THE BIOCOMPATIBILITY OF SURGICEL® AND BONE WAX®

Aspects of *In Vivo* Evaluation.

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To my parents,

with love and gratitude.

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PRECIS

Surgicel and Bone Wax are local haemostatic agents used in most branches of surgery including Oral Surgery. Surgicel is manufactured from cellulose, and is, therefore, of plant origin. Bone Wax is composed of 70% beeswax, the other 30% comprising olive oil and phenol.

The objectives of the present investigation were:

(1) To evaluate the biological effects of Surgicel and Bone Wax on rat striated muscle and bone,including haemopoietic bone marrow. Special attention was paid to the absorbability or non-absorbability of the test materials. In addition, an attempt was made to delineate the role of macrophages in the absorption of the implant materials by careful examination of those cells in close proximity to the test materials.

(2) To develop an experimental model by which the biocompatibility of implantable local haemostatic agents could be studied in vivo.

Forty-eight young male Sprague-Dawley Albino rats were used as the experimental animals. Teflon tubes, containing the test materials, were implanted in the muscles of the anterior chest wall and marrow space of the femurs of these rats. The animals were sacrificed at time periods ranging from two days to twenty-six weeks post-implantation. All the specimens obtained were processed for qualitative histologic examination. In addition, most of the soft tissue specimens were examined quantitatively.

The results of this investigation revealed that:

(1) Surgicel is an absorbable local haemostatic agent. It is absorbed from rat striated muscle within two weeks after placement into this tissue, and within four weeks after implantation into haemopoietic marrow. The absence of overt signs of inflammation occasioned by the implantation of Surgicel, and its complete absorption from the tissues into which it was implanted, indicate that, in rat tissues, it is a highly compatible material.

Vacuolated (foamy) macrophages were consistently seen in close relation to Surgicel. This finding strongly suggested that these cells could, in part, have played a role in the absorption of this implant material.

(2) Bone Wax is a non-absorbable local haemostatic agent. When implanted in rat striated muscle, Bone Wax becomes isolated from the surrounding tissues by a dense fibrous capsule twelve weeks after placement into this tissue. In bone, the wax becomes isolated from haemopoietic marrow by a distinct layer of woven bone by eight weeks post-implantation. The absence of overt signs of inflammation to implanted Bone Wax, and its isolation by a fibrous capsule and woven bone in muscle and haemopoietic marrow respectively, indicate that this implant material is compatible with rat tissues.

Vacuolated (foamy) macrophages were consistently seen at the Bone Wax-tissue interface, indicating continued phagocytic activity by these cells.

(3) The experimental model used in this investigation, appears to be suitable for evaluation of the biocompatibility of implantable local haemostatic agents in vivo.

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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 obtaining the Honours Degree of Bachelor of Science in Dentistry in 1979.

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.

V. GARACH.

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INTRODUCTION

Since ancient times, local haemostasis has played an important role in the control of haemorrhage following surgery or trauma (Evans:1977). Not infrequently, excessive bleeding is observed during dental or oral surgical procedures, as well as during the recovery period. In patients where excessive or uncontrollable haemorrhage is encountered, careful haematological evaluation should be undertaken in order to include or exclude the possibility of an underlying bleeding diathesis. In cases of excessive blood loss, due either to the presence or absence of a dysfunction of the haemostatic mechanism, the use of local styptic remedies forms an essential adjunct in the management of bleeding.

Over the years, a variety of techniques have been developed to aid haemostasis locally (Table 1).

TABLE 1: LIST OF LOCAL HAEMOSTATIC TECHNIQUES

1. Pressure (Lucas: 1966; Evans: 1977);
2. Temperature, e.g. cold packs (Lucas: 1966; Evans: 1977);
3. Cautery (Lucas: 1966; Shephard: 1976; Evans: 1977);
4. Vasoconstrictors, e.g. adrenalin (Lucas: 1966; Evans: 1977);
5. Caustics, e.g. silver nitrate (Lucas: 1966; Evans: 1977);
6. Adhesives, e.g. butyl cyanoacrylate (Bhaskar et al: 1967; Howard et al: 1973; Besserman: 1977);
7. Biological agents, e.g. Topical Thrombin (Lucas: 1966; Evans: 1977);
8. Implantable local haemostatic agents:
   (a) Gelfoam® (Correl et al: 1945; Correl and Wise: 1945; Light and Prentice: 1945a; 1945b; Guralnick and Berg: 1948; Benoit and Hunt: 1976; Evans: 1977);
   (b) Microfibrillar Collagen Haemostat (Benoit and Hunt: 1976; Mason and Read: 1976; Zucker and Mason: 1976; Evans: 1977);
   (c) Oxidized Cellulose (Frantz: 1943, Frantz et al: 1944; Frantz: 1945; Buchman and Blair: 1947; Gwinn et al: 1948; Sutherland: 1948; Gottlieb: 1949; Otenasek and Otenasek Jr.: 1968; Evans: 1977);
   (d) Starch Sponge (Lattes and Frantz: 1945; Korchin: 1956);
   (e) Absele® (Harris and Capperauld: 1978);
   (f) Surgicel®
   (g) Bone Wax®

There is widespread use of many implantable local haemostatic agents (as listed in Table 1) in millions of human patients, and a likely potential for future exponential increase in their use. It is surprising that there is little objective data available in the literature detailing the effects of these agents on host tissues, and how they should be studied in order to determine their biocompatibility.

In the present study, the biological effects of Surgicel® (Oxidized Regenerated Cellulose) and Bone Wax® were investigated. It was also the aim of this investigation to develop an experimental model by which the biocompatibility of implantable local haemostatic agents could be studied in vivo in the future.

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CHAPTER 1

SURGICEL AND BONE WAX

There are several local haemostatic agents available on the market (Table 2).

TABLE 2 : LIST OF LOCAL HAEMOSTATIC AGENTS

1.	Surgicel® (Oxidized Regenerated Cellulose)
2.	Bone Wax®
3.	Gelfoam®
4.	Avitene® (Microfibrillar Collagen Haemostat)
5.	Oxycel® (Oxidized Cellulose)
6.	Topical Thrombin®
7.	Cyanoacrylate Spray
8.	Absele®

Since Surgicel and Bone Wax form the basis of the present study, only these materials will be reviewed.

SURGICEL (OXIDIZED REGENERATED CELLULOSE)

Surgicel is a local haemostatic agent that is prepared from a pure form of ∝-cellulose (Degenshein et al: 1963). Cellulose itself is extensively found in the plant kingdom and it forms a protective coat around plant cells (Evans et al: 1980a). It is, unquestionably, the most abundant organic compound in the world, constituting 50% or more of all carbon in vegetation (White et al: 1978).

® Registered Trade Name.

Basically, cellulose is a carbohydrate (Evans et al: 1980b) with a molecular weight of between 50,000 and 400,000. It comprises roughly 300 to 2,500 glucose residues per molecule (White et al: 1978), and the molecular chain does not exhibit branching (Evans et al: 1980b). The molecular structure of cellulose is illustrated schematically in Figure 1. The molecule consists of glucose residues joined together by β-1,4 glucosidic linkages.

Surgicel is prepared by dissolving the cellulose in an alkalinized organic solvent. This solution is then forced through spinnerets into an acid bath, where the cellulose is regenerated as a continuous fibre which is knitted into a gauze, and then oxidized (Degenshein et al: 1963). In the process of oxidation, the alcohol radicals on carbon atom 6 (C6) of the cellulose molecule are oxidized to carboxyl groups (COOH), and are carefully purged of undesirable by-products by subsequent neutralization and washing (Lebendiger et al: 1960).

The fundamental unit of the Surgicel is termed polyanhydroglucuronic acid. It comprises molecules of β-glucose and α-glucose, joined by a β-1,4 glucosidic linkage, in which the primary alcohol groups of carbon atom 6 (C6) have been oxidized to a carboxyl group (Figure 2).

Figure 1 : Schematic diagram of the molecular structure of cellulose. The encircled figures represent the number of the carbon atom in the molecule.

Figure 2 : Schematic diagram of the molecular structure of polyanhydroglucuronic acid. The encircled figures represent the number of the carbon atom in the molecule.

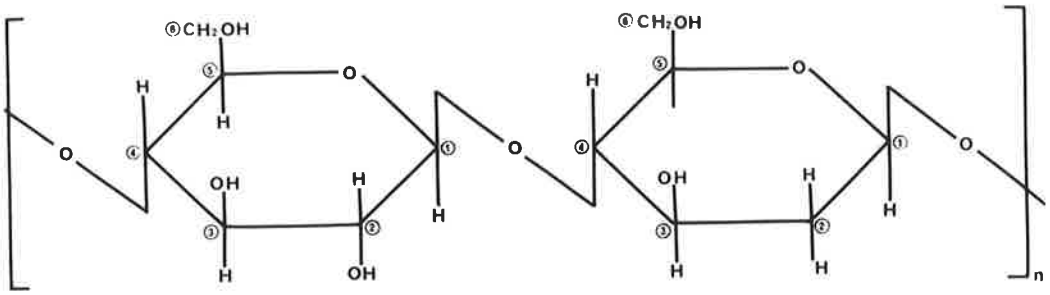

Figure 1

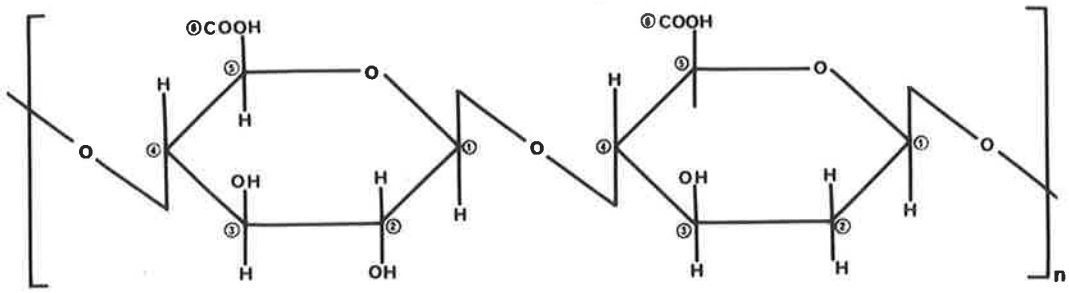

Figure 2

By this process, the degree of oxidation is 20%, and this can be achieved as the material is of uniform molecular weight and the fibres are of uniform diameter (Hurwitt et al: 1960).

The material itself is a shiny gauze (Degenshein et al: 1963) and the special characteristics which make this product useful as an absorbable haemostatic agent arise from the fact that the regenerated cellulose is oxidized to the true chemical compound, polyanhydroglucuronic acid, which, in addition to having styptic properties, is soluble in weak alkalis, and can be hydrolysed by living tissues (Hurwitt et al: 1960; Shea: 1962; Degenshein et al: 1963). Such chemical degradation, accomplished by enzymes of the carbohydrase system, contributes to the ease with which the material is absorbed in the body tissues and the reportedly minimal degree of tissue response occasioned by its implantation (Hurwitt et al: 1960; Shea: 1962).

When exposed to blood, either in vitro or under surgical conditions, the material, which has a pH of 3.5 to 4.5 (Hurwitt et al: 1960, Georgiade et al: 1961), turns very dark brown or black due to the formation of the acid, haematin (Hurwitt et al: 1960; Miller et al: 1961a Degenshein et al: 1963). The acidic nature of the material partly produces haemostasis by causing local blood vessel contraction and, as well, initiates local fibrin deposition (Degenshein et al. 1963).

In additon, haemostasis very probably depends, in part, upon the marked affinity of polyanhydroglucuronic acid for haemoglobin and other blood proteins (Hurwitt et al: 1960; Georgiade et al: 1961). According to Miller et al. (1961b), the haemoglobin is freed from the

red blood cells by the acidity produced by the gauze. When Surgicel combines with acid haematin, it becomes firmly gelatinous and adheres to the surrounding tissues (Blau et al: 1960; Degenshein et al: 1963). The basic factor necessary for the fibres to hydrate and form a gel is a slightly alkaline fluid. The formation of a salt of cellulose, by its union with calcium, also enhances the degree of gelation. (Miller et al: 1961b).

In vitro, the changes in hydrogen ion concentration produced by Surgicel will denature fibrinogen, although not irreversibly. In vivo, the lowered pH produced by the gauze is slight, local and transitory, and does not impair clotting of blood as demonstrated by the test using the gauze in freshly drawn blood. The acidic groups of the gauze apparently are rapidly neutralized by the excellent buffer systems of the blood. Red blood cells, platelets and fibrinogen are not necessary for the gauze to form a gel (Miller et al: 1961b), and thus, it does not enter into the normal clotting mechanism (Evans: 1977).

It is, therefore, effective in haemophilia and thrombocytopenia, and its action is not enhanced by the addition of thrombin (Hurwitt et al: 1960). However, Lucas (1966) refutes this claim and states that the action of Surgicel is enhanced by the topical application of thrombin. According to Miller et al. (1961a), Surgicel effects haemostasis within two minutes.

Hurwitt et al. (1960) implanted Surgicel in the abdominal subcutaneous tissues of rats. One hundred and sixty samples of the fabric were implanted,and an autopsy was performed upon each animal. In the majority of cases, the animals were sacrificed on the seventh and fourteenth, or fifteenth day post-operatively. In order to observe very early tissue responses, a limited number of rats were sacrificed at 1, 3, 5 and 7 days post-implantation. Tissues recovered from the implantation sites were stained with Haematoxylin and Eosin, Van Gieson's stain and Texchrome*. They evaluated their results histologically and their findings were as follows:

At day 1 post-implantation, the channel in the subcutaneous tissue, into which the pledget of Surgicel had been introduced, contained a considerable amount of translucent, viscous fluid, intermingled with bits of gelatinous material. Nothing resembling the original fabric was observed. Tissue sections stained with Texchrome revealed fragments of amorphous,lavender-coloured material associated with oedema, but showing little evidence of a cellular response.

At day 3 post-implantation, the subcutaneous channel was no longer apparent but the implant material could still be recognized macroscopically as a soft gelatinous mass. Microscopically, the cellular response was moderate and consisted mainly of large macrophages.

* A commercial dye used for the identification of fabrics.

At day 5 post-implantation, there was, macroscopically, a significant reduction in size of the gelatinous mass. Microscopically, the degree of oedema was lessened and evidence of fibroplasia was apparent.

At day 7 post-implantation, the mass bore no resemblance to the original fabric, but consisted, macroscopically, of an off-pink to white, soft, gelatinous material. Microscopically, the reaction was characterized by active fibroplasia with moderate numbers of macrophages. When stained with Haematoxylin and Eosin, the residue appeared as an amorphous eosinophilic mass, invaded by proliferating fibroblasts and large macrophages frequently containing pigment.

At day 15 post-operatively, the implant material was not identifiable microscopically. Fibroblasts predominated, and only a few mononuclear phagocytes were seen.

Surgicel was also implanted in the liver, kidney and brain of dogs (Hurwitt et al: 1960). Specimens from the experimental animals were examined clinically and histologically at periods varying from twenty-one to sixty-two days post-implantation. According to these authors, Surgicel caused no adverse effects at either the systemic or local level. In most of the animals, the gauze was not identifiable either grossly or microscopically at the post-operative observational time periods mentioned above. However, in one of the experiments, where Surgicel was implanted in the kidney of the dog, the fabric was still identifiable at thirty days post-operatively.

Hurwitt et al.(1960) also cited clinical situations in which Surgicel was used in over three hundred cases. According to these authors, the gauze was effective in the control of troublesome bleeding or oozing from raw surfaces, where no blood vessels could be clamped or controlled with electrocautery, and, in some instances, its use was considered to be life-saving.

Blau et al.(1960) used Surgicel as a post-operative dressing in various dermatological procedures, namely, dermabrasion, punch biopsy, excision biopsy, curettage and in finger- and toenail removal. Apart from controlling haemorrhage from its sites of application, they stated that the gauze had the property of being completely reabsorbed, and that frequent dressing of the wounds, following its placement, was not necessary.

Jantet and Rob (1960) implanted Surgicel into various implantation sites in the rat, namely, the abdominal subcutaneous tissues, intraperitoneally in the right iliac fossa, intramuscularly in the rectus abdominis muscle and in the liver. According to the authors, the gauze was rapidly absorbed from its sites of implantation - within five days from the subcutaneous tissues, muscle and the peritoneal cavity, but not until about two weeks, from the liver. The possible mechanism, according to Jantet and Rob (1960), by which Surgicel may have been resorbed from its sites of implantation, was by phagocytosis. This deduction was based on the fact that particles of the implant material were discernible within macrophages. Jantet and Rob (1960) also reported the successful use of Surgicel in various clinical procedures, examples of which were partial hepatectomy and partial nephrectomy, to which no untoward local reactions were reported.

Georgiade et al. (1961) performed 50 extractions on rats and packed the sockets with Surgicel. The animals were sacrificed at 3 day intervals, and sections obtained were examined histologically. The material appeared to be completely resorbed in 21 days, with no foreign-body reaction. The Surgicel was then used in patients for a variety of oral surgical procedures and no untoward reactions were noted.

Miller and Thomas (1961) used Surgicel on 14 mongrel dogs. They divided their animals into 3 groups:

Group Number	Number of Dogs	Type of Operation
1	6	Incision of left lobe of liver
2	6	Incision of spleen
3	2	Incision of abdominal wall

Groups 1 and 3 were given one million units of penicillin G post-operatively. Their histologic results were as follows:

Group 1

At 3 days, the liver showed a large defect with clotted blood and hyaline and fibrillar material. A small number of polymorphonuclear leukocytes were scattered throughout the area, and a thin layer of connective tissue separated this region from the cells of the liver. The dividing layer had a moderate number of polymorphonuclear leukocytes and lymphocytes scattered throughout it. Plasma cells and macrophages were also present, but giant cells were not seen.

At 10 days post-operatively, in addition to the picture at 3 days, giant cells were seen.

At 17 days post-operatively, the defect contained a clot which was separated from the cells of the liver by a narrow band of connective tissue. This area was infiltrated focally by lymphocytes and plasma cells.

Group 2

In this group, absorption of the gauze from the wounds of the spleen was more rapid than from the liver. At 10 days post-operatively, the clot had macroscopically disappeared.

The microscopic section of the spleen at 10 days revealed a wound extending deeply into the pulp. The central portion of the region was filled with a blood clot and hyaline, fibrillar material. A narrow zone of connective tissue containing leukocytes and lymphocytes separated this area from the cells of the spleen. Plasma cells and a few scattered giant cells were also present. In some regions, numerous macrophages, containing haemosiderin, were seen.

Group 3

In this group, no macroscopic or microscopic evidence of Surgicel was seen at 64 days, and no inflammation was noted.

Shea (1962) used Surgicel as a skin dressing to donor sites on 47 patients, in addition to the application of a non-adherent rayon mesh. Haemostasis was effected in 4 to 10 minutes. He concluded the following regarding the benefits of Surgicel:

(a) It prevented the excessive loss of blood;

(b) A moist wound was avoided;

(c) There was reduction in bacterial proliferation;

(d) Aftercare was minimised;

(e) There was a reduction of mobility, allowing effective re-epithelialization.

Degenshein et al. (1963) performed a partial thyroidectomy on mongrel dogs in which an area of 0.5cm was left oozing. Surgicel was effective in controlling the ooze when properly applied. Portions of the liver and spleen were also resected by these investigators and haemostasis was secured safely with the gauze. In one case, a hepatic lobectomy was performed and bleeding was controlled with three layers of the gauze as the sole method of haemostasis.

The above authors also used the gauze in a variety of clinical situations, including skin bleeding, breast surgery, head and neck surgery and surgery of the cardiovascular and pulmonary systems. They found Surgicel to be effective in the control of a sanguinous ooze. In order for the gauze to be used effectively, it had to be applied to a dry surface under pressure. If oozing of blood persisted, they recommended the application of an additional layer of the gauze.

Tierney (1964) used Surgicel in a series of ninety prostatectomies in humans. After the prostate gland was enucleated, a Foley catheter was inserted. Surgicel gauze was placed over the raw prostatic bed, and inflation of the bulb of the catheter held the gauze firmly in place. According to the results of Tierney (1964), post-operative bleeding was less than usually encountered. There was a considerable decrease in the need for irrigation of the catheters, and it was thought that the latter may have been responsible for the observed lower incidence of post-operative bladder spasm.

Cytoscopic examinations were made on thirty patients over a period ranging from three to six months post-operatively. No traces of Surgicel fibres were seen in any of the implantation sites (Tierney: 1964).

Skoog (1967) conducted experimental animal studies on rabbits to investigate the effects of Surgicel on bone healing. In this study, the fronto-nasal bones of the animals were exposed through a midline incision, which was then carried down to the periosteum. A subperiosteal pocket, measuring 7mm by 25mm, was created and packed with Surgicel. The periosteum was sutured with 6/0 catgut sutures and the skin with 4/0 mersilene. The animals were sacrificed at eight, fourteen and twenty-eight days post-operatively, and tissue specimens were processed and examined histologically. His results revealed the following:

At day 8 post-operatively, Surgicel took on a fibrillar appearance, and it was invaded by a massive proliferation of osteoblasts and fine vessels.

At day 14 post-operatively, Surgicel appeared in the form of an amorphous mass, surrounded by spicules of newly-formed woven bone.

At day 28 post-operatively, there was no microscopic evidence of the implant material. The area originally occupied by Surgicel was filled with newly-formed subperiosteal bone.

Skoog (1967) concluded from his study that Surgicel could be used as a subperiosteal implant, and that it aided in the formation of bone at its site of implantation. He subsequently used Surgicel in the repair of maxillary clefts in humans, and concluded from his results that the gauze was able to control the quantity and shape of new bone.

Huggins (1969) used Surgicel in various otorhinolaryngologic procedures in humans. The Surgicel was used to temporarily control bleeding and then removed to expose the area to the air for healing, or it was left in place over raw weeping or oozing areas. It was also used as a packing in the ear canal to keep grafts in place. According to Huggins (1969), there were no post-operative problems such as delayed healing, foreign-body reactions or serious discomfort to the patient.

Thilander and Stenström (1969) implanted Ivalon, Surgicel and Collagen Chips in the lower part of the premaxillo-maxillary suture of guinea pigs. A defect, measuring 3mm by 3mm, was made along the exposed suture using a bur, and the implant materials were inserted into this defect. An unfilled defect served as a control. The experimental animals were decapitated after one week, two weeks,

one month, two months and four months post-operatively, and the specimens obtained were prepared for histologic examination.

Their results indicated that, even after four months post-operatively, there was still microscopic evidence of Ivalon and Collagen Chips in the implantation sites. The implanted Surgicel had completely disappeared by this time, and the defect, into which it was placed, was filled with new bone. Thilander and Stenström (1969) concluded, from this study, that Surgicel had a beneficial effect on bone healing as opposed to Ivalon and Collagen Chips, which appeared to retard bone healing.

Engdahl (1972) implanted Surgicel in rabbits that had undergone a unilateral maxillary resection. The implant material was placed in the subperiosteal space created by the resection. Their results indicated that bone formation was delayed and incomplete, and that normal anatomy was rarely fully restored where the Surgicel was implanted,as compared with the control animals, in which no Surgicel was placed in the area of resection.

Rosenquist and Finne (1974) implanted Surgicel and fibrin foam in mandibular and tibial bone cavities of adult rabbits. An equal number of cavities, filled with blood clot, served as controls. The cavities were created using a dental bur. The effects that the implanted local haemostatic agents had on bone healing were studied by microradiography. Tissue specimens were examined in this way at two and four weeks post-implantation.

Their results indicated that, at two weeks post-implantation, both Surgicel and fibrin foam delayed healing of bone to an equal extent, as compared with the control series. At four weeks post-implantation, it was apparent, from the microradiographs, that Surgicel delayed bone healing to a lesser extent than did fibrin foam. Complete healing of the cortical defect was noted in 64% of the specimens implanted with Surgicel, whereas the figure was only 25% in specimens treated with fibrin foam. They concluded, from their study, that both Surgicel and fibrin foam were of no value in stimulating bone formation but, instead, appeared to delay the process somewhat.

Larsson et al (1978) studied the effects of Surgicel in the prevention of peritoneal adhesions in rats. In this investigation, forty animals were laparotomized and subjected to standardized caecal trauma. In half the number of rats, the injured caecum was embedded in Surgicel. In the remaining twenty rats, which served as controls, the caecum was left uncovered. The animals were sacrificed fourteen days post-operatively, and examined for peritoneal adhesions. Adhesions were found to be significantly less common in the group treated with Surgicel (1 in 20 for the Surgicel-treated specimens compared with 18 in 20 for the control specimens).

In a study by Uddströmer (1978), a total of 117 operations were performed on the tibia and skulls of 92 growing rabbits. A defect, accommodating a Teflon cup, was made in the bone. The cups were filled either with blood, haemopoietic marrow or Surgicel. The overlying periosteum was re-sutured over the Teflon cups. The rabbits were sacrificed two to fifteen weeks post-operatively, and some of the specimens were examined histologically. In the remaining specimens,

bone, that was produced in the Teflon cups, was ashed in a furnace. The amount of calcium present in the ashed sample was determined by spectrophotometry.

The amount of calcium produced in the cups filled with Surgicel was found to be one-third of that produced in the cups filled with blood and haemopoietic marrow. Uddströmer (1978), therefore, demonstrated quantitatively that Surgicel strongly retarded new bone formation.

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BONE WAX

On August 18th, 1891, a patient, who had been kicked on his left ear, was admitted to hospital. He had been suffering from rigors, vomiting, restlessness and a fever of 105°F. He was diagnosed as having a thrombophlebitis of his "jugular" vein. At operation, the named vein was ligated and the thrombus excised. Bone Wax was successfully used to control post-operative bleeding.

Parker (1892) published this case in the British Medical Journal, since it marked the first milestone in the use of Bone Wax as a local haemostatic agent. Parker was, however, uncertain of the composition of the wax, and stated this fact in his clinical report. Victor Horsley (1892) replied to his query in the following letter:

> "Sir, - The antiseptic wax which Mr. Rushton Parker refers to on page 1076 of the BRITISH MEDICAL JOURNAL of May 21st has the following composition: beeswax, 7 parts; almond oil, 1 part; salicylic acid, 1 per cent. It is the outcome of experiments made in 1885, when, remembering the practice of Magendie and others at the commencement of the century in stopping the sinuses with wax, I tried the effect of "smudging" modelling wax worked soft in the fingers on the free bleeding cut surface of the cranial bones in dogs. As such a proceeding instantly arrested the bleeding, I tried to make an antiseptic compound for operations on man, and the formula was published. It was not, however, satisfactory in my opinion, and I asked Mr. P.W. Squire kindly to make experiments so as to arrive nearly at the tenacity of modelling wax. This, I think, he has perfectly

succeeded in with the above formula. I have very often used it, and without the least inconvenience. I need hardly say that it is always sterilised by boiling before use, and kept in covered stoppered bottles. I am, etc.,

VICTOR HORSLEY".

Cavendish Square, W.

According to Geary and Frantz (1950), the Bone Wax, as described by Horsley (1892), is effective as a haemostatic agent by virtue of its tamponade action, but it has no inherent styptic properties. In addition, it is not soluble in the body fluids and thus remains at the site of implantation for long periods of time. That portion of it, which eventually is removed, is probably carried away through the action of phagocytic cells. As a result, the wax acts as a foreign body, tending to promote infection, and, in itself, causing a low-grade inflammatory reaction in the tissues about the site of implantation.

In order to overcome the difficulties of ordinary Bone Wax, as described above, Geary and Frantz (1950) experimented with a new "absorbable" haemostatic Bone Wax, which had the following composition:

Carbowax 1540	60%
Polyethylene Glycol 300	15%
Oxidized Cellulose	25%

In their study, Geary and Frantz (1950) implanted the new "absorbable" wax and ordinary Bone Wax (according to the formula of Horsley: 1892) in experimental rib fracture sites in dogs. The experimental sites were examined both roentgenographically and by histology from 2 to 120 days post-implantation.

Roentgenographically, the first definite evidence of calcium deposition occurred at 23 days post-implantation, and bony union, at all fracture sites, first occurred at 59 days post-implantation. The Roentgen rays showed that at 23 days post-implantation, the amount of calcified callus present at the sites where the new "absorbable" Bone Wax was used, was either about the same, or somewhat less, than the amount at the control fracture. Where ordinary Bone Wax was used, the amount of calcified callus was about the same, or occasionally somewhat greater, than in the control fractures, even though union of the fractures had not yet occurred because of interposition of particles of wax.

It was evident from the results that a delay in healing occurred with both types of wax, but the mechanism appeared to be different in the two cases. With ordinary Bone Wax, the delay was probably a result of mechanical factors. Particles of wax remained between the fragments and thus prevented union. With "absorbable" wax, however, the delay was probably due to the alterations in the pH of the tissues. The acidity of the Oxidized Cellulose undoubtedly lowered the pH of the tissues around the fracture sites, and thus delayed the "alkaline tide", which is essential for the activity of alkaline phosphatase, and the deposition of calcium (Geary and Frantz: 1950).

On microscopic examination, Geary and Frantz observed some irregular basophilic masses in the two-day post-implantation sites where the new "absorbable" Bone Wax was used. These masses were probably fragments of residue Oxidized Cellulose. This material was not seen in any of the later fractures. The inflammatory reaction was mild, and had completely disappeared after the second day.

The ordinary Bone Wax produced a very characteristic histological appearance and foreign-body reaction, which was easily recognizable as late as the fifty-ninth day post-implantation. Wherever the wax remained in the tissues, many irregular, clear spaces were present, and in these areas, numerous irregularly-shaped multinucleated giant cells were seen. Cells such as lymphocytes, plasma cells and mononuclear phagocytes were also present.

Geary and Frantz (1950) thus concluded from their study, that both forms of wax caused some delay in healing. They felt that this delay was not of any practical significance since the "absorbable" Bone Wax would not, in any event, be used in accidental fractures, where early bone repair is the first consideration, nor in weight-bearing bones.

They subsequently used the new form of Bone Wax in one hundred neurosurgical operations, and found no ill-effects or complications which could have been attributed to the use of the "absorbable" Bone Wax.

Following the experimental and clinical results reported by Geary and Frantz (1950), Douglas (1953) used the same "absorbable" haemostatic Bone Wax in dental and oral surgery in humans. His experiment was divided into four separate groups.

In the first group, the material was introduced into tooth sockets following normal, practically atraumatic extractions, where a similar tooth was indicated for extraction on the other side of the same jaw (used as a control). In the second group, more traumatic surgical operations were studied, and these were further subdivided into difficult lower third molar extractions, where a control could be used on the opposite side, and a large group of traumatic operations of all kinds, where no control was available. In the third category, all cases of expectant bleeding difficulties and actual post-operative haemorrhagic troubles were included; and, in the last, an effort was made to determine the efficacy of the Bone Wax in relieving the discomforts of "dry sockets".

His results indicated that, in the first group, more post-operative oedema was present in the sockets packed with Bone Wax. In the second group, where mandibular third molar teeth were removed, there was no difference in the amount of post-operative oedema observed between the empty control sockets, and those filled with the haemostatic Bone Wax. However, in half the number of cases in which both mandibular third molar teeth were removed simultaneously, the patients reported less pain in the sockets treated with the Bone Wax. Roentgenographic follow-ups showed no marked differences in healing on opposite sides. In the third group, the haemostatic wax was successfully used to control post-operative bleeding in all cases, and, in the fourth group, Bone Wax successfully controlled post-operative pain in all patients

afflicted with "dry sockets".

Bone Wax, that is currently used in clinical practice, has the following composition*:

Beeswax	-	7 parts
Olive Oil	-	2 parts
Phenol	-	1 part

Very little experimental and clinical data is available in the literature on the biological effects of this Bone Wax.

Howard and Kelley (1969) implanted this particular form of Bone Wax in rat tibial defects created with a dental bur. The experimental animals were sacrificed at time periods ranging from twelve hours to sixty days post-operatively, and tissue specimens were processed and examined histologically.
Their results revealed the following:

At twelve hours and day 1 post-implantation, there was a localized accumulation of erythrocytes, neutrophils, lymphocytes and fibrin at the interface between the Bone Wax implant and the surrounding haemopoietic marrow.

At day 3 post-implantation, a hyaline membrane separated the implant material (filling the defect) from the mild inflammatory reaction in the adjacent marrow space.

* Courtesy: Pharmacy Department, Royal Adelaide Hospital, Adelaide, South Australia.

At day 6 post-implantation, the bone lesion was devoid of osteoblastic activity, except in the adjacent marrow space.

At days 12 and 20 post-implantation, a thin fibrous and histiocytic membrane at the face of the Bone Wax implant represented the total reparative response. There was an accumulation of a few foreign-body giant cells and foamy macrophages in the fibrous and histiocytic membrane.

At days 31, 42 and 60 post-implantation, the Bone Wax was still present at its sites of implantation, and there was very little evidence of osteogenesis at the interface between the implant material and the haemopoietic marrow.

Howard and Kelley (1969) concluded from their study that Bone Wax completely inhibited the formation of new bone at its site of implantation. The wax, apparently, represented an inert, physical barrier to the reparative process.

Seldon (1970) used Bone Wax as a temporary haemostatic agent during periapical surgical procedures on twenty-eight different teeth. After access to the surgical sites was gained, Bone Wax was firmly applied to the area around the apices of the teeth to control bleeding locally from the surrounding bone. After the surgical procedure was completed, all the Bone Wax was removed and the wound re-sutured.

His study indicated that, by controlling haemorrhage locally around the operative sites, the use of Bone Wax produced a bloodless operative field. In this way, the surgical procedure was easier to perform. He also reasoned that Bone Wax offered a wider margin of safety to control bleeding locally, as opposed to strong chemical vasoconstrictors with their inevitable systemic side-effects.

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It is apparent that quantitative data on tissue responses to Surgicel are lacking. Uddströmer (1978) was the only researcher to conduct experiments on Surgicel in which the results were interpreted both qualitatively and quantitatively. Surgicel was, therefore, selected for this study in order to quantitate tissue responses to its presence, and to develop an experimental model which can, in future, be used to study the biocompatibility of similar materials.

The literature on the biological effects of Bone Wax currently in clinical use is scanty, and no long-term studies, to date, have been conducted using this material. It was, therefore, also selected for the present study to evaluate its biocompatibility.

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CHAPTER 2

EVALUATION OF BIOCOMPATIBILITY

The development of new surgical biomaterials is a long and complex process, the most important aspect of which is the biocompatibility testing of the final product (Gourlay et al: 1978). At present, there are no universal and standard means of evaluating the biological acceptability of new candidate materials (Langeland and Cotton: 1977). In fact, the confusing array of test approaches and the lack of co-ordination and completeness of testing efforts has become one of the key factors limiting further progress in materials research, making it difficult for materials scientists and engineers to determine whether their product is likely to be of use in human patients, whether its performance is better or worse than similar products already developed, and hence, its potential as a marketable product (Hegyeli: 1971).

The most productive method of assessing biocompatibility would be to evaluate all new candidate materials in acute and chronic experiments in large numbers of human volunteers, taking into account the intended site and function of the implant, individual variations in patient responses, sex, age and health conditions. Such an experimental approach is very old, having been used as far back as 2700 B.C. According to records found on Chinese bamboo slips, Shen Nung, a Chinese Physician, experimented at one time with about a hundred drugs a day, using three thousand condemned criminals as research patients. On the basis of his experimental results, he was able to introduce therapeutic drugs, some of which are still potent therapeutic agents today, four thousand seven hundred years later. For legal, ethical, religious and other reasons, society today is not conducive to the use of human volunteers for the testing of new biomedical materials (Hegyeli: 1971).

Current techniques for the evaluation of the biohazards and

biocompatibility of materials are by nature indirect and are limited in their predictive value for the following reasons:

(1) There is no agreement as to what constitutes a valid biological test and its interpretations;

(2) There has been an overemphasis on studies of the effect of materials on the biological environment at the expense of bioengineering and biodegradation studies;

(3) Except in cases of obvious problems, there has been relatively little follow-up of the large numbers of patients receiving implanted materials.

(Hegyeli; 1971)

A protocol for the testing and evaluation of biomaterials must allow maximum control over variables which can affect the reaction. As some amount of surgical trauma is necessary for implanting any material, all of the factors which affect general inflammation and wound healing must be considered and controlled if valid conclusions are to be drawn.

According to Coleman et al (1974), the basic criteria that should be met by any implantation study are:

(1) The elimination and/or standardization of variables which might affect the tissue reaction to the implant material;

(2) Adequate controls to eliminate the variability of tissue reactions due to unusual or unique individuals in the test population;

(3) Test animals should be relatively inexpensive and easily cared for;

(4) Implantation sites should be easily accessible surgically, should provide a homogeneous environment for the implant and should be as mechanically inactive as possible (unless mechanical trauma is being studied);

(5) Proper sterile and pyrogen-free technique should be used during implantation;

(6) Experimental design should be such that results are reproducible within experimental limits; and

(7) Ideally, the results should be quantitative rather than purely qualitative.

In the past, biocompatibility of implant materials has been evaluated most often by limited animal implantation studies followed by tentative clinical application (Homsy: 1970). The criterion for rejection was usually gross tissue reaction adjacent to the implant material (Homsy: 1970; Coleman et al: 1974).

Hegyeli (1971) listed desired characteristics of a material following its implantation into a subject, namely:

(1) It should be non-toxic;

(2) It should not elicit an inflammatory response;

(3) It should be free of an allergic effect;

(4) It should not be carcinogenic;

(5) It should have no other adverse effects on cells or body fluids.

The importance of finding methods to quantitate tissue reactions cannot be overemphasised (Coleman et al: 1974). More importantly, if reliable methods of objective evaluation could be devised to predict accurately the ultimate biocompatibility of candidate materials tested in the early phases of development, time and money could be concentrated on those materials which were non-toxic in these screening tests, rather than be wasted on the further development of materials that might later prove unacceptable for implantation in the body (Gourlay et al: 1978).

Currently, the evaluation methods relating to the compatibility of bio-implants fall into two major groups, namely, *in vitro* tests and *in vivo* tests.

While both groups of methods have their advantages, neither of them is ideal in the study of biocompatibility pertaining ultimately to human subjects. *In vitro* tests correlate rather poorly with *in vivo* results (Homsy: 1970; Hegyeli: 1971). The reason for this shortcoming is that the physiological conditions in the body are not adequately duplicated. Parameters such as gas atmosphere, acidity, evaporation and body temperature are not adequately controlled. This is particularly important in the case of studies involving implant candidate materials since it has been demonstrated that these factors can influence the interaction between the implant material and the biological environment (Hegyeli: 1971). Moreover, tissue culture techniques, while most valuable in predicting possible toxicity, cannot by nature, provide information on complex polymer-tissue interactions such as might occur *in vivo* (Salthouse: 1976; Langeland: 1978). Spångberg (1978), however, refutes the claim that *in vitro* results correlate poorly with *in vivo* results. In order to prove his claim, he implanted eight root canal filling materials in approximately two hundred animals and examined the tissue specimens obtained at two and twelve weeks post-implantation, histologically. Each of the implant materials was ranked from 1 to 8, depending on the degree of tissue irritation that they caused. A ranking of 1 indicated least toxicity, while a material with a ranking of 8 was regarded as being most toxic. The criteria that he used to evaluate irritancy were not mentioned in his article. The same eight root canal filling materials were then subjected to an *in vitro* test and Spångberg (1978) found the *in vivo* results to correlate very well with the *in vitro* results.

Besides ranking materials according to their toxicity, *in vitro* tests allow the testing of components of materials. Thus, a manufac-

turer may, by this method, be able to choose the least toxic component if he, during the development of the material, has a choice of various chemical compositions while maintaining the optimal physical properties of the material (Langeland: 1978). Furthermore, it is known that freshly prepared, unset materials may have high initial toxic effects, which may decrease rapidly when the setting of the material is completed. These findings emphasize the need for a test method whereby the initial reaction of a tissue to a material may be assessed (Tronstad et al: 1978). By using these initial in vitro toxicity profile screening tests, only candidates of great compatibility potential would need to be evaluated in definitive animal implantation studies and, thereby, sharply reduce the number of animals required for biocompatibility testing of these materials (Homsy: 1970).

In vivo testing of biomaterials is advantageous in the sense that the materials being tested are in direct contact with viable, living tissue (Langeland: 1978). However, with this method, several factors beyond the control of the operator such as experimental trauma, uncontrolled or undetected infections, and response by the host often confuse the interpretation of the results (Spångberg: 1978). Species differences between experimental animals and man are a further disadvantage of in vivo experimentation (Hegyeli: 1971). To be able to critically grade in vivo responses in quality and quantity, the experiment has to be properly designed (Spångberg: 1978).

Under ideal circumstances, the study of the effects of a material on biological systems should not be limited to one or two tests, but rather, a battery of tests of increasing severity should be employed. These tests should preferably be performed in more than one animal system and it should be determined that the particular organ system being studied is similar in anatomy and physiology to the

corresponding system in humans. It is important to include both *in vitro* tests, determining the effect on specific cellular and molecular subsystems, as well as systems exploring the actual utilization of the material *in vivo* (Hegyeli: 1971). Although it would be desirable to advocate multiple tests in order to achieve a more comprehensive result, a practical and realistic approach must be taken. It is important that the tests for the materials be simple, easily reproducible and inexpensive to perform, but still reliable and adequately protective to the public (Langeland and Cotton: 1977).

Table 3 lists some of the tests that are currently used in the evaluation of the biological acceptability of test materials.

TABLE 3: RECOMMENDED STANDARD PRACTICES FOR THE BIOLOGICAL EVALUATION OF TEST MATERIALS.

A: Initial Tests

(1) Acute Systemic Toxicity Tests - Oral Route (Weil:1952; Langeland and Cotton:1977);

(2) Acute Systemic Toxicity Tests - Intraperitoneal Route (Langeland and Cotton:1977);

(3) Inhalational Toxicity Tests (Gage:1959; Langeland and Cotton:1977);

(4) Hemolysis Test (Langeland and Cotton:1977);

(5) Ames' Mutagenicity Test (Langeland and Cotton:1977);

(6) In Vitro Cytotoxicity Tests (Kawahara et al:1968; Powell et al:1970 Spångberg:1973; Wennberg:1980).

B: Secondary Tests

(1) Intradermal Test (Powell et al:1970);

(2) Dermal Irritation Test (Powell et al;1970);

(3) Mucous Membrane Irritation Test (Powell et al:1970);

(4) Subcutaneous Implantation Tests (Browne and Friend:1968; Truelove et al:1971; Wolfson and Seltzer:1975; Langeland and Cotton:1977; Haugen and Mjör:1978; Olsson et al:1981b);

(5) Intramuscular Implantation Tests (Sewell et al:1955; Ferguson et al:1960; Laing et al:1967; Wood et al:1970; Salthouse and Williган:1972; Matlaga et al:1976; Salthouse:1976; Wennberg et al:1978; Wennberg:1980);

(6) Intra-osseous Implantation Tests (Spångberg:1968;1969; Olsson et al:1981a);

(7) Intramedullary Implantation Tests (Neuman et al:1975; Zartner et al:1976; Laskin et al:1981).

C: Usage Tests

Tests on primates or humans to evaluate clinical performance of:

(1) Restorative Materials;

(2) Pulp Capping and Pulpotomy Materials;

(3) Endodontic Materials;

(4) Endosseous Implant Materials.

(Olsson et al:1981b)

Initial tests provide a general toxicity profile for implant materials (Olsson et al: 1981b). Should these tests indicate that a particular material exhibits low toxicity, it is then subjected to secondary tests to evaluate its local toxicity (Spångberg: 1978; Olsson et al: 1981b). Initial and secondary tests, at most, rank materials with regard to their cytotoxicity under the testing conditions in question (Langeland: 1978). Thus, no conclusions may be drawn as to the possible toxicity of materials in their actual use. For this reason, the employment of usage tests is imperative. In these tests, the materials being examined, are handled accurately following the directions of the manufacturer for the intended clinical use of the materials. Handling of the materials and the clinical steps necessary for their application, may be as important for the biologic response as the materials themselves (Langeland: 1978; Olsson et al: 1981b). To fulfill the requirements of a true usage test, it should ideally be performed in human oral tissue. However, because this is often not possible, primates are the preferred test animals (Langeland: 1978).

The world literature on test procedures for the evaluation of biocompatibility of implant materials is vast (Hegyeli: 1971). A large proportion of these test approaches have been devised by dental materials researchers, particularly in the field of endodontics. Therefore, in the ensuing discussion, examples of test procedures (Table 3), most of which have been conducted on dental materials, will be presented.

