The Fate of Intraosseous Anorganic Bone Implants in the Calvaria of Rabbits

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THE FATE OF INTRAOSSEOUS ANORGANIC BONE IMPLANTS
IN THE CALVARIA OF RABBITS

by

John M. Sachs

A Thesis Submitted to the Faculty of the Graduate School of
Loyola University in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

June
1961
"If your own performance of a job looks perfect to you, it isn't because you've done a perfect job. It's only because you have imperfect standards!"

Anonymous
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For their love and encouragement, I am indebted to my mother, wife and daughter. The completion of this work will temporarily restore to them a son, husband and father.
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INTRODUCTION

Anorganic bone, bone from which the organic matrix has been extracted with ethylenediamine (NH₂ CH₂ CH₂ NH₂), has been in use for nearly a decade. There is abundant literature concerning the use of this material, yet little has been written concerning the fate of the anorganic implants.

Previous investigators have based their results on radiographic examination of implants and the histologic study of decalcified sections. Through the study of undecalcified sections, stained by the method of von Kossa, it is hoped that further information as to the fate of this material when implanted in bone will be gained.
REVIEW OF LITERATURE

The literature on bone grafting ranks among the most voluminous in medicine. Chase and Herndon (1955) include 855 references in their review. The works of Keith (1919), Ham (1934), Jacob et al (1959), and Woodruff (1960) made a major contribution in indexing the literature on transplantation and grafting.

The point of greatest controversy in bone grafting history is that of the osteoblastic capacity of periosteum. This controversy started with Duhamel (1741) and Ollier (1867) who diligently tried to prove that periosteum formed bone; and Goodsir (1845), Macewen (1912) and Gallie and Robertson (1918) who felt that the periosteum was merely a limiting membrane with no osteogenic qualities. Clark (1952) states, "This controversy still exists today, although there can be little doubt that periosteum in its proper environment has osteogenic properties". This is further elaborated by Weinman and Sicher (1955) who state, "...periosteum is not osteogenic if it does not already contain osteoblasts or pre-osteoblasts".

The fate of the bone graft has been described variously as dying in its entirety (Haldeman 1933 and Levander 1938), remaining viable in part (Abbott 1947), and being replaced by "creeping substitution" (Phemister 1914).
The theory that inducer substances can initiate the formation of bone in soft tissue led to the use of calcium salts in dogs by Murray (1930), and the injection of alcoholic extracts of bone into the soft tissues of rabbits by Levander (1938) and Lacroix (1951) in an effort to produce bone. These attempts met with some success, their results being repeated with alcohol alone by Annersten (1940).

Most of these experiments were carried out on rabbits, which can produce ectopic bone under a variety of nonspecific conditions, leading to confusing results (McLean and Urist 1955). Ray et al (1952) injected calcium phosphate apatite crystals resembling the inorganic crystals of bone into the anterior chamber of the eye of rats all of which became necrotic and were eventually sloughed. Cohen (1957) using radioactive isotopes showed that the calcium incorporated in the callus of a healing graft came by way of the circulating blood from skeletal sources situated throughout the body, rather than through diffusion of the graft.

The search for a universally tolerated and widely available grafting material has continued. Bone has been boiled (Groves 1917), frozen (Ollier 1867), freeze-dried (Cooksey 1954).
It has been stored in merthiolate (Kromer 1960) and preserved in plasma (Tucker 1953). Orell (1934) advocated the use of "os purum" and "os novum", and reported good results with these materials. However, it was not until 1954 that bone extracted with ethylenediamine (80%), which removes most of the organic matrix, was produced by Williams and Irvine. Losee and Hurley (1956) at the Naval Medical Research Institute in Bethesda modified the procedure of Williams and Irvine by using 95% ethylenediamine, and called the resulting product ANORGANIC bone.

Criteria for the usefulness of anorganic bone, set forth by Losee and Hurley (1956) are: "1. Lack of foreign body reaction; 2. Minimal callus formation; 3. Mineral bonding between donor and host bone; 4. Revascularization; and 5. Remodelling (perivascular absorption and accretion)."

Anorganic bone has been used primarily in the filling of bony defects, as its weight bearing qualities are poor. (Boyne 1956 and 1958, Boyne and Losee 1958, Boyne and Lyon 1959, Hayward et al 1958, Hurley et al 1959, Losee and Boyne 1957, Lyon et al 1959 and Ray et al 1957.) Woodruff (1960), described it as being useful where strength is not one of the requirements.

