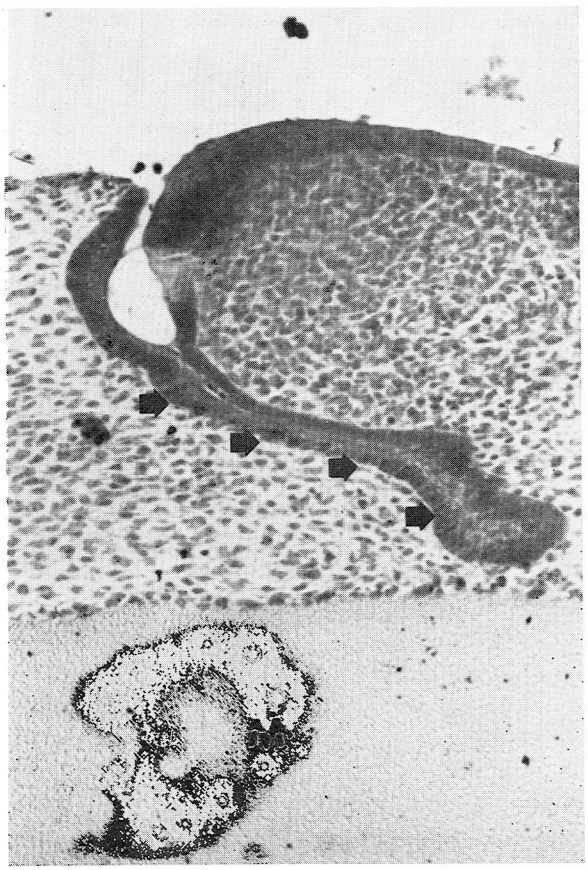

FIGURE 4. Cleft shelves from 17/16 embryo, cultured for 72 hrs. Arrows show area of laminated fusion. × 80.

age 17/16, ossification has commenced in the mesenchyme of the shelves which have a very slight amount of keratinization of the covering epithelium. After *in vitro* culture for 72 hours of cleft shelves from embryos, age 17/16, it was noted that the ossification of the mesenchyme had continued, however ossification had not occurred near the midline at the area of fusion. The covering epithelium was keratinized. The process of fusion was similar to that described for the fusion of normal 15/16 palatal shelves excepting that the epithelial barrier was wider and more persistent.

In normal 20/16 embryos the palate has a continuous layer of bone across the midline. The oral surface of the palatal vault is covered by a slightly keratinized, stratified squamous epithelium. The cleft shelves are small as compared to the alveolus, have a central core of bone and are covered by a stratified squamous epithelium which is heavily

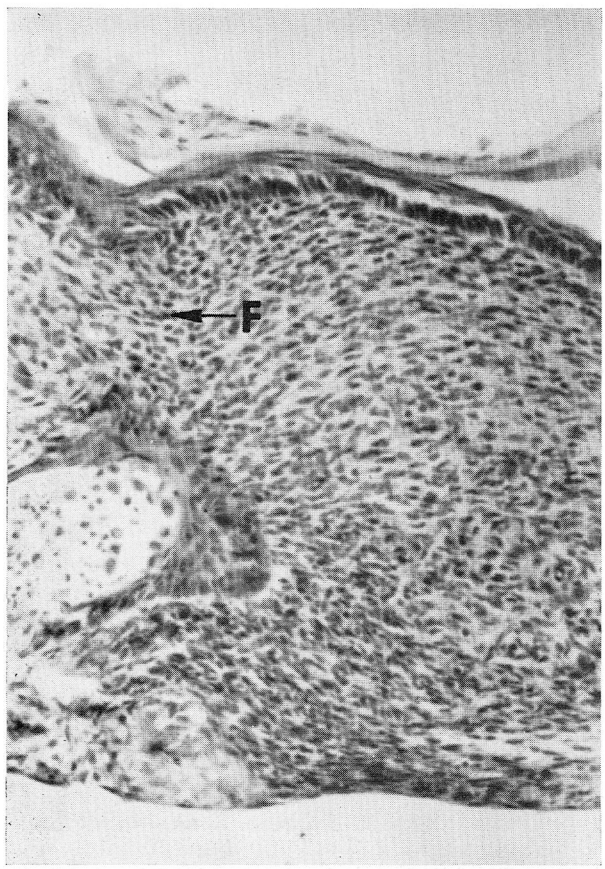

FIGURE 5. Cleft shelves from 17/16 embryo, cultured for 72 hrs. F shows area of mid-line fusion. × 80.

keratinized (Figure 6). The cleft shelves which had been cultured for nine days were similar to those which had been cultured for 72 hours.

All the cultured tissues in this study showed continued differentiation and formation of specialized tissues such as tooth buds and bone (Figure 7). There was no evidence of cellular degeneration or pyknosis.

Discussion

Successful in vivo palatal closure requires harmonious integration of a number of different growth and developmental factors. Too little or too much growth at a number of sites in the facial complex will result in palatal cleft. It is not known whether in the event of a growth disharmony resulting in failure of the shelves to come into apposition a subsequent compensation with shelf contact may allow for successful palatal closure.