A: Initial Tests

(1) Acute Systemic Toxicity Test by the Oral Route (Oral LD_{50} Test)

The purpose of the LD_{50} test is to determine the smallest dosage of a test substance that would be expected to kill 50% of the animals that received it (Weil:1952). In this test, liquids and powders, dissolved or suspended in an appropriate vehicle (usually cottonseed oil), are administered as such while powders are dispersed in cottonseed oil in a 10% concentration. Each test material is administered by a stomach tube to a group of ten rats, weighing between 200 and 300 gr. at the following dose levels:

0.05gr/kg body weight
0.5 gr/kg body weight
1.0 gr/kg body weight
2.0 gr/kg body weight

All animals are observed for up to fourteen days for death or other signs of toxicity. If the LD_{50} is 1.0 gr/kg body weight or greater, the material shall fulfill the requirement of the Oral LD_{50} Test.

(Langeland and Cotton:1977)

(2) Acute Systemic Toxicity Test by the Intraperitoneal Route (IP LD_{50} Test)

In this test, liquid products and pastes are administered as such, while powders are dispersed in cottonseed oil in a 10% to 20% concentration. Each test material is administered intraperitoneally to groups of six laboratory Albino rats at the following levels:

0.025gr/kg body weight

0.05 gr/kg body weight

0.5 gr/kg body weight

1.0 gr/kg body weight

Animals are observed for seven days for death or other signs of toxicity. The appropriate lethal dose is considered as that dose which kills three or more animals, but none in the immediate lower dose level.

(Langeland and Cotton:1977)

(3) Inhalational Toxicity Test

Forty male and forty female experimental rats, within a weight range of 160gr to 200gr, are exposed to test or control atmospheres for six hours daily, five days per week, in exposure chambers described by Gage (1959). The length of the test period, that is, the number of test weeks, can be selected according to whether acute or chronic effects are sought. Twenty male and twenty female control rats are similarly treated in exposure chambers under ambient atmospheric conditions. The animals are examined daily for clinical signs, and haematology and urinalysis are performed weekly (Langeland and Cotton:1977). Interpretation of this test shall be based on the LD_{50} calculation (Weil:1952).

(4) Haemolysis Test

In this test, 5gr of material, cut if necessary into small pieces, are placed in a test tube containing 0.2ml of diluted rabbit blood, mixed gently and incubated for sixty minutes. A positive control containing 0.2ml of the diluted rabbit blood, and a negative control (10ml of normal saline in 0.2ml of diluted rabbit blood), are also incubated. After

sixty minutes of incubation, all tubes are centrifuged for five minutes at 500 X G. The supernatant is carefully removed and subjected to spectrophotometric analysis to determine the density of the supernatant as a measure of erythrocyte haemolysis (Langeland and Cotton:1977).

(5) Ames' Mutagenicity Test

This test is specifically designed to test for chemical mutagens. Mutants of Salmonella Typhimurium, which are unable to survive on a histidine-free diet, are used in this test. If these bacteria are treated with a mutagen, then, by chance, there will sooner or later be a mutation which will replace the abnormal base in the "histidine" gene, and it will become functional again. The bacteria in which this mutation has occurred will now grow on a histidine-free medium. This phenomenon is the basis of the Ames' Test. If such a mutation does occur using a test chemical, then there is a 90% (or greater) chance that this chemical will be mutagenic to mammalian cells (Langeland and Cotton:1977).

(6) 'In Vitro' Cytotoxicity Tests

A number of tissue culture methods have been developed to assess the "cytotoxicity" of materials. Meaningful information has been obtained in studies with the use of these techniques.

One of these techniques is the 'hanging drop cuture test' (Kawahara et al:1968). They tested a group of thirty two metals, examples of which were copper, zinc, silver, gold, using this technique. A test piece of each metal was placed as near as possible to an explant from the spleen and heart of chick embryos, nine to fifteeen days old. Migration and transformation of mononuclear phagocytes from the explant were observed by phase

contrast microscopy and relative outgrowth values (R.O.V.) were established by measuring areal enlargement of fibroblastic outgrowth from the explant with a planimeter. The inhibition index of various test pieces upon the fibroblastic outgrowth was calculated by a method described by Kawahara et al(1968).

Kawahara et al(1968) described another in vitro test procedure in which a similar group of metals, examples of which were listed above, were placed singly in test tubes containing 1.5ml of a cell suspension of mouse fibroblast strain L-cells, and incubated at 37^{o} C. The aim of the test was to determine the effect that these metals had upon cell multiplication. The following method was used: the numbers of cell nuclei were determined on day 0, 4 and 7 of cultivation from the average of three culture tubes using a haemocytometer. The inhibition index of the various materials on cell multiplication was calculated by by a method described by Kawahara et al(1968). The results obtained for each metal indicated its cytotoxic effects.

Kawahara et al(1968) described another test procedure in which the bio-adaptability of metals (examples of which were listed above) was determined. For the purpose of deciding bio-adaptability by means of tissue culture, they used profiles of L-cells which contacted the surfaces of the metals (listed by them in their article) in special mosaic glass chambers. In these chambers, the floor (bed) was constructed from the metal being tested. The L-cells were allowed to sink to the floor (bed) of the chambers and they contacted the metal being tested at various contact angles. The smaller the contact angle, the more bio-adaptable was the test material regarded.

Powell et al(1970) tested various solid (for example, vinyl and plastic tubing) and liquid (for example, araldite and 2-chloroethanol) materials using monolayer tissue culture techniques. A layer of agar was placed on a monolayer of L 929 mouse cells (L-cells). All solid test materials were placed directly on the agar overlay while liquids were placed on sterile paper discs which were then placed on the agar. The plates were then incubated for a period of twenty-four hours and examined for zones of toxicity around the implants. The toxic materials where characterized by relatively clear, colourless zones of dead cells while the non-toxic materials were distinguished by an even pink colour of the cell monolayers.

This particular test has the disadvantage that materials or components of materials have to diffuse through the agar overlaying the monolayer of cells. Thus, materials which do not dissolve in or diffuse through agar will not cause cellular damage, although they could, nevertheless, be toxic. The test would, in such cases, give a false negative result (Langeland:1978).

Spångberg(1973) used chromium-labelled HeLa cells in a test tube to evaluate the toxicity of phenol. The toxicity of this substance was evaluated quantitatively on the basis of cell damage caused by the test material by measuring the release of ^{51}Cr from the target cells. In this test, 1ml of the solution to be tested was mixed with 1ml of the labelled cell suspension. The mixture was incubated at $37^{o}C$ for one, two, three, four and, in some experiments, twenty-four hours. The amount of ^{51}Cr released was measured using a gamma counter.

The main advantage of this test over the monolayer method is that it allows direct cell-material contact in dissolved materials as well as in semi-solid, setting or set materials. In addition, the test uses objective counting rather than subjective observation and evaluation. A disadvantage is that the chromium method is more expensive because the technique is more involved, and because it depends on the availability of a gamma counter. Also, chromium is bound rather than released in the initial phase of a toxic reaction (Langeland:1978).

Wennberg(1980) tested, in vitro, the cytotoxicity of AH 26, U of P sealer, Kloroperka, N-O and Riebler's Paste by employing a monolayer of mouse fibroblast cells (L 929) cultured on millipore filter discs. The filters were placed on an agar medium bed, cell side down, and the test specimens were placed on top of the filters for two hours. The test specimens were then removed, and the cells, still attached to the millipore filters, were incubated for cytochemical demonstration of succinate dehydrogenase (SDH) activity. After an incubation period of three hours at 37^{o}C, the filters were washed in distilled water, air dried and stained. The filters were examined macroscopically, and any changes in the staining intensity of the cell monolayer at the test specimen-agar contact area were registered. If an unstained zone was present, the diameter was measured. The larger the diameter, the more toxic was the test material regarded.

B: Secondary Tests

Powell et al(1970) used several tests (Intradermal test, Dermal Irritation Test, Mucous Membrane Irritation Test) to examine the irritant effects, if any, of several liquid materials (for example, araldite, methyl-methacrylate, 2-chloroethanol). These tests will be described briefly:

(1) Intradermal Test

In this test, Albino rabbits with hair on the dorsal surface, previously clipped, are used as the test animals. Test liquids (examples of which were mentioned above) are injected intradermally in a dose of 0.2ml into two or more sites on the back of the animal. A negative control solution (normal saline) and a positive control (20% ethyl alcohol) are also injected into several sites in the same animal. Fifteen minutes after the last injection, trypan blue solution (1%) is injected into the marginal ear vein. At 30 seconds and 1,5,10,15,30,45 and 60 minutes, the sites of the injections are examined carefully and compared with those of the controls. Tissue damage around the injection sites is apparent by blue coloration. The degree of coloration and size of the zones can be used to score the tissue response.

(2) Dermal Irritation Test

In this test, hair on the dorsal surface of Albino rabbits is shaved and cotton patches, to which 0.2ml of the test liquid is added, are placed on the cleanly-shaven skin. The patches are held in place with cotton gauze and polyethylene strips. Sodium lauryl sulfate (8% solution) is used as a positive control while normal saline is included

as a negative control. The animals are placed in restraining devices for twenty-four hours at which time the various covers are removed and the skin examined for signs of irritancy (erythema, blistering, oedema, burning). The response is graded as follows:

0 - No irritation, cannot distinguish from negative control;

± - Questionable response;

1 - Very mild erythema, somewhat spotty and does not cover the entire area under the patch;

2 - Erythema, response stronger, but no blistering, oedema or "burn effect";

3 - Erythema extremely strong, blistering, oedema and "burn effect" apparent to some.

(Powell et al:1970)

(3) Mucous Membrane Irritation Test

In this test, the eyes of rabbits are used to determine the irritancy of certain liquids. The test liquid (noted above) is administered in a dose of 0.1ml directly onto the superior temporal quadrant of the right eye of Albino rabbits. The left eye is left untreated. Observations of the eye are made at thirty minute intervals up to two hours, then after twenty-four hours. Irritational responses are scored according to signs of conjunctival and palpebral irritation, iritis and an oedematous, closed eye, with or without a purulent discharge.

(Powell et al:1970)

(4) Subcutaneous Implantation Tests

The implantation of materials into the connective tissues of small animals is considered a suitable test for the evaluation of the biocompatibility of various dental materials

(Olsson et al:1981b). Several methods, using the subcutaneous tissues, have been described. Truelove et al(1971) implanted pellets of freshly-mixed Durelon and Zinc Oxide-Eugenol cement directly into the subcutaneous tissues of rats. The severity of the response of the tissues to the test materials was assessed by histological observation. The criteria that they used to evaluate the irritancy of the materials were not mentioned.

Wolfson and Seltzer(1975) implanted Gutta Percha directly into the subcutaneous tissues of rats. They graded the reaction to this material as mild, moderate or severe on the basis of the average number of cells visible in fields of vision (at a magnification of 315 X) and on the thickness and staining characteristics of the capsule surrounding the implant at 0,2,4,8,15,32 and 64 days post-implantation.

The disadvantage of placing materials directly into tissues, as was done in the studies just described, is that the materials are capable of undergoing fragmentation with subsequent dispersion of the material into the tissue. Moreover, the implant is prone to excessive movement,and both of these factors can produce an erroneous histological picture by causing a tissue reaction beyond the confines of the implant and by mechanical trauma. Further, it is difficult to implant a constant volume of the test substance using this method, with the result that the collection and collation of quantitative data is impossible.

In order to obviate the problem of non-standardized volumes of the test materials in direct implantation methods,and of the possibility of dispersion of the materials into the surrounding tissues, Torneck(1966) suggested that polyethylene tubes could

be used as carriers of test substances for implantation studies. Polyethylene tubing has subsequently been used by Browne and Friend(1968) and Haugen and Mjör(1978) to study the biological effects of root canal filling materials and periodontal dressings respectively on subcutaneous tissues.

Teflon tubing, as carriers of test materials, has increased in popularity in recent years and it has been used extensively in the study of biocompatibility of various implant materials (Spångberg:1968;1969; Uddströmer:1978; Olsson et al:1981a;1981b). It is one of the most inert plastics available and it can be autoclaved (LeVeen and Barberio:1949).

Langeland and Cotton(1977), in the F.D.I. document which they authored, suggested the use of Teflon tubing (inner diameter of 1.3mm and a length of 10mm), filled with the test materials, for the testing of dental materials in the subcutaneous tissues of guinea pigs. According to these authors, the interface area between the test material and the connective tissue would provide an interface for microscopic assessment of parameters, such as the presence of necrosis or inflammation, the intensity of inflammation, the extent of inflammation and other pertinent changes such as resorbability of the implant material. The tissue response along the mid-section of the Teflon tube serves as a control in this system. According to Olsson et al(1981b), the chief advantage of this method is the standardization of the tissue-material contact area for detailed cellular analysis.

(5) Intramuscular Implantation Tests

Muscle, as an implantation site, is widely used for the study of the biological effects of implant materials (Sewell et al:1955; Ferguson et al:1960; Laing et al:1967; Wood et al: 1970; Salthouse and Willigan:1972; Matlaga et al:1976; Salthouse:1976; Wennberg et al:1978; Wennberg:1980) as it is readily accessible and provides a homogeneous environment for the implant (Colemen et al:1974).

Sewell et al(1955) devised a method for quantitating the tissue reaction to sutures of ovine catgut and bovine catgut. The number of different types of cells and their concentrations were assigned an arbitrary value. The summation of the various factors measured offered a means of grading tissue reactions from very slight (0 to 16 points) to extensive (greater than 112 points) with six grades in between. Gourlay et al(1978) modified this system slightly to study the effects of various polymers on rat muscle. Differentially weighted scores were used to grade different indicators of tissue response to the implanted polymers approximating the original method. The tissue response indicators used in Gourlay's method were:

(a) The degree of muscle cell damage;
(b) The total thickness of the reaction;
(c) The overall cell density;
(d) The number of polymorphonuclear leukocytes, erythrocytes, eosinophils, lymphocytes, foreign-body giant cells, fibrocytes and mononuclear phagocytes.

The grade for each of the indicators was multiplied by the empiric weighting factor to derive a score; the sum of the scores was the Tissue Reaction Score (TS) for that material. The higher the TS, the more toxic was the test material regarded.

Ferguson et al(1960) implanted various metals into the paravertebral muscles of rabbits. The concentration of trace elements found in tissues surrounding the metal implants of different composition were compared with the normal level of these elements in the tissue using spectrographic methods.

Enzyme histochemistry is another tool that can contribute valuable data to supplement other methods in the evaluation of materials for surgical implantation (Salthouse and Williganː1972). The least irritating materials evoke only a minimum level of lysosomal enzyme activity in the cell population at the implant site and such activity usually diminishes to undetectable levels by the third to fourth week after implantation. Higher levels of hydrolase activity in the macrophage population appear to be related to a greater degree of fibrosis surrounding the implant later. Implants containing toxic or irritating additives appear to stimulate a higher output of aminopeptidase activity. Such activity is indicative of a higher level of proteolytic activity in the area and may be associated with increased phagocytic activity of damaged or necrotic tissue. Conversely, an inhibition of both dehydrogenase and oxidase activity is observed in cells affected by the presence of toxic materials (Salthouseː1976).

Enzyme histochemical methods formed the basis of the studies by Wennberg et al(1978) and Wennberg(1980) in which the initial toxic effects of endodontic materials were evaluated by a two-stage implantation test. Specially designed Teflon bodies were implanted in the thigh muscle of rabbits. After at least 6 weeks, the implants were removed, and an intact, non-epithelialized tissue surface was exposed.

The shape of the Teflon bodies made indentations in the tissue at each implantation site. The test materials were placed in the indentations and allowed to influence the tissue for 15 minutes. After this time, the specimen was processed for histochemical demonstration of succinate dehydrogenase (SDH) activity. The width of inhibition of succinate dehydrogenase around the implant materials was measured in millimeters and the results were scored. A decrease in the amount of this enzyme would indicate initial toxicity of the test material.

(12) Intra-osseous* Implantation Tests

Intra-osseous implants have a distinct advantage over subcutaneous implants for the reason that the former only infrequently result in mechanical displacement of material into the surrounding tissue. However, the intra-osseous implantation method requires a more precise technique and is more time-consuming (Olsson et al:1981a). This technique has largely been used for the study of the biological effects of endodontic materials which, in man, are used in close proximity to bone.

Spångberg(1968) implanted Teflon and Gutta Percha into the cortical bone of the mandible of rats. His results were evaluated qualitatively by histological description and quantitatively by measuring the thickness of the fibrous capsule around the implants, and by determining the presence or absence of bone adjacent to the implants.

Spångberg(1969) further developed a standardized technique for studying the effects of root canal filling materials. A Teflon applicator was designed, and it

* Refers to implantation into cortical bone as opposed to bone marrow.

consisted of a cylinder open at one end with its outer surface provided with a spiral ridge to anchor it firmly after insertion. The cylinder was 2mm long and had an inner diameter of 1.3mm and an outer diameter of 2mm. Before its use, it was sterilized by autoclaving. The burs that were used were also designed to produce a standardized cavity. By the use of stop collars on spiral and fissure burs, the depth of the cavity was standardized. Using the mandibles of guinea pigs, various endodontic materials were implanted in bone using Teflon cups as carriers. One sort of filling material was implanted in one half of the mandible and another sort in the other half. The animals were sacrificed at two and twelve weeks post-implantation, and the tissue responses on each side of the mandible were compared. The criteria that were used in the assessment of irritancy of the materials were as follows:

(a) Leukocyte infiltration;

(b) Tissue necrosis and sequestrum formation along the face of the implant material;

(c) Absence of cellular proliferation and tissue organization between root canal filling material and bone;

(d) Thickness of the capsule along the face of the root canal filling material;

(e) Large amounts of small vessels and cells in tissues under the root canal filling materials;

(f) The presence of mono- and multinuclear macrophages;

(g) Osteoclasia.

The first five changes, and particularly the first three, were regarded as the strongest signs of irritation, the last two being the least important. All specimens were examined twice, and the

difference, if any, in the reaction of the tissue to the implant materials on one side and to that on the other side, was graded as moderately stronger (+) or much stronger (++). In the absence of any demonstrable difference, the result was designated by the symbol (0).

Olsson et al.(1981a) used the same technique, as described by Spångberg (1969), to evaluate the biocompatibility of various root canal filling materials, namely, Kloroperka, Kerr Sealer and AH 26. The experimental animals were sacrificed at 30, 90 and 180 days post-implantation. The criteria used to assess the tissue responses were similar to those used by Spångberg(1969).

(13) Intramedullary Implantation Tests

Since most dental materials, for example, local haemostatic agents, endodontic materials and metallic implants, come into direct contact with the bone marrow in a clinical situation, the study of the biological effects of implant materials on bone marrow is especially important.

There are two basic approaches to the use of haemopoietic bone marrow as an implantation site, namely, by direct placement of the test material through a cavity drilled into cortical bone, or by placement of the material into the marrow cavity via a carrier, for example, Teflon or Polyethylene tubing. The former test method was used by several researchers. Neuman et al.(1975) implanted Teflon, Titanium and Vitallium into the maxillae of guinea pigs. Half of the implants were exposed to the oral cavity and the other half were left unexposed. The experimental

animals were sacrificed at two and twelve weeks post-implantation and tissue specimens obtained were processed for histological examination. The criteria that they used for the assessment of tissue responses were the same as those employed by Spångberg (1969). Zartner et al.(1976) implanted Amalgam and a freshly-mixed Polycarboxylate Cement into small holes drilled in the tibia of rats. Each of the holes contained one of the test materials and the third hole served as a control. The irritancy of the test materials were assessed histologically on the basis of the viability of osteocytes adjacent to the implanted materials.

Using a similar approach, Laskin et al.(1981) studied the effects of a granular gelatin preparation and Gelfoam powder upon the healing of cortical defects cut into the tibia of rats. After the holes were drilled in the tibia, the test materials were implanted in the marrow space. The experimental animals were sacrificed at 3, 7, 14 and 49 days post-implantation, and the specimens obtained were prepared for histologic examination. The area of the experimental bone defect was examined for the following:

(a) Inflammatory cell infiltrate;

(b) Presence of foreign material;

(c) Giant cell infiltrate;

(d) Abscess formation;

(e) Necrosis at the site of injury;

(f) Periosteal reactive bone formation;

(g) Healing of the defect with endosteal bone.

When test materials are implanted directly into a tissue, for example, in the experiments of Zartner et al.(1976) and Laskin et al.(1981), as described above, there is no control over the volume of the test material implanted. While, in such cases, it would be possible to evaluate the results by qualitative means, it would be impossible to derive any quantitative data because of this variable.

In order to minimise the problem of variability of implant material volume, tubes (Polyethylene or Teflon) may be used as carriers of implant materials. A further advantage of placing test materials into tubes is that the original implantation sites are easily identifiable. This is especially important when absorbable materials are studied.

Teflon is one of the most chemically inert plastics yet discovered. An unusual property of Teflon is that it cannot be wet with water and nothing will adhere to it. It is semi-flexible and can easily be cut and shaped with a knife (Le Veen and Barberio: 1949). Because of its inert nature and the inability of proteins to adsorb onto its surface (Le Veen and Barberio: 1949), Spångberg (1968; 1969) and Olsson et al.(1981a; 1981b) suggested that Teflon could be used as a carrier of test materials in the study of biocompatibility. In addition, it can be autoclaved and rendered sterile (Le Veen and Barberio: 1949).

It was on this basis that Teflon was selected over Polyethylene as a carrier of Surgicel and Bone Wax used in this study.

****

CHAPTER 3

MATERIALS AND METHODS

INTRODUCTION

In a previous and preliminary study (Garach, 1978), the haemostatic agents Surgicel®, Gelfoam® and Bone Wax® were implanted in surgical defects cut into the femurs of Male Sprague-Dawley Albino rats using a number 8 round bur (Figure 3). The animals were sacrificed at 2,4,7,10,14 and 21 days post-implantation and the implant sites dissected out, processed for histological examination and examined microscopically.

While this study produced some interesting results regarding the responses of bone and marrow tissues to the implanted materials, it became evident that the method of placement of the materials was unsatisfactory for the following reasons:

(1) The quantity of local haemostatic agent implanted in the surgical defects in the femurs was not constant;

(2) The original implant sites, especially over long periods of time, were not always identifiable as the resorbable agents often disappeared and there was complete healing of the cortical defect in the bone at the later stages of the experiment.

(3) Quantitation of cellular and tissue responses could not be reliably undertaken since no single and constant area in the experimental implant sites could be identified in the different specimens.

In an attempt to improve the method of implantation of the materials used in the present study (viz. Surgicel and Bone Wax) and to reduce the limitations indicated above, a series of pilot studies were undertaken.

The first of these involved the procurement of a suitable carrier for the test materials. Teflon (polytetrafluorethylene) tubing (Figure 4) having an outer diameter of 1.7mm and an inner diameter of 1.3mm, was ultimately chosen for this purpose.

Pilot implant studies were next undertaken using short lengths of Teflon tubing, containing the materials to be tested, and these were inserted into the femurs of anaesthetized rats. The essential features of these techniques are illustrated in Figures 5, and 6. Of these methods, the technique used in Figure 5 proved unsatisfactory for the reason that the lengths of Teflon tubing were found to dislodge themselves from the bone defect. The method used in Figure 6 also proved unsatisfactory for the following reasons:

1. The diameter of the Teflon tube proved too small for the easy and complete filling of the tube with Surgicel.
2. The tube seldom remained *in situ*.
3. In most cases, it was difficult to make a shallow groove in the cortical plate of bone without causing a perforation into the marrow.

The method illustrated in Figure 7 where a short length of Teflon containing the test materials was inserted into the bone marrow cavity via a defect cut through the cortex of the rat femur, proved, on both implant retention and histological grounds, to be the most satisfactory method. This method was thus chosen for the bone implants in this study. Since, however, this particular method involved placement of the test materials into cellular haemopoietic marrow containing a variety of white blood cells in various stages of maturity, it was considered necessary to also implant the test materials into soft tissue sites where cellular responses, particularly those related to inflammation, if any, to these materials, could be more readily identified if required. For this purpose, the muscles of the anterior chest wall were chosen. These muscles are accessible surgically, large enough to house the implants and they provided a homogeneous environment.

Figure 3: Diagram illustrating the method of placement of the test materials into the femur of the rat in a pilot study undertaken by Garach (1978).

Figure 4: Photograph of Teflon (polytetrafluorethylene) tubing of outer diameter 1.7mm and inner diameter 1.3mm, used in the present investigation.

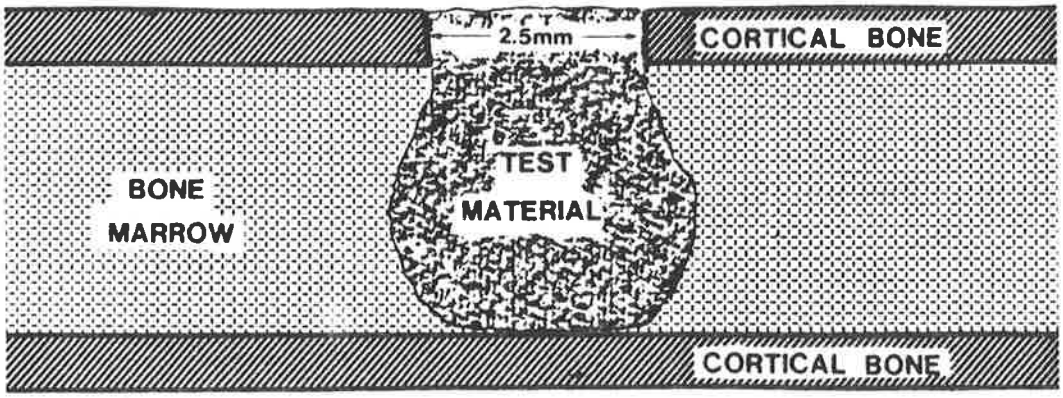


Figure 3

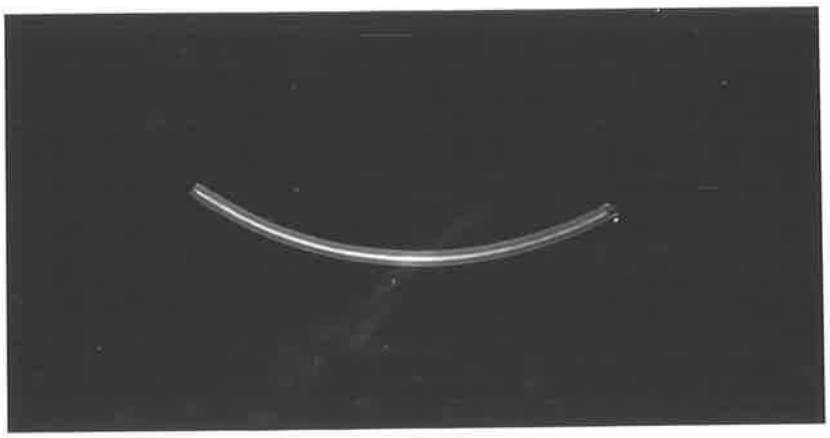


Figure 4

Figure 5: Diagram illustrating the vertical placement of the Teflon tube, containing the test material, in one of the pilot studies undertaken. The bone defect was produced using a number 5 round dental bur. The Teflon tube used had an outer diameter of 1.7mm and an inner diameter of 1.3mm.

Figure 6: Diagram illustrating the horizontal placement of a length of Teflon tube, containing the test material, in a groove created on the surface of the cortical bone of the femur of the rat. The tube had an outer diameter of 1.5mm and an inner diameter of 1.2mm. The length of the tube was 3mm.

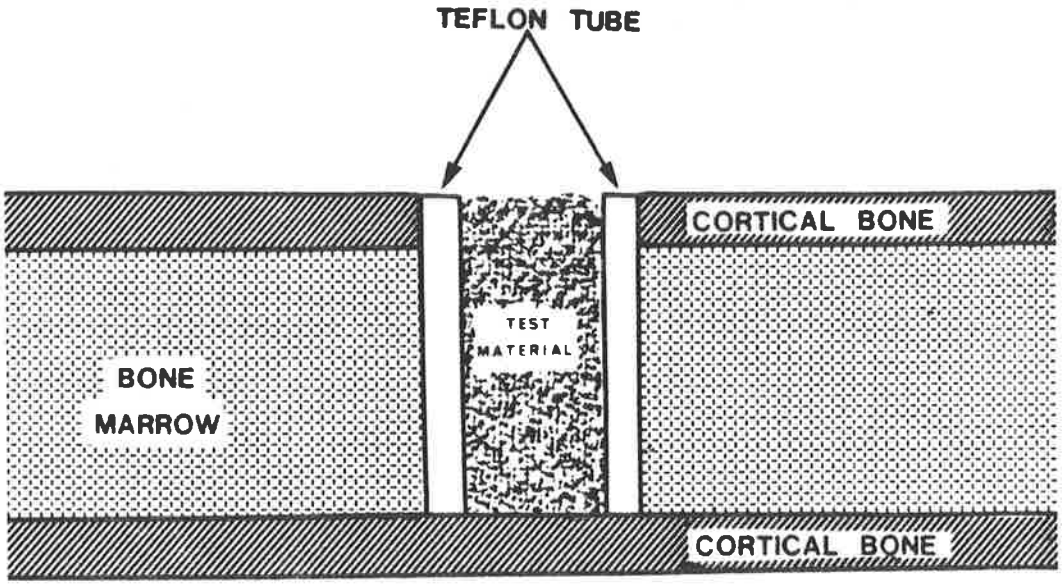


Figure 5

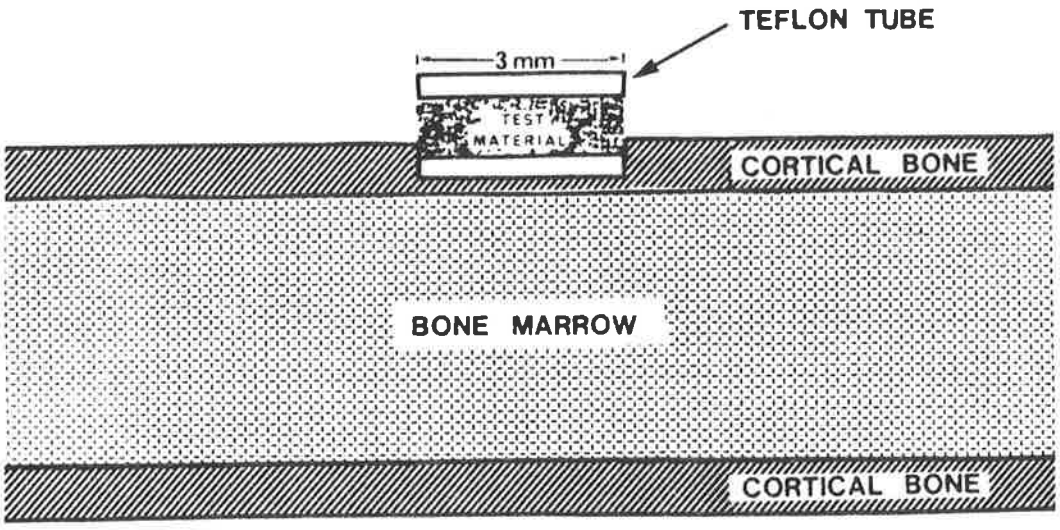


Figure 6

Figure 7: Diagram illustrating the placement of a short length of Teflon tube, with or without the test material, into the haemopoietic marrow space of the femur of the rat. The tube had an outer diameter of 1.7mm and an inner diameter of 1.3mm. To place the Teflon tubing in the position illustrated in the diagram, a defect in the cortical bone was cut using a number 5 round dental bur on a dental handpiece. The tube was then placed into the marrow space via the defect and subsequently pushed horizontally until it was completely covered by intact cortical bone. The reason for placing the tube in this position was to prevent it from dislodging itself from the marrow space. T = Teflon Tube

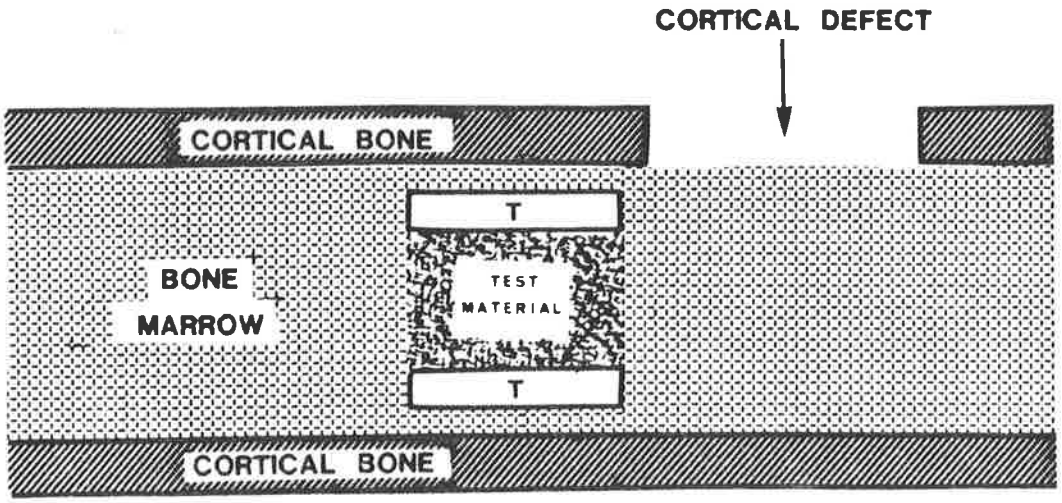

CORTICAL DEFECT
CORTICAL BONE
T
BONE
MARROW
TEST
MATERIAL
T
CORTICAL BONE

Figure 7

DETAILED DESCRIPTION OF MATERIALS AND METHODS

A: THE IMPLANT MATERIALS

Oxidized Regenerated Cellulose (Surgicel)

Surgicel is manufactured by Surgicos (U.S.A.), a member of the Johnson and Johnson Group of Companies. It is presented as a sterile gauze prepacked in foil overwrap (Figure 8). It is a whitish-yellow knitted material (Figure 9) with a faint caramel-like smell. The Surgicel used in this study came prepacked as strips measuring 1.3cm by 5.1cm (6.5 sq.cm.).

Bone Wax

Bone Wax is manufactured by Ethicon Ltd. (Australia) and it is presented in a prepacked sterile form (Figure 10). It comes in the form of a flat rectangular strip of material, 2.5gr in weight. It is white in colour and extremely pliable. (Figure 11).

B: PRESENTATION OF TEFLON TUBING AND TEST MATERIALS FOR IMPLANTATION

As already described, lengths of Teflon* tubing were used as carriers for the Surgicel and Bone Wax in the present study. Prior to placing the test materials into the tubing, lengths of Teflon tube measuring 6cm were placed in Guardian Hospital Autoclave Bags and autoclaved in a Smith Pressure Sterilizer. Pressure Sensitive Autoclave Tape (Scotch :Australia) was used as an indicator of adequate steam penetration. The tubes were stored in these paper wrappers until subsequently used.

Surgicel and Bone Wax cannot be autoclaved. In order to maintain aseptic conditions, all presurgical procedures involving the handling and placement of the test materials into the Teflon tubing were carried out in an

* Brand number T21196-0047 : Supplied by John Morris (Pty.) Ltd., Sydney

Oliphant Laminar Flow Unit (Model HLF-6-L) (Figure 12). Special body drapes and sterile gloves were worn during the handling of the haemostatic agents.

Short lengths of Surgicel-filled Teflon tubing were prepared for implantation as follows: the Surgicel gauze was first cut in half (Figure 13). A 000 black silk suture was threaded through the gauze and then both ends of the suture were passed through the entire length of the tube. These were gently pulled and the Surgicel was drawn into the empty tube (Figure 14). Once the tube was completely filled with the gauze, it was cut into lengths measuring 2mm and 3mm with a scalpel. This filling procedure was repeated until a sufficient number of Surgicel-filled tubes for the entire experiment were obtained. The latter were all prepared on the same day and were subsequently stored in sterile plastic containers.

The preparation of the Bone Wax-filled tubes was undertaken as follows: 2.5gr of the wax was gently heated in a beaker over a hot plate (Figure 15). An 18-gauge needle (Terumo), attached to a 10ml syringe (A-Sik, Denmark), was fixed to one of the open ends of the pre-autoclaved Teflon tubes. When the wax reached a molten state, it was drawn into the tube using the syringe and needle described above (Figure 16). The wax assumed its usual solid state almost immediately and lengths of Bone Wax-filled tubes, measuring 2mm and 3mm, were prepared in a manner similar to the Surgicel filled specimens in the laminar flow unit.

Empty Teflon tubes measuring 2mm and 3mm were similarly prepared from pre-autoclaved lengths of tubing in the laminar flow unit and stored as described above.

In order to ensure sterility of the tubes to be implanted, the Federation Dentaire Internationale (F.D.I.) recommends that random samples of the carriers be cultured aerobically for 4 days at 37^{o}C. In

the present investigation, random samples of the prepared tubes were placed in Brain Heart Infusion culture medium (Appendix II) at the time of preparation and one week* following their preparation, and incubated aerobically for 4 days at 37°C. None of the samples yielded any positive cultures.

It has previously been mentioned that lengths of Teflon tubing, 2mm and 3mm, were prepared. The 3mm lengths of tubing were implanted intramuscularly and the 2mm tubes were implanted intrafemorally. The reason for placing the shorter lengths of tubing intrafemorally was that a smaller wound (defect) in the cortical bone sufficed for its placement. The larger the cortical wound within the femur, the greater was the likelihood of a fracture of this bone with subsequent loss of the specimen.

* All the operative experimental procedures on the rats were completed within ten days.

Figure 8 : Photograph showing a package of Surgicel.

Figure 9 : Photograph of unwrapped Surgicel.
Note the knitted nature of the gauze.

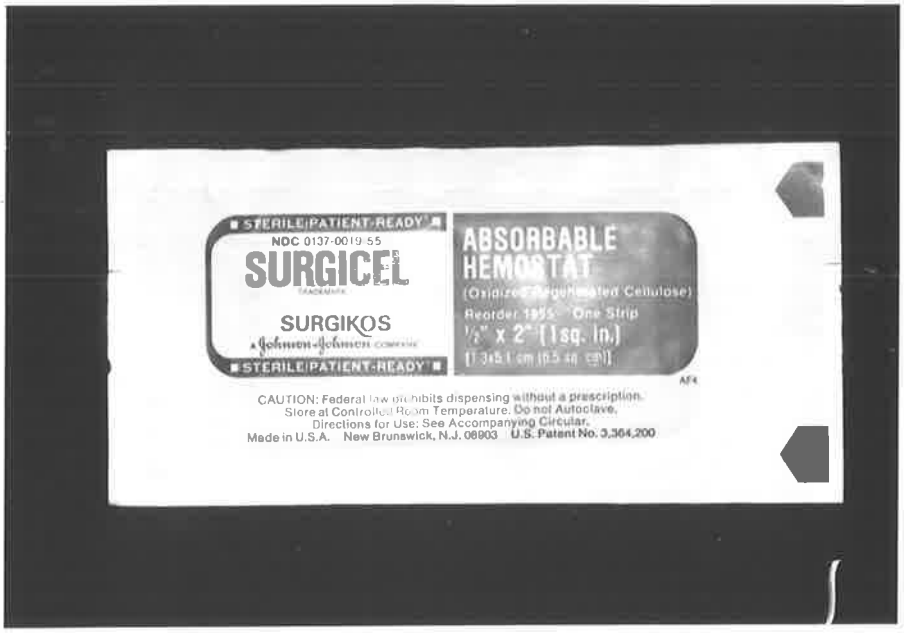


Figure 8

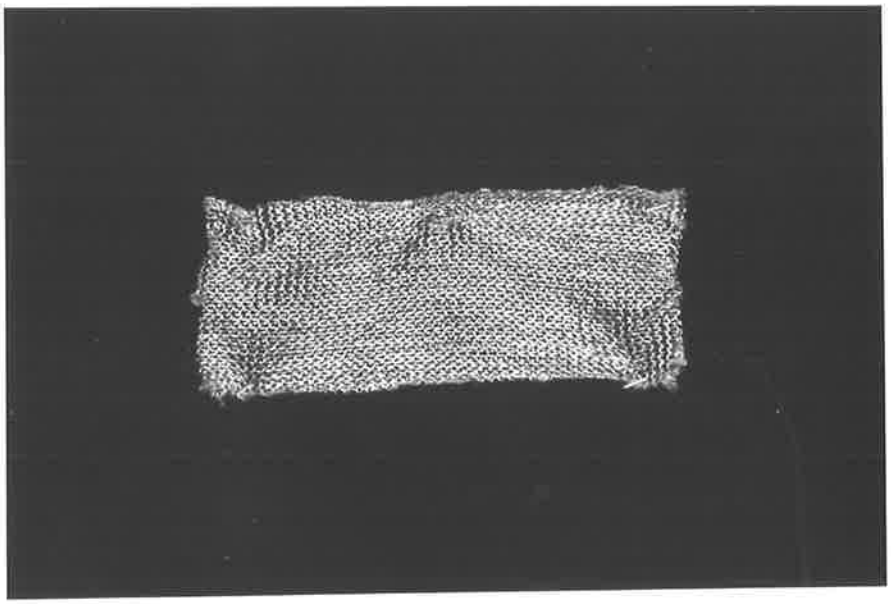


Figure 9

Figure 10: Photograph showing a package of Bone Wax.

Figure 11: Photograph of a sheet of Bone Wax, 2.5gr in weight, after removal from its packaging.

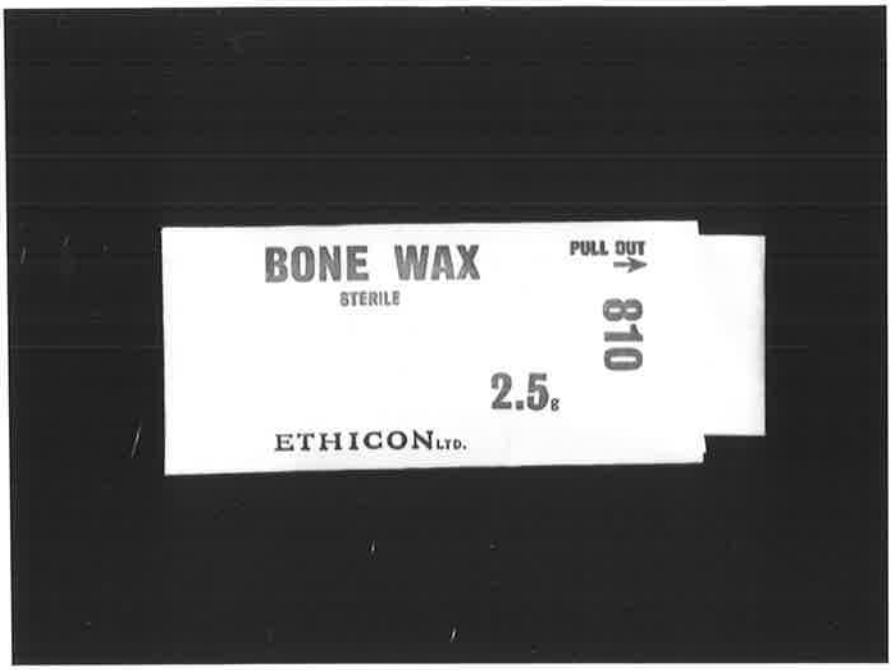


Figure 10

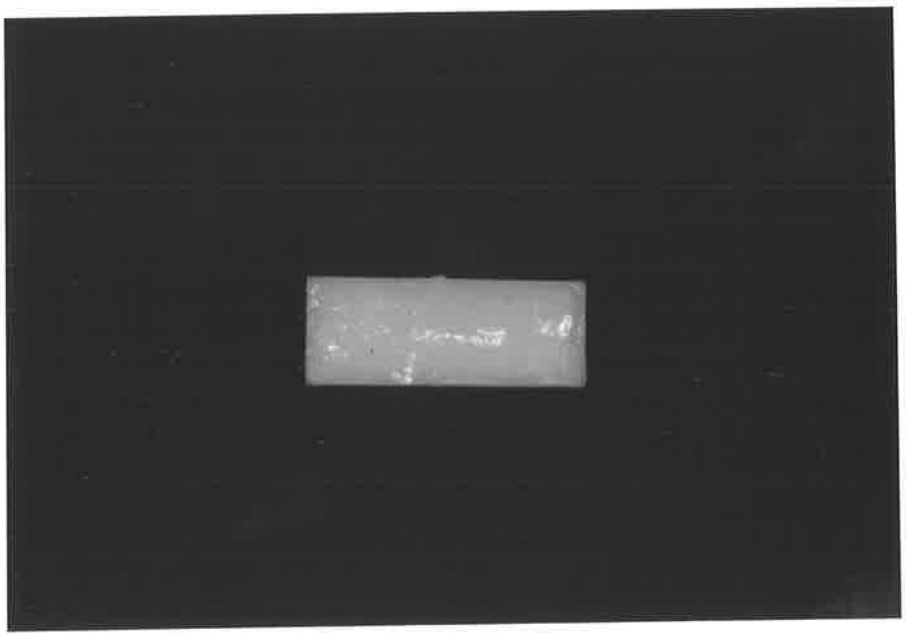


Figure 11

Figure 12: Photograph of the Oliphant Laminar Flow Unit used during the preparation of the specimens for implantation.