* Os purum is bone freed of fat and connective tissue by extraction with potassium hydroxide followed by extraction with ether. When os purum is implanted lateral to the tibia, the resulting new bone formation between the implant and the tibia is called os novum.
When implanted in soft tissues (Bell 1959 and Giannini 1961), anorganic bone becomes encapsulated by a fibrous connective tissue capsule. Bell (1960) showed that the anorganic implants are present radiographically sixteen (16) months after implantation.

Murray et al (1960) have shown their presence histologically up to three (3) years after implantation.
MATERIAL AND METHOD

This study is based on an investigation of implants of anorganic bone, autogenous bone and boiled beef bone into the calvaria of young, male, New Zealand White rabbits.

The animals were anesthetized with intravenous injection of 3% solution of pentobarbital sodium (Abbott), using one milliliter of solution per kilogram of body weight. As the amount of pentobarbital sodium was found to be extremely critical, only a heavy sedation was attained.* This was supplemented by the infiltration of one per cent (1%) procaine at the site of surgery. The head was shaved between and anterior to the ears, and the shaved area prepared with seventy per cent (70%) alcohol.

A four centimeter (4 cm.) incision was made in the midline over the frontal and parietal bones, and the skin reflected laterally. The periosteum was incised in the midline and reflected laterally with a periosteal elevator. Four plugs, two millimeters (2 mm.), were removed just lateral to the midline, using a trephine in the dental engine.

* Krause (1884) already called attention to the risk involved in anesthetizing rabbits with ether or chloroform as this frequently resulted in an early death.
The trephine was fabricated from a flame-shaped acrylic bur, which had the temper removed, a two millimeter (2 mm.) hole drilled into the center, and then retempered by heating and quenching in oil. Great care was exercised during the trephination in order not to perforate the dura mater.

After the trephination, the entire area was irrigated with liberal amounts of normal saline to remove bone debris formed during the procedure. Three of the defects received implants, the fourth remained empty to serve as a control. The following materials were implanted:

1. Anorganic bone (bovine) 40 mesh
2. Boiled beef bone, ground to resemble anorganic bone particles in size
3. Autogenous bone: one of the plugs removed by the trephine was replaced

The periosteum and skin were approximated and sutured with 000 silk suture. Following surgery all the animals were given a single dose (300,000 U.) procaine penicillin administered intra-muscularly.

The animals were sacrificed at the following intervals:

1. Twenty-four (24) hours post-operatively
2. Seventy-two (72) hours post-operatively
3. One (1) week post-operatively
4. One (1) month post-operatively

5. Three (3) months post-operatively

6. Six (6) months post-operatively.

The specimens were removed en bloc and immediately fixed in fresh ten percent (10%) neutral formalin. The undecalcified specimens were embedded in acrylic after Yaeger (1958) and Loe (1959) and sections were cut on the metallurgic microtome, R. Jung, model K; the most satisfactory results were obtained at eight (8) to ten (10) microns.

The sections were washed in acetone overnight to remove the acrylic, and then stained. Staining was done with hematoxylin and eosin for routine examination, von Kossa's silver stain for inorganic mineral salts, and periodic acid-Schiff reagent for mucopolysaccharides.

It might be well to state here that sections prepared in the above manner do not lend themselves well to the study of cellular detail.
FINDINGS

Healing of the surgical defect was uneventful in all cases. After one month it became increasingly difficult to locate the operative sites clinically. No distinction could be made clinically between the implant sites and the control defect.

Histologically, at twenty-four hours, the defects were filled with a blood clot that contained formed elements of the blood with no signs or organization. Sections of all implants stained positively (black to dark brown) for phosphates and (reddish-pink) for mucopolysaccharides. The implants of anorganic bone were surrounded by many dust like particles, rubbed off at the time of implantation. The autogenous bone and boiled beef bone did not show any such particles. However, grindings of the rabbit bone produced during the trephining procedure were found in all implant sites. The cellular detail was not clear, as was anticipated, due to the embedding procedure; but nuclei of osteocytes of the host bone could be discerned.

The seventy-two hour specimen showed organization of the clot. Few polymorphonuclear leukocytes were seen, mainly about the boiled beef bone implant. Staining with von Kossa's silver stain and periodic acid-Schiff reagent show nearly equal acceptance of stain by all the implants.
At one week proliferation of capillaries and differentiation of fibroblasts with the production of few delicate collagenous fibers in the blood clot were observed. In all sections there was some endosteal apposition of bone on the lamellae adjacent to the cut surfaces. Staining with special stains failed to reveal any significant differences from that of the twenty-four hour specimens.