This study shows that cleft shelves from the rat embryo maintain the capability to fuse *in vitro* for at least twenty four hours after the

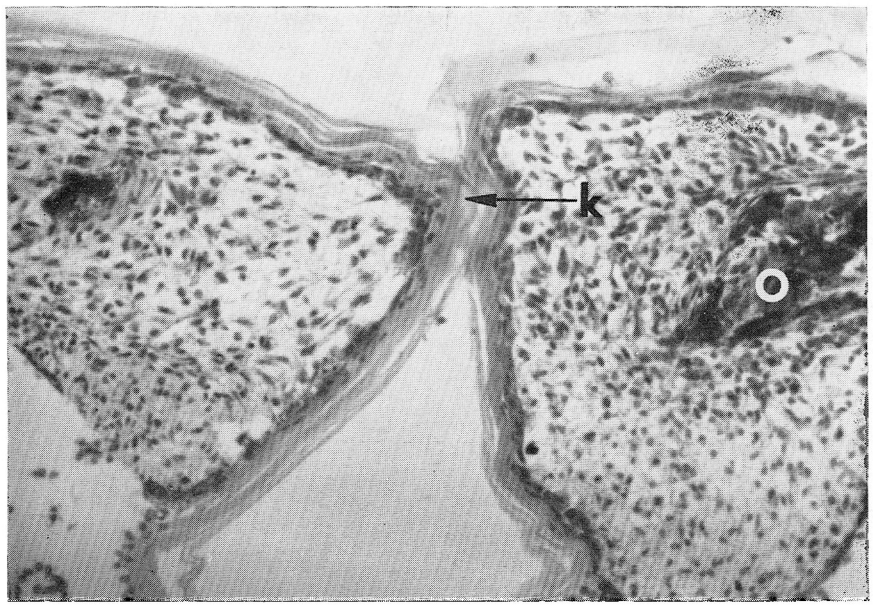

FIGURE 6. Cleft shelves from 20/16 embryo, cultured for 72 hrs. K shows fairly heavy keratinization, O shows area of ossification.

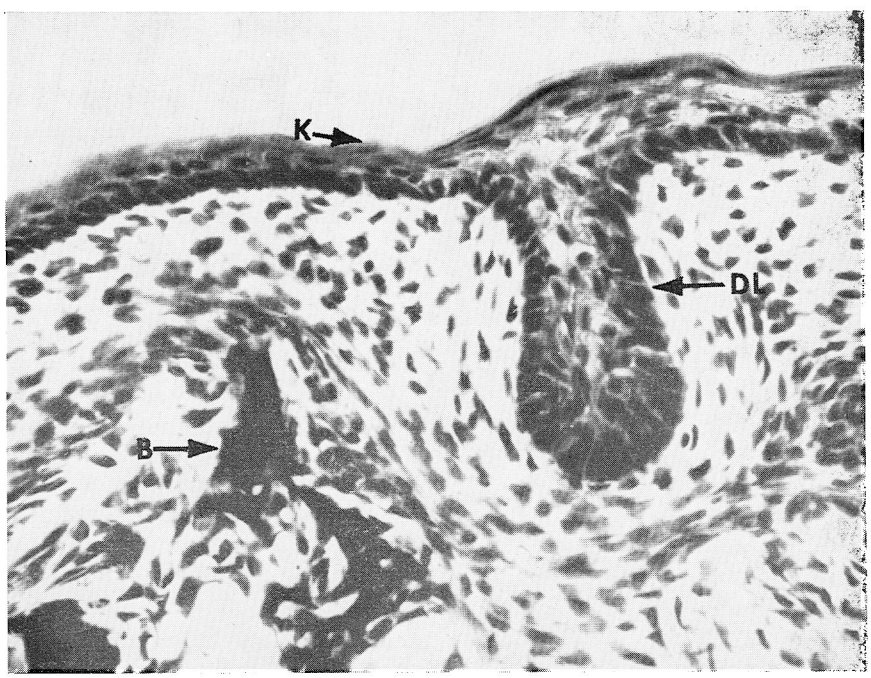

FIGURE 7. Lateral portion of cleft shelf from 17/16 embryo, cultured for 72 hrs. K shows slight amount of keratinization, DL shows the developing dental lamina, and B shows area of bone formation.× 160.

normal time of *in vivo* fusion. However this capability to fuse is lost prior to ninety-six hours after the normal *in vivo* fusion time. These findings, coupled with those of Pourtois (*4*) concerning the *in vitro* potentiality of palatal shelves to fuse prior to the normal *in vivo* time of fusion, show that in the rat there is a period of at least sixty hours in which palatal fusion will occur should the shelves be in contact. Both before and after this period the shelves fail to fuse *in vitro* even if they are maintained in contact.

The tissues involved in fusion from both normal 15/16 and cleft 17/16 are similar in that the covering epithelium is thin with that of the cleft 17/16 being only slightly keratinized. The underlying mesenchyme is undifferentiated although in cleft 17/16 shelves bone formation has commenced. This bone formation in the lateral edges of the shelves is some distance from the site of fusion. The cleft 20/16 palatal shelves are different in that they contain well differentiated mesenchymal tissue with bone formation close to the site of fusion. The epithelium has differentiated into a keratinized, stratified squamous type. It is not known whether the failure of fusion was caused by the mechanical presence of the keratinization, or from a change in behavior of the more specialized epithelium or mesenchyme. Certainly for fusion to occur an interrelationship is required between two epithelial covered mesenchymal tissues, for there is failure of fusion *in vitro* when a single epithelial covered piece of mesenchymal tissue is placed against bare mesenchyme. The nature of this relationship is at present unknown.

In this study it was not possible to ascertain the influence of the differentiating mesenchyme on the fusion process. Previous studies by Bodner, Goss and Avery (*10*) suggest that the degree of fusion is approximately inversely proportional to the stage of differentiation of the mesenchymal tissue in the area of fusion.

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