Figure 13: Photograph illustrating the dimensions of the Surgicel gauze prior to being inserted into lengths of Teflon tubing.

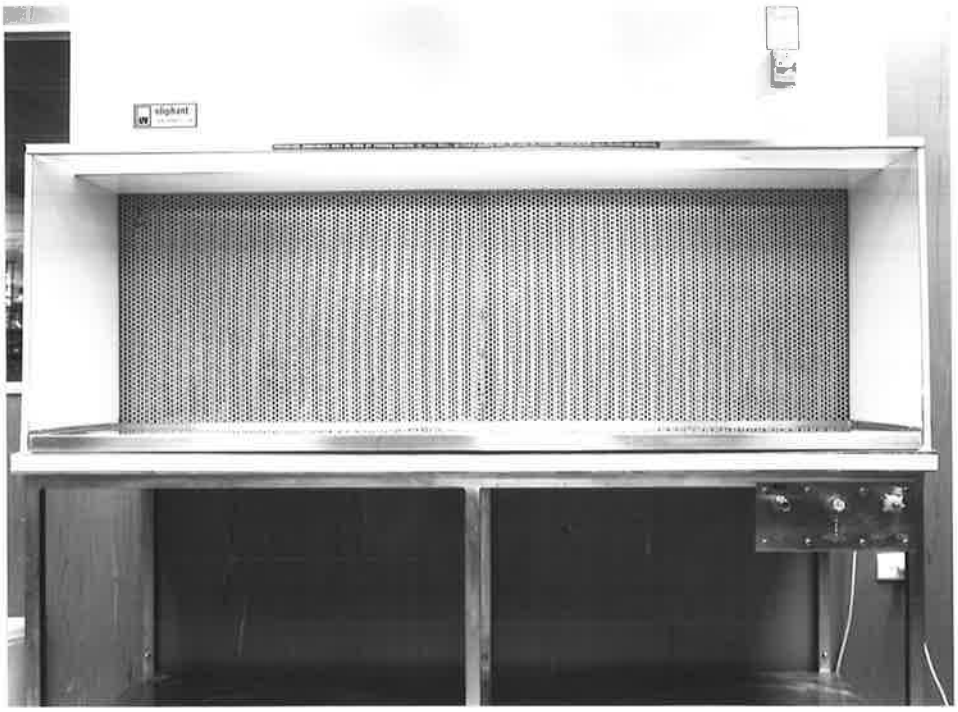


Figure 12

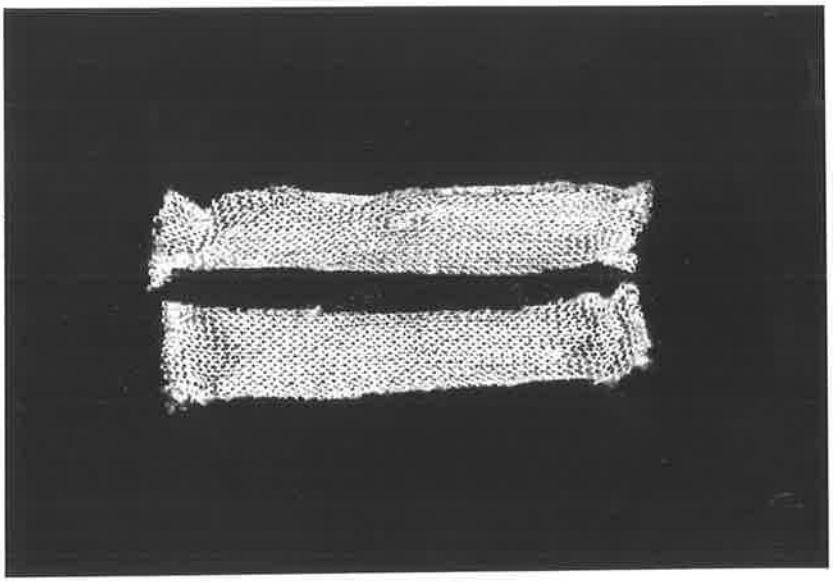


Figure 13

Figure 14: Photograph illustrating how the Surgicel gauze was drawn into the Teflon tubing using 000 black silk sutures.

Figure 15: Photograph showing how the Bone Wax was heated prior to being drawn into lengths of Teflon tubing.

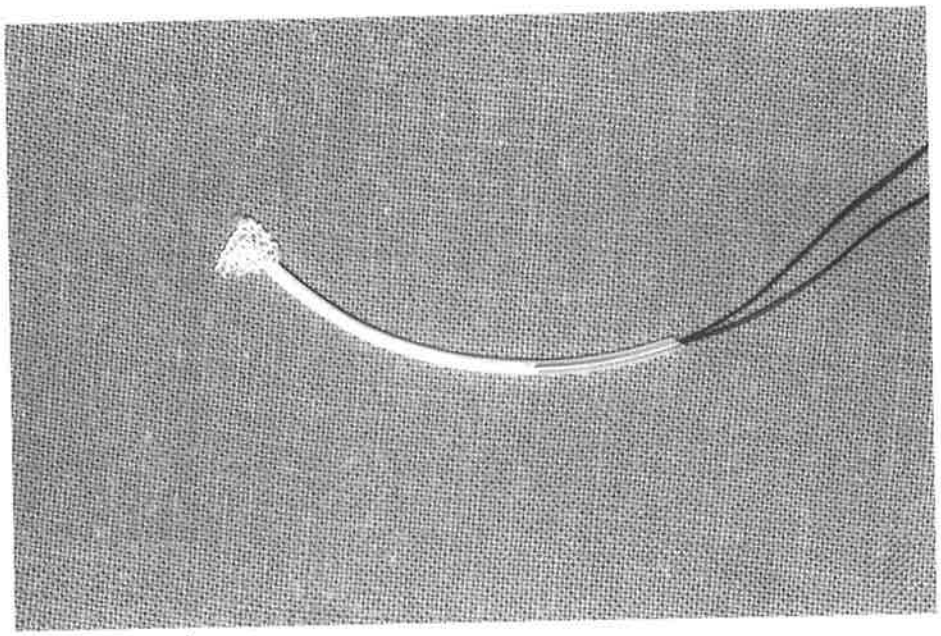


Figure 14

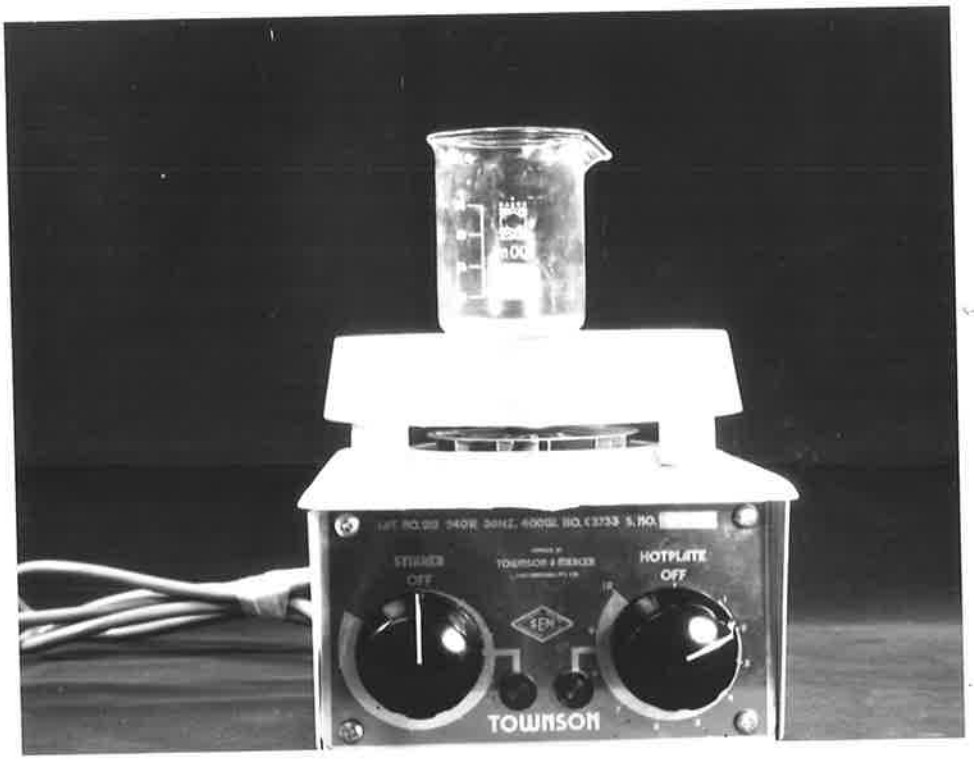


Figure 15

Figure 16: Photograph illustrating the method of drawing the molten Bone Wax into lengths of Teflon tubing.

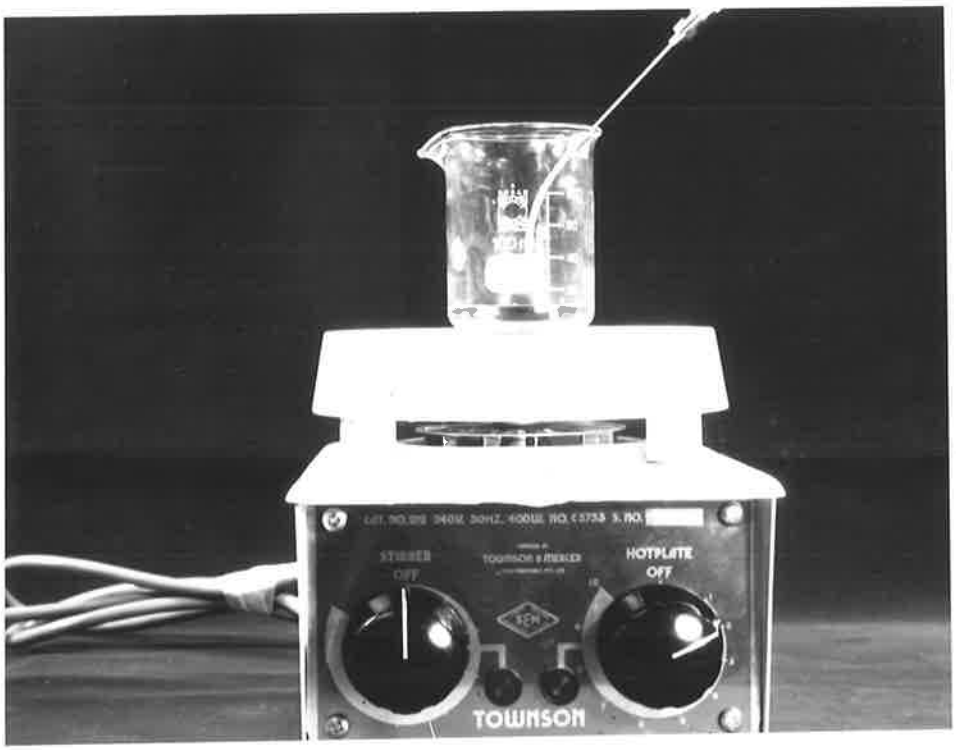


Figure 16

C: THE EXPERIMENTAL ANIMALS

The rat was chosen as the experimental animal in the present study. The reason for the choice of this animal related to their availability in adequate numbers, the suitability of adequate housing facilities and their appropriate size for the surgical procedures involved in this study.

Young male Sprague-Dawley Albino rats weighing between 200 and 250 gr. were used in this study. The number of animals used at the various experimental observation periods is shown in Table 4.

TABLE 4: THE NUMBER OF EXPERIMENTAL ANIMALS AT EACH POST-OPERATIVE OBSERVATIONAL TIME PERIOD

POST-OPERATIVE OBSERVATION TIME	TOTAL NUMBER OF ANIMALS PER TIME PERIOD
2 Days	6
7 Days (1 Week)	6
14 Days (2 Weeks)	6
21 Days (3 Weeks)	6
28 Days (4 Weeks)	6
42 Days (6 Weeks)	6
56 Days (8 Weeks)	6
84 Days (12 Weeks)	3
182 Days (26 Weeks)	3
TOTAL =	48 Rats

During the course of the experiment, four animals were housed per cage, fed a standard diet (Appendix I) and provided with water and libitum.

D: THE SURGICAL IMPLANTATION TECHNIQUE

Method of Anaesthesia:

Induction of anaesthesia was achieved in an ether chamber saturated with ether fumes. Anaesthesia was maintained with an injection of Ketalar* (ketamine hydrochloride) in the muscles of the upper limb of the rat in a dose of 0.2ml/100gr. body weight.

Operative Technique:

When the experimental animal was fully anaesthetized, it was placed on a wooden operating table, and held in place with elastic bands that extended from the fore- and hindlimbs to hooks on the operating table. The entire ventral surface of the rat was cleansed and sterilized with a sterile aqueous solution of 0.5% citrimide and 0.05% chlorhexidine**.

Throughout the experiment, Surgicel implants, both bone and soft tissue, were placed on the right side of the animal. The Bone Wax implants were placed on the left side. For each of the post-operative observational time periods (excluding twelve and twenty six weeks post-implantation), six experimental animals were

* Manufactured by Parke-Davis, Sydney, Australia.

** Manufactured by the Pharmacy Department, Royal Adelaide Hospital, Adelaide, Australia.

used. In four of these animals at each post-operative observational time period, the Surgicel and Bone Wax implants were placed on their respective sides as mentioned above, giving a total of four implants in soft tissue and bone for each of the test materials at each time period. The remaining two animals served as controls, and empty Teflon tubes were implanted bilaterally in the muscles of the anterior chest wall and in the femurs, giving, as well, a total of four control specimens in soft tissue and bone at each post-operative observational time period. At twelve and twenty-six weeks post-implantation, half the number of animals were operated upon, yielding half the number of specimens in each of the three groups.

Separate animals were chosen as controls on the basis of the results of pilot studies which showed that the basic reactions occurring within empty tubes was the same regardless of whether or not empty control tubes were placed in experimental animals or in separate control animals. Such a protocol also eliminates the possibility of any variables being created in experimental animals where both the experimental and control tubes are implanted in the same animal. Further, it is also economic of animals in terms of numbers required.

Table 5 illustrates the number and distribution of Surgicel, Bone Wax and control implants, used in this study.

TABLE 5: THE NUMBER AND DISTRIBUTION OF SURGICEL, BONE WAX AND CONTROL IMPLANTS AT EACH OF THE POST-OPERATIVE OBSERVATIONAL TIME PERIODS.

	SURGICEL SERIES		BONE WAX SERIES		CONTROL SERIES	
POST-OPERATIVE OBSERVATION TIME (DAYS)	TOTAL NUMBER OF IM* IMPLANTS	TOTAL NUMBER OF IF** IMPLANTS	TOTAL NUMBER OF IM* IMPLANTS	TOTAL NUMBER OF IF** IMPLANTS	TOTAL NUMBER OF IM* IMPLANTS	TOTAL NUMBER OF IF** IMPLANTS
2 Days	4	4	4	4	4	4
7 Days (1 Week)	4	4	4	4	4	4
14 Days (2 Weeks)	4	4	4	4	4	4
21 Days (3 Weeks)	4	4	4	4	4	4
28 Days (4 Weeks)	4	4	4	4	4	4
42 Days (6 Weeks)	4	4	4	4	4	4
56 Days (8 Weeks)	4	4	4	4	4	4
84 Days (12 Weeks)	2	2	2	2	2	2
182 Days (26 Weeks)	2	2	2	2	2	2
TOTAL NUMBER OF SPECIMENS = 192						

* IM = INTRAMUSCULAR; ** IF = INTRAFEMORAL

The instruments used in the operative procedure are listed and illustrated in Appendix III. Prior to their use, all the instruments were autoclaved in a Smith Pressure Sterilizer, and stored in Guardian Hospital Autoclave Bags. During the operative procedure, the instruments were always immersed in a 70% alcohol solution.

Insertion of the intramuscular implants: Following a skin incision, the muscles of the anterior chest wall were exposed, using a Metzenbaum Scissors. A small incision, running parallel to the muscle fibres, was then made to create a crevice between the fibres (Figure 17).

The appropriate Teflon tube, with or without the test material, was placed in this crevice (Figure 18) using College Tweezers. The muscle containing the implant was then sutured with 6/0 nylon sutures. This suture served two purposes:

(1) It prevented the tube from being expelled from the muscle during muscular contraction;
(2) It aided in the localization of the tube at the time of collection of the specimen from the animal.

The skin wound was sutured with 3/0 black silk sutures.

Insertion of the intrafemoral implants: An incision was made along the animal's thigh with Metzenbaum Scissors. The femoral neurovascular bundle was exposed and retracted. The shaft of the femur was then exposed by cutting into the overlying muscle and periosteum with a scalpel.

A cortical defect, measuring approximately 4mm in length, was created along the shaft of the femur (Figure 19) with a dental bur to expose the medullary cavity. The appropriate Teflon tube was placed into this defect (Figure 20) with College Tweezers, and inserted into a position in the shaft of the femur (as illustrated diagrammatically in Figure 7), using a flat plastic instrument. The overlying muscle and skin were closed with 3/0 black silk sutures.

Figure 17: Photograph showing the creation of a crevice between the muscle fibres of the anterior chest wall of the rat.

Figure 18: Photograph showing placement of Teflon tube into the crevice between the muscle fibres.

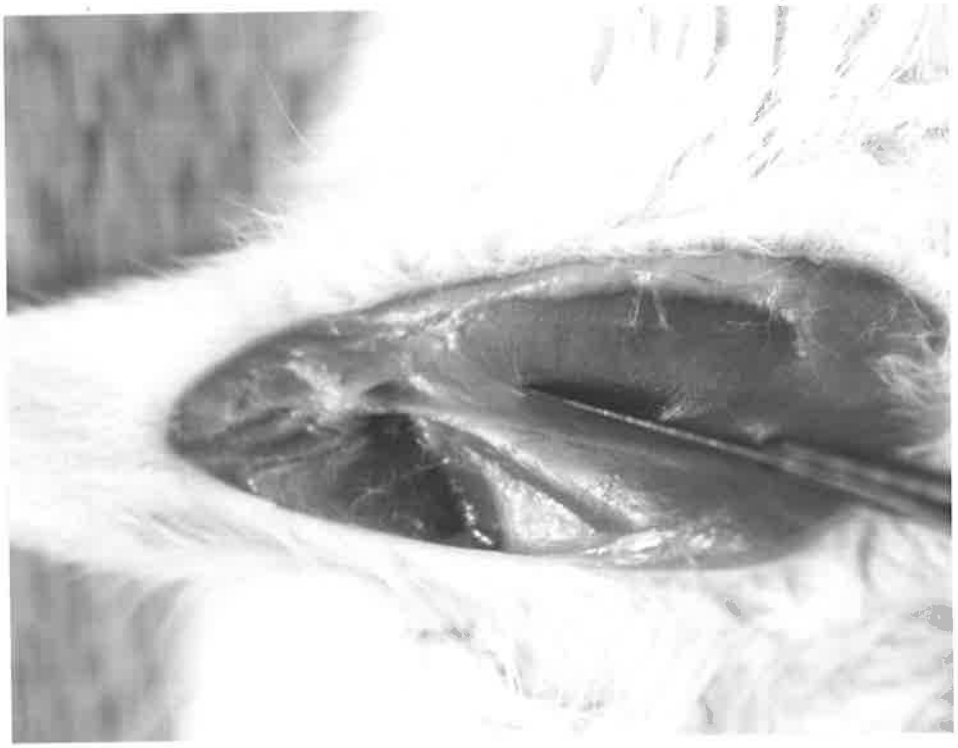


Figure 17

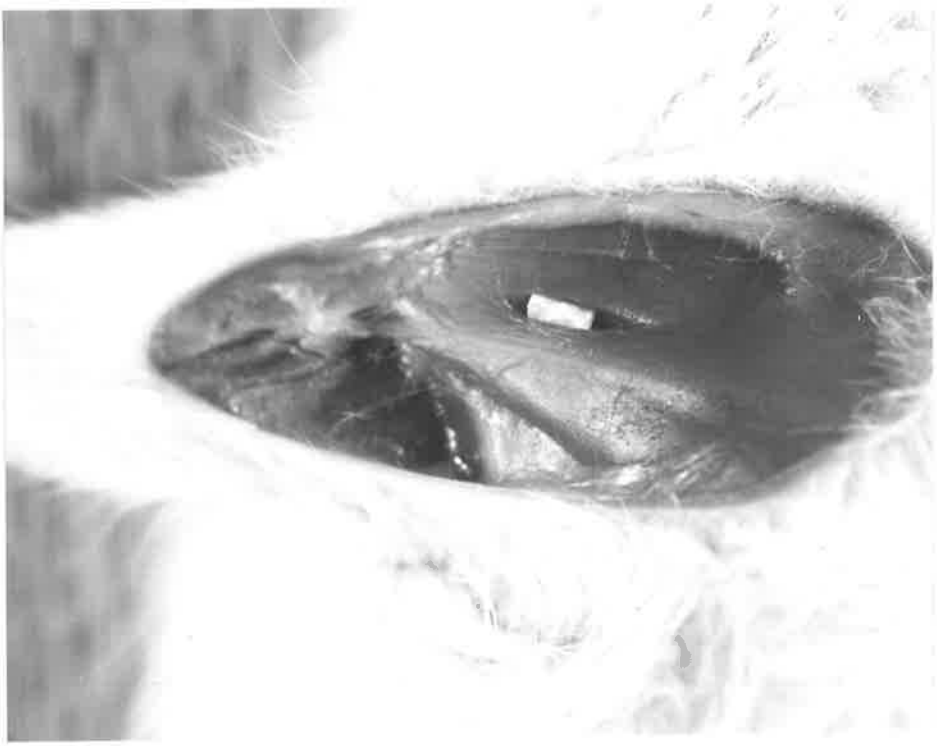


Figure 18

Figure 19: Photograph showing a cortical defect in the shaft of the femur of the experimental animal through which the Teflon tube was inserted into the marrow space.

Figure 20: Photograph showing placement of the Teflon tube into the cortical defect, after which it was inserted, with the aid of a flat plastic instrument, into the shaft of the femur.

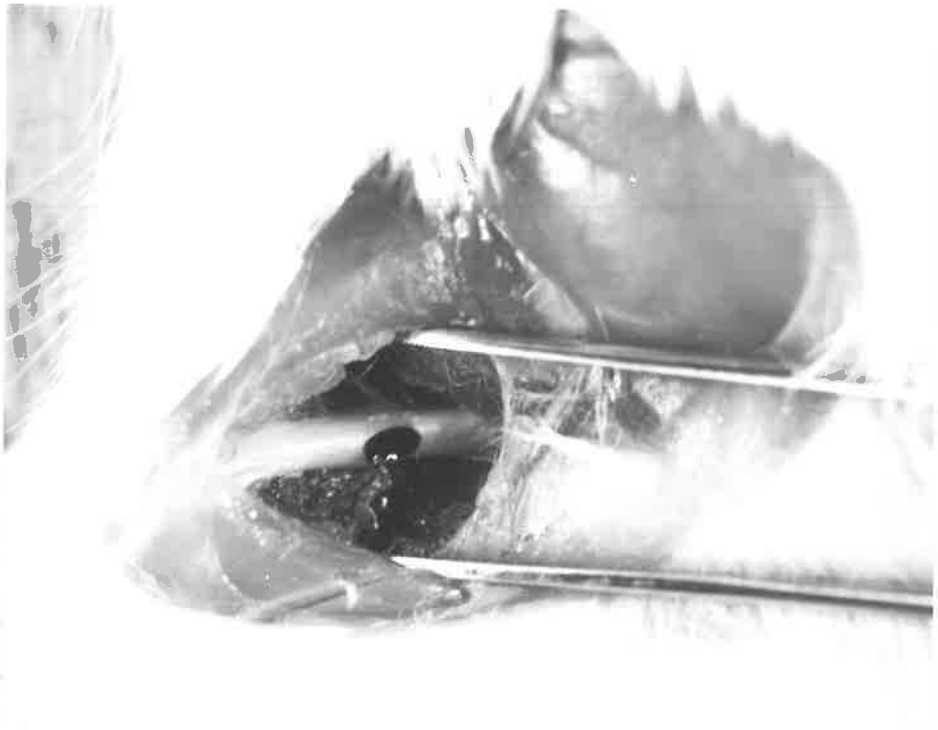


Figure 19

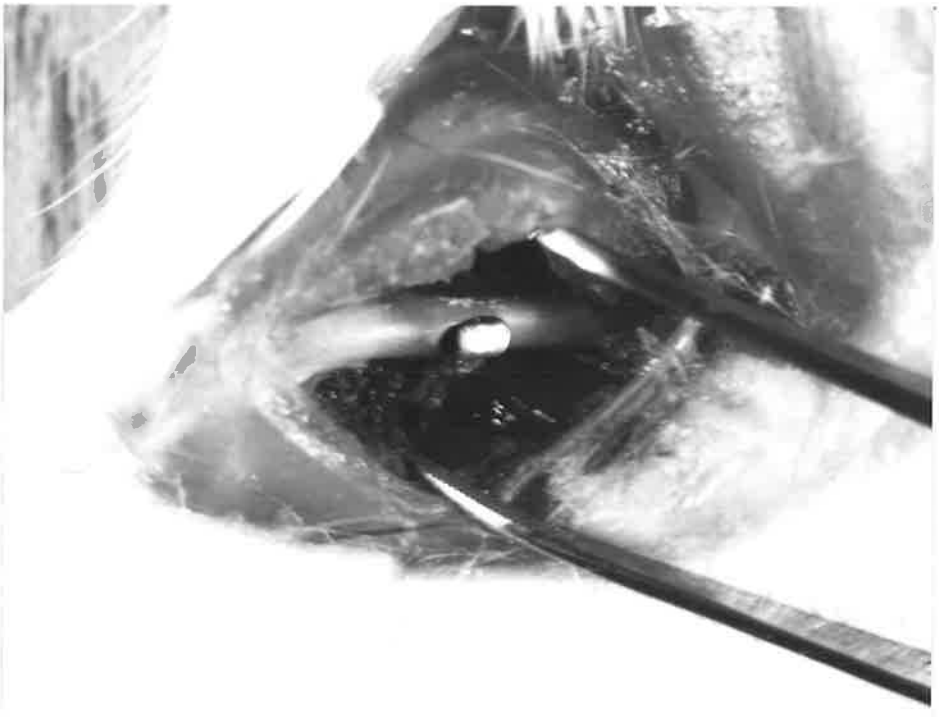


Figure 20

E: COLLECTION OF SPECIMENS

At the appropriate times, the animals were sacrificed using an ether overdose.

The Intramuscular Implants

Access to the sites of implantation was gained by a skin incision over the anterior chest wall using a Metzembaum Scissors. The implant was removed with a large amount of surrounding muscle. The specimen was immediately fixed in neutral buffered formalin (Appendix IV).

The Intrafemoral Implants

After access was gained to the femur, it was disarticulated from the hip and knee joints and removed en bloc with its attached muscles. These specimens were fixed in 10% neutral buffered formalin for 7 days after which time, the femur was stripped of its attached muscles. Both ends of the femur were then trimmed away with a rotary electric saw. After fixation, the femoral specimens were decalcified in Decal (Appendix IV) prior to being processed for histology.

F: PREPARATION OF HISTOLOGIC MATERIAL

All the specimens in this study were processed for histology using the double embedding technique (Appendix IV) and blocked in Paraplast + at $56^{o}C$.

After the specimens were blocked, serial sections, 7u in thickness, were cut using a Leitz Rotary Microtome (Model 1212, Germany) for all the experimental and control specimens. The sections were cut in a plane parallel to the long axis of the tube (Figure 21). In some cases, the specimens had to be reblocked to ensure proper alignment of the Teflon tubes with respect to the plane of section.

Short ribbons containing 3 sections were mounted on glass slides. Approximately 30 sections* were obtained from each specimen, giving a total of 10 slides per specimen. These slides were numbered from 1 to 10, slides 5 and 6 corresponding approximately to the centre of the tube. The latter were stained with Haematoxylin and Eosin and slide number 4 was stained using the van Gieson method. In addition, a selected number of slides for the various post-implantation time periods were stained using the Mallory's Phosphotungstic Acid Haematoxylin (PTAH) stain to demonstrate striated muscle fibres. This stain, however, was only used to confirm the presence of striated muscle fibres in the Teflon tube and the slides for this purpose were randomly selected. All specimens containing empty control and Surgicel-filled tubes were stained with Safranin-Aniline Blue in an attempt to identify Surgicel in the Surgicel-filled tubes. The technical details of the various staining procedures are described in Appendix V.

* Whilst it was theoretically possible to obtain many more sections for each specimen, it was extremely difficult to obtain more than 30 usable sections, especially in the soft tissue Surgicel and control implants. The reason for this is as follows: the ingrowth tissue into the tubes assumed an hour-glass shape (Figure 22). When such a section is theoretically viewed in cross-section (Figure 23), it becomes evident that the tissue in the tube occupies only a small portion in the centre of the tube. It can, therefore, be seen why fewer sections were obtained. For all specimens, only 30 sections from the centre of the tube were obtained in order to standardize the number of sections per specimen.

Figure 21: Diagram illustrating the plane of histologic section of specimens with respect to the Teflon tube.

Figure 22: Diagram showing the shape of the tissue ingrowth (B) into the lumen of the Teflon tube.

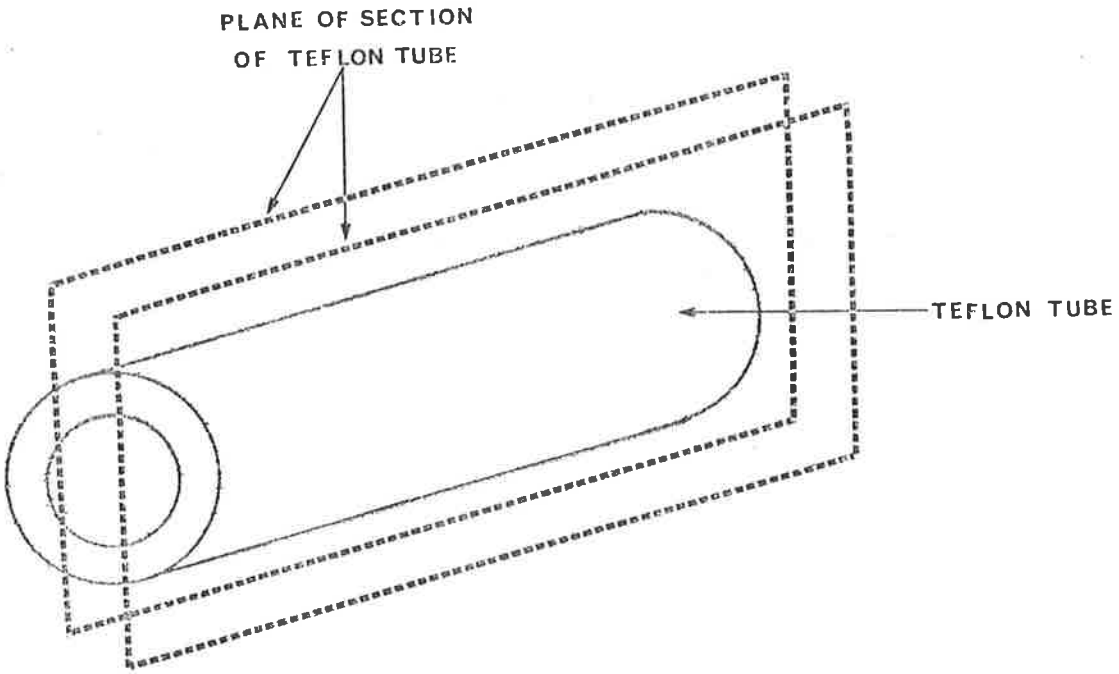


Figure 21

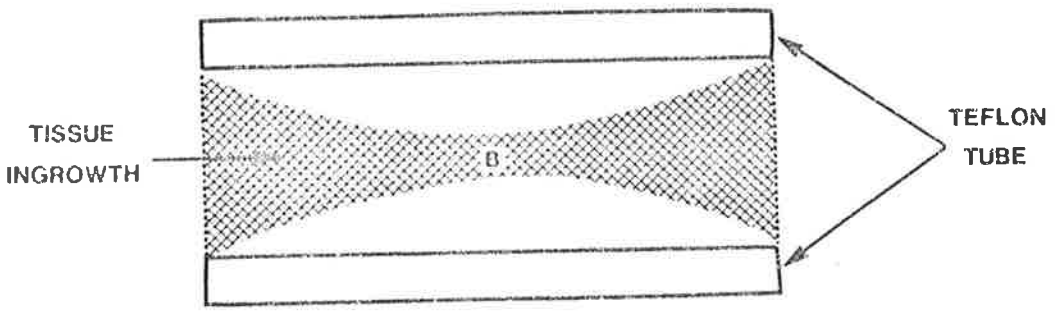


Figure 22

Figure 23: Diagram showing the location of tissue ingrowth in the Teflon tube, in cross-sectional view, with respect to the wall of the tube in the intramuscular control and Surgicel implants. Since the tissue ingrowth in the tube occupied a small portion of the centre of its lumen, a limited number of usable sections were obtained.

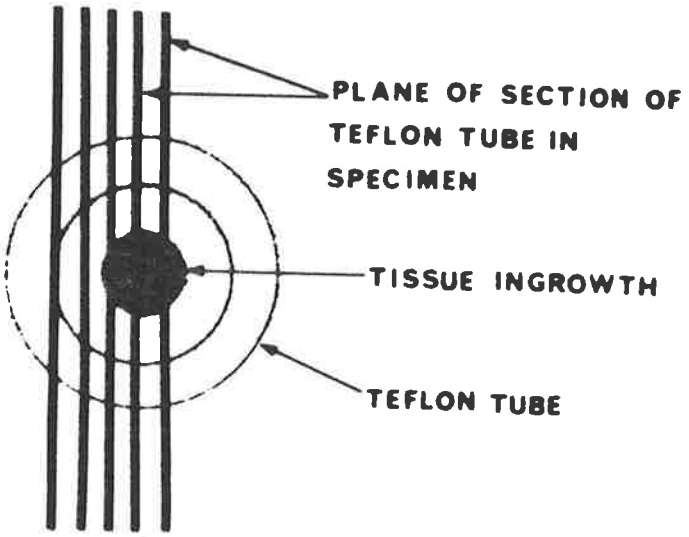

PLANE OF SECTION OF
TEFLON TUBE IN
SPECIMEN
TISSUE INGROWTH
TEFLON TUBE

Figure 23

G: ANALYSIS OF THE HISTOLOGICAL MATERIAL

The histological material obtained was analysed qualitatively and quantitatively. The quantitative assessment was confined to the intramuscular implants as described in the section below.

1. Surgicel and Control Series

(a) The Qualitative Assessment of the Tissue Reactions

Histological observations were confined to that zone within the Teflon tube, i.e. in Zone A in Figure 24.

The features assessed were:

(1) The presence or absence of inflammation. If present, the nature of the cellular infiltrate was assessed.

(2) The intensity and nature (viz. acute-chronic) of the inflammatory response.

(3) The presence or absence of macrophages. Particular attention was paid to signs of phagocytic activity by these cells as evidenced by the presence of cytoplasmic vacuolation and/or particulate material.

(4) The presence or absence of multinucleate foreign-body giant cells.

(5) The resorption and possible replacement of the test material.

(6) The nature of the tissues replacing the test materials.

The reaction outside the tube was not assessed in detail.

(b) The Quantitative Assessment of the Tissue Reactions.

In an attempt to quantify the rate of tissue ingrowth into the tubes containing the resorbable Surgicel and the empty control tubes, the following method of analysis was used: three sections from the centre of the tube of each specimen were projected onto a piece of paper on a wall using a Leitz Right Angle Prism

on a microscope. The outline of the Teflon tube and its contents was then traced. The total inner area of the tube and its contents was determined by using a planimeter.* The area of the tube was represented by the sum of areas A', B and A''; and the area of tissue ingrowth was represented by area B (Figure 25). The total area of the tube was designated as 'A_{tube}' and the area of the tissue occupying the tube designated as "$A_{tissue\ ingrowth}$". The percentage ingrowth of tissue into the tube was calculated as follows:

$$\frac{A_{tissue\ ingrowth}}{A_{tube}} \times 100$$

The steps involved in the statistical analysis of the Surgicel and control specimens are illustrated in Appendix V. The Student's t-Test was used to determine whether there was a statistically significant difference between the experimental and control tubes.

II Bone Wax Series

(a) Qualitative Assessment of the Tissue Reactions

This comprised a histological observation of the tissue reactions between the Bone Wax and the nearest muscle fibres, i.e. in Zone B (Figure 26). In the intrafemoral implants, the area between Bone Wax and haemopoietic marrow was examined. The histologic features assessed were the same as those for Surgicel.

(b) Quantitative Assessment of the Tissue Reactions

The Poisson distribution method was used in the analysis of the Bone Wax series. This form of analysis is a discrete frequency distribution of the number of times a rare event occurs. The purpose of fitting a Poisson distribution to numbers of rare events in nature is to test whether the events occur independently with respect to each other. If they do, they will follow the Poisson distribution. If the occurrence of one event enhances

* Type KP27, Manufactured by Koizumi, Japan.

the probability of a second such event, one obtains a clumped or contiguous distribution. If the occurrence of one event impedes that of a second such event in the sampling unit, a repulsed or spatially uniform distribution is obtained. In this study, the Poisson distribution was used to determine the effect of Bone Wax on the distribution of foamy macrophages in the tissues adjacent to the implant face.

From the data obtained by cell counts in the specimens, the Coefficient of Dispersion (C.D.) was determined. This value will be near 1 in distributions that are essentially Poisson; will be > 1 in clumped samples and < 1 in cases of repulsion (Sokal and James Rohlf: 1969). The details of this analysis are presented in Appendix V.

Whilst the C.D. indicated whether the foamy macrophages were distributed in a Poisson fashion or not, another form of analysis was undertaken to elaborate numerically on how these cells were distributed within the confines of the Olympus Eyepiece Micrometer (i.e. within the area of 260 microns by 260 microns). For this purpose, the area under examination was divided into 5 equal zones which run parallel to the margin of the Bone Wax, and these were termed zones 1, 2, 3, 4 and 5 (Figure 27). Using this form of analysis, it was possible to determine the number of foamy macrophages that occurred in each of the zones and, thereby, elaborate on the C.D. for each of the post-implantation periods.

The range of distances from the Bone Wax implant that each zone represented in this latter analysis, is illustrated in Table 6.

TABLE 6: RANGE OF DISTANCES FROM BONE WAX IMPLANT THAT EACH ZONE REPRESENTS.

ZONE	DISTANCE (IN MICRONS) FROM BONE WAX
1	0 to 52
2	53 to 104
3	105 to 156
4	157 to 208
5	209 to 260

****

Figure 24: Diagram illustrating the zone within the lumen of the Teflon tube (Zone A) of the intramuscular control and Surgicel implants that was assessed qualitatively.

Figure 25: Diagram illustrating the outline of the Teflon tube and its contents. The area of the inner aspect of the tube, represented by the sum of areas A', B and A"; and the area of tissue ingrowth, represented by area B, were determined using a planimeter.

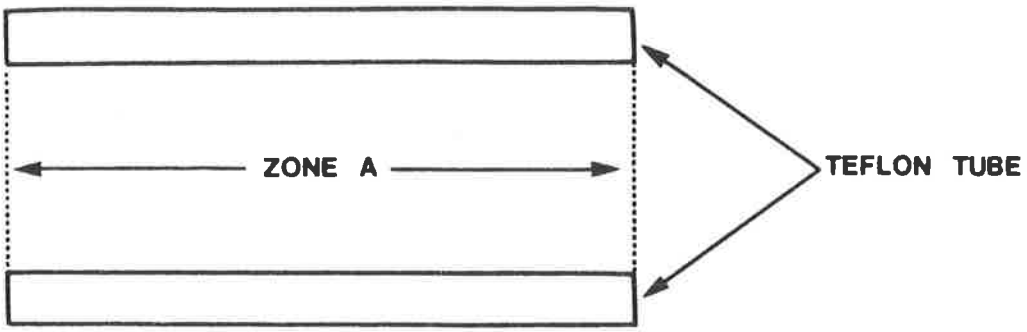


Figure 24

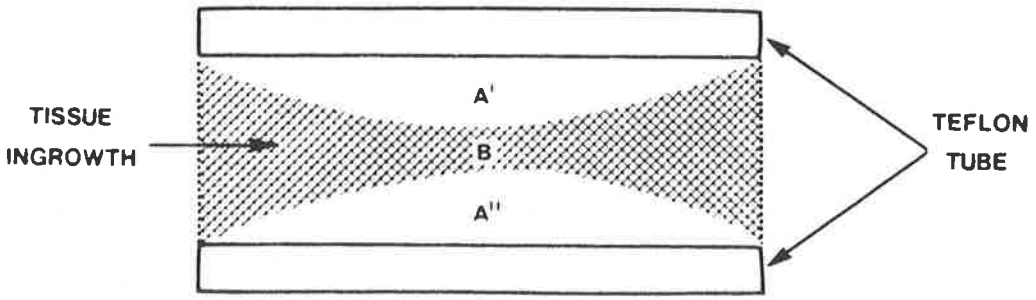


Figure 25

Figure 26: Diagram illustrating the area adjacent to Bone Wax (Zone B) that was assessed histologically.

T = Teflon tube.

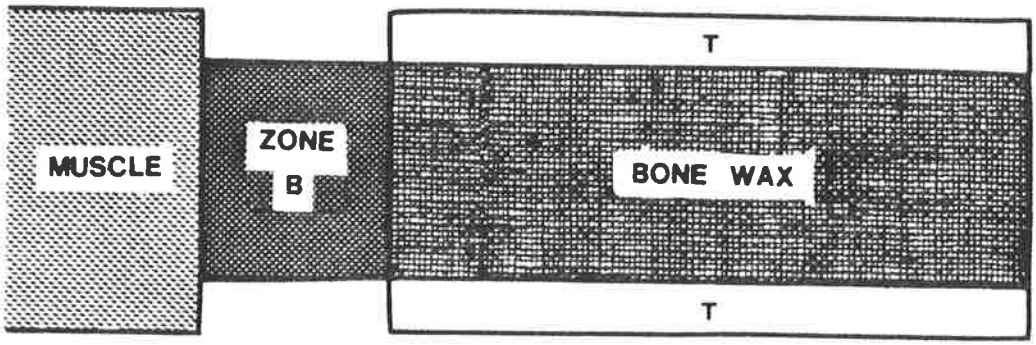


Figure 26

Figure 27: Diagram illustrating the division of the area within the confines of the Olympus Eyepiece Micrometer (260 microns by 260 microns), adjacent to Bone Wax, into five equal zones (Zones 1, 2, 3, 4, 5) which run parallel to the implant material.

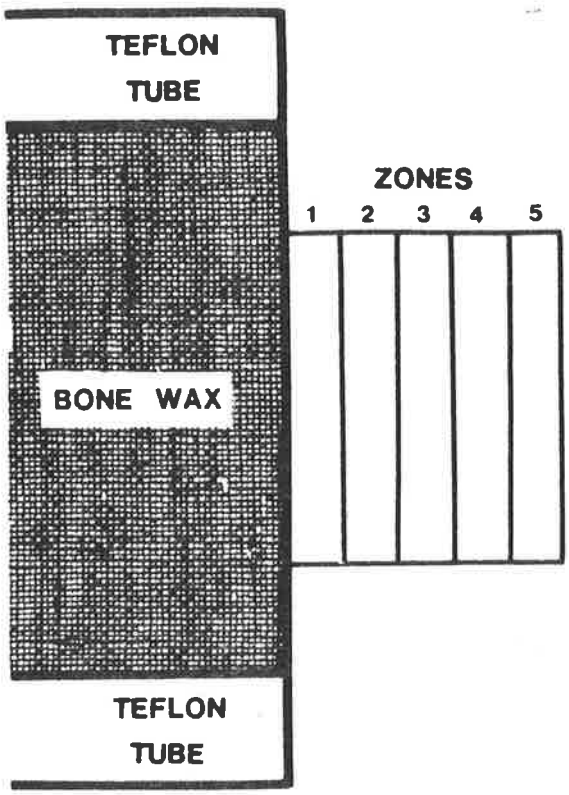


Figure 27

CHAPTER 4

RESULTS

Out of a total number of forty-eight rats, three died post-operatively of unknown causes. The remaining forty-five rats remained in an excellent state of health and no post-operative infections were seen at the sites of implantation.