One month post-operatively the surgically created defects were covered with periosteum. This had proliferated about the implanted particles which are now contained within a fibro-vascular network. Apposition of new bone at the cut edges of the defects was seen at this time. This was most conspicuous in the control defect, which prior to this time had maintained the sharp outlines created by the trephination. Staining with periodic acid-Schiff reagent showed all the implants to contain relatively equal amounts of PAS positive material.

Boiled beef bone implants at one month showed some evidence of resorption and an occasional osteoclast was seen. Inflammatory cells, polymorphonuclear leukocytes and lymphocytes, were present about the implant; but not elsewhere in significant numbers. A dense fibrous capsule surrounded these implants.
The implanted anorganic bone particles were surrounded by a dense fibrous capsule. Neither osteoblasts or osteoclasts were present in this area. The capsule surrounding the anorganic particles is distinguished by the density of its fibers, compared with the cellularity of the stroma surrounding the boiled beef implants. Staining with von Kossa's silver stain revealed some anorganic particles stained less intensely than previously. When compared with the densely stained black to brown host bone, these particles now appeared light brown to yellow.

At one month, the autogenous graft implant had become fused to the host bone by the apposition of new bone on the surface of the host bone and the implant.

At three months the density of the fibrous capsule had increased with a notable decrease in its vascular elements. Around the boiled beef bone implants a few polymorphonuclear leukocytes were still present. There was evidence of osteoclastic activity, and of osteoblastic apposition of new bone. Around the anorganic bone particles, there was continued maturation of the capsule, with no evidence of a foreign body reaction. Loss of mineralization of some of the implanted anorganic bone particles was evident by the yellow-brown stain in the von Kossa silver stained sections.
Six months post-operatively there was no clinical evidence of the previous surgery. Microscopic examination however clearly showed the areas of the implants, as well as the control defect. Apposition of bone upon the cut margins of all defects had occurred, but in no case had this completely obliterated the defect. Collagenization of the capsule surrounding the boiled beef bone and anorganic bone implant had continued, and the capsule now had a dense fibrous appearance. Only few of the anorganic bone particles showed a further reduction in mineralization when stained with von Kossa's silver stain. However, the cytoplasm of some connective tissue cells were seen only in the vicinity of the anorganic bone implants. Infrequently observed were single particles of anorganic bone bound by fibrous connective tissue to the host bone, but in only one instance anorganic bone was completely entrapped by new bone.

The site of implant of boiled beef bone, at six months, showed some evidence of chronic inflammation and some giant cells were seen around the implanted bone particles. Staining with von Kossa's technic demonstrated very little difference in intensity when compared with the twenty-four hour specimen.

At six months, sections of all the implants stained positively for mucopolysaccharides with the periodic acid-Schiff reagent.
DISCUSSION

The implantation of anorganic bone into the calvaria of rabbits offers an excellent opportunity for the study of these implants. The cutting of undecalcified sections from these implants, followed by staining for phosphates to which calcium is bound in the apatite crystals of bone presents a method for analyzing the demineralization of these implants by histologic means.

Resorption of bone is mediated by the osteoclast as first described by Koelliker (1873). Failure to demonstrate osteoclasts in the area of the implanted anorganic bone leads one to believe that the organic matrix of bone plays an important role in osteoclastic resorption. Although anorganic bone has some residual matrix, it is denatured by ethylenediamine in the extraction process, therefore, may resist resorption. The presence of von Kossa positive granules in the cytoplasm of histiocytes adjacent to the anorganic implants indicates that these cells may take an active part in the transport of calcified particles in the absence of osteoclasts. But since there were many non-phosphate positive cells, it is not likely that they play a significant role in the removal of particles of anorganic bone.
These findings are at some variance with those of the early investigators of anorganic bone implants. Williams and Irvine (1954), state that "Anorganic bone implanted in dogs is disposed of in a manner metabolically similar to that of fresh bone.....". No reference is made in their report as to how these conclusions were reached. Hurley in 1957, reported ".....remodelling of anorganic bone in a manner similar to that occurring with autogenous grafts", his results being based on radiographic studies. Losee and Hurley (1956) implanted anorganic rat femur and bovine femur into the tibias of dogs. Their results showed invasion of host tissue into the spaces formerly occupied by the organic matter; this process, they described as revascularization; they also report absorption of implants without the presence of osteoclasts. Their finding, that absorption of anorganic implants was followed by perivascular new bone formation was not corroborated by the present study.