SECTION I: HISTOLOGICAL ASSESSMENT OF THE INTRAMUSCULAR IMPLANTS

(A) Control Implants

Microscopic observations of the empty control Teflon tubes at the various post-implantation periods revealed the following:

Two days post-implantation: The lumina of the Teflon tubes were filled with an eosinophilic fibrillar coagulum and inflammatory cells (Figure 28). The predominant cell type seen was the mononuclear phagocyte (macrophage). These cells were scattered randomly between fibrin strands. Occasionally, polymorphonuclear leukocytes were seen, but many of these were in the process of undergoing degeneration. A few erythrocytes were also present between the strands of fibrin.

Seven days post-implantation: The most striking histologic feature was the presence of a triangularly-shaped ingrowth of granulation tissue into both ends of the Teflon tube (Figure 29). This granulation tissue contained large numbers of erythrocytes, macrophages and plump fibroblasts. A few lymphocytes were also evident among these cells. Some of the macrophages were large and foamy, while others were normal in size and appearance (Figure 30). The presence of newly-synthesized collagen was revealed in van Gieson-stained sections. Fibrillar material occupied the remainder of the lumen of the tube.

Two weeks post-implantation: Microscopic observations of sections from this time period revealed that maturing granulation tissue and

young fibrous tissue had grown through the entire length of the lumina of the Teflon tubes (Figure 31). This ingrowth had an hour-glass appearance. On either side of the granulation tissue, fibrillar material was still evident. Macrophages and fibroblasts formed the chief cell types present, although a few lymphocytes were also seen. Many blood vessels, filled with erythrocytes, were noted in the substance of the granulation tissue.

An interesting feature noted at this observational time period in Haematoxylin and Eosin-stained material was the presence of striated muscle fibres and cells within the granulation tissue ingrowth into the Teflon tubes. The presence of muscle cells was confirmed using PTAH-stained sections (Figure 32). In some areas, the muscle fibres were arranged in the form of bundles, while in other areas, they occurred singly.

Three weeks post-implantation: The amount and nature of the tissue ingrowth into the lumina of the tubes was not markedly different from that observed at two weeks post-operatively (Figure 33). Mature fibrous tissue containing bundles of striated muscle fibres comprised the main elements of the hour glass-shaped contents of the tube. Macrophages remained prominent, especially towards the periphery of the tissue ingrowth. Fibrillar material was noted between the periphery of the tissue ingrowth and the inner wall of the Teflon tube.

Four weeks post-implantation: The histological picture was essentially the same as that observed at three weeks except that some adipose tissue was now seen within the substance of the tissue ingrowth.

Six and eight weeks post-implantation: Observation of histologic specimens at these time periods revealed that the tissue ingrowth into the lumina of the Teflon tubes was largely composed of striated muscle fibres (Figure 34). At the same time, there was an increase in the amount of adipose tissue present in the tube. Fibrillar material could still be seen on either side of the tissue ingrowth.

Twelve weeks post-implantation: The most striking histological feature observed at this time period was the replacement, to a great extent, of the striated muscle within the tube by adipose tissue (Figure 35). Bundles of striated muscle fibres were still seen between fat cells and van Gieson-stained sections revealed the presence of collagen fibres along the periphery of the tissue ingrowth. The fibrillar material, noted earlier, still featured prominently on either side of the tissue ingrowth which still maintained an hourglass shape.

Twentysix weeks post-implantation: The contents of the tubes were composed largely of adipose tissue although bundles of striated muscle fibres still remained (Figure 36).

Figure 28: Intramuscular Control Implant 2 days:
Photomicrograph showing fibrin and inflammatory cells (F) in the lumen of the Teflon tube. T represents artefact space due to loss of the Teflon tube during section preparation. Inflammation (I) of the striated muscle (SM), caused by surgical trauma, can be seen outside the wall of the Teflon tube (T). H & E x 40

Figure 29: Intramuscular Control Implant 7 days:
Photomicrograph showing a triangularly-shaped cellular granulation tissue ingrowth (TI) into the lumen of the Teflon tube. Fibrillar material (F) occupies the remainder of the lumen of the Teflon tube (T). H & E x 40

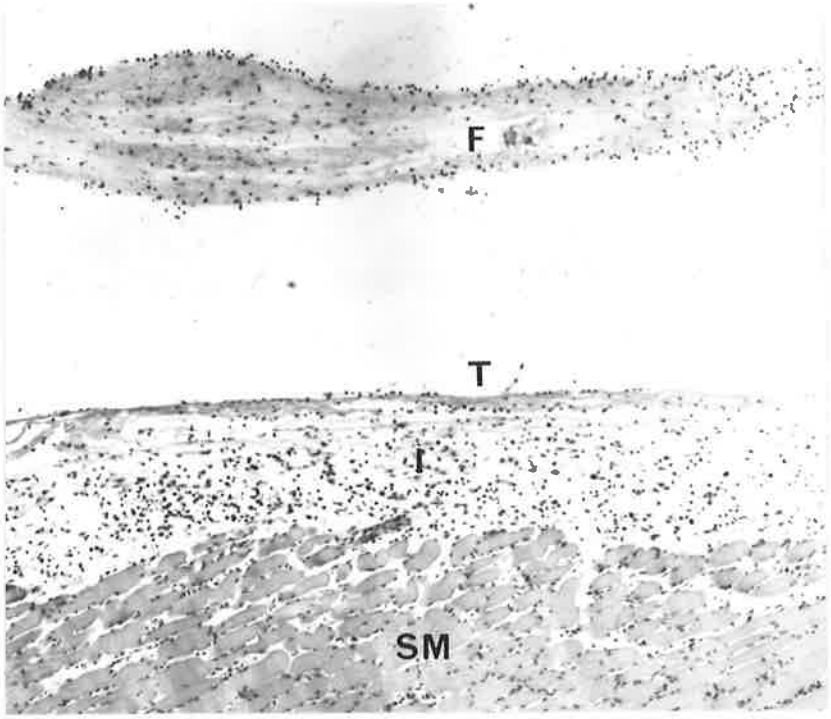


Figure 28

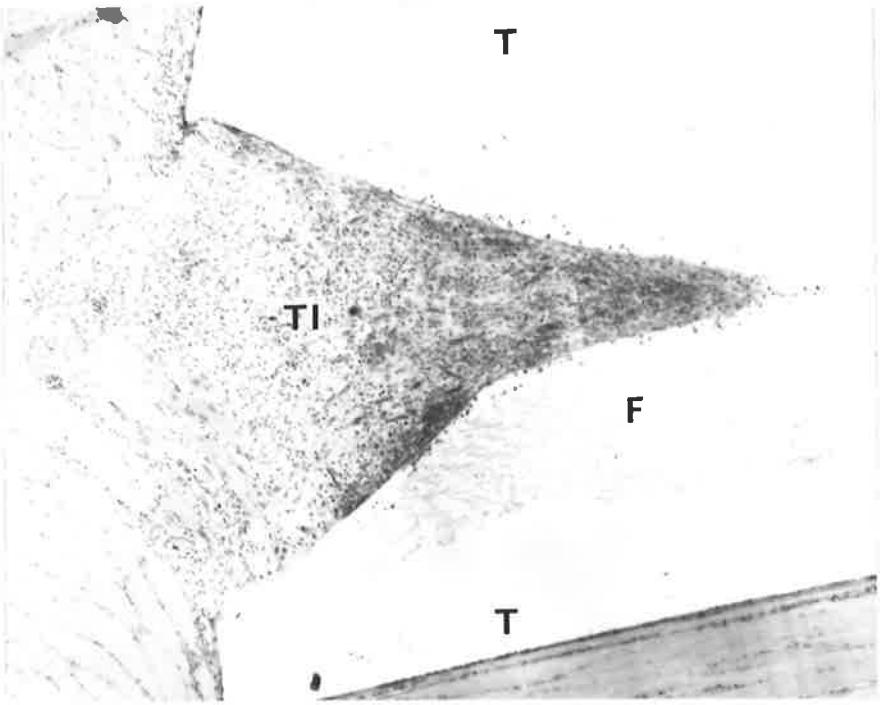


Figure 29

Figure 30: Intramuscular Control Implant 7 days:
High power photomicrograph of an area in Figure 2 showing the cellular nature of the tissue ingrowth into the tube. Macrophages (FM) can be seen along the margin of the tissue ingrowth. H & E x400

Figure 31: Intramuscular Control Implant 2 weeks:
Photomicrograph showing the ingrowth of tissue (TI) throughout the entire length of the lumen of the Teflon tube (T). Note the hourglass shape of the tissue ingrowth (TI). Fibrillar material (F) can be seen on either side of this ingrowth. H & E x 25.

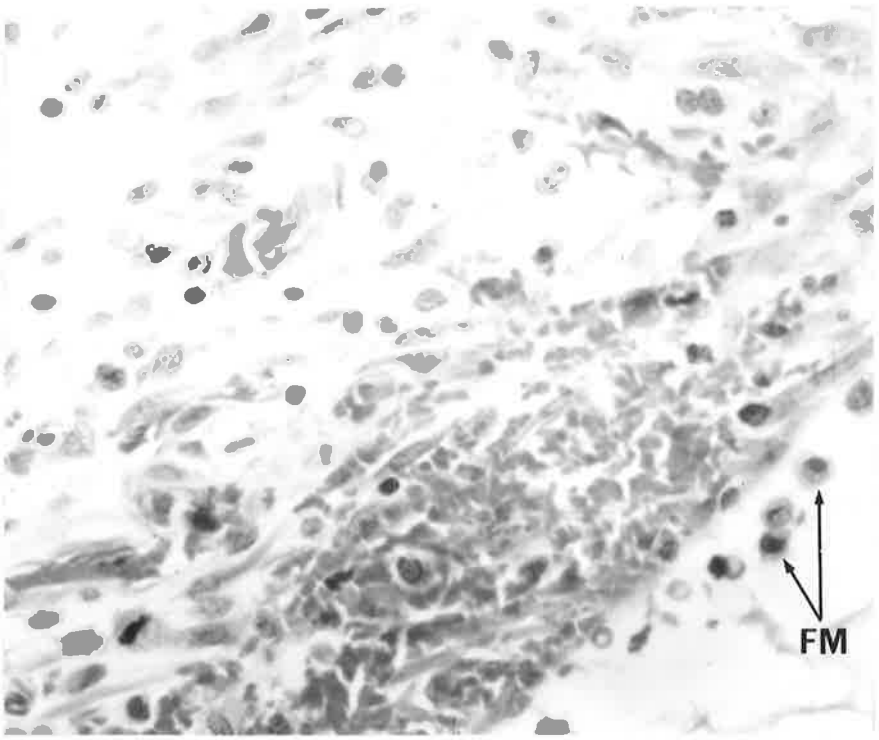


Figure 30

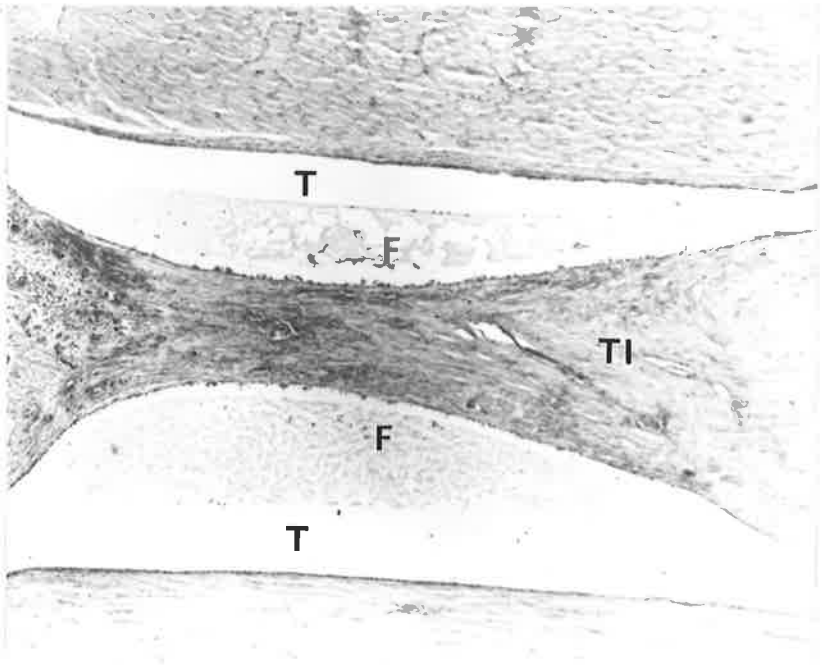


Figure 31

Figure 32: Intramuscular Control Implant 2 weeks:
High power photomicrograph of an area in Figure 31 showing striated muscle fibres (SM) within the tissue ingrowth. PTAH x 400

Figure 33: Intramuscular Control Implant 3 weeks:
Photomicrograph illustrating tissue ingrowth (TI) into the lumen of the Teflon tube (T). Fibrillar material (F) is present on either side of this tissue ingrowth. H & E x 25

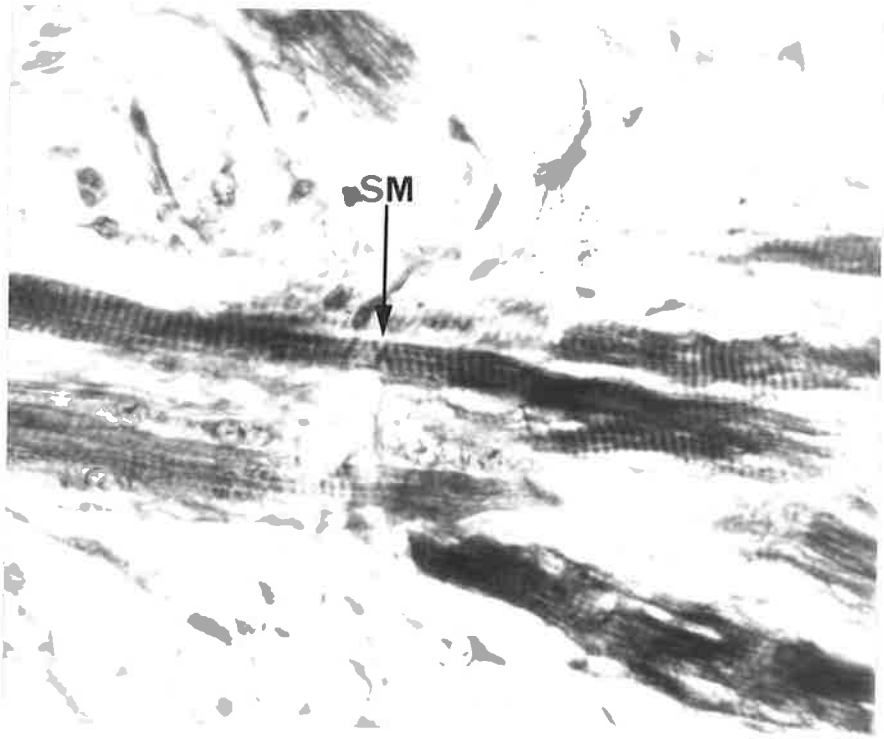


Figure 32

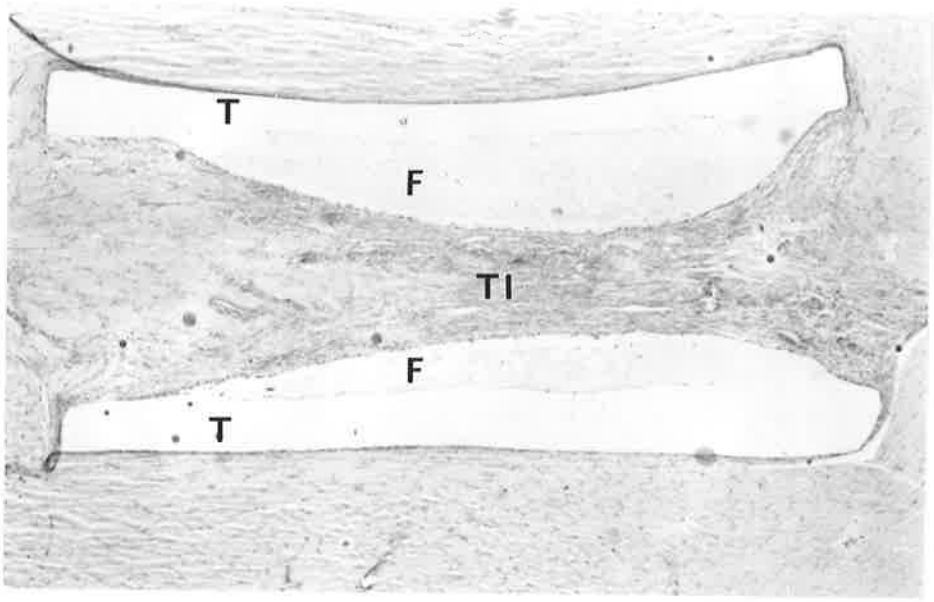


Figure 33

Figure 34: Intramuscular Control Implant 8 weeks:
Photomicrograph showing striated muscle fibres (SM) and adipose tissue (AT) within the lumen of the Teflon tube (T). Fibrillar material (F) can still be seen on either side of the tissue ingrowth. H & E x 25

Figure 35: Intramuscular Control Implant 12 weeks:
Photomicrograph showing striated muscle fibres (SM) and adipose tissue (AT) comprising the tissue ingrowth seen at this time period. Collagen (CF) can be seen along the periphery of the tissue ingrowth and fibrillar material (F) is noted on either side between the ingrowth and the inner wall of the Teflon tube (T).
van Gieson x 25.

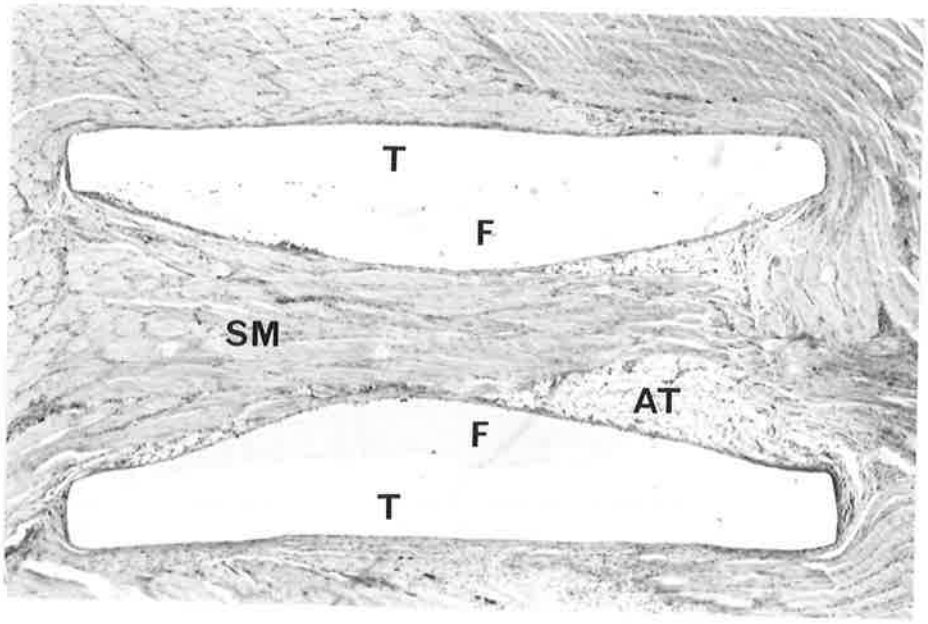


Figure 34

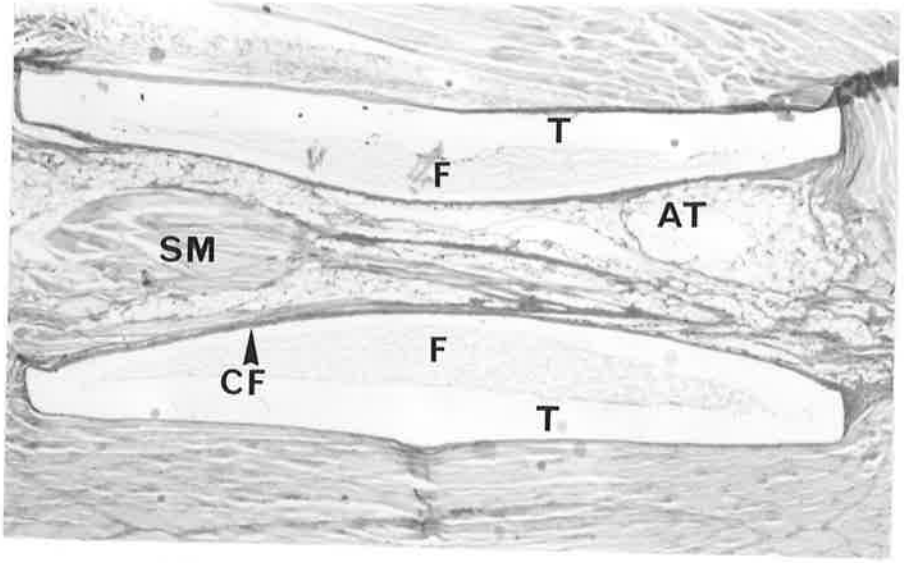


Figure 35

Figure 36: Intramuscular Control Implant 26 weeks:
Photomicrograph illustrating the predominance of adipose tissue (AT) within the lumen of the Teflon tube (T). A few bundles of striated muscle fibres (SM) still remain. H & E x 25

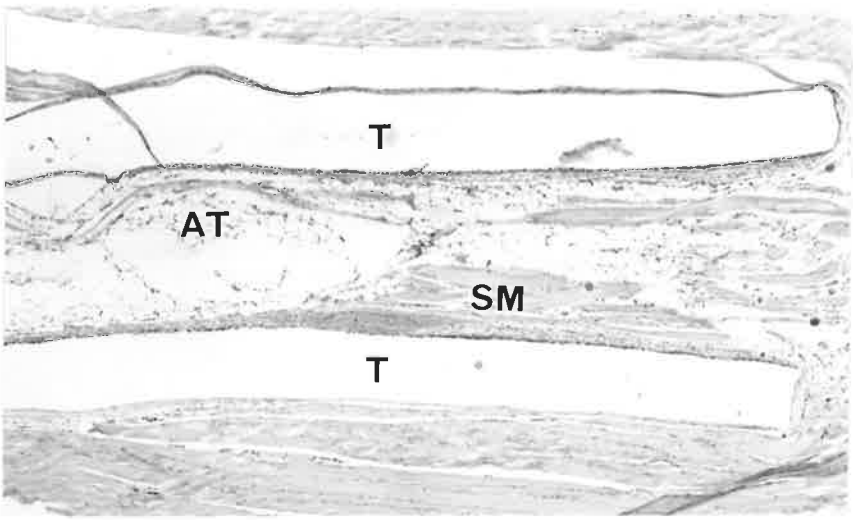


Figure 36

(B) Surgicel Implants

Microscopic observation of all the intramuscular Surgicel implants revealed that Surgicel was not clearly identifiable in a morphologic form similar to its original knitted appearance (Figure 37) or as discrete fibres (Figure 38). However, in the intrafemoral Surgicel implants, the fibrous nature of the implant material was clearly identifiable at two and seven days post-implantation (Figures 69 and 72).

Histologic examination of all the specimens at the various post-implantation periods revealed the following:

Two days post-implantation: Microscopic observation of all sections at this time period revealed that the lumina of the Teflon tubes contained an eosinophilic fibrillar coagulum containing cells (Figure 39). Detailed examination of the tube contents demonstrated the presence of large numbers of polymorphonuclear leukocytes and macrophages, as well as eosinophilic fibrillar material (Figure 40). This eosinophilic coagulum was morphologically different from fibrin and was interpreted as being altered Surgicel in these two day post operative group of specimens. Some of the macrophages exhibited a foamy appearance, while others were normal in size and shape. Many of the polymorphonuclear leukocytes had pyknotic nuclei.

Seven days post-implantation: A wedge-shaped ingrowth of tissue was noted at both ends of the Teflon tube (Figure 41). The eosinophilic fibrillar material noted at two days post-operatively, still occupied the remainder of the lumen of the tube (Figure 42). The granulation tissue was characterised by an abundance of erythrocytes, macrophages and plump fibroblasts. Many of the macrophages had a foamy appearance but no particulate or stainable matter was identifiable within these cells. A few polymorphonuclear leukocytes were

seen. Plasma cells and lymphocytes were also present in small numbers.

Two weeks post-implantation: At this time period, the tissue ingrowth extended along the entire length of the tubes and had an hour-glass appearance (Figure 43). The ingrowth consisted of large numbers of fibroblasts in a collagenous matrix, and macrophages. Polymorphonuclear leukocytes were absent. Fibrillar material, indistinguishable from the fibrillar material noted in the corresponding control tubes, could be seen between the tissue ingrowth and the inner surface of the Teflon tube. Striated muscles fibres were observed within the substance of the tissue ingrowth and were demonstrated using PTAH-stained sections (Figure 44).

There was no clear evidence of readily identifiable Surgicel in the tissues or within macrophages.

Three weeks post-implantation: The ingrowth within the tube exhibited an hour-glass shape (Figure 45) and consisted mainly of striated muscle fibres and connective tissue (Figure 46). Fibrillar material was seen between the tissue ingrowth and the inner surface of the Teflon tube. This material was indistinguishable from the fibrillar material noted in the control series. Adipose tissue was observed within the substance of the tissue ingrowth and, scattered among the fat cells, were macrophages and occasional lymphocytes. Flattened fibroblasts were noted along the outer margin of the tissue ingrowth.

Four weeks post-implantation: The reaction observed was essentially the same as that seen at three weeks post-operatively except that a greater quantity of striated muscle fibres and adipose tissue was noted in the tissue ingrowth.

Six weeks post-implantation: Striated muscle fibres and adipose tissue were seen in increasing quantities in the tissue ingrowth, while there was a corresponding decrease in the amount of fibrous tissue (Figure 47). A small amount of fibrillar material was still present on either side of the tissue ingrowth.

Eight weeks post-implantation: The striated muscle within the tissue ingrowth was largely replaced by adipose tissue (Figure 48). Flattened fibroblasts and prominent collagen fibre bundles were seen along the outer margin of the tissue ingrowth, and the fibrillar material, previously described, was still present (Figure 49).

Twelve and twentysix weeks post-implantation: Observation of histologic specimens at these time periods showed that the tissue ingrowth into the lumen of the Teflon tube consisted mainly of adipose tissue. Apart from the relative loss of some striated muscle fibres and an increase in the amount of adipose tissue, the histologic picture seen was identical to that observed eight weeks post-operatively.

Figure 37: Photomicrograph of unused and unstained Surgicel. Note the knitted structure of the gauze.

Figure 38: Photomicrograph of Surgicel embedded in Bacto-agar. Note its fibrous appearance. Safranin-Aniline Blue x 25.

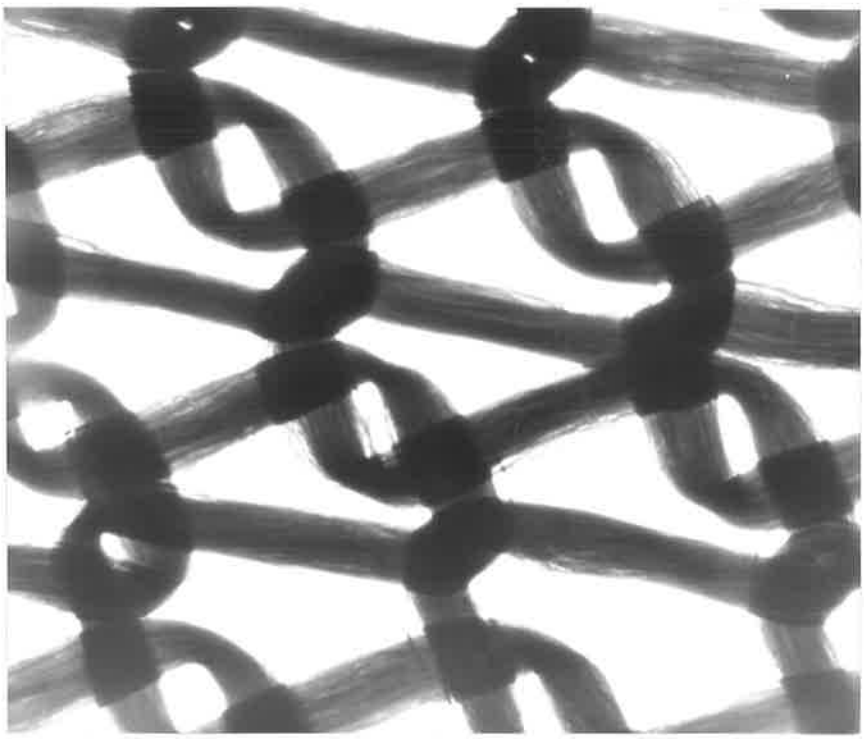


Figure 37

Figure 38

Figure 39: Intramuscular Surgicel Implant 2 days:
Photomicrograph showing Teflon tube (T) containing exudate and inflammatory cells (IE) within its lumen. The area (S) differs in composition and appearance from the other tube contents and represents altered Surgicel.
H & E x 25

Figure 40: Intramuscular Surgicel Implant 2 days:
High power photomicrograph of an area in Figure 39 showing fibrillar material (F) and inflammatory cells.
PAS x 100

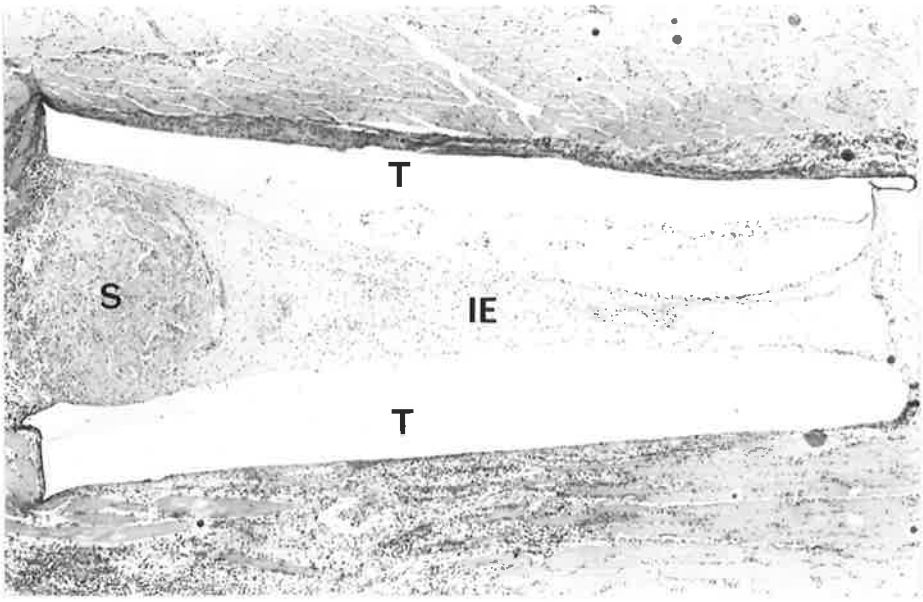


Figure 39

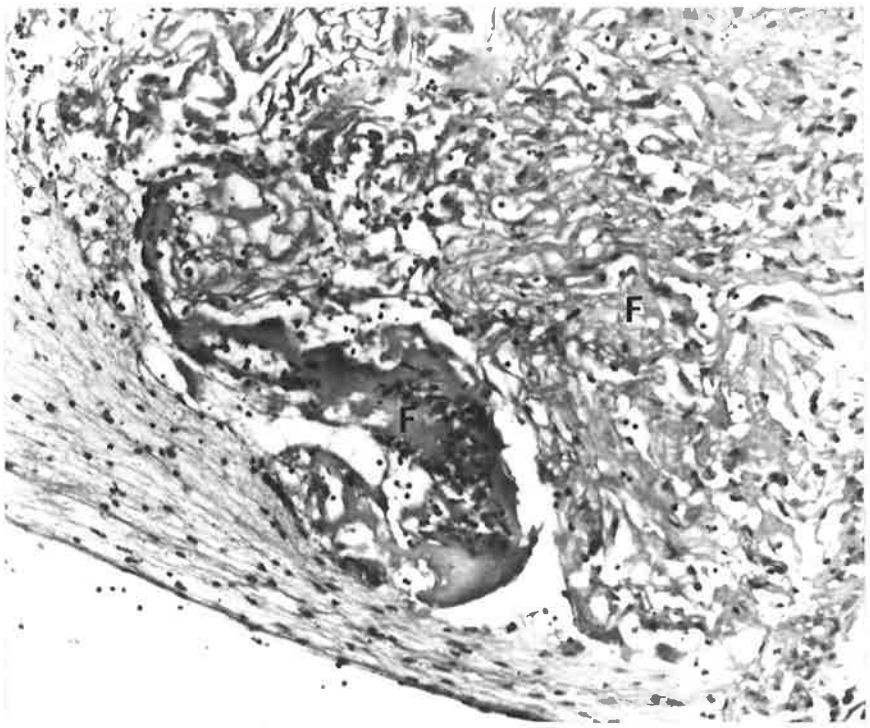


Figure 40

Figure 41: Intramuscular Surgicel Implant 7 days:
Photomicrograph showing a wedge-shaped ingrowth of tissue (TI) into the lumen of the Teflon tube (T). The remainder of the tube is filled with a fibrillar material (F). Striated muscle fibres (SM) can be seen on the left.
H & E x 40.

Figure 42: Intramuscular Surgicel Implant 7 days:
Photomicrograph showing coarse fibrillar material (F) adjacent to the tissue ingrowth (TI).
PAS x 100.

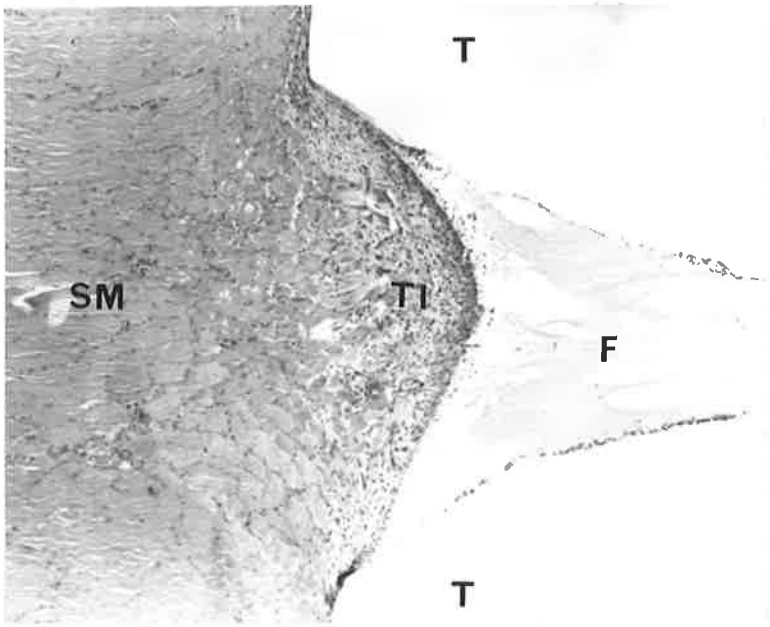


Figure 41

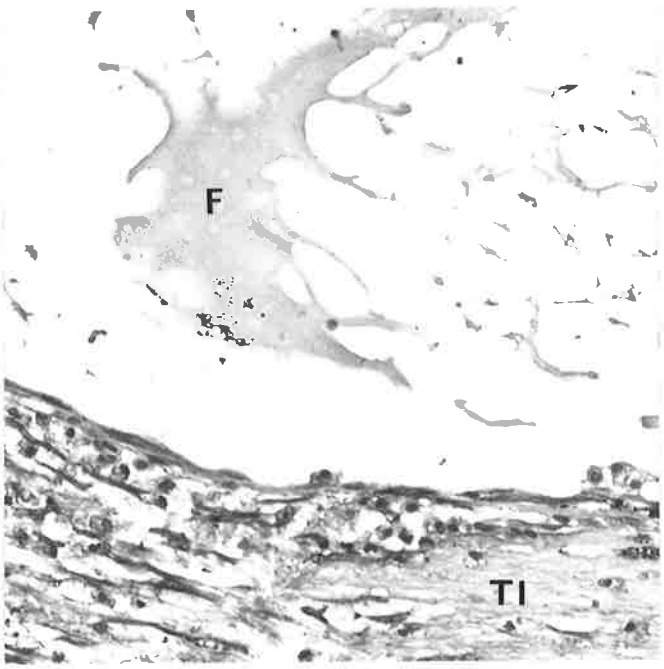


Figure 42

Figure 43: Intramuscular Surgicel Implant 2 weeks:
Photomicrograph showing an hour-glass shaped tissue ingrowth (TI) into the lumen of the Teflon tube (T). Fibrillar material (F) can be seen on either side of the tissue ingrowth. H & E x 25

Figure 44: Intramuscular Surgicel Implant 2 weeks:
High power photomicrograph of an area in Figure 43 showing striated muscle fibres (SM) within the substance of the tissue ingrowth in the Teflon tube. PTAH x 400

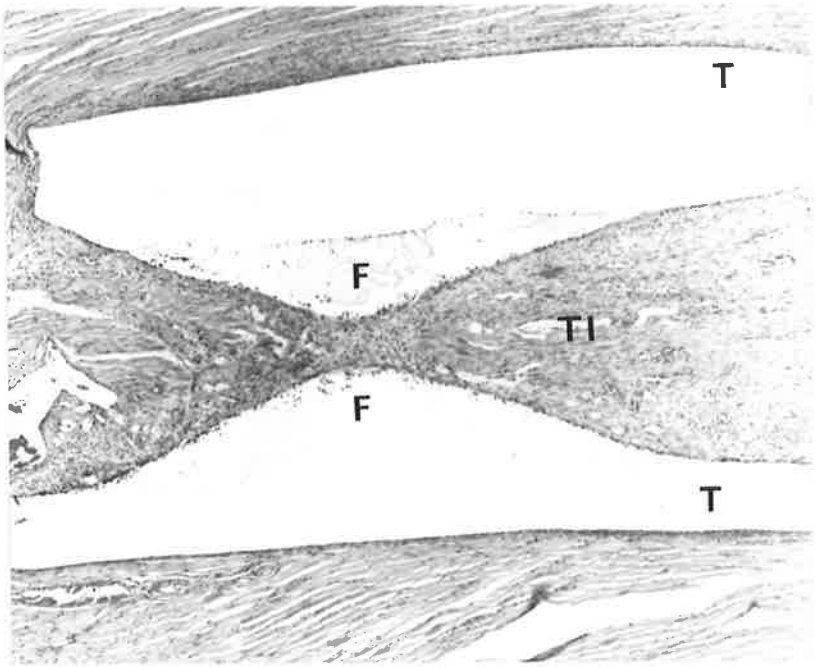


Figure 43

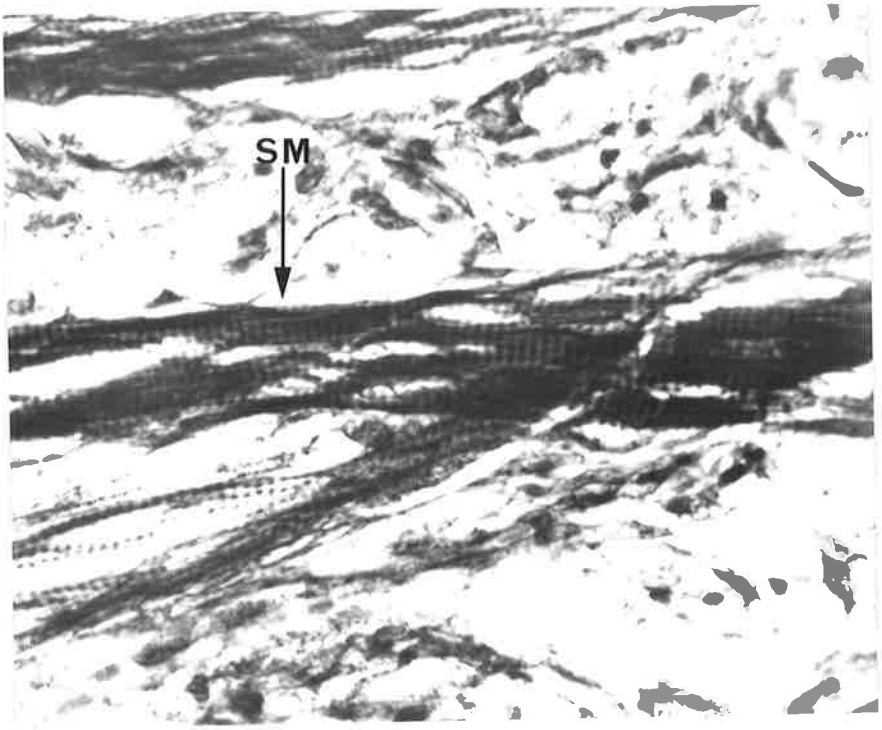


Figure 44

Figure 45: Intramuscular Surgicel Implant 3 weeks:
Photomicrograph showing an hour-glass shaped tissue ingrowth (TI) into the lumen of the Teflon tube (T). H & E x 25.

Figure 46: Intramuscular Surgicel Implant 3 weeks:
High power photomicrograph of an area in Figure 45 showing striated muscle fibres (SM) and connective tissue (CT) within the substance of the tissue ingrowth in the Teflon tube. Fibrillar material (F) can be seen alongside the tissue ingrowth. H & E x 100.

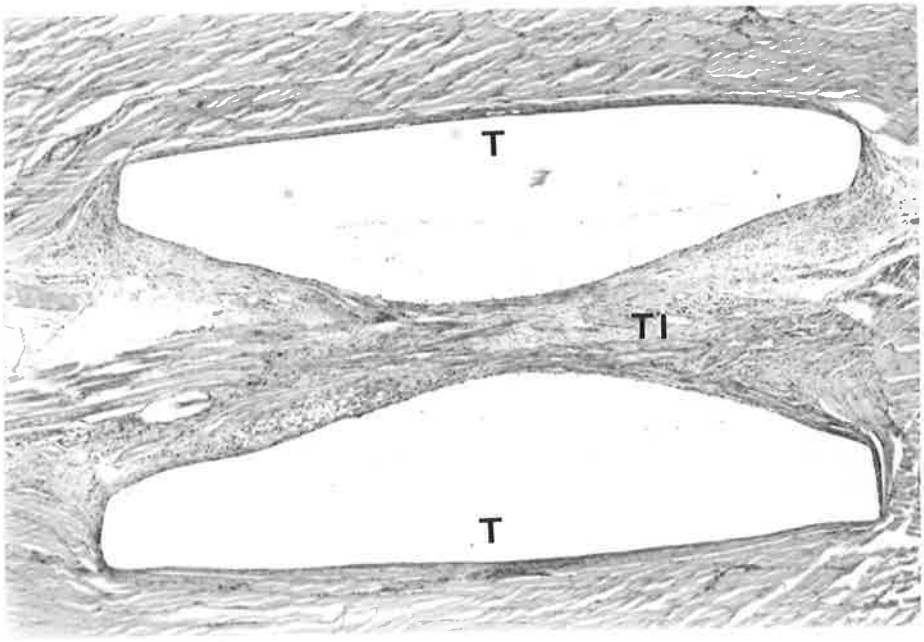


Figure 45

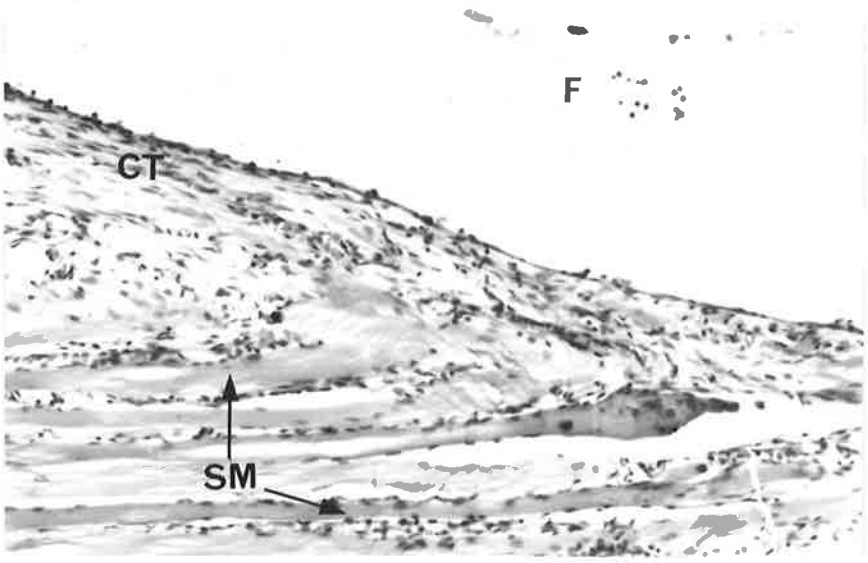


Figure 46

Figure 47: Intramuscular Surgicel Implant 6 weeks:
Photomicrograph showing tissue ingrowth into the Teflon tube (T) consisting mainly of striated muscle (SM) and adipose tissue (AT). H & E x 25

Figure 48: Intramuscular Surgicel Implant 8 weeks:
Photomicrograph showing tissue ingrowth into the Teflon tube (T) consisting mainly of striated muscle (SM) and adipose tissue (AT). H & E x 25

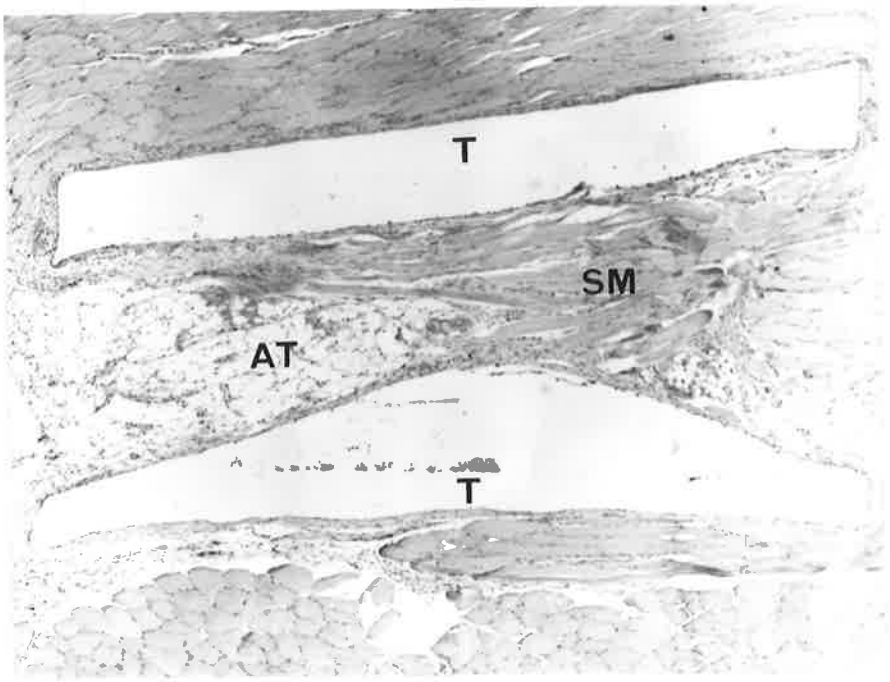


Figure 47

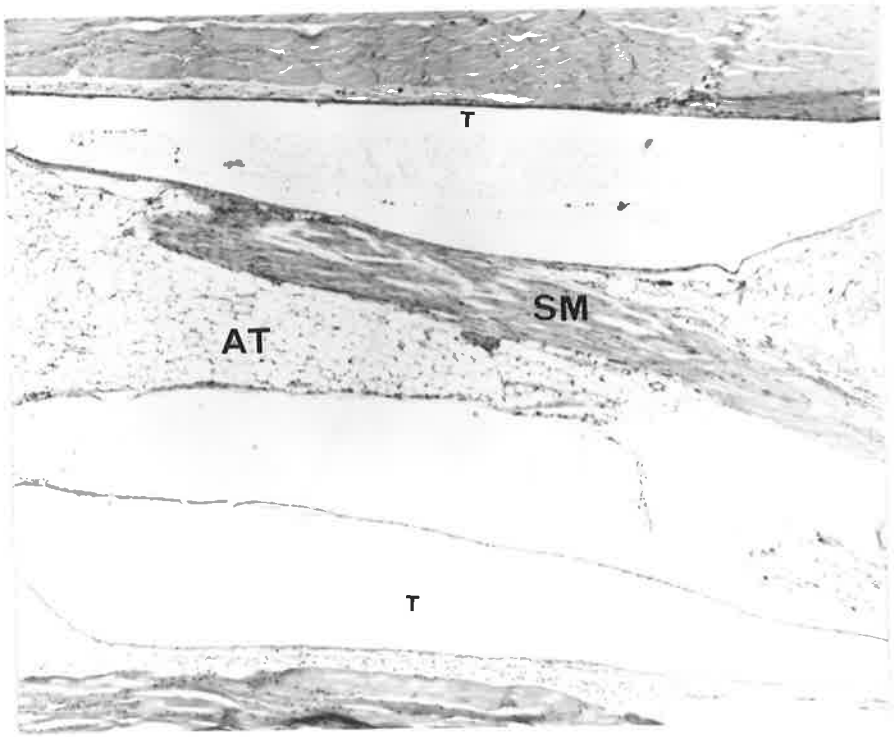


Figure 48

Figure 49: Intramuscular Surgicel Implant 8 weeks:
High power photomicrograph of an area in Figure 48 showing flattened fibroblasts (FB) in a thin fibrous capsule (FC) along the outer margin of the tissue ingrowth into the Teflon tube. Fibrillar material (F) can be seen outside the tissue ingrowth between it and the inner wall of the Teflon tube. H & E x 250

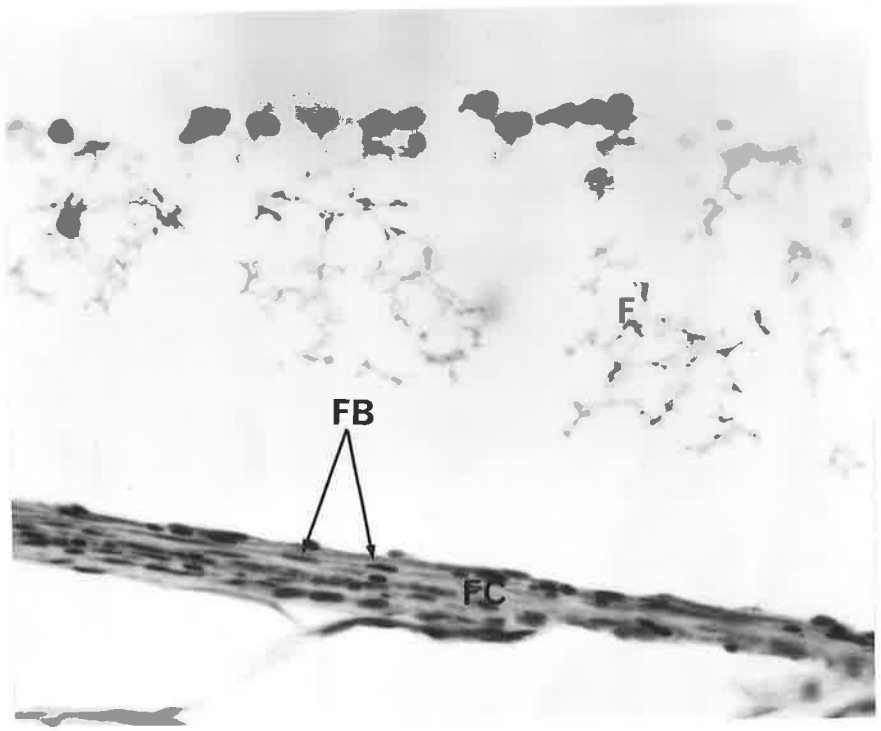


Figure 49

Comparison of Tissue Ingrowth into Surgicel-Containing and Control Tubes.

The percentage tissue ingrowth into the Surgicel-containing and empty control tubes was determined for all the post-operative observational time periods, excluding the 12 and 26 week post-implantation series. The method by which these calculations were made has been described in detail in Chapter 3 and in Appendix V.

Table 7 illustrates the mean percentage ingrowth of tissues into the lumina of the Teflon tubes in experimental and control implants.

TABLE 7 : MEAN PERCENTAGE INGROWTH OF TISSUE INTO SURGICEL-FILLED AND CONTROL TEFLON TUBES

POSTOPERATIVE OBSERVATIONAL TIME PERIODS	MEAN PERCENTAGE INGROWTH OF TISSUES INTO THE LUMEN OF THE TEFLON TUBE	
	SURGICEL SERIES	CONTROL SERIES
2 Days	0	0
7 Days (1 week)	20.96	34.66
14 " (2 weeks)	48.29	53.49
21 " (3 ")	58.83	53.20
28 " (4 ")	55.77	58.77
42 " (6 ")	61.84	62.06
56 " (8 ")	63.86	65.41
84* " (12 ")		
182* " (26 ")		

* Because of the small number of animals used, no reliable statistical data could be obtained for these specimens.

The figures in Table 7 were subjected to the Student's t-Test. The results obtained indicated that, at no stage, was there a statistically significant difference between the Surgicel and control implants (at the 5% level).

****

(C) Bone Wax Implants

During the preparation of the specimens containing the Bone Wax implants for histologic examination, the wax was lost, leaving an empty space at its site of implantation. However, in the histologic description to follow, the name of the implant will still be used.

Histologic examination of tissue sections containing the Bone Wax implants revealed the following at the various observational time periods:

Two days post-implantation: There was a very clear demarcation between Bone Wax and the surrounding tissues. Immediately adjacent to the implant, was a cellular zone (Figure 50). Peripheral to this zone was a prominent oedematous area (Figure 51) containing diffusely scattered inflammatory cells. A detailed examination of the cellular zone, mentioned above, revealed the presence of large numbers of polymorphonuclear leukocytes, macrophages and erythrocytes. Many of the polymorphonuclear leukocytes appeared to be undergoing degeneration as evidenced by pyknosis of their nuclei.

Seven days post-implantation: Microscopic observation of tissue sections at this time period showed that the Bone Wax was still present in the lumen of the Teflon tube as evidenced by the clear space in the tube lumina. Several histologic zones were distinguishable adjacent to the implant (Figure 52): immediately adjacent to the Bone Wax was a fine fibrillar zone and peripheral to this was a cellular layer, one to six cell layers in thickness, consisting of darkly stained cells. These cells were mainly lymphocytes and macrophages. Between this cellular layer and the striated muscle bundles, was a zone of immature fibrous granulation tissue. Van Gieson-stained sections demonstrated the presence of collagen in this tissue. Cells

identified as foamy macrophages (Figure 53) were evident, but no particulate or stainable material was identifiable within these cells. Occasionally, multinucleate giant cells were seen. These tended to occur in close proximity to the striated muscle bundles rather than to the implant. Polymorphonuclear leukocytes were conspicuously absent.

Two weeks post-implantation: The histologic picture seen at this stage was essentially the same as that observed at 7 days post-operatively, except that van Gieson-stained sections revealed the presence of an increased amount of collagen fibres adjacent to the implant face.

Three weeks post-implantation: The Bone Wax was clearly evident and showed no signs of undergoing resorption and replacement by tissue (Figure 54). An interesting feature noted at this stage in some specimens was the presence of adipose tissue in that zone between the implant and striated muscle. Detailed examination revealed the persistence of a fibrillar layer immediately adjacent to the Bone Wax (Figure 55). Peripheral to the fibrillar layer was a zone of young fibrous tissue containing macrophages and lymphocytes. Between this zone and the striated muscle, adipose tissue was evident. Many of the macrophages had a foamy appearance. Occasionally, multinucleate giant cells were scattered between the fibroblasts and macrophages.

Four and six weeks post-implantation: The most striking histologic feature noted at these post-operative time periods was a reduction in the overall cell density adjacent to the implant (Figure 56). The cells that were present consisted mainly of flattened fibroblasts, and macrophages, many of which exhibited a foamy appearance (Figure 57). There was no indication of resorption of the Bone Wax, and in some specimens, the fibrillar layer, previously described, was not present. Fat cells were still present adjacent to the striated muscle bundles but no giant cells were noted in any of the specimens.

Eight and twelve weeks post-implantation: Microscopic observation of the histologic sections demonstrated the presence of a thin fibrous capsule around the implant (Figure 58). A very thin fibrillar zone was still noted immediately adjacent to the Bone Wax but there was a marked decrease in the number of macrophages in relation to the implant. Peripheral to the fibrillar zone, was a fibrous capsule containing flattened fibroblasts and between this capsule and the striated muscle bundles, were several layers of fat cells.

Twentysix weeks post-implantation: The Bone Wax was surrounded by a well-defined fibrous capsule. Between the fibrous capsule and the striated muscle, was a well-defined layer of adipose tissue (Figure 59). Immediately adjacent to the Bone Wax, remnants of a fibrillar zone were identifiable in all the specimens examined and peripheral to this zone, was a thin cellular zone (Figure 60). Cells in this zone comprised fibroblasts and cells identified as macrophages.

Figure 50: Intramuscular Bone Wax Implant 2 Days:
Photomicrograph showing the cellular zone (CZ) adjacent to the Bone Wax (BW) implant. H & E x 25

Figure 51: Intramuscular Bone Wax Implant 2 Days:
High power photomicrograph of an area in Figure 50 illustrating an oedematous area (O) peripheral to the cellular zone (CZ). (BW = Bone Wax) H & E x 100

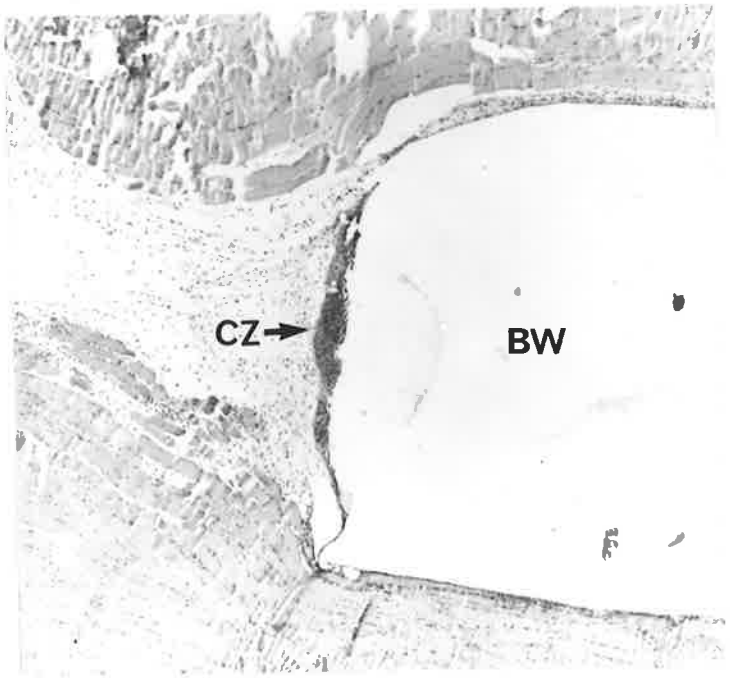


Figure 50

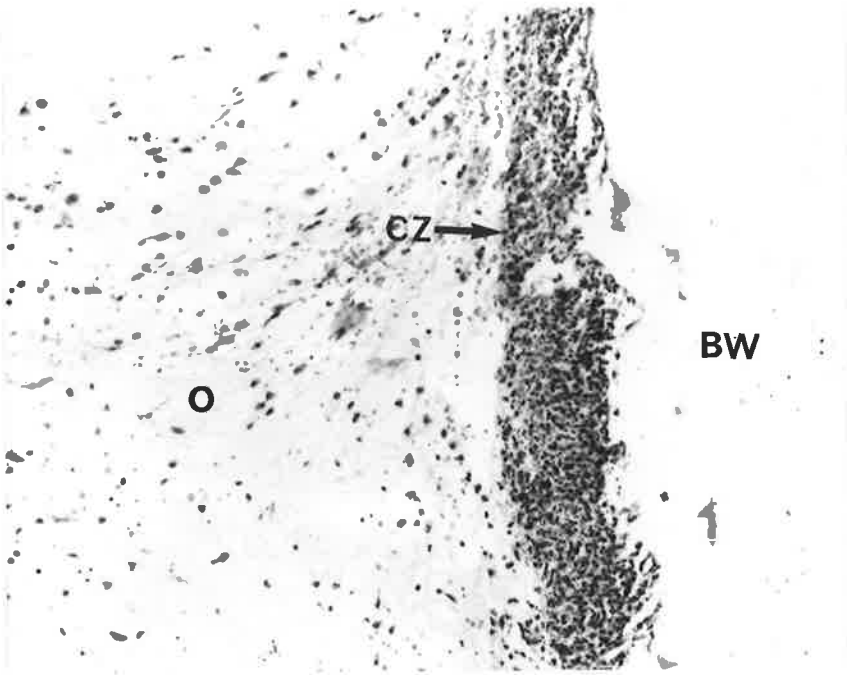


Figure 51

Figure 52: Intramuscular Bone Wax Implant 7 Days:
Photomicrograph showing fibrillar material (F) immediately adjacent to the Bone Wax (BW). Peripheral to the fibrillar zone, is a discrete zone of darkly stained cells (arrow). A multinucleate giant cell (GC) can be seen in close proximity to the striated muscle bundles.
H & E x 250

Figure 53: Intramuscular Bone Wax Implant 7 Days:
High power photomicrograph of an area in Figure 52 showing foamy macrophages (arrows) in close proximity to the Bone Wax (BW). H & E x 400

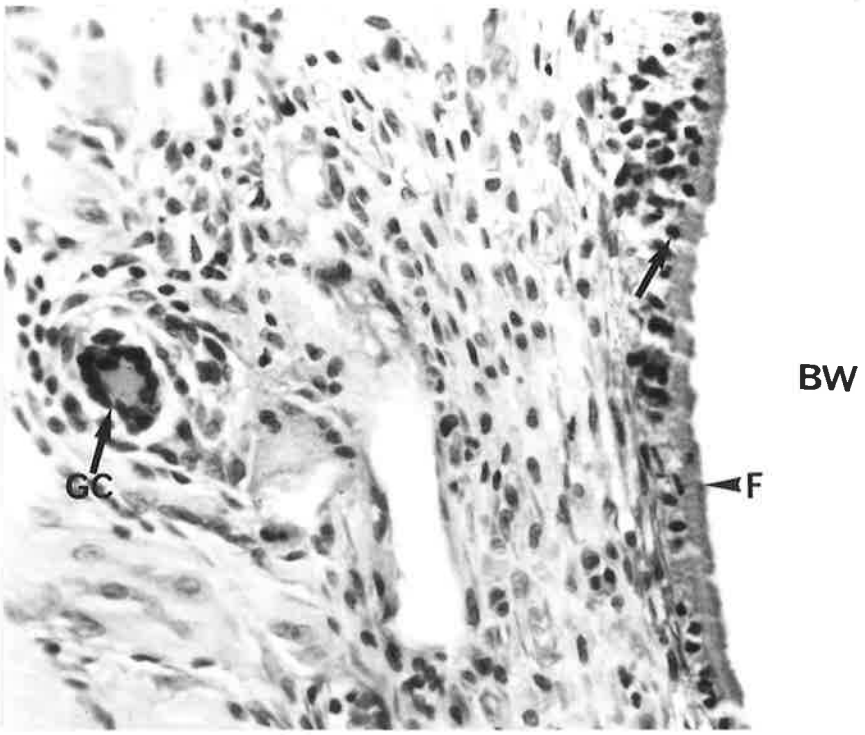


Figure 52

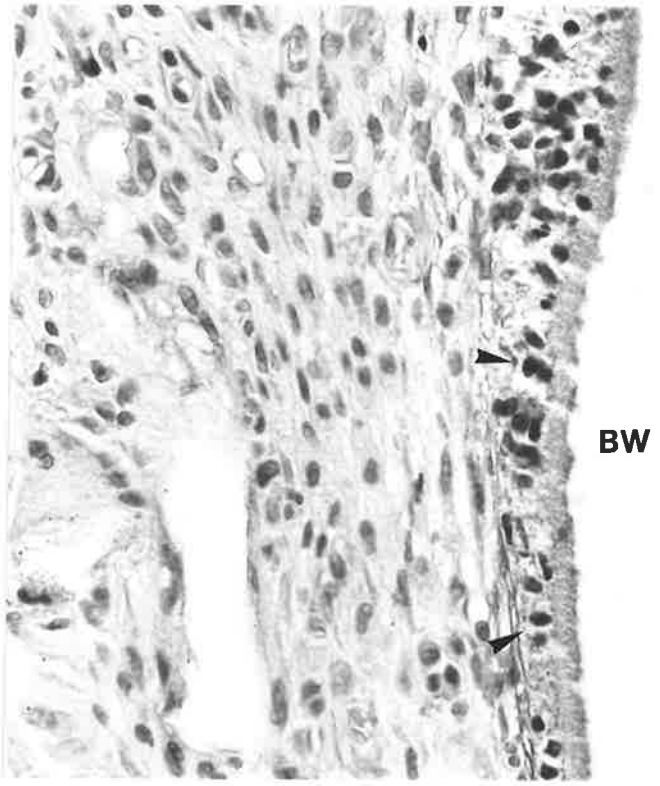


Figure 53

Figure 54: Intramuscular Bone Wax Implant 3 Weeks:
Photomicrograph illustrating the presence of the Bone Wax (BW) implant. A cellular zone (CZ) and adipose tissue (AT) can be seen between the Bone Wax (BW) and the striated muscle bundles (SM). Note the flat interface between the Bone Wax space and the adjacent tissue. H & E x 40.

Figure 55: Intramuscular Bone Wax Implant 3 Weeks:
High power photomicrograph of an area in Figure 54 showing the fibrillar layer (F) immediately adjacent to the Bone Wax (BW). Peripheral to the fibrillar layer (F), is a thin zone of darkly stained cells (arrow) and between this zone and the adipose tissue (AT), is a highly cellular zone consisting of macrophages, flattened fibroblasts and a few lymphocytes. H & E x 250.

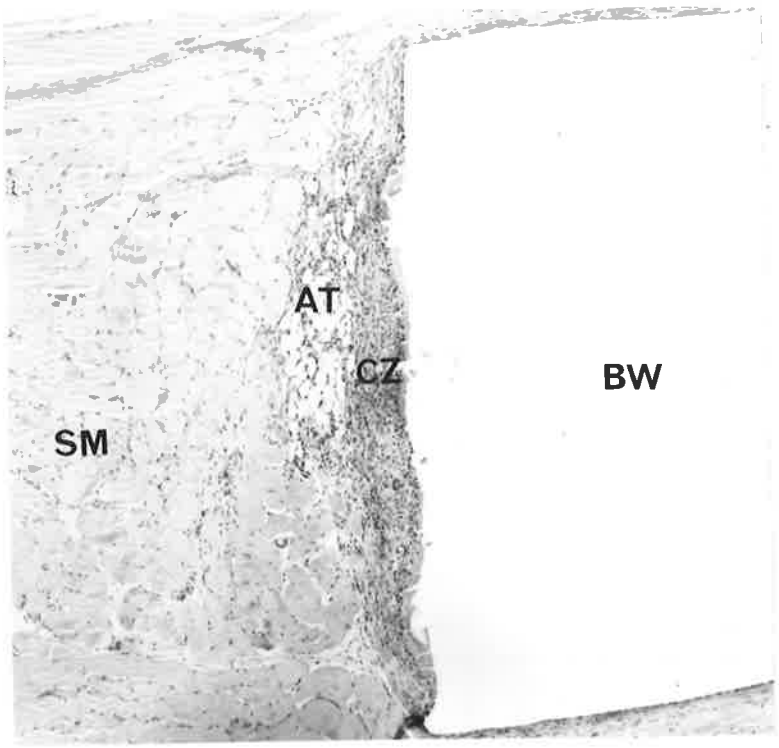


Figure 54

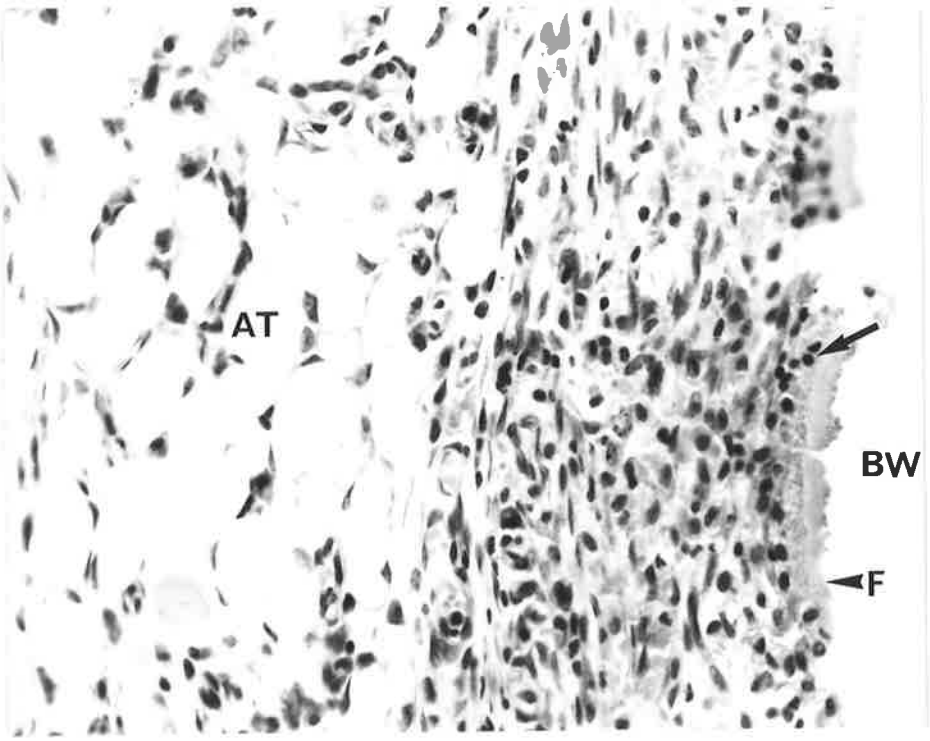


Figure 55

Figure 56: Intramuscular Bone Wax Implant 6 Weeks:
Photomicrograph illustrating a reduction in the overall cells density adjacent to the Bone Wax (BW).
(AT = Adipose Tissue). van Gieson x 250

Figure 57: Intramuscular Bone Wax Implant 6 Weeks:
High power photomicrograph of an area in Figure 56 illustrating macrophages (arrows) close to the Bone Wax (BW) implant. van Gieson x 400

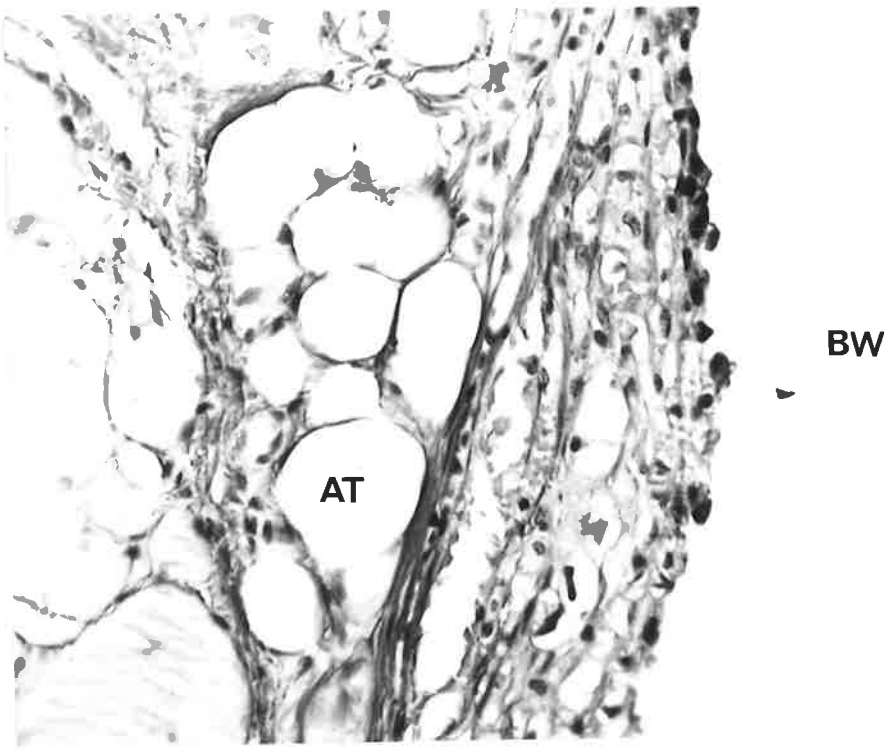


Figure 56

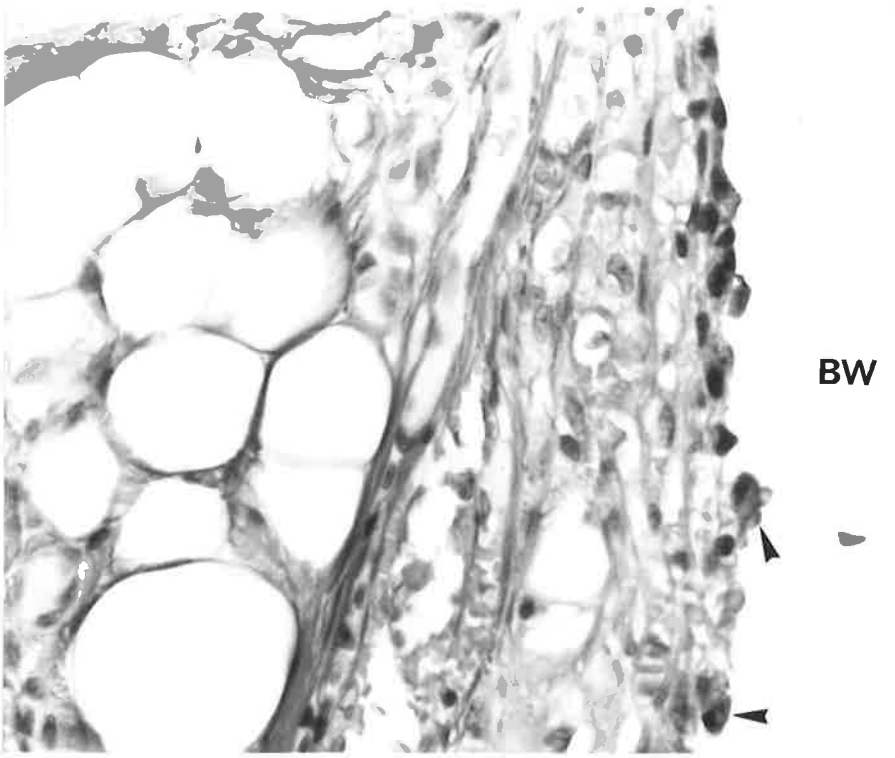


Figure 57

Figure 58: Intramuscular Bone Wax Implant 12 Weeks:
Photomicrograph showing a thin fibrous capsule (FC) adjacent to the Bone Wax (BW) implant. A very thin fibrillar layer (F) separates the fibrous capsule (FC) from the implant (BW). Note adipose tissue (AT) peripheral to the capsule. H & E x 400

Figure 59: Intramuscular Bone Wax Implant 26 Weeks:
Photomicrograph showing a well-defined fibrous capsule (FC) adjacent to the Bone Wax (BW). Adipose tissue (AT) can be seen between the fibrous capsule (FC) and striated muscle (SM). H & E x 25

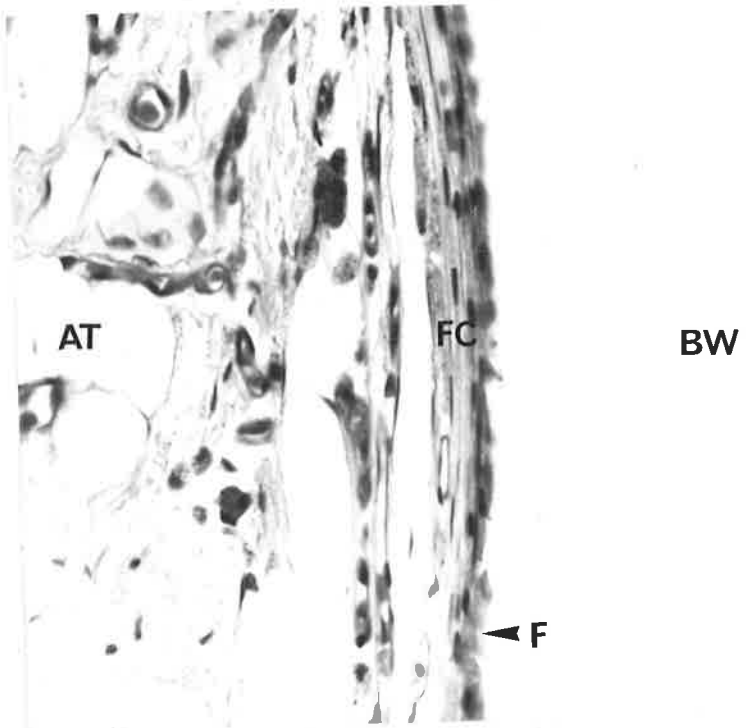


Figure 58

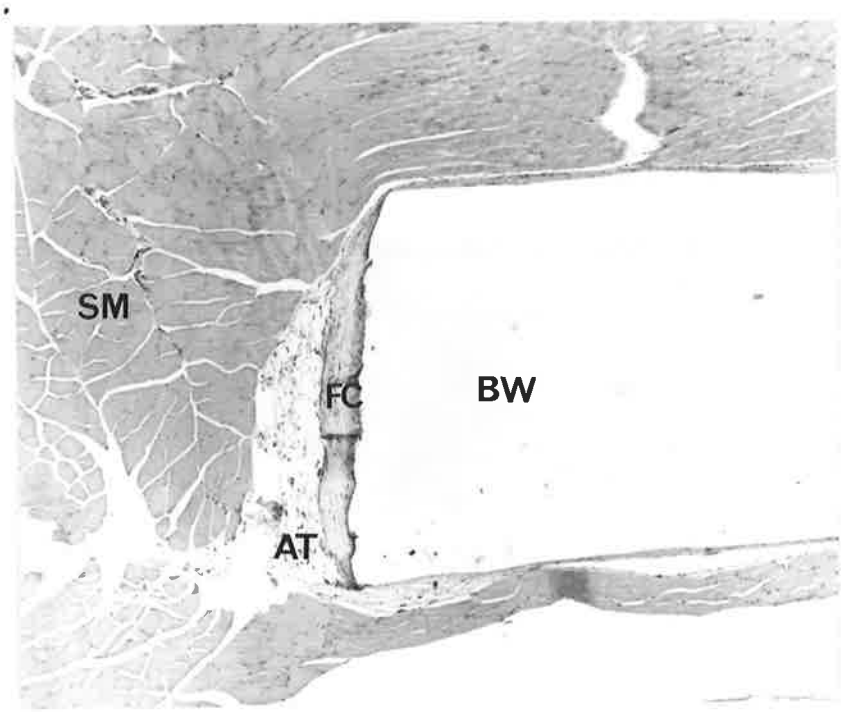


Figure 59

Figure 60: Intramuscular Bone Wax Implant 26 Weeks: High power photomicrograph of an area in Figure 59 showing a well-defined fibrous capsule (FC) adjacent to the Bone Wax (BW). Many flattened fibroblasts can be seen within the capsule. Note the relatively cellular zone (CZ) between the Bone Wax and capsule. H & E x 250.

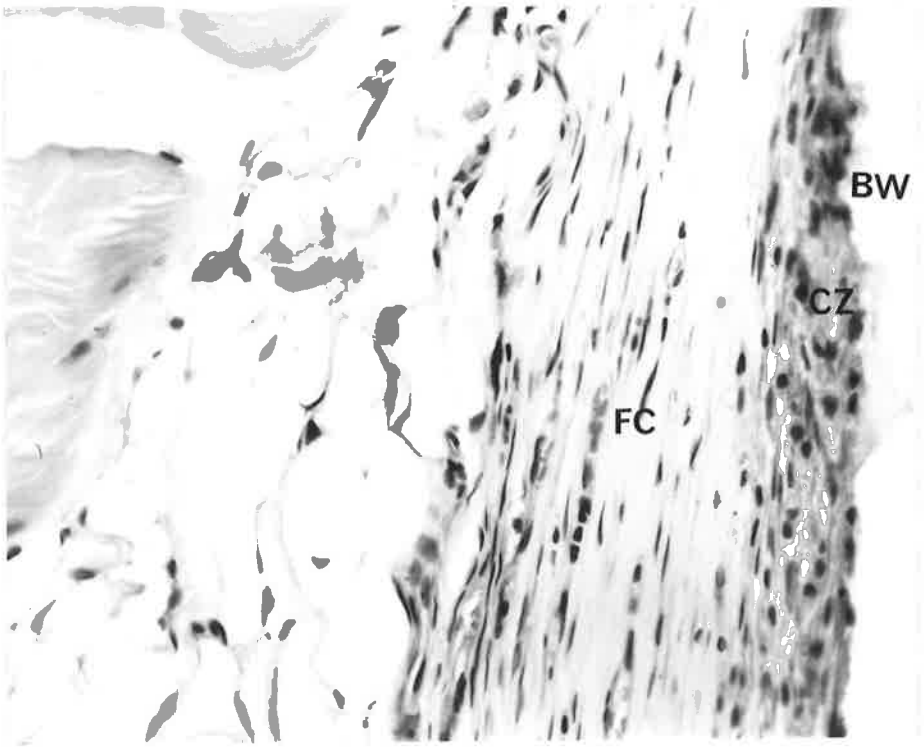


Figure 60

Poisson Analysis of the Intramuscular Bone Wax Implants

The purpose of this method of analysis was to determine whether the implanted Bone Wax brought about the aggregation of foamy macrophages in close proximity to its surface. The persistence of these cells around the implant would most likely indicate continued phagocytosis, and hence, a continued attempt by the mononuclear phagocytic system to remove the Bone Wax. The ultimate aim of the Poisson analysis is to calculate the Coefficient of Dispersion (C.D.) of histologically prepared specimens by determining the distribution of given cell types in a predetermined area. The C.D. will be near 1 in distributions that are essentially Poisson; will be > 1 in clumped samples and < 1 in cases of repulsion. In this study, the C.D. was calculated to determine the distribution of foamy macrophages in relation to the Bone Wax implants. The method by which these calculations were made have been described in detail in Chapter 3 and in Appendix V.

Table 8 illustrates the C.D. that was calculated for each of the post-operative implantation time periods:

TABLE 8: COEFFICIENT OF DISPERSION (C.D.) FOR FOAMY MACROPHAGES FOR EACH OF THE POST-IMPLANTATION TIME PERIODS IN INTRAMUSCULAR BONE WAX IMPLANTS.

POST-OPERATIVE OBSERVATIONAL TIME PERIOD	COEFFICIENT OF DISPERSION (C.D.)
2 Days	1.15
7 Days (1 Week)	1.00
14 " (2 Weeks)	1.57
21 " (3 ")	1.51
28 " (4 ")	2.47
42 " (6 ")	1.65
56 " (8 ")	1.71
84 " (12 ")	1.40
182 " (26 ")	1.50

Whilst the C.D. indicated whether the foamy macrophages were clumped around the surface of the Bone Wax or not, this figure provided no numerical data on the actual distribution of these cells in relation to the implant. Therefore, another form of analysis was undertaken, as described in detail in Chapter 3. In this method of analysis, the area under examination was divided into five equal zones, each running parallel to the surface of the Bone Wax, and the number of foamy macrophages in each of these zones was calculated. The results of this analysis are illustrated in Table 9 .

TABLE 9: NUMERICAL DISTRIBUTION AND PERCENTAGE DISTRIBUTION OF FOAMY MACROPHAGES IN EACH OF THE FIVE CONTIGUOUS ZONES ADJACENT TO THE BONE WAX IMPLANT.

POSTOPERATIVE OBSERVATIONAL TIME PERIOD	TOTAL NUMBER OF FM*	NUMBER OF FM** IN EACH ZONE					PERCENTAGE OF FM** IN EACH ZONE				
		1	2	3	4	5	1	2	3	4	5
+ 2 Days	1128	332	116	212	202	226	28	15	19	18	20
7 Days(1 Week)	1191	454	452	366	382	337	23	23	28	29	17
14 " (2 Weeks)	1407	493	416	235	155	108	35	29	17	11	8
+21 " (3 ")	857	330	235	124	81	87	39	27	15	9	10
+28 " (4 ")	582	388	123	44	25	2	67	21	8	4	0
42 " (6 ")	279	182	60	34	3	0	65	22	12	1	0
56 " (8 ")	255	172	69	14	0	0	67	27	6	0	0
+84 " (12 ")	16	16	0	0	0	0	100	0	0	0	0
182 " (26 ")	24	24	0	0	0	0	100	0	0	0	0

* Represents the total number of foamy macrophages occurring in all specimens at each observational time period.

** Foamy macrophages.

\+ Indicates 1 specimen lost either through death of animal or during histological processing.

SECTION II: HISTOLOGICAL ASSESSMENT OF THE INTRAFEMORAL IMPLANTS

When the femoral specimens were collected at the time of sacrifice of the experimental animals, thirteen out of a total of ninety femurs were found to be fractured. These were discarded and the remaining specimens were processed for histological examination.

(A) Control Implants

Microscopic observations of the empty control Teflon tubes at the various post-implantation periods revealed the following:

Two days post-implantation: Blood clot filled the lumina of all the Teflon tubes (Figure 61). Polymorphonuclear leukocytes and macrophages were seen in large numbers between erythrocytes. In addition, fibroblasts, which were elongated in shape, appeared at the open ends of the Teflon tube but no collagen formation was seen (Figure 62).

Seven days post-implantation: A granulation tissue ingrowth into into the lumen of the Teflon tube was observed. The ingrowth exhibited an hour-glass shape (Figure 63). On either side of this ingrowth, blood clot was still clearly visible. Bone spicules were evident within the lumina of some of the tubes. Some of this bone exhibited histologic features consistent with that of new woven bone while some spicules appeared to be bone chips formed as a consequence of the surgical procedure. (Figure 64). New bone formation was prominent adjacent to the Teflon tubes (Figure 63).

Two weeks post-implantation: Haemopoietic bone marrow on the side of the tube away from the wound in the cortical bone occupied varying proportions of the tube lumina (Figure 65). On the side of the cortical wound, the haemopoietic marrow was less established. Spicules

of woven bone were noted along the inner surface of the Teflon tube as well as within the lumen of the tube itself (Figure 66).

Three and four weeks post-implantation: Only a small amount of woven bone was seen within the lumina of the Teflon tubes. Prominent bone, however, lined the inner aspect of the tubes. The lumina of the tubes were occupied by abundant haemopoietic marrow (Figure 67).

Six to twenty-six weeks post-implantation: Microscopic examination of all the histologic specimens during these post-operative time periods revealed that the haemopoietic bone marrow had completely re-established itself within the tubes (Figure 68). The Teflon tubes tended to be completely "encapsulated" by woven bone. Some areas of the haemopoietic marrow contained fat cells.

Figure 61: Intrafemoral Control Implant 2 Days:
Photomicrograph showing the Teflon tube (T) within the femur. Cortical bone (CB) is visible on either side of the tube. A blood clot (BC) can be seen along the inner aspect of the Teflon tube (T). H & E x 25

Figure 62: Intrafemoral Control Implant 2 Days:
High power photomicrograph of an area in Figure 61 showing elongated fibroblasts (arrows) appearing at the open end of the Teflon tube (T). H & E x 400

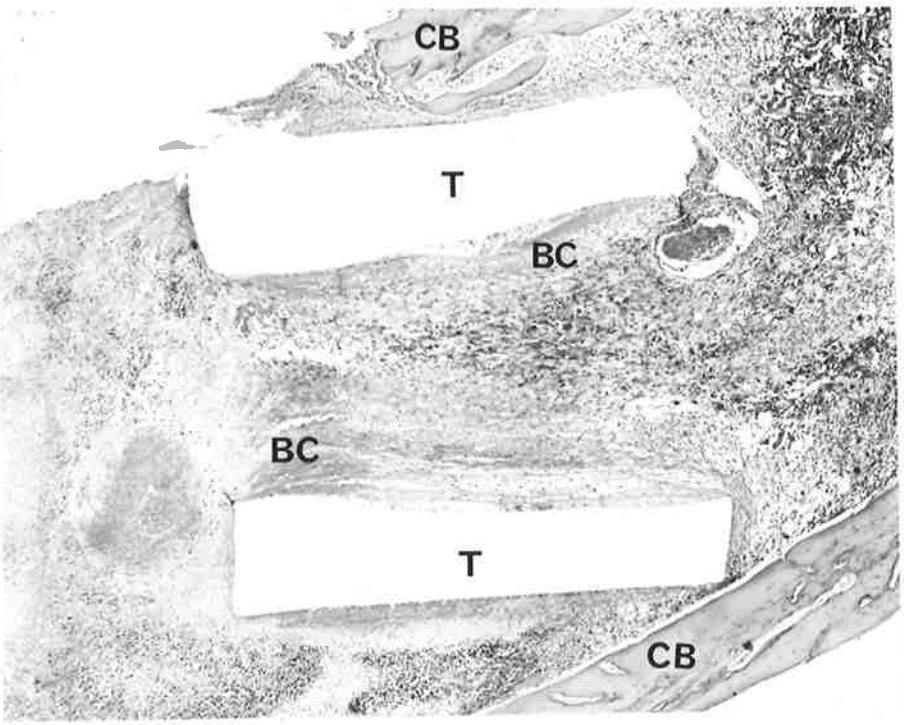


Figure 61

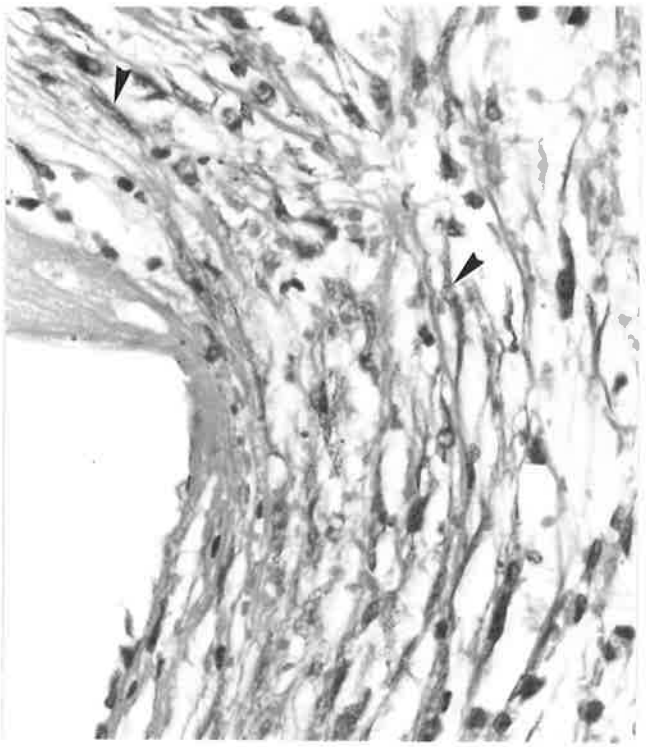


Figure 62

Figure 63: Intrafemoral Control Implant 7 Days:
Photomicrograph showing an hour-glass shaped granulation tissue ingrowth (TI) into the lumen of the Teflon tube (T). Blood clot (BC) is observable on either side of the tissue ingrowth. The cortical bone of the femur (CB) can be seen on either side of the tube. New woven bone (WB) and haemopoietic bone marrow (BM) can be seen on the left and right sides of the tube respectively.
H & E x 25.

Figure 64: Intrafemoral Control Implant 7 Days:
Higher power photomicrograph of an area in Figure 63 showing spicules of bone in the lumen of the Teflon Tube. Some of the bone is woven bone. H & E x 100

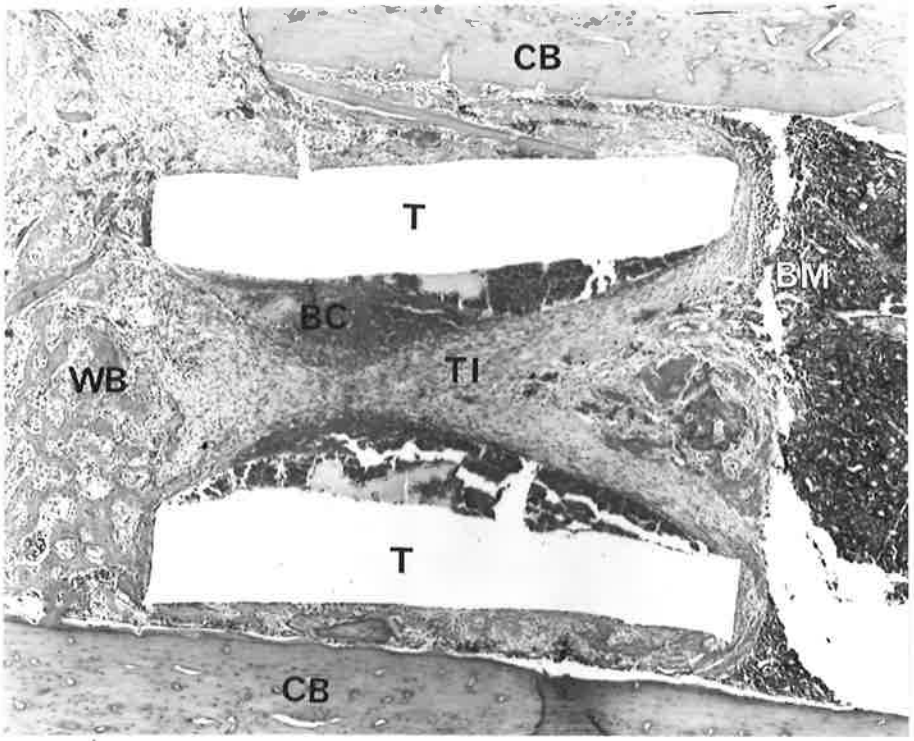


Figure 63

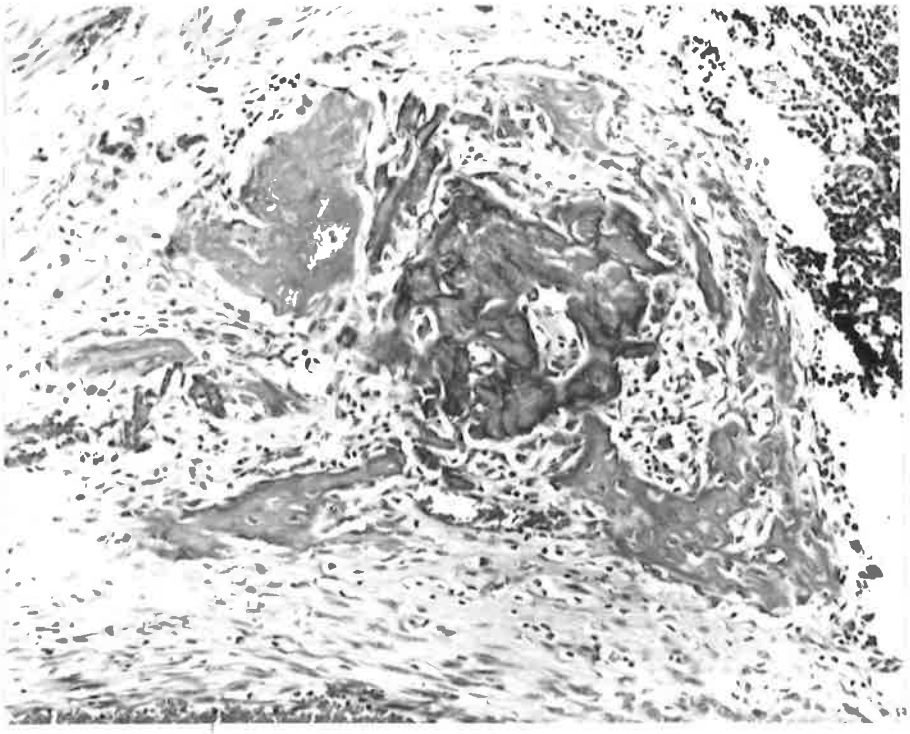


Figure 64

Figure 65: Intrafemoral Control Implant 2 Weeks:
Photomicrograph showing re-establishment of the haemopoietic bone marrow (BM) in the side of the tube away from the cortical wound (CW). Woven bone (WB) can be seen within the lumen of the Teflon Tube (T) as well as lining its inner surface. Cortical bone (CB) is seen on either side of the tube. H & E x 25

Figure 66: Intrafemoral Control Implant 2 Weeks:
High power photomicrograph of an area in Figure 65 showing the woven bone (WB) and haemopoietic marrow within the Teflon tube. H & E x 400

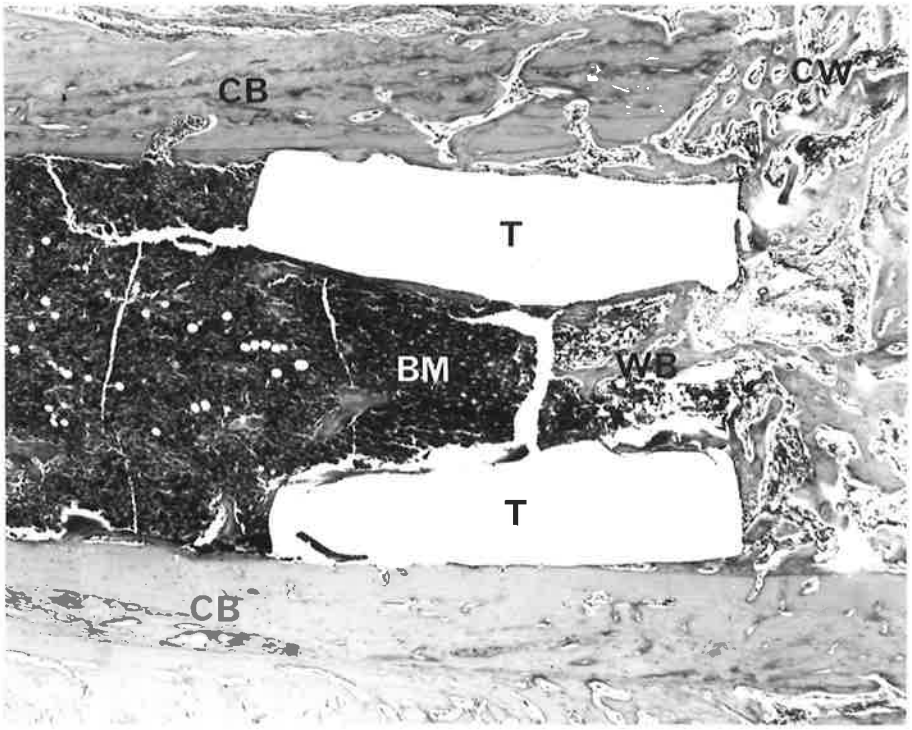


Figure 65

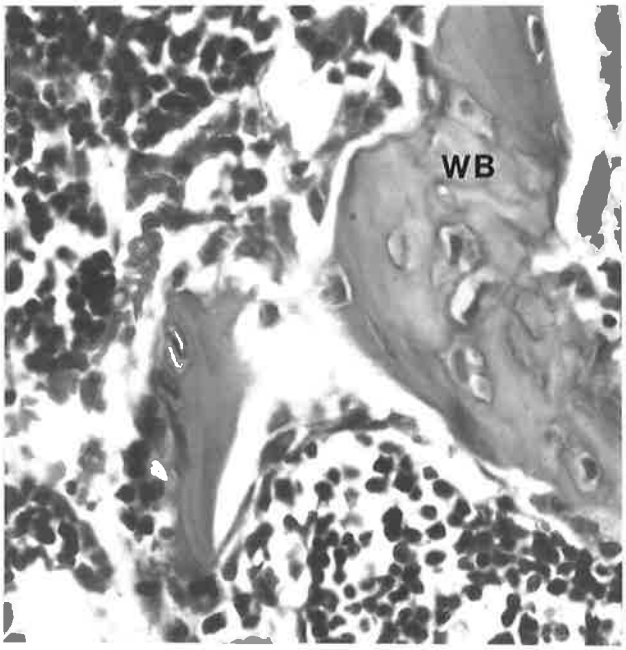


Figure 66

Figure 67: Intrafemoral Control Implant 4 Weeks:
Photomicrograph showing spicules of woven bone (WB) lining the inner surface of the Teflon tube (T) as well as being present within its lumen. The re-establishment of the haemopoietic bone marrow (BM) is clearly evident.
H & E x 25

Figure 68: Intrafemoral Control Implant 8 Weeks:
Photomicrograph showing a re-established haemopoietic bone marrow (BM) within the lumen of the Teflon tube (T). Fat cells can be seen within the marrow. The surfaces of the Teflon tubing (T) appear to be encapsulated by woven bone (arrows). CB = Cortical Bone. H & E x 25.

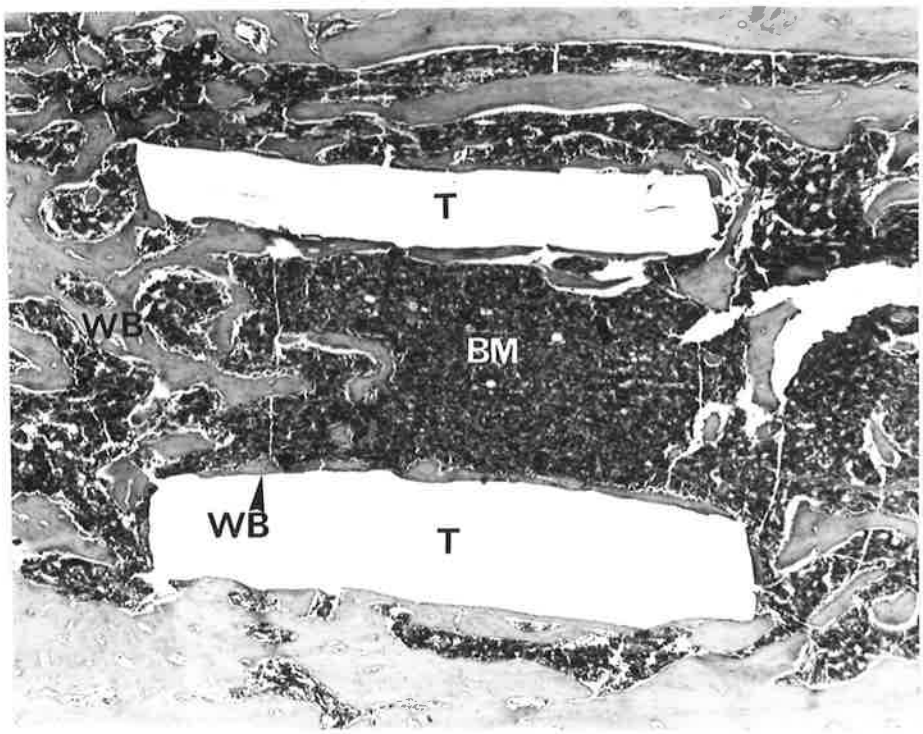


Figure 67

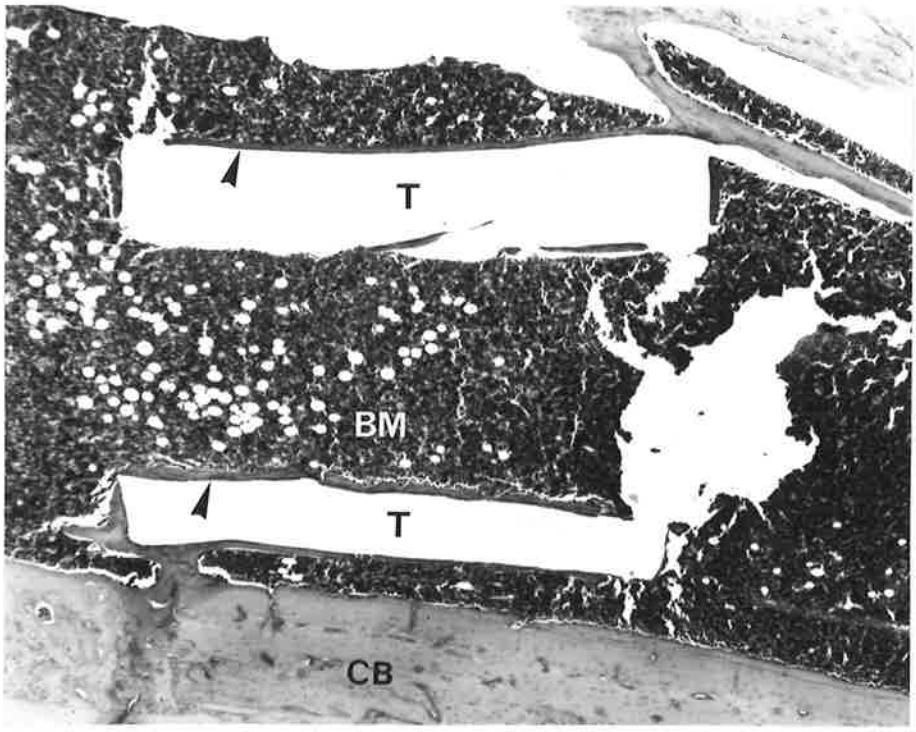


Figure 68

(B) Surgicel Implants

Histologic examination of the tissue sections containing the Surgicel-filled tubes at the various post-implantation periods revealed the following:

Two days post-implantation: Large Surgicel fibres were clearly evident within the lumina of the Teflon tubes of all the specimens (Figure 69). Blood clot was noted along the inner surface of the tubes. Detailed examination of all the tissue sections at this post-operative time period revealed the presence of erythrocytes, mononuclear cells and polymorphonuclear leukocytes among the fibres of the implant material. Many of the macrophages exhibited a foamy appearance (Figure 70), while some were normal in size and appearance. Polymorphonuclear leukocytes were present in very small numbers (Figure 71) and many of these were in the process of degenerating as evidenced by the presence of pyknotic nuclei.

Seven days post-implantation: Large Surgicel fibres were still clearly visible within the lumina of the tubes of all the tissue specimens. However, a greater part of the contents of the tubes was characterised by the presence of granulation tissue (Figure 72). Macrophages and erythrocytes were seen in large numbers among the Surgicel fibres and within the granulation tissue ingrowth. Many of the macrophages exhibited a foamy appearance (Figure 73) but no discretely stainable material or particulate matter could be discerned within these cells. Plump elongated fibroblasts were evident in the interstices of the Surgicel fibres (Figure 74).

Two weeks post-implantation: Microscopic examination of all the tissue specimens revealed that Surgicel fibres in the form noted at two and seven days post-operatively, were not identifiable at the

fourteen day observation period. On the side of the tube away from the cortical wound, haemopoietic marrow was observed within the lumina of the Teflon tubes (Figure 75). Bone spicules were also noted among marrow cells and along the walls of the tubes. On the side of the Teflon tube adjacent to the cortical wound of all the tissue sections examined, pale-staining eosinophilic material was evident. Detailed examination of the latter (Figure 76) revealed that it contained many inflammatory cells. On either side of the amorphous material, spicules of woven bone were evident. Macrophages constituted the main cell type within the amorphous substance although a few lymphocytes were also seen. Many of the macrophages noted still had a foamy appearance but no particulate matter could be identified within these cells (Figure 77).

Three weeks post-implantation: At this time period, spicules of woven bone were prominent within the lumina of the tubes. In addition, a thin layer of bone also lined the surfaces of the Teflon tubes. Normal haemopoietic marrow comprised the bulk of the tissues within the tubes (Figure 78). Surgicel fibres were not seen in any of the tissue sections examined but foci of fibrillar material were identified among marrow cells and woven bone. Detailed examination of these foci (Figure 79) indicated that they were characterised by the presence of numerous macrophages, some of which exhibited a markedly enlarged, foamy appearance.

Four and six weeks post-implantation: Examination of specimens from these time periods revealed essentially similar features and thus, they are described together. Microscopic observation of all tissue sections indicated that haemopoietic marrow occupied the lumina of all the tubes (Figure 80). The surfaces of the walls of the Teflon tubes were lined by a thin layer of bone. There were no signs of

the implant material but many macrophages of large size were seen among cells of haemopoietic marrow (Figure 81).

Eight weeks post-implantation: Haemopoietic marrow and fat cells constitited the main contents of the lumina of the Teflon tubes in all the specimens examined (Figure 82). A thin layer of bone lined the surfaces of the walls of the tubes. In all the sections examined, there was no evidence of the implant material or the foamy macrophages noted earlier.

At subsequent post-operative time periods, the histologic pictures seen were identical to those observed eight weeks post-implantation.

Figure 69: Intrafemoral Surgicel Implant 2 Days:
Photomicrograph showing Surgicel fibres (S) within the lumen of the Teflon tube (T). Blood clot (BC) can be seen along the inner surface of the tube. CB = Cortical Bone; CW = Cortical Wound. H & E x 25

Figure 70: Intrafemoral Surgicel Implant 2 Days:
High power photomicrograph of an area in Figure 69 showing foamy macrophages (arrows). H & E x 400

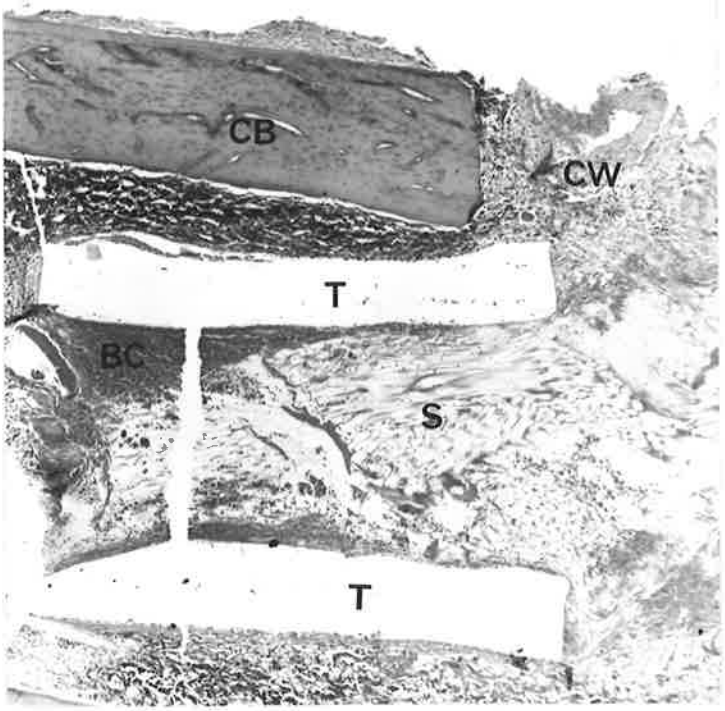


Figure 69

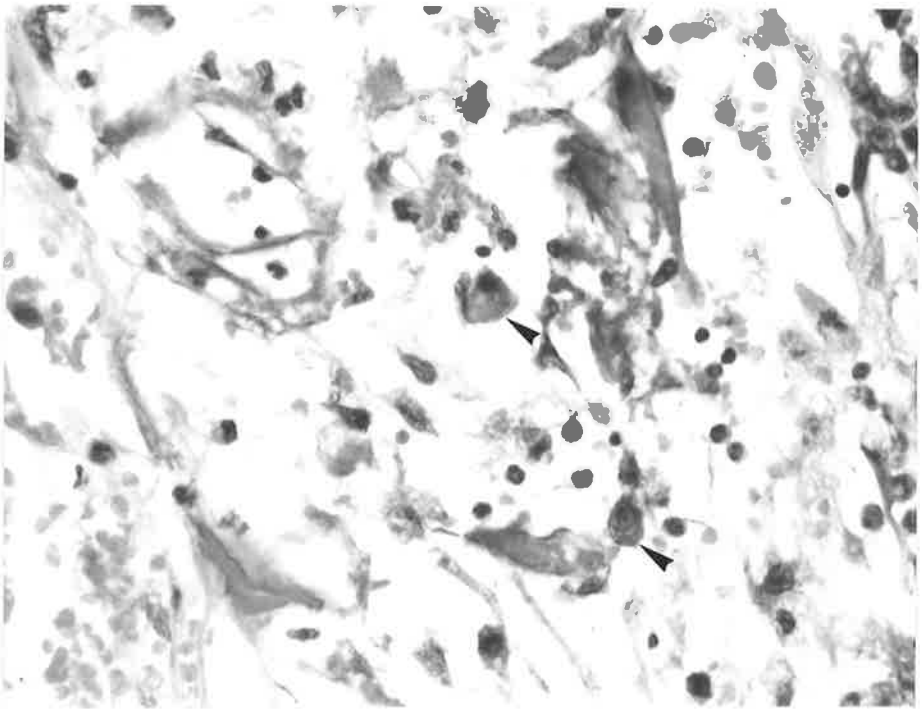


Figure 70

Figure 71: Intrafemoral Surgicel Implant 2 Days:
High power photomicrograph of an area in Figure 69 showing Polymorphonuclear leukocytes (arrows) between Surgicel fibres (S). H & E x 400.

Figure 72: Intrafemoral Surgicel Implant 7 Days:
Photomicrograph showing granulation tissue ingrowth (TI) into the lumen of the Teflon tube (T). The Surgicel (S) has been extruded from one end of the tube. CB = Cortical Bone. H & E x 25.

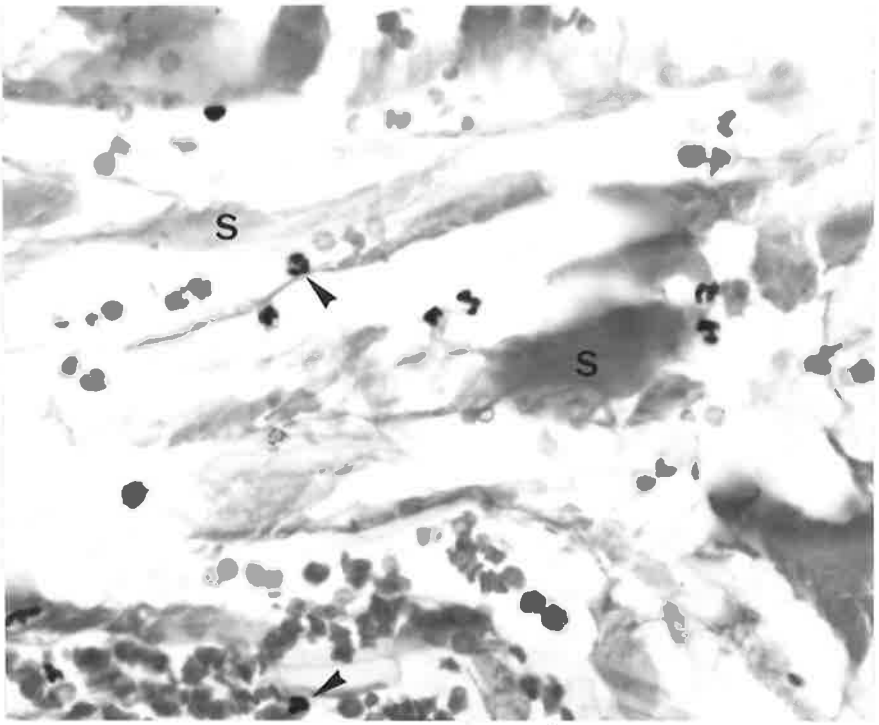


Figure 71

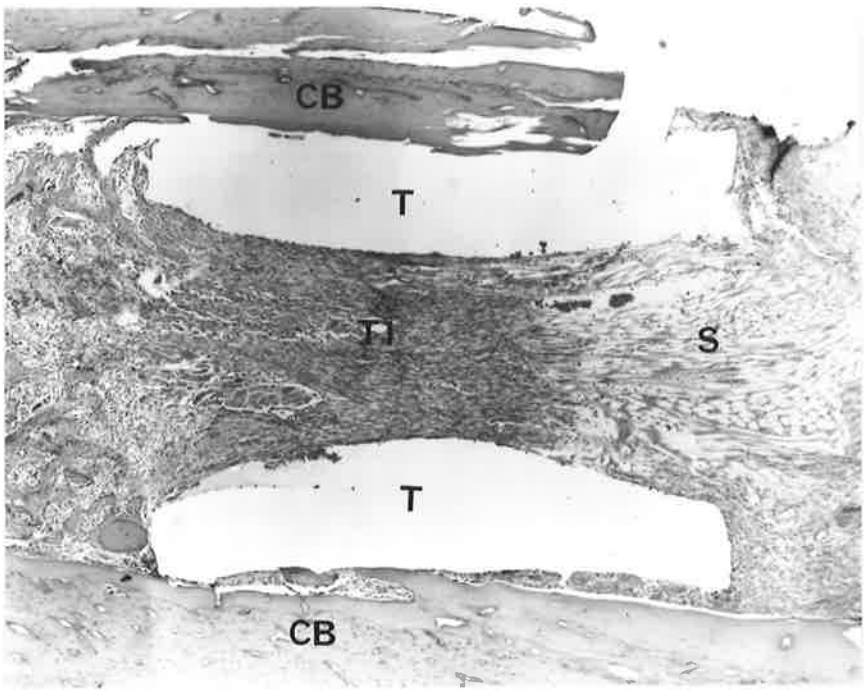


Figure 72

Figure 73: Intrafemoral Surgicel Implant 7 Days:
High power photomicrograph of an area in Figure 72 showing foamy macrophages (arrows). H & E x 400

Figure 74: Intrafemoral Surgicel Implant 7 Days:
High power photomicrograph of an area in Figure 72 showing fibroblasts (arrows) invading the interstices of the Surgicel fibres (S). H & E x 250

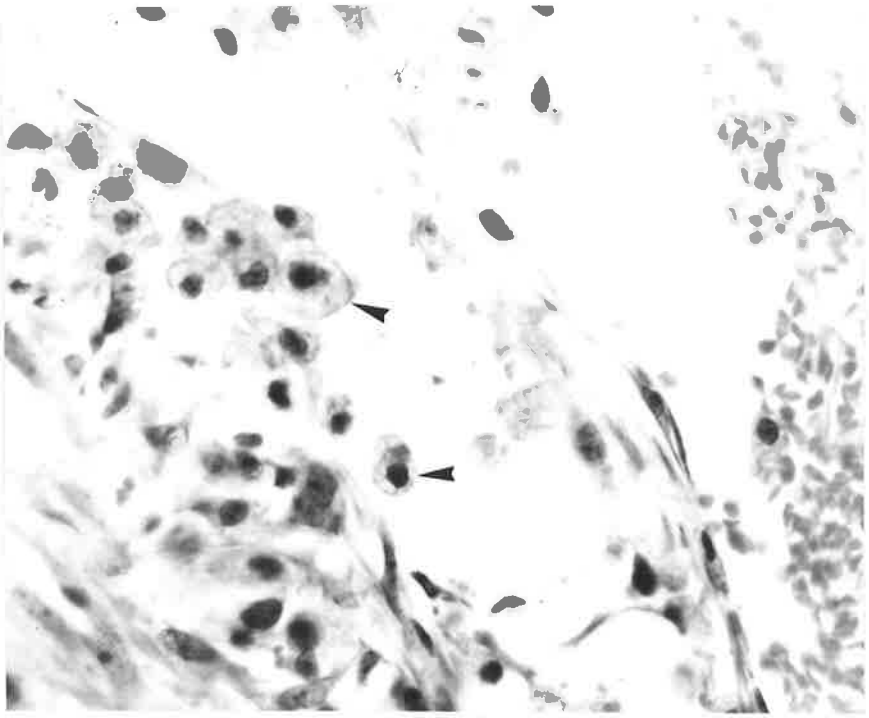


Figure 73

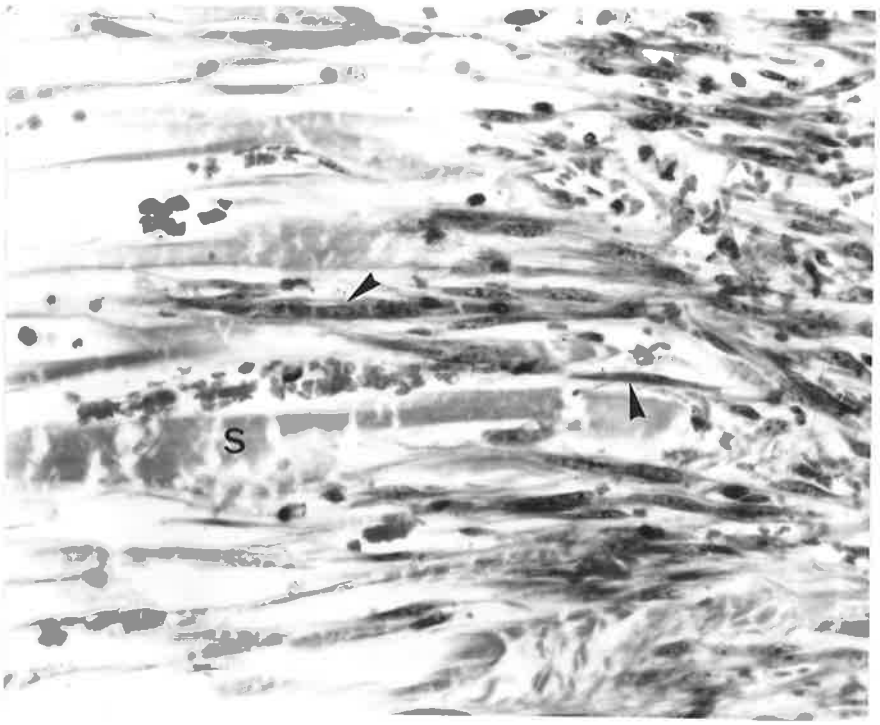
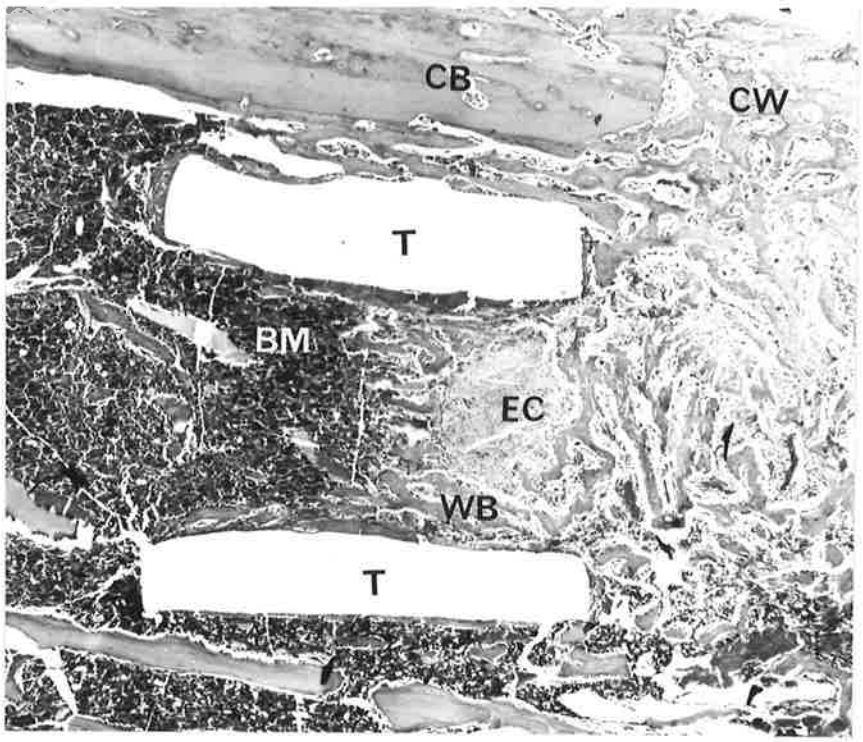


Figure 74

Figure 75: Intrafemoral Surgicel Implant 2 weeks:
Photomicrograph showing the re-establishment of the haemopoietic marrow (BM) in the side of the tube away from the cortical wound (CW). Woven bone (WB) and a pale-staining esoinophilic coagulum (EC) can be seen in the lumen of the Teflon tube (T) on the side of the cortical wound. CB = Cortical Bone. H & E x 25

Figure 76: Intrafemoral Surgicel Implant 2 weeks:
Higher power photomicrograph of an area in Figure 75 showing the cellular eosinophilic amorphous material (EC) surrounded by spicules of woven bone (arrows). H & E x 100



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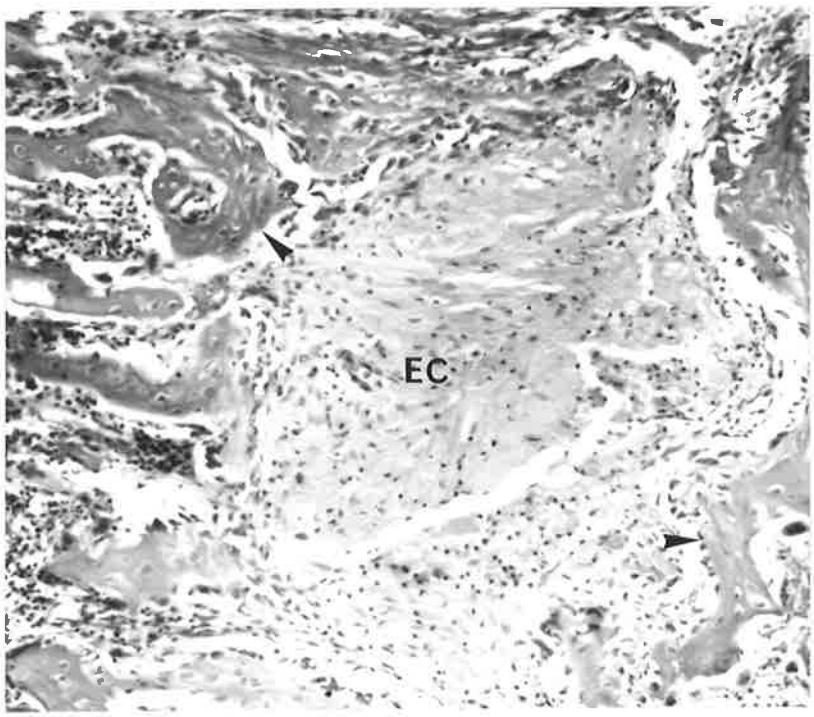


Figure 76

Figure 77: Intrafemoral Surgicel Implant 2 weeks:
High power photomicrograph of an area in Figure 76 showing foamy macrophages (arrows) in the eosinophilic amorphous material. H & E x 400.

Figure 78: Intrafemoral Surgicel Implant 3 weeks:
Photomicrograph showing haemopoietic marrow (BM) within the lumen of the Teflon tube (T). Woven bone (WB) can be seen between the marrow cells and along the surfaces of the tube. C B = Cortical Bone. H & E x 25.

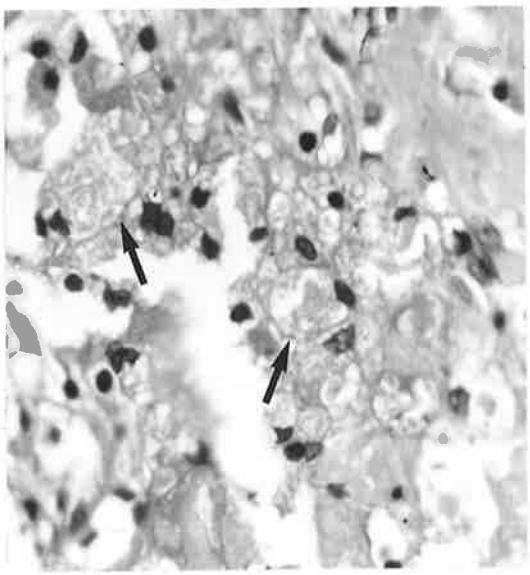


Figure 77

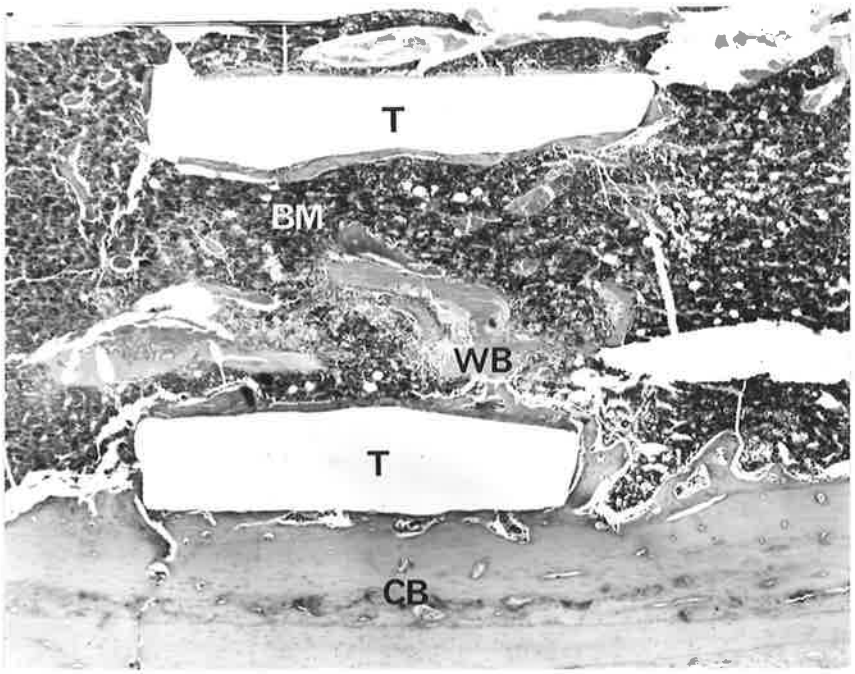


Figure 78

Figure 79: Intrafemoral Surgicel Implant 3 weeks:
High power photomicrograph of an area in Figure 78 showing foci of delineated Fibrillar material (F) which contains cell nuclei. Foamy macrophages (arrows) can be seen in proximity to the fibrillar material. The macrophage cytoplasm exhibits a similar morphology to the larger masses. WB = Woven Bone. H & E x 400.

Figure 80: Intrafemoral Surgicel Implant 6 weeks:
Photomicrograph showing haemopoietic marrow (BM) within the lumen of the Teflon tube (T). Woven Bone (arrows) lines the surfaces of the tube. CB = Cortical Bone. H & E x 25.

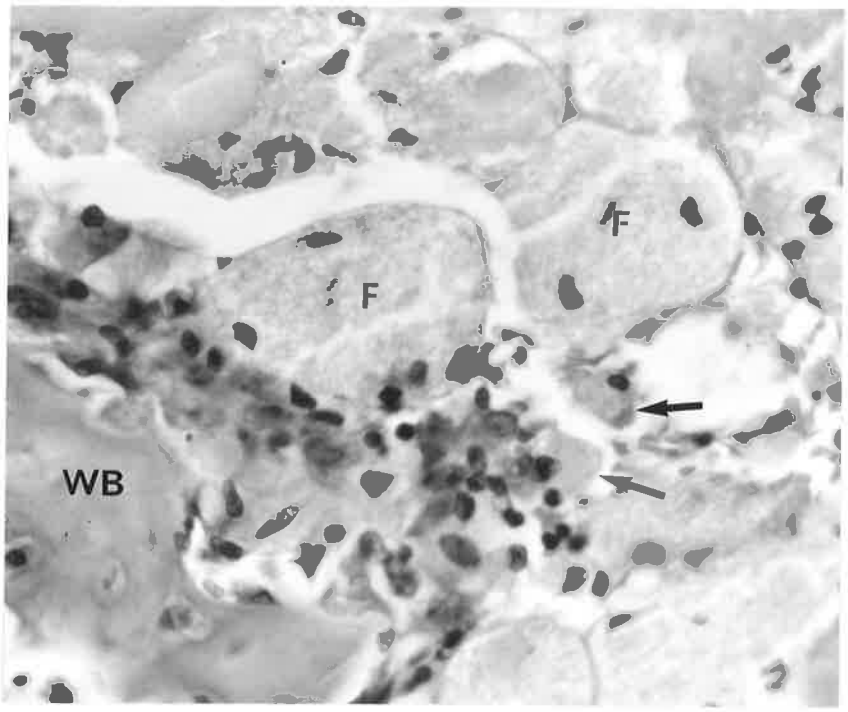


Figure 79

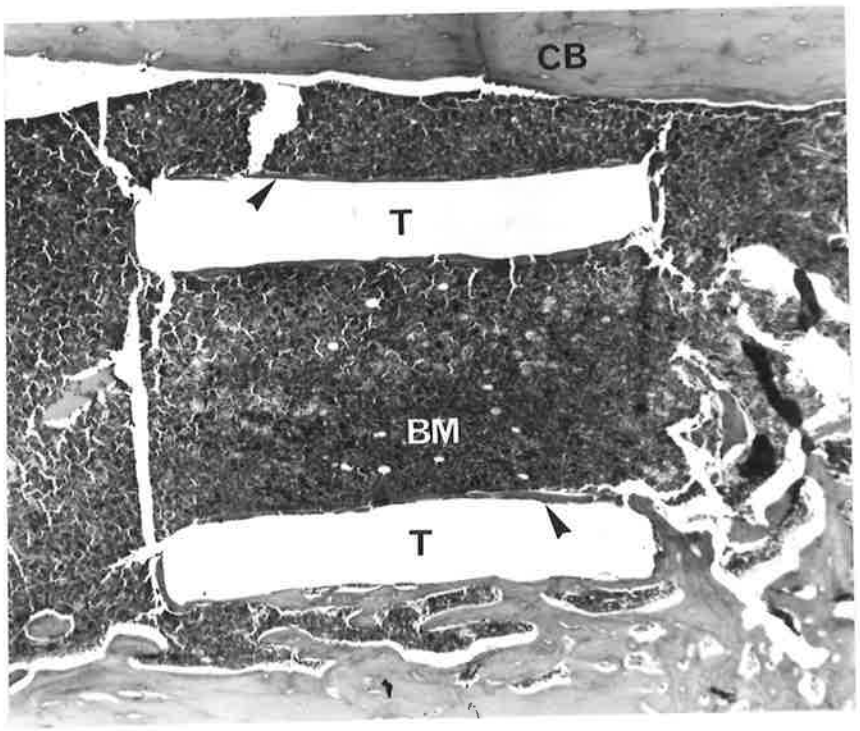


Figure 80

Figure 81: Intrafemoral Surgicel Implant 6 weeks:
High power photomicrograph of an area in Figure 80 in the lumen of the tube showing large foamy macrophages (arrows). H & E x 400.

Figure 82: Intrafemoral Surgicel Implant 8 weeks:
Photomicrograph illustrating normal haemopoietic marrow with fat cells throughout the femur. Woven Bone (arrows) lines the surfaces of the Teflon tube (T). Cortical bone (CB) can be seen on either side of the tube.
H & E x 25.

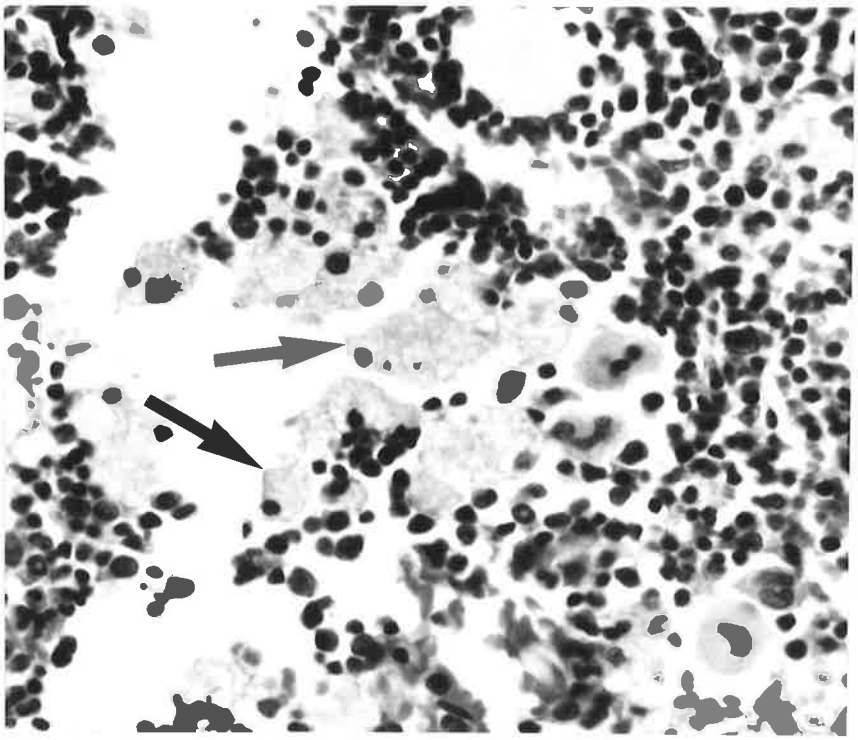


Figure 81

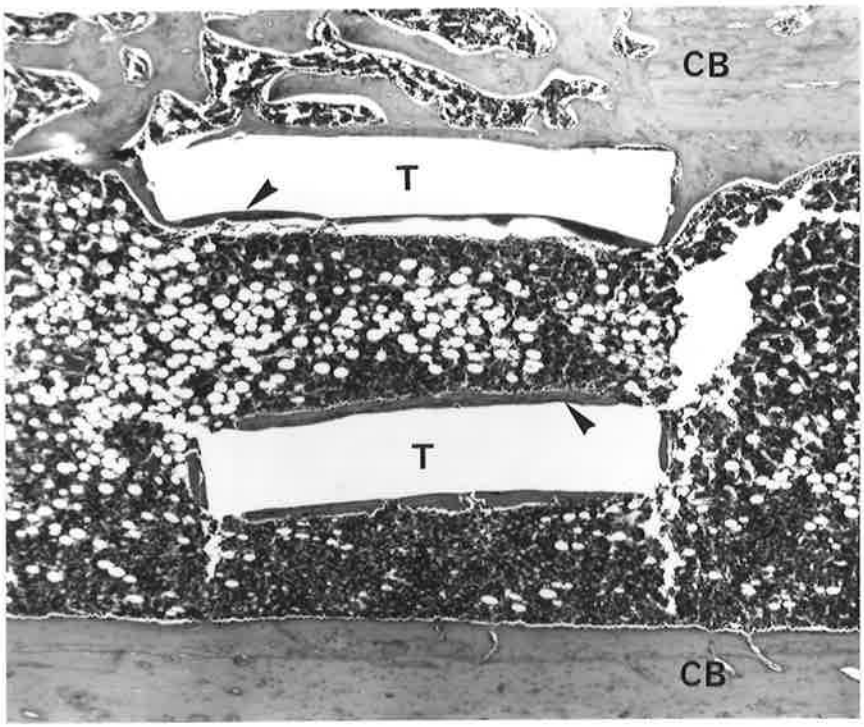


Figure 82

(C) Bone Wax Implants

During the preparation of the tissue specimens for histological examination, the Bone Wax was lost due to dissolution of the material during processing. As a result, a clear space was seen histologically in all specimens in the area occupied by the Bone Wax in the tubes. For descriptive purposes, however, the term "Bone Wax" will be used to indicate the space representing the implant material in the histological sections.

Microscopic observation of the tissue specimens containing the Bone Wax implants at the various post-implantation periods revealed the following:

Two days post-implantation: The implant material was clearly identifiable within the femur (Figure 83). Detailed examination of the Bone Wax-tissue interface revealed that the implant material was separated from the adjacent tissue reaction by prominent cell layer which was associated with a fine fibrillar layer at the Bone Wax face (Figure 85). Peripheral to this layer was a zone of inflammatory cells consisting mainly of polymorphonuclear leukocytes and macrophages. Many of the macrophages noted exhibited a foamy appearance (Figure 85) and they were concentrated close to the implant material. Oedematous granulation tissue, characterised by the presence of polymorphonuclear leukocytes and occasional lymphocytes, comprised the bulk of the tissue between the implant and pre-existing marrow tissue.

Seven days post-implantation: In all the tissue specimens examined, the Bone Wax was clearly visible and showed no signs of being resorbed. Detailed examination of the interface between the implant mat-

erial and the surrounding tissue demonstrated that the Bone Wax was separated from the adjacent tissues by a distinct fibrillar layer (Figure 86). Peripheral to this layer, maturing granulation tissue and some fibrous tissue were seen. Macrophages, some of which exhibited a foamy appearance, were noted close to the implant. Polymorphonuclear leukocytes were conspicuously absent. Peripheral to the zone of young fibrous tissue, new bone formation was evident.

Two weeks post-implantation: Microscopic examination of all the tissue specimens revealed that the implant material was still clearly visible in the femurs of the experimental animals and showed no signs of resorption (Figure 87). The implant itself was surrounded by a cellular zone and by a zone of woven bone. Detailed examination of the implant-tissue interface (Figure 88) illustrated the presence of a fine fibrillar layer between the implant material and the adjac- cellular zone. The cellular zone consisted of fibrous tissue containing macrophages, and a very small number of lymphocytes. The macrophages were difficult to identify in the dense fibrous tissue. Those macrophages identified, however, were characterised by a relatively small size and by vacuolation. In some areas, ossifying cartilage was noted in direct contact with the Bone Wax (Figure 89).

Three weeks post-implantation: Histologic observation of all tissue specimens demonstrated the presence of the implant material in the femurs of the rats. It still showed no signs of undergoing resorption. At this post-operative time period, the Bone Wax interface was characterised by woven bone (Figure 90) in close proximity to the wax space. Peripheral to the woven bone, normal haemopoietic marrow was evident. The woven bone was separated from the implant material by a thin cellular layer. Detailed examination of the implant-tissue interface demonstrated the presence of a fine fibrillar

layer immediately adjacent to the Bone Wax and a cellular zone, in which macrophages were prominent (Figure 91). Many of the macrophages exhibited a foamy appearance. In some areas, spicules of woven bone were separated from the implant material by two or three layers of cells, while in other areas, five to twelve layers of cells were seen between the Bone Wax and the adjacent woven bone.

Four weeks post-implantation: Examination of all histologic specimens indicated that the implant material was still present at its sites of implantation in the experimental animals. The Bone Wax showed no signs of being resorbed. The histologic features observed at this post-operative time period were essentially the same as those observed at three weeks post-implantation. Detailed examination of the Bone Wax-tissue interface revealed the presence of a fibrillar layer immediately adjacent to the implant (Figure 92). Macrophages and woven bone were evident peripheral to the fibrillar layer. Peripheral to the macrophages and woven bone, haemopoietic marrow was evident. The macrophages were relatively large and foamy.

Six to twentysix weeks post-implantation: Microscopic observation of all tissue specimens from six weeks post-operatively onwards exhibited the same histologic features and, thus, they are described together. Examination of all tissue sections revealed that the implant material was clearly visible and showed no signs of resorption. The Bone Wax appeared to be encapsulated by a layer of woven bone (Figure 93). Detailed observation of the implant-tissue interface revealed the presence of enlarged foamy macrophages and a layer of woven bone adjacent to the implant material (Figure 94). Peripheral to the zone of macrophages and woven bone, haemopoietic marrow was noted. Some macrophages were in direct contact with the Bone Wax and most of them exhibited an enlarged, foamy appearance.

Figure 83: Intrafemoral Bone Wax Implant 2 Days:
Photomicrograph showing Bone Wax (BW) implant in femur of rat. T = Teflon tube; BM = Haemopoietic Bone Marrow; CB = Cortical Bone; CW = Cortical Wound. H & E x 25

Figure 84: Intrafemoral Bone Wax Implant 2 Days:
Higher power photomicrograph of an area in Figure 83 showing fibrillar layer (F) immediately adjacent to the Bone Wax (BW) implant. Peripheral to this layer, a palisading layer of cells (arrows), apparently macrophages, can be seen. Inflammatory cells in an oedematous granulation tissue stroma comprise the cell and tissue component peripheral to the palisading cells.

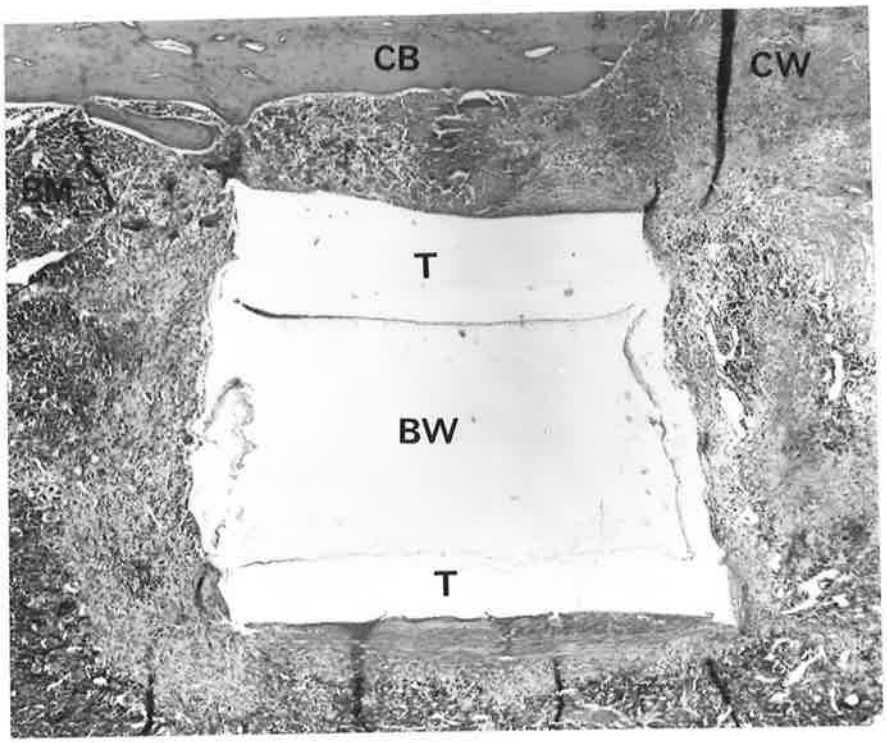


Figure 83

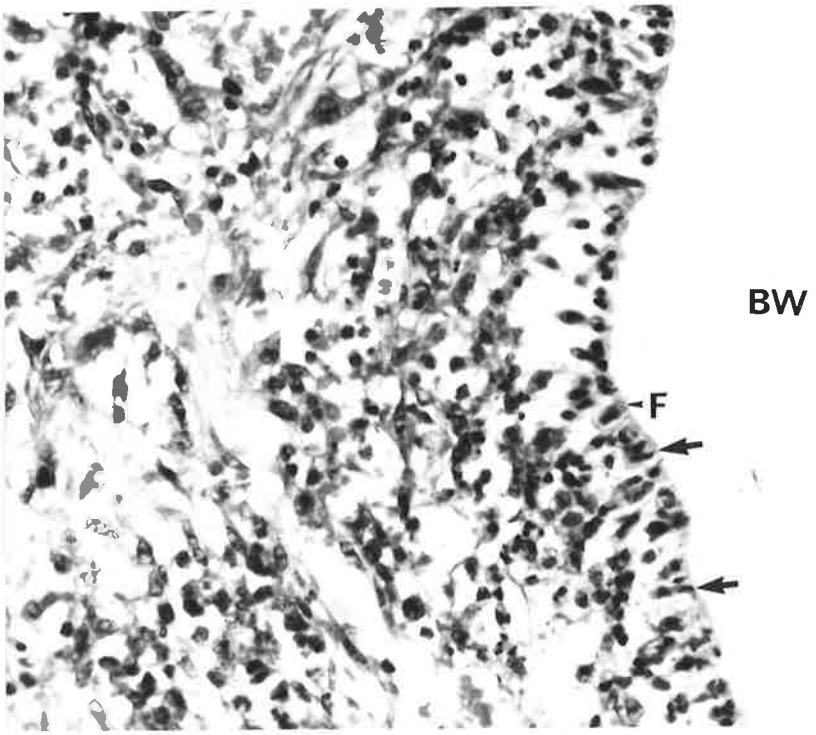
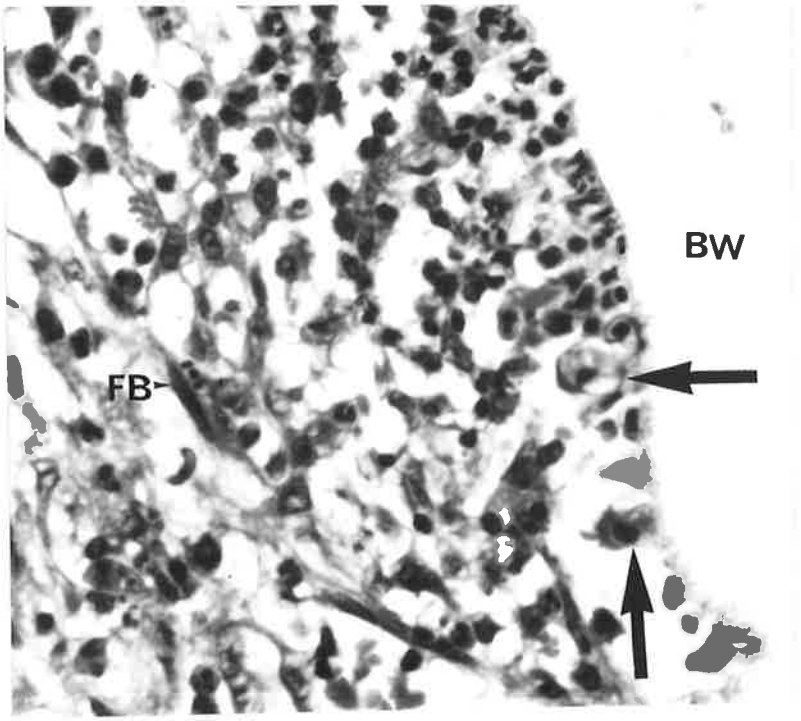


Figure 84

Figure 85: Intrafemoral Bone Wax Implant 2 Days:
High power photomicrograph of an area in Figure 84 illustrating foamy macrophages (arrows) adjacent to the Bone Wax (BW) implant. FB = Fibroblast. H & E x 400

Figure 86: Intrafemoral Bone Wax Implant 7 Days:
Photomicrograph showing fibrillar layer (F) immediately adjacent to the Bone Wax (BW) implant. H & E x 250.



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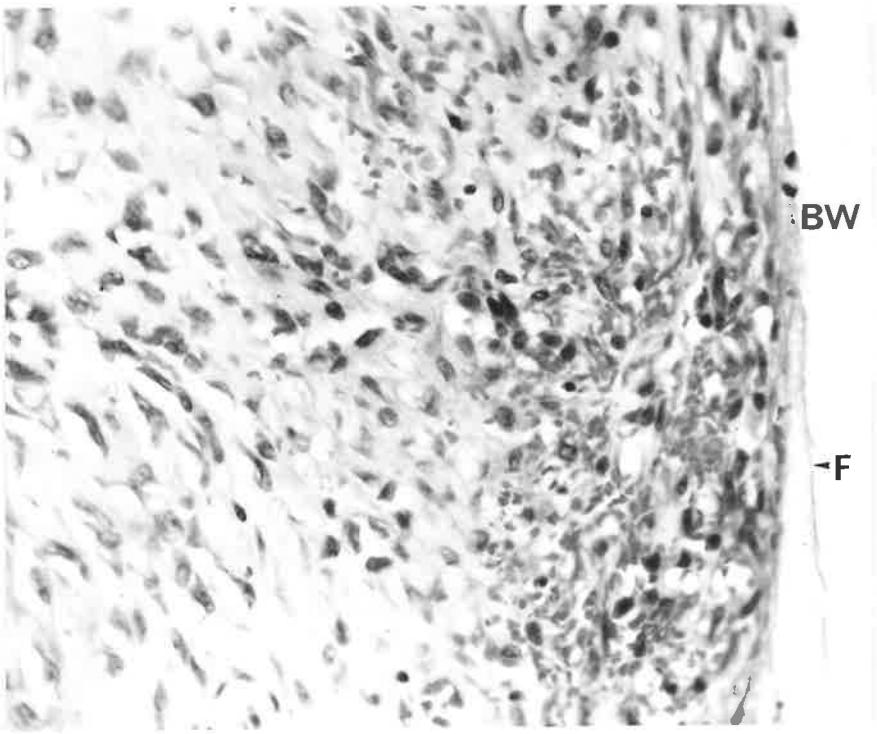

Figure 86

Figure 87: Intrafemoral Bone Wax Implant 2 Weeks:
Photomicrograph showing the Bone Wax (BW) implant in the femur of the rat. The implant is separated from woven bone (WB) by a cellular zone (arrow). T = Teflon tube; CB = Cortical Bone. H & E x 25

Figure 88: Intrafemoral Bone Wax Implant 2 Weeks:
High power photomicrograph of an area in Figure 87 showing a cellular zone (CZ) between Bone Wax (BW) and woven bone (WB). Fibrillar material (F) can be seen immediately adjacent to the implant. H & E x 400

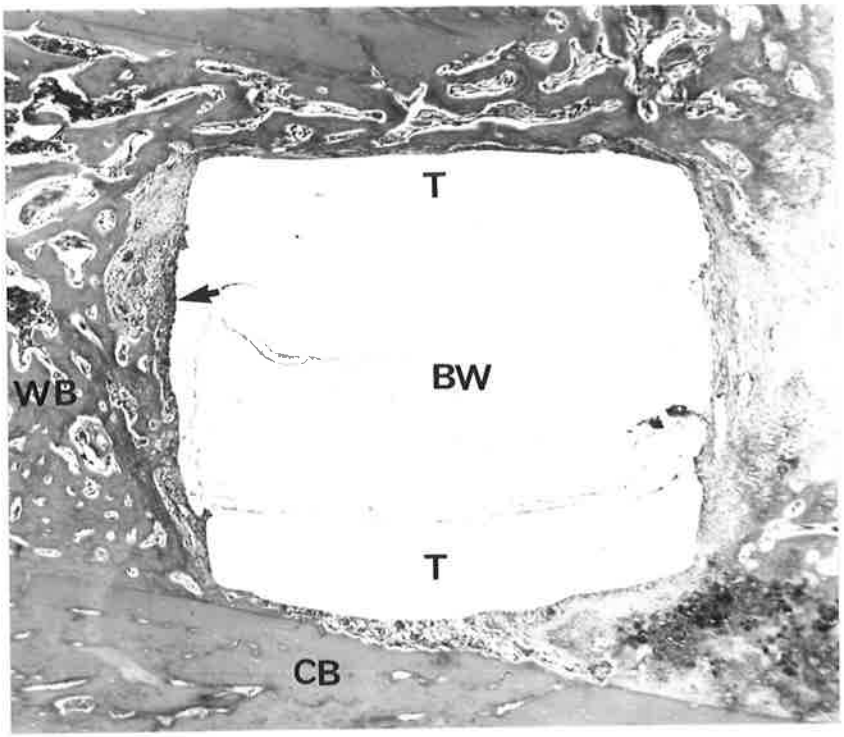

Figure 87

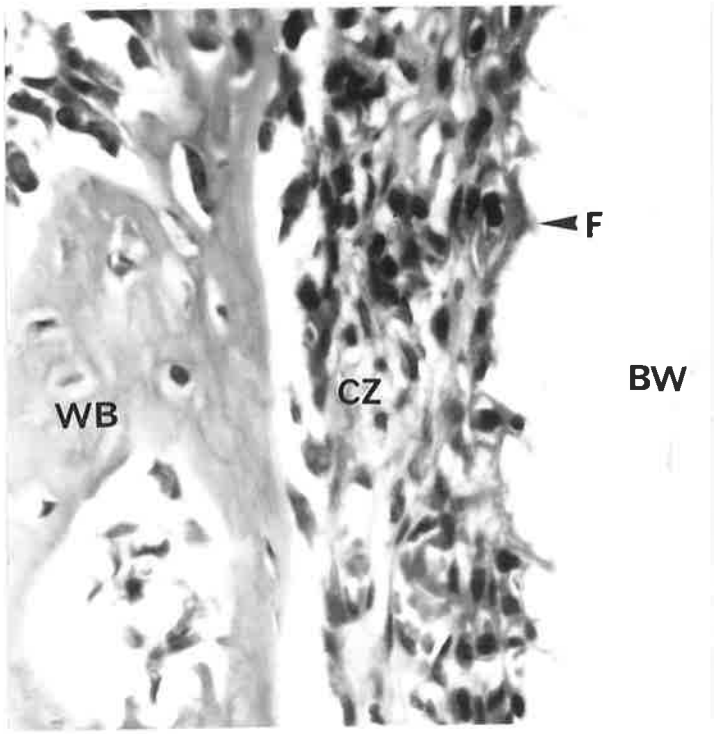

Figure 88

Figure 89: Intrafemoral Bone Wax Implant 2 Weeks:
High power photomicrograph of an area in Figure 87 showing ossifying cartilage (OC) in direct contact with Bone Wax (BW). H & E x 250

Figure 90: Intrafemoral Bone Wax Implant 3 Weeks:
Photomicrograph showing cellular zone (arrow) and woven bone (WB) between Bone Wax (BW) implant and haemopoietic bone marrow (BM). H & E x 40

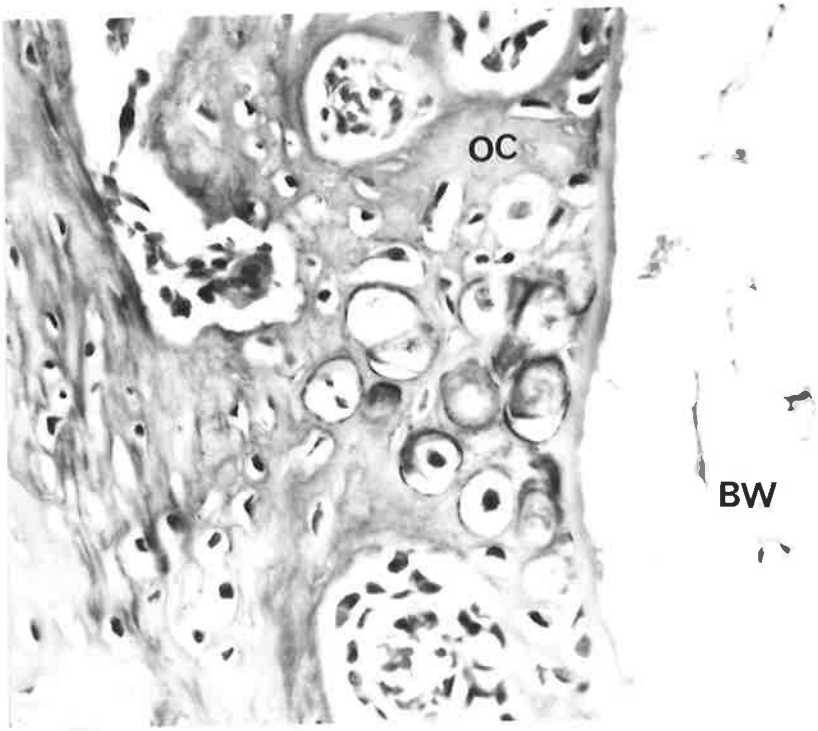

Figure 89

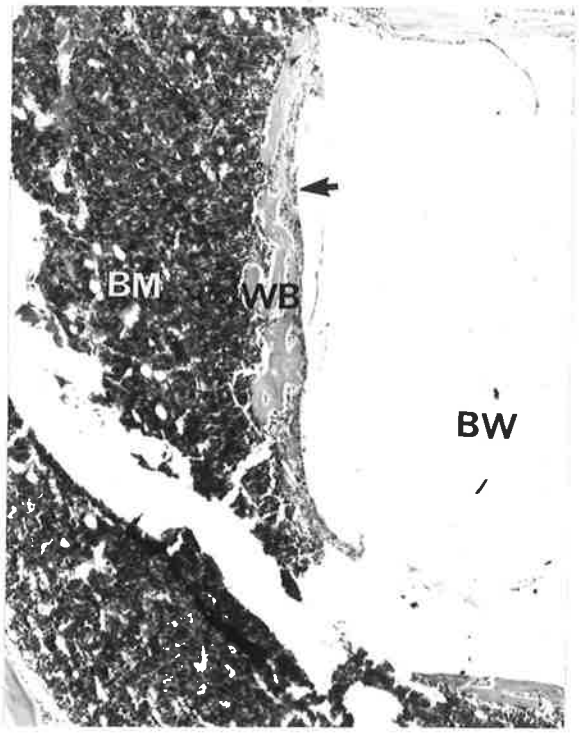

Figure 90

Figure 91: Intrafemoral Bone Wax Implant 3 Weeks:
High power photomicrograph of an area in Figure 90 showing zone of macrophages (arrow) between Bone Wax (BW) and woven bone (WB). A thin fibrillar layer (F) is present immediately adjacent to the implant. H & E x 400

Figure 92: Intrafemoral Bone Wax Implant 4 Weeks:
Photomicrograph showing zone of macrophages (arrow) between the haemopoietic marrow (BM) and Bone Wax (BW) implant. Woven Bone (WB) can also be seen in direct contact with the implant. F = Fibrillar material. H & E x 400

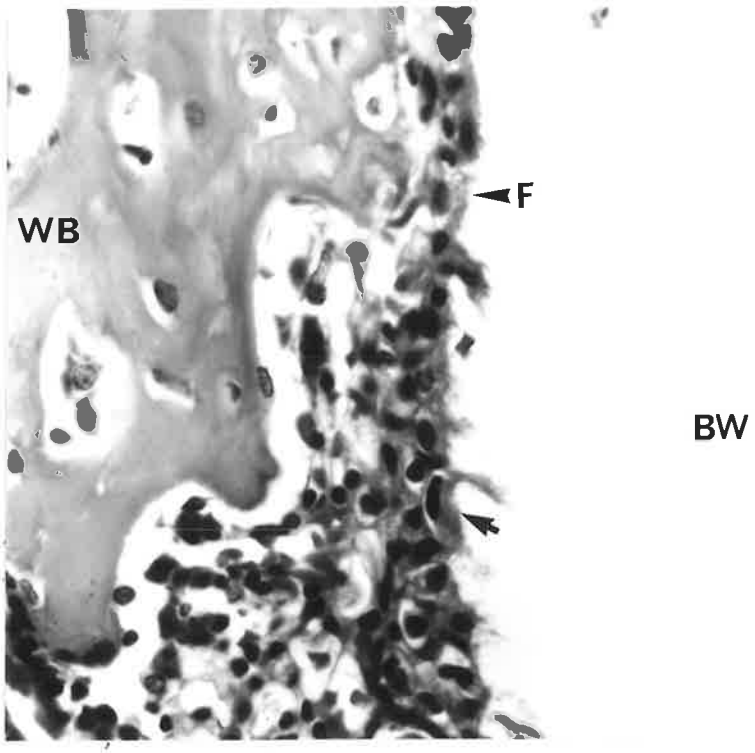

Figure 91

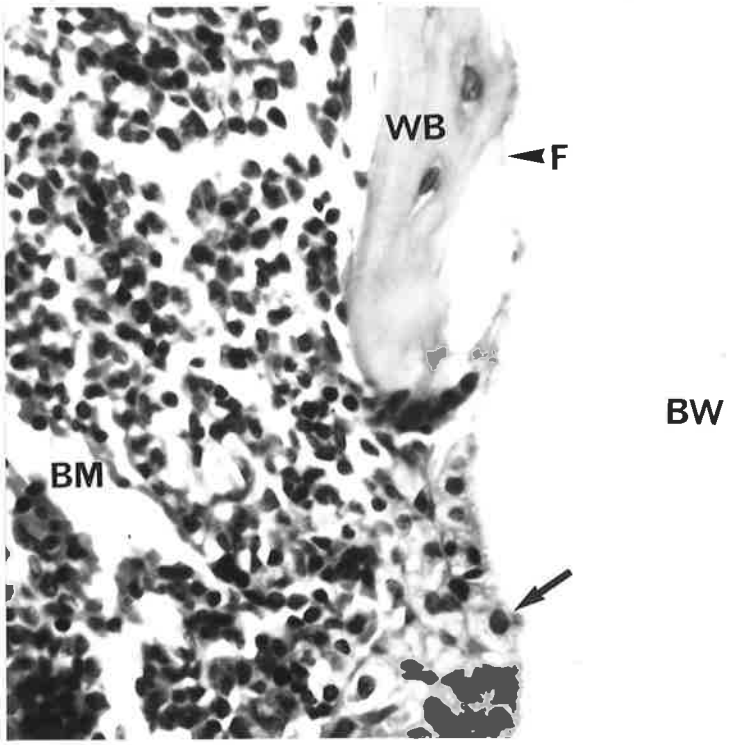

Figure 92

Figure 93: Intrafemoral Bone Wax Implant 8 Weeks:
Photomicrograph showing a layer of woven bone (arrow) between Bone Wax (BW) and haemopoietic bone marrow (BM). CB = Cortical Bone. H & E x 25.

Figure 94: Intrafemoral Bone Wax Implant 12 Weeks:
Photomicrograph showing foamy macrophages (arrows) and woven bone (WB) adjacent to Bone Wax (BW). BM = Haemopoietic Bone Marrow. H & E x 400.

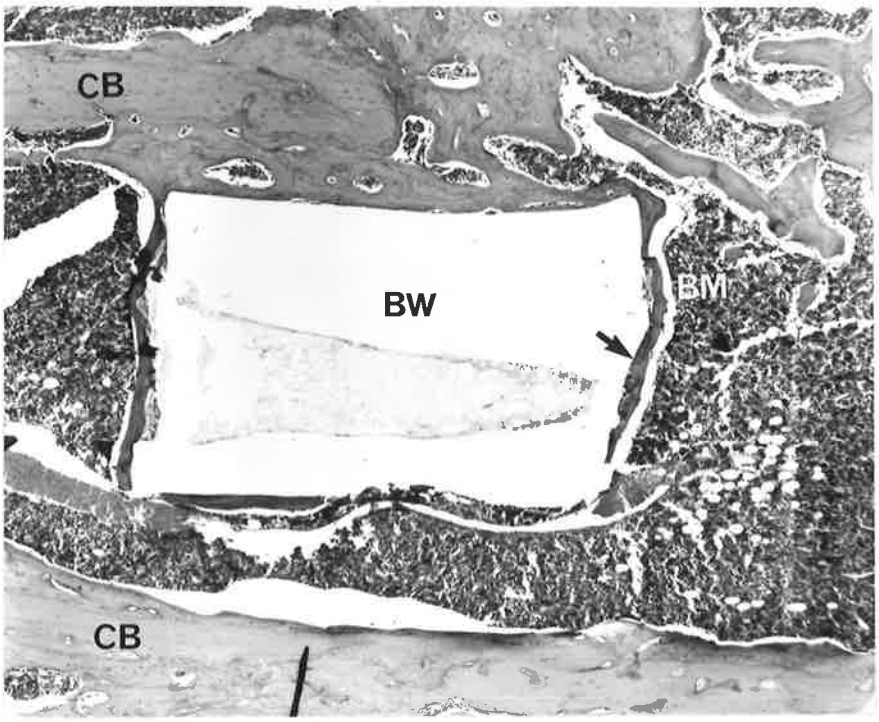

Figure 93

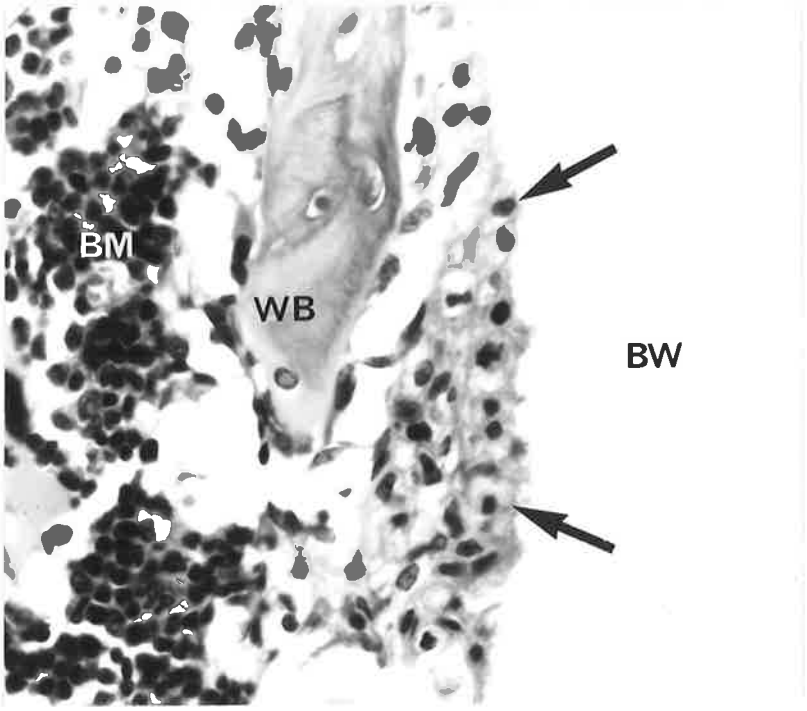

Figure 94

CHAPTER 5

DISCUSSION

A. General

Since ancient times, local haemostasis has played an important role in controlling haemorrhage following surgical procedures and trauma (Evans:1977). A variety of techniques and agents have been developed and utilized for styptic purposes. The employment of local haemostatic agents forms an essential aspect of treatment in the presence of abnormal platelet function, lack of an essential coagulation factor in blood, a structural defect in walls of blood vessels (Lucas:1966) and for patients on long-term anticoagulants (Evans:1977).

Modern industrial technology has, among other things, introduced haemostatic agents as solutions to some of the medical and surgical problems (Hegyeli:1971). The development of new surgical biomaterials is a lengthy and arduous process, the most important aspect of which is biocompatibility testing of the final product (Gourlay et al:1978). Currently, there is a lack of agreement as to what constitutes a valid test for implant compatibility studies and its interpretations (Hegyeli: 1971; Langeland and Cotton:1977).

In the present study, the biological effects of Surgicel and Bone Wax on rat muscle and haemopoietic bone marrow were investigated. The experimental model, based on the concept of inserting test materials into tubes prior to their placement into implantation sites in animals (Torneck:1966; Browne and Friend: 1968; Spångberg:1968; Friend and Browne:1969 Spångberg:1969; Langeland and Cotton:1977; Haugen and Mjör:1978; Wenger et al:1978; Olsson et al:1981a; 1981b), was selected for this study.

In the past, the biological effects of Surgicel have been studied largely by qualitative histologic observation of tissue responses that it elicited in experimental animals (Hurwitt et al: 1960; Jantet and Rob:1960; Georgiade et al:1961; Miller and Thomas: 1961; Skoog:1967; Thilander and Stenström:1969; Larsson et al: 1978) and by an assessment of its clinical efficacy in humans by observing its styptic properties (Lebendiger et al:1960; Hurwitt et al:1960; Jantet and Rob:1960; Blau et al:1960; Miller et al:1961a; Georgiade et al:1961; Shea:1962; Degenshein et al:1963; Tierney:1964; Lucas: 1966; Skoog:1967; Huggins:1969). To date, Uddströmer(1978) has been the only researcher to conduct a study on the biological effects of Surgicel where results were interpreted both qualitatively and quantitatively. Due to the paucity of data available regarding the effects of implanted Surgicel in experimental animals, this implant material was chosen for study in an attempt to augment currently available data, and to develop an experimental model which could allow quantitation of some of the tissue responses.

The literature on the biological effects of Bone Wax is scanty, the only experimental implantation study to date being conducted by Howard and Kelley(1969). In addition, no long-term studies, using this implant material, have been reported. For these reasons, Bone Wax was chosen for this study.

Polytetrafluorethylene (Teflon) tubing, with an outer diameter of 1.7mm and an inner diameter of 1.3mm, was used as a carrier of the test materials. The inertness of Teflon and the fact that it can be autoclaved (LeVeen and Barberio:1949) make it ideal as a carrier of implant materials (Spångberg:1968; 1969; Olsson et al 1981a; 1981b).

The advantage of placing Surgicel and Bone Wax into Teflon tubing is that the original implantation sites of the haemostatic agents remained identifiable clinically and microscopically. This was of especial importance in the case of Surgicel, which is generally regarded as an "absorbable" material. The reaction to Surgicel could be easily assessed by examining the tissue responses within the lumina of the tubes. In addition, ingrowth of tissue into the tubes could be quantified, and the validity of claims regarding the "absorbable" nature of Surgicel, could either be substantiated or refuted.

In the Bone Wax implants, the use of Teflon tubing allowed for the standardization of the tissue-material contact area. The tissue responses to the implant material could be assessed both qualitatively using histology, and quantitatively by using statistical means of analysis. The latter method was used only in the intramuscular Bone Wax implants.

Smaller tubes (2mm in length) were implanted in the femurs of the rats, compared with the 3mm tubes that were inserted intramuscularly. The reason for placing tubes of shorter length in the femurs was that a smaller cortical wound was desirable for placement of the implants to reduce the incidence of post-operative fracture. Despite this precaution, thirteen out of ninety femurs were found to be fractured at the time of sacrifice of the experimental animals, and these had to be discarded.

Surgicel and Bone Wax are marketed in sterile packages and a pyrogen-free transfer of these agents into the Teflon tubing was achieved by using an Oliphant Laminar Flow Unit. Samples of tubing, with and without the haemostatic agents, were cultured aerobically for four days. This procedure is recommended by the F.D.I. Brain Heart Infusion culture medium was used for this purpose. None of the samples yielded any bacterial colonies, indicating the sterility of the implants employed in this study. The importance of a sterile technique in implantation studies has been emphasized by Coleman et al(1974).

Muscle of the anterior chest wall was chosen as the soft tissue implantation sites since they were easily accessible. According to Coleman et al(1974), muscle is a suitable site for implantation studies since it provides a homogeneous environment.

After fixation in neutral buffered formalin for at least one week, the bone specimens were decalcified in Decal solution. Since decalcification is rapid when using this solution, care had to be taken not to "over decalcify" the tissues. Specimens were radiographed at regular intervals to avoid such overexposure to the Decal solution. Initially, the bone specimens were radiographed three hours after placement into the Decal solution, and, thereafter, at one-hourly intervals. Complete decalcification was achieved in five hours.

All bone and soft tissue specimens were subjected to a double-embedding procedure, with the view that better sections for histologic examination could be obtained.

When the soft tissue specimens were cut to obtain sections for histology, two major difficulties were, however, encountered. The first of these was incorrect alignment of the Teflon tubes with respect to the plane of section of the specimens. The second of the difficulties encountered was the tethering of sections, which appeared to be related to inadequate infiltration of the specimens by wax. In order to overcome these difficulties, the specimens were replaced in molten wax (Paraplast +) until all the original hardened wax had been removed. Specimens were subsequently placed in a vacuum chamber until adequate re-infiltration by wax was ensured. The specimens were re-blocked and cut. Better quality sections were obtained for histologic examination after re-infiltration and re-blocking of specimens.

In the bone specimens, the only difficulty encountered was tethering of sections. Re-infiltration and re-blocking of specimens, as performed on the soft tissue specimens, overcame this difficulty.

B. Surgicel Implants

In all the intramuscular control and Surgicel implants, the tissue responses noted in the tube lumina were essentially similar. The general tissue responses noted in the tube lumina of all the intramuscular control and Surgicel implants were as follows:

- by seven days post-implantation, granulation tissue ingrowth into both ends of the tubes was observed. Overt evidence of active acute inflammation was lacking.
- at two weeks post-implantation, the ingrowth of tissue extended along the entire length of the tube in the shape of an hour-glass; striated muscle fibres were noted in the tissue ingrowth and persisted, in part, six months post-operatively.
- at three weeks post-implantation, fat infiltration was noted in the tissue ingrowth. At subsequent post-operative time periods, adipose tissue gradually replaced both fibrous tissue and striated muscle in the tissue ingrowth.

The ingrowth of granulation tissue, noted initially at seven days post-implantation, could have occurred by either of two methods described by Torneck (1966). Fibrin meshwork in the serous fluid filling the tubes, a phenomenon noted in two and seven day post-implantation control and Surgicel implants, provides a scaffolding along which fibroblasts and blood vessels can extend. An alternative explanation for the ingrowth of granulation tissue into the tubes is the direct extension of endothelial cells of blood vessels and of fibroblasts into the lumina of the Teflon tubes (Torneck:1966). According to Walter and Israel (1975), endothelial cells are capable of forming pseudopodia which anastomose with their neighbours to form a series of vascular arcades. These arcades provide active fibroblasts with a blood supply for nutrition, as well as a scaffolding along which they can migrate (du Plessis:1970; Forrester:1976).

At two weeks post-implantation, striated muscle fibres and cells were noted in the tissue ingrowth of the intramuscular control and Surgicel-filled tubes. The muscle fibres persisted, in part, six months post-operatively. A possible causal mechanisms underlying the process of muscle regeneration observed in the present study, is that described by Carlson(1973) and Järvinen and Sorvari(1975). According to these investigators, there is, between the basement membrane and the sarcolemma of a muscle fibre, a mononucleated cell called the satellite cell. Satellite cells are normally spindle-shaped and often tend to be flattened along one axis. The long axis of these cells is normally oriented parallel to that of the muscle fibre and they are frequently embedded in depressions in the surface contour of the muscle fibre itself.

It has been postulated by Carlson(1973) that satellite cells represent a population of cells which serve as a cellular reserve and could be mobilized to repair damage to the muscle fibres themselves. Recognition of satellite cells in striated muscle has rekindled the old controversy of whether the cells of highly differentiated tissues can act on their own behalf in post-traumatic repair, or whether an infusion of new cells from a normally dormant reserve population is necessary. According to the former viewpoint, nuclei, surrounded by a thin rim of cytoplasm, break off from damaged muscle fibres, and, after becoming completely invested with a cell membrane, serve as the cellular source of regenerating muscle fibres. This is called the "dedifferentiation" of muscle. According to the second viewpoint, nuclei of the damaged muscle fibres are unable either to reactivate the synthetic ability required of a myogenic cell, or even to survive. Therefore, they would be

excluded from participation in any subsequent regenerative process following injury. In their stead, a population of undifferentiated cells, with myogenic potential (satellite cells), is activated to proliferate and later, differentiate into muscle. The concept is supported by the experiments of Layman et al(1980), who demonstrated an increase in the amount of DNA in rat striated muscle for up to 165 days post-partem.

Historically, cells, other than satellite cells, have been considered to represent reserve populations in connective tissue. These have been primarily cells of the perivascular connective tissue and, at times, even nucleated blood cells (Carlson:1973). However, the role of satellite cells in muscle regeneration is receiving the greatest attention since these cells are capable of mitotic division (Carlson:1973; Rayne and Crawford:1975).

The frequency of occurrence of satellite cells, in adults of most species, has been found to be in the range of 5% and 10% of the nuclei present within the basement membranes of the muscle fibres. In the muscles of newborn animals, up to one-third of the nuclei may belong to the satellite cells (Carlson:1973). The fact that young rats were used our study, possibly explains, in part, the reason for the rapid ingrowth of striated muscle fibres into the lumina of the Teflon tubes.

From three weeks post-operatively onwards, in the intramuscular control and Surgicel-filled tubes, there was a progressive increase in the amount of adipose tissue in the tissue ingrowth, associated with a corresponding decrease in the number of striated muscle fibres present. The appearance of adipose tissue in intraluminal tissues, according to Selye et al(1959), may be explained in the following terms: After implantation, a considerable negative pressure develops in the lumina of tubes as a result of the absorption of air introduced at the time of implantation, or of subsequently formed exudate. In such cases, the surrounding tissue is "sucked into" the lumina of tubes and then becomes transformed into typical adipose tissue. It is, therefore, assumed that suction itself is partly responsible for the appearance of adipose tissue. This mechanism would not, on the basis of observations made in the present study, adequately explain the observed phenomena related to tissue ingrowth.

Adipose tissue replacement of striated muscle and granulation tissue, noted in our study from six weeks post-operatively onwards, could also be explained by a mechanism other than suction. According to Selye et al(1959) and Torneck(1966), the rate of metabolism of intraluminal tissues is lower than that of normal tissue from which it is derived. This is probably due to a reduction in blood supply to intraluminal tissues. Consequently, the latter undergoes fatty degeneration (Selye et al:1959).

Another explanation for the occurrence of adipose tissue inside the tubes also has to be considered. It is possible that newly-formed striated muscle fibres, noted in the intraluminal

tissue ingrowth in our specimens, were afunctional. Carlson(1973) and Järvinen(1975) have stated that muscle fibres that regenerate, must become functional if they are to persist in the tissues. Therefore, any newly-formed muscle should be of direct functional value to the subject to maintain its structural integrity. The possible lack of function of intraluminal muscle, noted in the specimens in this study, possibly accounts for its degeneration and subsequent replacement by adipose tissue.

When Surgicel implants in this study were examined histologically, the most striking difficulty encountered was positive identification of the implant material. This difficulty was more apparent in the intramuscular Surgicel implants. Since the chemical structure of Surgicel is akin to that of cellulose (Figures 1 and 2), a staining method, normally employed to demonstrate cellulose (viz. Safranin-Aniline Blue), was used in an attempt to stain Surgicel in sections of all specimens containing Surgicel-filled tubes. The implant material failed to stain in all specimens. In an attempt to account for this apparent anomaly, two sets of slides were prepared as follows: In one set, unused Surgicel was embedded in Bacto-agar*, and sections were cut and mounted on glass slides. In the other set, Surgicel was fixed in neutral buffered formalin for twenty-four hours and subsequently embedded in Bacto-agar embedding medium. Sections were prepared and mounted on glass slides. Slides from both sets were stained using the Safranin-Aniline Blue method (Appendix IV). Only the Surgicel embedded directly in the agar medium, that is, without fixation in neutral buffered formalin, stained positively.

* Manufactured by Difco Laboratories., U.S.A.

This result indicates that histologic processing of Surgicel possibly alters its chemical structure. Further, Surgicel obviously underwent a rapid physical change, especially in intramuscular implants. Both of these factors made its positive identification extremely difficult. Further investigations regarding the exact chemical composition of Surgicel and its behaviour in a tissue environment or following exposure to chemicals involved in tissue processing,should be undertaken in order to clarify these problems.

At two days post-implantation, Surgicel had lost its woven appearance (compare with Figure 37). However, an eosinophilic, coarse fibrillar mass was identified in the tube lumina of all the specimens. This mass was identified as altered Surgicel since a similar mass was not identifiable in any of the control specimens at this post-operative time period. Many enlarged, foamy macrophages were noted in close relation to the implant material but no particulate matter or stainable material could be identified within these cells.

Polymorphonuclear leukocytes were noted both at two and seven days post-implantation,but not at fourteen days post-implantation,in the soft tissue specimens. This observation is consistent with the view that surgicel is not, per se, particularly "irritating",and that its presence does not induce a marked acute inflammatory reaction. It was possible that surgical trauma could have accounted for the presence of these cells.

At seven days post-implantation, triangularly-shaped ingrowths of granulation tissue were noted to arise from both ends of the Teflon tubes in all intramuscular specimens. On either side of the tissue ingrowth, coarse fibrillar material, noted at two days post-implantation, was seen.

At fourteen days post-implantation, the tissue ingrowth had extended throughout the entire length of the Teflon tube. The fibrillar material, noted on either side of the tissue ingrowth, was histologically indistinguishable from the fibrillar material noted in the corresponding intramuscular control implants. It is, therefore, likely that, by fourteen days post-implantation, implanted surgicel had been resorbed. This experimental finding and conclusion is supported by the results of Hurwitt et al(1960) and Jantet and Rob(1960), who reported that Surgicel was resorbed from the subcutaneous tissues of rats within fourteen days post-implantation.

Absorption of Surgicel from the livers of experimental animals takes up to seventeen days post-implantation (Jantet and Rob:1960; Miller and Thomas:1961). At no stage in our experimental study, did Surgicel elicit a foreign-body giant cell reaction, but Miller and Thomas(1961) did note such a response, at ten days post-operatively, to Surgicel implanted in the liver and spleen of dogs.

When the figures of the mean percentage ingrowth of tissue in the intramuscular control and Surgicel-filled tubes were examined, the most striking result was seen at seven days post-operatively.

The mean percentage ingrowth of tissue, at this post-implantation period, in the Surgicel-filled tubes, was 20.96%, and for the control tubes, the figure was 34.66%. The differences noted between the control and Surgicel-filled tubes at subsequent post-implantation time periods, were very small and attributable, most probably, to artifacts during histologic processing of the specimens. No statistical analyses were performed on the twelve and twenty-six week post-operative specimens as the number of animals used was too small to derive any reliable statistical data.

From the statistical data obtained, it is possible that Surgicel delayed the ingrowth of tissue into the tube lumina only during the first week post-implantation, the period during which the implant material was apparently undergoing resorption. All the results obtained for the mean percentage ingrowth into tubes of the intramuscular control and Surgicel implants, were subjected to the Student's t-Test. This test indicated that, at no stage, was there a statistically significant difference (at the 5% level), between figures obtained for the control and Surgicel implants.

In the intrafemoral control implants which served as negative controls for the Surgicel and Bone Wax bone implants, several phases of repair of haemopoietic bone marrow were noted, namely, haematoma and clot formation, the appearance of elongated young fibroblasts, osteoblastic proliferation and cancellous bone formation, the reappearance of haemopoietic marrow cells and resorption of cancellous bone. These findings on the healing of bone marrow are consistent with the reports by Brånemark et al(1964), Pritchard(1964), Amsel et al(1969), Maloney and Patt(1969), Van Dyke and Harris(1969),

Patt and Maloney(1972), Chapelle et al(1973), Patt and Maloney(1975), Owen(1978).

In the intrafemoral Surgicel implants, the Surgicel was clearly identifiable in the tube lumina at two and seven days post-implantation. This finding differed somewhat with the histologic observation made in the intramuscular Surgicel implants, in which the implant material had lost its original woven structure and appeared as an amorphous, eosinophilic fibrillar mass. The reasons for these differences in the morphology of Surgicel between the two implantation sites are not known.

At two weeks post-implantation, Surgicel was not identifiable in the morphologic form noted at two and seven days post-implantation. Instead, an eosinophilic coagulum, containing inflammatory cells, was noted between spicules of woven bone in the tube lumina. At three weeks post-operatively, a fine fibrillar material, containing many inflammatory cells, was present between spicules of woven bone in the lumina of the Teflon tubes. Since the material, noted above at two and three weeks post-implantation, was not present in the corresponding control implants, it was likely that it represented Surgicel which had undergone morphologic alterations in vivo.

From four weeks post-operatively onwards, there was no histologic evidence of the implant material.

Polymorphonuclear leukocytes were seen only at two days post-implantation, indicating that an acute inflammatory reaction subsided within the first week after placement of the gauze.

The Polymorphonuclear response, in large part, was probably a response to tissue injury resulting from surgical trauma.

Large, foamy macrophages were consistently seen between fibres of the implant material at two and seven days post-implantation. At two weeks post-operatively, these cells were noted within the substance of the eosinophilic coagulum, described above, and, at three weeks post-implantation, they were conspicuous among the fine fibrillar material, described above, and identified as Surgicel.

Despite the fact that Surgicel was not histologically identifiable from four weeks post-operatively onwards, large, foamy macrophages persisted among cells of the haemopoietic bone marrow up to six weeks post-implantation. These cells were not present in the corresponding intrafemoral control implants.

Georgiade et al(1961) performed fifty extractions on rats and packed the sockets with Surgicel gauze. According to these authors, the gauze was completely reabsorbed microscopically by three weeks post-implantation. Their experimental findings are open to question as there are two possible mechanisms whereby the Surgicel could have been removed. Firstly, this could have been achieved by the inherent biological mechanisms within bone, that is, by phagocytosis and enzyme degradation. Secondly, it was likely that the healing processes, within the tooth sockets, could have displaced the Surgicel physically from the sockets, aided by the cleansing effect of the saliva.

Skoog(1967), Thilander and Stenström(1969), Engdahl(1972) and Uddströmer(1978) implanted Surgicel in compact bone. According to Skoog(1967), the implant material was completely reabsorbed within four weeks. Thilander and Stenström(1969) claimed that Surgicel disappeared after two weeks post-operatively. They also stated that Surgicel had a beneficial effect on bone healing, but Engdahl(1972) and Uddströmer(1978) claimed that the implant material retarded bone healing. The results of the present study with regard to the persistence of Surgicel in bone wounds are thus in general accord with the findings of others. There was, however, no overt evidence that Surgicel either retarded (Engdahl:1972; Uddströmer:1978), or enhanced bone healing (Skoog:1967; Thilander and Stenström:1969).

The process by which Surgicel is resorbed from tissues is not clear. The slight alkalinity of body fluids (Hurwitt et al: 1960; Shea:1962; Degenshein et al:1963) and the enzymes of the carbohydrase system (Hurwitt et al:1960; Shea:1962) may, in part, be responsible for the absorption of Surgicel. The role of the mononuclear phagocyte (macrophage) in the absorption of Surgicel is not clear. Hurwitt et al(1960) and Lebendiger et al(1960) described the presence of "large macrophages" in close relation to implanted Surgicel. In addition, Jantet and Rob(1960) observed Surgicel particles in macrophages of subcutaneous tissues and muscle of rats five days after implantation of the gauze and suggested that these cells were responsible for phagocytosis of Surgicel particles.

In a wound healing situation, macrophages play an important role in the ingestion of foreign material (Ross and Odland:1968 ; Leibovich and Ross:1975). As a result of phagocytic activity, these cells develop an enlarged vacuolated appearance (Piller:1978). According to Piller and Clodius(1979), an increase in size and vacuolation of macrophages can be taken to represent past or present phagocytic activity of these cells. The increase in vacuolation of the macrophage cytoplasm is related to non-selective release of lysosomal contents (Piller and Clodius:1979). According to the concepts of Piller and Clodius(1979), large, vacuolated (foamy) macrophages, noted in relation to Surgicel in our study, probably indicate phagocytosis of Surgicel particles by these cells. However, no particulate matter or stainable material could be identified within these cells. Further in vivo and in vitro studies of the macrophage response to Surgicel will have to be undertaken, preferable at an ultrastructural level, to clarify the role of macrophages in the absorption of Surgicel.

C. Bone Wax Implants

Throughout the entire six-month post-operative observation time period in this study, Bone Wax remained at its sites of implantation in soft tissue and bone, without showing any signs of undergoing resorption. This finding supports the results of Geary and Frantz(1950) and Howard and Kelley(1969). The failure of Bone Wax to undergo resorption, is, according to Howard and Kelley(1969), related to its "impenetrable nature".

The effects of Bone Wax in a soft tissue environment have not previously been reported. The reason for implanting Bone Wax

into muscle, was to observe, in detail, the cellular responses that this implant material might elicit. Special attention was paid to the presence of vacuolated (foamy) macrophages at the implant-tissue interface and to the presence or absence of acute inflammatory changes. Such an observation would have been extremely difficult if haemopoietic bone marrow were used on its own as the implantation site because of the multitude of marrow cells present.

At two days post-implantation, an acute inflammatory cell reaction, consisting mainly of polymorphonuclear leukocytes and macrophages, was observed at the Bone Wax-tissue interface. Polymorphonuclear leukocytes were not observed at seven days post-operatively in any of the specimens examined at this post-implantation time period, indicating that the acute inflammatory response had subsided by seven days. An essentially similar histologic picture with respect to polymorphonuclear leukocytes and signs of acute inflammation was also exhibited by intrafemoral Bone Wax specimens. These findings are consistent with the view that the acute inflammatory response noted was probably a consequence of the surgical procedure rather than a response to the Bone Wax.

A feature noted in all specimens, from two days to twenty-six weeks post-implantation, was the presence of vacuolated (foamy) macrophages at the Bone Wax-tissue interface. These cells occurred in greater numbers at seven days post-operatively. It was likely that the presence of the implant material on one hand, and post-surgical healing of the implantation site on the other hand, accounted for the large number of foamy macrophages seen at this post-operative time period. The macrophages adjacent to the Bone Wax were, undoubtedly, involved in phagocytosis of the Bone Wax. However, as in the case of

Surgicel, no particulate or stainable material could be detected in the macrophages adjacent to the Bone Wax. Further studies, for example, ultrastructural studies, are indicated.

From two weeks post-operatively onwards, there was a steady decline in the number of foamy macrophages at the implant-tissue interface. However, only after six weeks post-implantation, was there a statistically significant reduction* ($0.025>p>0.01$), at the 5% level, in the numbers of these cells in the area examined. The reason for this result appeared to be related to the development of a fibrous capsule at the Bone Wax-tissue interface. At three weeks post-implantation, the capsule comprised immature fibrous tissue. By twenty-six weeks post-operatively, the capsule was a well-defined, densely collagenous structure. The presence of the dense capsule probably acted as a relatively impenetrable barrier to phagocytic cells towards the implant material.

From seven days to three weeks post-implantation, foreign-body giant cells were noted occasionally in the area between Bone Wax and striated muscle. Some of these cells were evident in granulation tissue and young fibrous tissue at the implant-tissue interface, while others were noted in close relation to striated muscle fibres. There are two possible explanations for the presence of foreign-body giant cells in the areas mentioned. These cells are usually formed in response to insoluble foreign material in tissues (Walter and Israel:1975). Therefore, one explanation for their existence is possibly related to the presence of insoluble Bone Wax implanted in muscle.

* Using the Student's t-Test

A second explanation for the presence of foreign-body giant cells, especially those in close proximity to striated muscle, may be related to muscle regeneration. According to Allbrook et al(1966), fibroblasts increase in number along with muscle regeneration. The network of collagen fibres, deposited by fibroblasts, sometimes impede the longitudinal growth of the regenerating muscle fibres and results in the formation of "muscle giant cells" (Allbrook et al:1966; Järvinen: 1975). Some of the giant cells noted in the intramuscular Bone Wax implants may not have been true foreign-body giant cells, but "muscle giant cells".

In the intramuscular Bone Wax implants, another interesting feature was noted between the implant material and striated muscle from three weeks post-operatively onwards, namely, the presence of adipose tissue. Adipose tissue formation in relation to non-resorbable implants, such as Bone Wax, may be explained by a mechanism different from the way in which it is formed in tube lumina, as noted in the intramuscular control and Surgicel implants, in this study.

According to Kaminski et al(1977), the presence of adipose tissue in relation to non-resorbable implant materials, has special significance. Adipose fat cells are primarily storage cells with relatively few protective qualities. Consequently, these cells could be expected to be more susceptible to the presence of toxic materials. From a functional standpoint, the appearance of adipose tissue in the protective fibrous capsule around implanted materials indicates decreasing need for "protection". The early appearance of adipose cells thus indicates low implant toxicity and, thus,

greater biocompatibility.

In this study, adipose tissue was noted from three weeks post-operatively onwards between the implant material and striated muscle. According to Kaminski et al(1977), implant materials of low toxicity begin to exhibit adipose tissue formation, in relation to their face, by six weeks post-implantation. According to this concept, Bone Wax should be regarded as a highly compatible implant material. However, it should be noted that Bone Wax still elicited a low grade cellular response as evidenced by the persistence of normal and enlarged foamy macrophages and occasional lymphocytes up to twenty-six weeks post-implantation.

The distribution of foamy macrophages, in an area measuring 260 microns by 260 microns immediately adjacent to Bone Wax, was determined by using the Poisson method of analysis. This method of analysis indicated that, only at seven days post-implantation, were foamy macrophages distributed in a Poisson fashion, that is, randomly. At the other post-operative time periods, the C.D. was always greater than 1, indicating that foamy macrophages, in the area examined, were not distributed in a Poisson fashion and that there was a factor causing their deliberate aggregation. Detailed analysis of the numerical distribution of foamy macrophages, in zones running parallel to the face of the implant material, supports the view that Bone Wax caused the aggregation of these cells.

At two and seven days post-implantation, foamy macrophages were distributed fairly evenly in all five zones. However, from four weeks post-operatively onwards, there was clear evidence that phagocytic foamy macrophages were aggregating in close proximity to Bone Wax. At this post-implantation time period, 67% of these cells were noted in Zone 1 (that is, within 52 microns of the implant material). At twelve and twenty-six-weeks post-implantation, all foamy macrophages, in the area examined, were noted in Zone 1. This result implied that phagocytic activity of macrophages was concentrated immediately adjacent to the implant material.

No foreign-body giant cells were seen in relation to the face of the intrafemoral Bone Wax implants, in our study, at any of the post-implantation time periods. This finding differed from the results of Howard and Kelley(1969), who noted the presence of foreign-body giant cells, at ten and twelve days post-operatively, to Bone Wax implanted in the tibia of rats.

A consistent finding in all intrafemoral Bone Wax specimens throughout the entire post-operative observational time period, was the presence of vacuolated (foamy) macrophages at the implant-tissue interface. These cells were also noted in the study by Howard and Kelley(1969). The persistence of foamy macrophages, even up to six months post-implantation, indicated continued phagocytic activity by these cells and this feature,observed in the bone implants,was consistent with that found in the soft tissue implants.

Certain histologic features, observed in specimens, suggested adequate biological acceptability of implanted Bone Wax by the femurs of rats. One of these features, noted first at two weeks post-operatively, was the presence of ossifying cartilage in direct contact with a small area of the Bone Wax surface, without the intervention of a zone of inflammatory cells or fibrous tissue. Woven bone was similarly noted in areas along the face of the implant material, apparently in contact with the Bone Wax, at four and six weeks post-operatively.

Another interesting feature, observed from eight weeks post-implantation onwards, was the presence of a "capsule" of woven bone along the entire face of the Bone Wax implant. This phenomenon was possibly an attempt by the local environment to isolate the implant material from the surrounding haemopoietic bone marrow. In this way, it would be possible for the Bone Wax to exist in a quiescent state at its site of implantation. The presence of the bone "capsule" could also represent a functional modification of the femur to the implant itself (i.e. the Teflon tube plus contents).

The isolation of Bone Wax by a fibrous capsule and woven bone in muscle and haemopoietic marrow, respectively, and the existence of this implant material in a quiescent state at its sites of implantation, indicates that, while Bone Wax is non-absorbable, it is compatible with rat tissues.

Since Bone Wax was found to be a non-absorbable material, the use of a positive control, employing a material of similar nature and behaviour to Bone Wax, would, in retrospect, have been desirable, in addition to the use of empty Teflon tubes as controls, especially in the intrafemoral Bone Wax implants.

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SUMMARY

In the present investigation, the biological effects of Surgicel and Bone Wax on rat striated muscle and haemopoietic bone marrow were investigated.

The test materials were inserted into Teflon tubing, while empty Teflon tubes served as controls.

Forty-eight young male Sprague-Dawley Albino rats were used as the experimental animals. The appropriate Teflon tubes, with or without the test materials, were implanted in the muscles of the anterior chest wall of the rats, as well as in the marrow space of their femurs. The animals were sacrificed at 2 days, 7 days, 2, 3, 4, 6, 8, 12 and 26 weeks post-implantation. All the specimens obtained were processed for histological examination.

In all the Surgicel and control implants (that is, soft tissue and bone), the area within the lumina of the Teflon tubes was assessed qualitatively. The histologic features assessed were:

(1) The presence or absence of inflammation. If present, the nature of the cellular infiltrate was assessed;

(2) The intensity and nature (viz. acute/chronic) of the inflammatory response;

(3) The presence or absence of macrophages. Particular attention was paid to signs of phagocytic activity by these cells as evidenced by the presence of cytoplasmic vacuolation and/or particulate material.

(4) The presence or absence of multinucleate foreign-body giant cells;

(5) The resorption and possible replacement of the test material;

(6) The nature of the tissues replacing the test materials.

In all the intramuscular Bone Wax implants, histological observation of tissue reactions was confined to the area between Bone Wax and the nearest muscle fibres. In the intrafemoral Bone Wax implants, the area between Bone Wax and haemopoietic marrow was examined. The histologic features assessed were the same as those for Surgicel.

In addition, most of the intramuscular Surgicel and control implants were subjected to a quantitative form of analysis. This was done by measuring the percentage ingrowth of tissue into the tubes of both groups of specimens with a planimeter. In all the intramuscular Bone Wax implants, the occurrence of vacuolated (foamy) macrophages in the histologic area examined, were subjected to the Poisson analysis.

The conclusions that can be drawn from this study are:

(1) Surgicel is an absorbable local haemostatic agent. This finding is in accordance with the results obtained by Hurwitt et al.(1960), Jantet and Rob (1960), Georgiade et al.(1961), Miller and Thomas (1961), Skoog (1967), Thilander and Stenström (1969) and Larsson et al. (1978). Its absorption from muscle appears to be more

rapid than it is from haemopoietic bone marrow. The implant material was found to be completely absorbed from striated muscle in rats by two weeks post-implantation. This result is in agreement with the findings of Hurwitt et al.(1960) and Jantet and Rob (1960). Surgicel was found to be completely reabsorbed from haemopoietic bone marrow by four weeks post-implantation, which is in agreement with the results obtained by Skoog (1967).

The absence of overt signs of inflammation occasioned by the implantation of Surgicel, and its complete absorption from the tissue into which it was placed, indicate that in rat tissues, it is a highly compatible material.

The process by which Surgicel is resorbed from tissues is not clear. Hurwitt et al(1960) and Shea (1962) suggested that Surgicel was degraded by enzymes in tissues, while Jantet and Rob (1960) found evidence of phagocytosis of Surgicel particles by macrophages. In our study, no conclusions regarding the mechanism(s) by which Surgicel is resorbed from rat tissues, can be drawn, since no enzyme histochemical assays were performed to indicate altered enzyme activity in response to implanted Surgicel, nor could particles of the implant material be demonstrated in macrophages. According to Piller and Clodius (1979), an increase in size and vacuolation of macrophages can be taken to represent past or present phagocytic activity of these cells. Enlarged, vacuolated (foamy) macrophages were clearly seen in relation to implanted Surgicel in our study, indicating that macrophages could have played a role in the absorption of this implant material. However, electron microscopic studies will have to be undertaken in the future to delineate the role of mononuclear

phagocytes in the absorption of Surgicel.

(2) Bone Wax is a non-absorbable local haemostatic agent. This result is consistent with the findings of Howard and Kelley (1969). When implanted in rat striated muscle, Bone Wax becomes isolated from the surrounding tissues by a dense fibrous capsule twelve weeks after its implantation.

In bone, the wax becomes isolated from haemopoietic marrow by a distinct layer of woven bone by eight weeks after its implantation.

In both soft tissue and bone, Bone Wax elicited an acute inflammatory response to its presence only at two days post-implantation. The absence of overt signs of inflammation occasioned by the implantation of the Bone Wax, and the fact that, with time, the wax becomes "isolated" from rat muscle by a dense fibrous capsule, and from haemopoietic marrow by a layer of woven bone, indicate that Bone Wax can exist in a quiescent state at its site of implantation over long periods of time, and that it, therefore, is compatible with rat tissues. This statement holds true for implanted Bone Wax that has been isolated from the external environment.

The Poisson analysis of the intramuscular Bone Wax implants indicated that phagocytic activity of macrophages adjacent to the implant material, as evidenced by the presence of large vacuolated mononuclear phagocytes, decreased with time. Also, such activity appeared to be concentrated as close as possible

to the face of the Bone Wax implant. However, no particulate material was identifiable within these cells.

The persistence of vacuolated macrophages at the implant face, even at twenty-six weeks post-implantation, suggests that there is a continued attempt by the rat mononuclear phagocytic system to resorb the implant material.

(3) The experimental model, used in this investigation, appears to be suitable for the study of the biological effects of implantable local haemostatic agents.

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APPENDICES

APPENDIX I

HOUSING OF EXPERIMENTAL ANIMALS

All experimental animals were housed in RB 3 polypropylene cages with stainless steel tops. The dimensions of the cages were 45cm x 28cm x 22cm, and they were manufactured by North Kent Plastic Cages Ltd., England. A total of four rats were placed in each cage.

DIET OF EXPERIMENTAL ANIMALS

All experimental animals were fed on Charlick's Feeds Mouse Ration, manufactured by Wm. Charlick Ltd., South Australia.

Contents:

Minimum Crude Protein	21.0%
Minimum Crude Fat	3.5%
Maximum Crude Fibre	6.0%
Minimum Vitamin A Content	10000.0 I.U./kg
Minimum Vitamin D Content	2000.0 I.U./kg
Maximum Added Salt	0.5%
Ethoxyquin	100 p.p.m.

Ingredients

Wheat, Barley, Bran, Pollard, Lucerne, Meat Meal, Soyabean Meal, Yeast, Cottonseed Meal, Fish Meal, Salt, Dicalcium Phosphate, Vitamins, Minerals and Antioxidant.

APPENDIX II

COMPOSITION OF THE CULTURE MEDIUM

Samples of the Teflon tubes containing Surgicel and Bone Wax, as well as samples of empty tubes, were cultured aerobically for 4 days in Brain Heart Infusion culture medium. It is manufactured by Oxoid Limited, England, and has the following formula:

(1)	Calf brain infusion solids	12.5gr
(2)	Beef heart infusion solids	5.0gr
(3)	Proteose peptone (Oxoid L46)	10.0gr
(4)	Sodium chloride	5.0gr
(5)	Dextrose	2.0gr
(6)	Disodium phosphate anhyd.	2.5gr
	pH 7.4	

Add 37gr to 1 litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121^{o} for 15 minutes.

APPENDIX III

INSTRUMENTS USED IN THE OPERATIVE PROCEDURE

(1) Gillies Tissue Forceps

(2) Metzembaum Scissors

(3) College Tweezers

(4) No. 3 Scalpel Handle

(5) No.15 Scalpel Blade

(6) Mayo Needle Holder

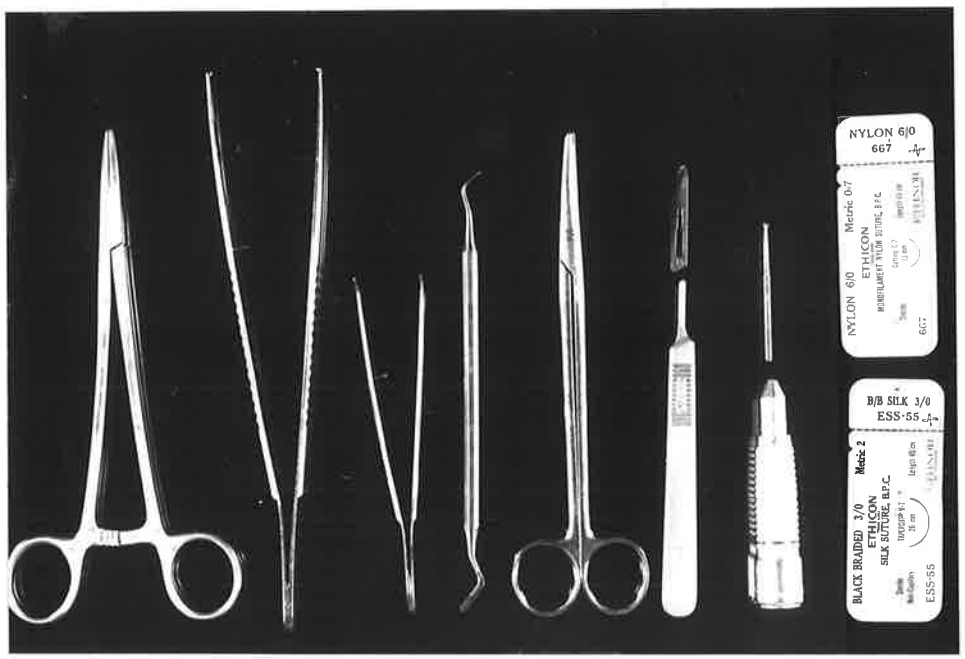
(7) Flat Plastic Instrument

(8) Straight Dental Handpiece

(9) No. 5 Round Dental Bur

(10) 6/0 Nylon Sutures

(11) 3/0 Black Silk Sutures



APPENDIX IV

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

(A) Tissue Fixation

All specimens were fixed in neutral buffered formalin.

Recipe:

Formalin	500mls
Tap Water	4500mls
Acid Sodium Phosphate Monohydrate	20gr
Anhydrous Disodium Phosphate	32gr

(B) Decalcification of Bone Specimens

All bone specimens were decalcified in Decal solution. They were placed in circular plastic containers with compartments, and placed in Decal solution. The solution was constantly stirred to prevent the accumulation of calcium around the specimens.

The femurs were radiographed three hours after placement into Decal, and, subsequently, at one-hourly intervals, to determine the endpoint of decalcification. After decalcification of the femurs, they were washed in tap water and replaced in jars containing neutral buffered formalin until they were ready for the embedding process.

(C) Embedding Procedure

All hard and soft tissue specimens went through the following reagents:

(1)	Alcohol 70%	Overnight
(2)	Absolute Alcohol	2 Hours
(3)	Absolute Alcohol	2 Hours
(4)	Absolute Alcohol	2 Hours
(5)	Absolute Alcohol	2 Hours
(6)	Methyl Salicylate and Absolute Alcohol (50:50)	Overnight
(7)	Methyl Salicylate	8 Hours
(8)	Methyl Salicylate	8 Hours
(9)	Methyl Salicylate and Celloidin 1%	2 Days
(10)	Methyl Salicylate and Wax (50:50)	1 Hour
(11)	Wax 100%	2 Hours
(12)	Wax 100%	2 Hours
(13)	Wax 100%	2 Hours

All specimens were placed in a vacuum chamber for 1 hour for the evacuation of air bubbles, and subsequently blocked in Paraplast + .

(D) Staining Procedures Used In The Present Investigation

The following staining procedures were used:

(i) Haematoxylin and Eosin Stain;

(ii) Mallory's Phosphotungstic Acid Haematoxylin Technique (PTAH),

(iii) van Gieson's Stain.

(iv) Safranin - Aniline Blue Stain.

(i) Haematoxylin and Eosin Stain

Method

(1) Xylol 2 to 5 minutes

(2) Xylol 2 to 5 minutes

(3) Absolute alcohol 2 to 5 minutes

(4) Absolute alcohol 2 to 5 minutes

(5) Dip in tap water

(6) Harris' Haematoxylin 3 to 5 minutes

(7) Tap water 5 minutes

(8) Differentiate in 0.5 to 1.0% hydrochloric acid in 70% alcohol for 30 seconds

(9) Immerse in running tap water for 10 minutes

(10) Eosin 45 seconds

(11) Absolute alcohol 1 minute

(12) Absolute alcohol 1 minute

(13) Xylol 1 minute

(14) Xylol 1 minute

(15) Mount in Depex

Results

Nuclei	: Blue to blue black
Karyosomes	: Dark blue
Cartilage	: Pink or light blue
Cement lines of bone	: Blue
Calcium and calcified bone	: Purplish blue
Basophil cytoplasm	: Purplish
Red blood cells, eosinophil granules, zymogen granules	: Bright orange red

(ii) Mallory's Phosphotungstic Acid Haematoxylin (PTAH) Stain

Method

(1) Xylol 2 to 5 minutes
(2) Xylol 2 to 5 minutes
(3) Absolute alcohol 2 to 5 minutes
(4) Absolute alcohol 2 to 5 minutes
(5) Sections to distilled water
(6) Mordant overnight in dichromate solution
(7) Rinse in distilled water; treat with Lugol's iodine for 5 minutes
(8) Decolourize in 5% hypo or 95% alcohol
(9) Wash in distilled water
(10) Oxidize in 0.25% potassium permanganate for 3 minutes
(11) Rinse in distilled water
(12) Decolourize in 5% oxalic acid for 5 minutes
(13) Wash in distilled water and then in tap water for 10 minutes
(14) Stain in a closed coplin jar of PTAH
(15) Differentiate in 95% alcohol, dehydrate and clear in xylene and mount.

Results

Nuclei, centrosomes, mitochondria, neuroglial fibres, muscle striations, fibrin	: Deep blue
Collagen, reticulin, bone, ground substance	: Red
Myelin	: Light blue

(iii) van Gieson's Stain

Method

(1) Xylol 2 to 5 minutes

(2) Xylol 2 to 5 minutes

(3) Absolute alcohol 2 to 5 minutes

(4) Absolute alcohol 2 to 5 minutes

(5) Sections to water

(6) Stain nuclei with Weigert's iron haematoxylin for 5 to 15 minutes

(7) Wash well in tap water followed by a rinse in distilled water

(8) Stain in van Gieson's solution for 2 to 5 minutes

(9) Rinse in distilled water

(10) Rinse in 95% alcohol

(11) Dehydrate in absolute alcohol, clear in xylene and mount.

Results

Nuclei	: Brown black to black
Collagen	: Deep Red
Muscle, cytoplasm, erythrocytes, fibrin	: Yellow

(iv) Safranin - Aniline Blue Stain

Solutions Required:

A: Safranin 1% Aqueous;

B: Aniline Blue 1% in Absolute Alcohol.

Method

(1) Xylol 2 to 5 minutes

(2) Xylol 2 to 5 minutes

(3) Absolute alcohol 2 to 5 minutes

(4) Absolute alcohol 2 to 5 minutes

(5) Dip in distilled water

(6) Stain for 15 minutes in the Safranin solution

(7) Wash in distilled water

(8) Rinse in 70%, then 90% alcohol

(9) Rinse in absolute alcohol

(10) Stain for 2 minutes in the Aniline Blue solution

(11) Rinse quickly, but thoroughly, with two changes of absolute alcohol

(12) Xylol 1 minute

(13) Xylol 1 minute

(14) Mount in Depex

Results

Cellulose : Blue

APPENDIX V

QUANTITATIVE ASSESSMENT OF TISSUE REACTIONS IN THE INTRAMUSCULAR IMPLANTS

(A) Surgicel and Control Implants

In the analysis of the Surgicel and control implants, the percentage ingrowth of tissues into the Teflon tubes was calculated using the following formula:

$$\frac{A_{\text{tissue ingrowth}}}{A_{\text{tube}}} \times 100$$

where A means 'area'.

The area of tissue ingrowth into the tubes, and the area of the inner aspect of the tubes (Figure 25), were determined by using a planimeter.

The aim of calculating the percentage ingrowth of tissue into the Surgicel-filled tubes and empty control tubes, at the various post-implantation time periods, was to determine whether a difference in the percentage ingrowth between the two groups existed, and, if so, whether this difference was statistically significant. In this way, it was possible to determine whether Surgicel retarded or expedited tissue reactions to its presence.

Three sections from the centre of the tube in each specimen* were analysed in this way. For each of the post-implantation time periods, twelve sections were analysed.

In order to illustrate the method of statistical analysis of the intramuscular Surgicel and control implants, data obtained from two week post-implantation specimens will be used.

The four specimens in each group (i.e. Surgicel and control) will be designated A,B,C and D. The average percentage ingrowth of tissue into the tube of each specimen (i.e., the figures obtained from three sections and divided by three) was as follows:

	Surgicel-filled tubes	Control tubes
Specimen A	65.11	58.97
B	43.57	60.01
C	48.70	45.78
D	35.79	49.20
Mean percentage ingrowth ($\bar{X}$) =	48.29	53.49
Standard deviation (SD) =	$\pm$ 12.40	$\pm$ 7.08
Variance =	153.87	50.13

* Specimens from the 12 and 26 week post-implantation time periods were excluded from the quantitative form of analysis since no reliable statistical data could be obtained. The reason for this was related to the smaller number of animals used.

The figures above were used to perform the Student's t-Test. The mathematical steps involved in the performance of this test were described by Swinscow(1980). In the example presented above, the Student's t-Test indicated that $P>0.50$, indicating that the difference between the tissue ingrowth into the Surgicel-filled tubes and the empty control tubes, at two weeks post-implantation, was not statistically significant (at the 5% level).

(B) Bone Wax Implants

The Poisson analysis was performed on all the intra-muscular Bone Wax implants. The essence of this form of analysis was discussed in detail in Chapter 3. However, the mathematical steps involved in the performance of the Poisson analysis will be presented here, and one of the two-week post-implantation specimens will be used to illustrate these steps.

The first step in the analysis involved the determination of the number of vacuolated (foamy) macrophages in a given area. For this purpose, an Olympus Eyepiece Micrometer was used in the eyepiece of an Olympus light microscope. The micrometer consisted of one hundred squares, and, at a magnification of four hundred times (400 X), the total area that the micrometer represented was 260 microns by 260 microns (Figure 95). Therefore, the area analysed corresponded to the area of the micrometer.

The total number of foamy macrophages that were present within the confines of the micrometer (i.e., within a hundred small squares shown in Figure 95), adjacent to the Bone Wax implant, was determined. Since three sections from the centre of the tube in each specimen were analysed in this way, the number of foamy macrophages occurring in three hundred small squares was calculated for each specimen.

The next step in the analysis involved the determination of the number of small squares containing no cells (foamy macrophages), 1 cell, 2 cells, 3 cells, etc. These were termed the 'Observed Frequencies' (O). In one of the two week post-implantation specimens (used as an example to illustrate the method of calculation), the following were the 'Observed Frequencies' (O):

Number of squares containing no cells	= 221
Number of squares containing 1 cell	= 55
Number of squares containing 2 cells	= 17
Number of squares containing 3 cells	= 7
Total number of squares	= 300

From the above, it can be seen that the total number of cells (foamy macrophages) occurring in 300 squares was 110.

The next step was to determine the mean number of cells per square. This was done as follows:

$$\text{Mean}(\bar{x}) = \frac{\text{Total number of cells} \quad (Tc)}{\text{Total number of squares} \quad (n)} = \frac{Tc}{300}$$

$$= \frac{110}{300}$$

$$= 0.37$$

In order to calculate the expected frequencies (E) of the number of squares containing no cells (Eo), the following formula is used:

$$Eo = n\left(\frac{1}{e^{\bar{x}}}\right)$$

where e is the base of the natural or Naperian logarithm, a constant whose value, accurate to 5 decimal places, is 2.71828. Thus:

$$Eo = n\left(\frac{1}{2.71828^{\bar{x}}}\right) \text{ or } Eo = n(2.71828^{-\bar{x}})$$

$$= 300(2.71828^{-\bar{x}})$$

The Biometrica Tables for Statisticians, edited by Pearson and Hartley (1966), provide in table 39, relative expected frequencies up to a mean of 15. Thus, the figure obtained for $2.71828^{-\bar{x}}$ where $\bar{x} = 0.37$ is readily available in these tables and has been found to be 0.6914694. Thus:

$$Eo = 300(0.6914694)$$

$$= 207.44$$

The observed frequency (O) of squares containing no cells was 221 and the expected frequency (Eo) was calculated to be 207.44.

Consequently, E_1(squares with 1 cell), E_2(squares with 2 cells), etc. are calculated as follows:

$$E_1 = Eo(\frac{\bar{x}}{1}) = 207.44 \quad (0.37) = 76.75$$

$$E_2 = E_1(\frac{\bar{x}}{2}) = 76.75 \quad (\frac{0.37}{2}) = 14.20$$

$$E_3 = E_2(\frac{\bar{x}}{3}) = 14.20 \quad (\frac{0.37}{3}) = 1.75$$

etc.

(Sokal and James Rohlf:1969)

The chi-square test (X^2-test) was then used to determine if there was a statistically significant difference between the observed frequencies (O) and the expected frequencies (E) as follows:

$$P = \epsilon(\frac{(O - E)^2}{E})$$

If the difference is statistically significant, the implication is that the cells are not distributed in a Poisson fashion and that there is a factor causing their deliberate aggregation or dispersion. In the example shown, $P < 0.001$, and, at the 5% level, the difference was statistically significant, implying that the cells were not distributed in a Poisson fashion.

Since, in the present example, the cells were not distributed in a Poisson fashion, the next step involved the determination of whether the cells (foamy macrophages) were dispersed or clumped. For this purpose, the Coefficient of Dispersion (C.D.) was calculated as follows:

$$C.D. = \frac{\text{Variance}}{\text{Mean}}$$

$$= \frac{0.487}{0.37} = 1.316$$

A C.D. greater than 1 implies that cells are clumped. In the example shown, the C.D. = 1.316. From the numerical Figures obtained on the number of foamy macrophages occurring in the small squares adjacent to the Bone Wax implant, it was evident that these cells aggregated as close as possible to the implant material.

****

Figure 95 : Schematic diagram illustrating the relationship of the Olympus Eyepiece Micrometer to the Bone Wax implant for cell counting. The total area represented by the micrometer, at a magnification of four hundred times (400 X), is 260 microns by 260 microns. The micrometer itself is made up of 100 small squares and the number of foamy macrophages occurring in each of these small squares was determined using an Olympus light microscope.

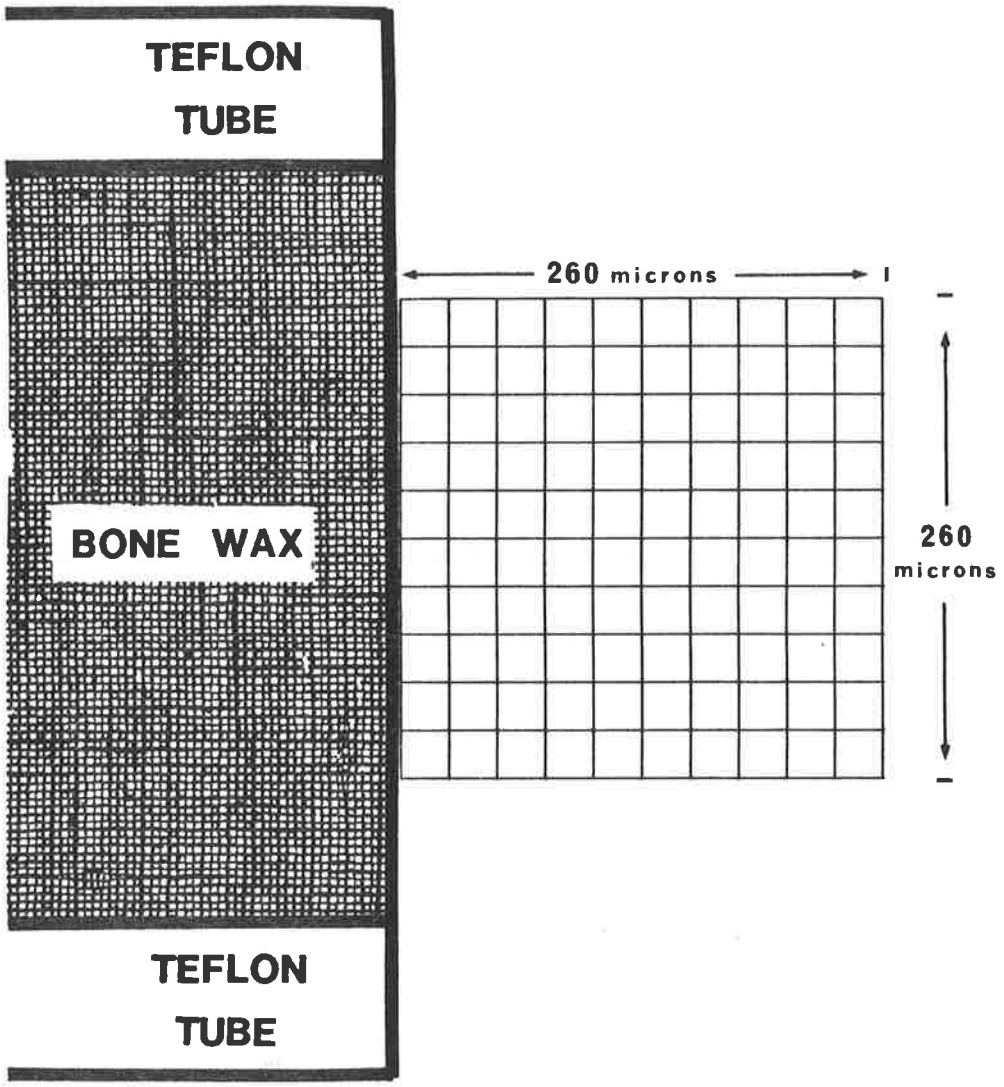


Figure 95

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