Boyne and Losee (1957) in reporting their results of anorganic bovine bone implants in eighteen human subjects following oral surgery procedures state, "post-operative roentgenograms indicate a pattern of host acceptance similar to that found in animal experimentation (Boyne and Losee 1958). Boyne in 1956 stated that "Roentgenographic evidence indicated an early institution of osteogenic activity" following implants of anorganic bone in extravasation cysts of the mandible.
Bell (1959) states, "The success of a bone graft is dependent on rapid resorption of the graft material and the replacement with new host osseous tissue". His studies on the resorption characteristics of various implant materials (Bell 1959 and 1960) show anorganic bone to be the most slowly resorbed implants. Giannini (1961) in his study of subcutaneous anorganic bone implants also indicates that little resorption is taking place.

From this study it may be concluded that healing of the defects appeared to proceed in spite of the implants rather than because of it. Murray et al (1960) implanted anorganic bone into the calvaria of seventy-four (74) rats; of these, sixty-four (64) were found to be encapsulated in a dense fibrous capsule. Only ten (10) of the grafts were found attached to the host parietal bone. Ray and Holloway (1957) in an identical experiment concluded that "the presence of inorganic salts would appear to impede rather than accelerate the process of replacement". Hurley (1957) reporting the use of anorganic bone in the repair of cranial defects in dogs found total reconstitution of the grafts in one (1) year and showed the presence of marrow within the implants. He concluded that the porosity of this material, and the ease of revascularization, made it an ideal implant.

No osteogenic activity could be demonstrated near the anorganic bone implants in this study, these findings comparing favorably with the most recently
published reports of long term investigations of anorganic bone implants. Boyne and Lyon (1959) found no osteogenic activity around anorganic bone particles implanted into alveolar bone defects in man; particles found under the muco-periosteum were surrounded by a capsule consisting of fibrous connective tissue. Lyon et al (1959) also found residual, unresorbed anorganic bone particles surrounded by a fibrous connective tissue capsule one (1) year after implantation for the correction of maxillo-facial defects in Rhesus monkeys.

In all of the above studies, as well as this study, anorganic bone whether implanted in bone or soft tissue was accepted with a minimal inflammatory reaction, and no demonstrable foreign body reaction. Boiled beef implants cause an inflammatory reaction that persists until the implant is completely resorbed. In the six (6) months covered by this study resorption was far from complete. Boiling of bone denatures the organic matrix or otherwise so alters it that it resists osteoclastic resorption. Foreign body giant cells are a frequent finding.

Most rapid and uneventful healing takes place in the areas of the autogenous implants. The findings of this study are in complete accord with the literature on this point.
The healing of the untreated defect takes place by osteoblastic activity at the margins of the defect. No osteoblastic activity could be observed from either the dura mater or the pericranium. This finding differs from those of Berezowsky (1899) and Sirola (1960) who maintain that cranial defects in rabbits remain patent if the dura mater is removed, thereby ascribing osteogenetic properties to the dura. Kochiyama (1908) however, found that the repair of trephination defects in the calvaria of rabbits took place mostly from the diploe and only to a minor degree from the dura mater and the peristeum.
SUMMARY AND CONCLUSION

Anorganic bone, boiled beef bone and autogenous bone were implanted in trephination defects in the calvaria of rabbits. Histologic examination of undecalcified sections cut from acrylic embedded material were done at intervals from twenty-four (24) hours to six (6) months post-operatively. In addition to hematoxylin and eosin, these sections were stained with von Kossa's silver stain impregnation and periodic acid-Schiff reagent.

Within seventy-two (72) hours after implantation the anorganic implants are surrounded by a fibro-vascular network. As this capsule matures, the vascularity decreases and the fibrous elements become more densely arranged. Concurrently there is a decrease in the initial rate of demineralization of the anorganic bone implants, as seen in the sections stained with von Kossa's stain. At six (6) months, the anorganic bone particles are present in the defect with little change from their appearance at one month. At no time were either osteoblasts or osteoclasts visualized around the anorganic bone implants.

Anorganic bone implants seem to be rather inert, inducing neither differentiation of osteoclasts nor osteoblasts from the surrounding connective tissue of the host.
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Figure 1

Rabbit, showing trephination defects.
Figure 2

Rabbit, showing implants in place. From anterior: boiled beef bone, autogenous bone, control (empty) and anorganic bone.
Figure 3

Metallurgic microtome, R. Jung.
Boiled beef bone at 24 hours (von Kossa $\times 100$).
Figure 5

Anorganic bone at 24 hours (von Kossa) x100.
Figure 6

Boiled beef bone at 72 hours (H&E) x100.
Figure 7

Anorganic bone at 72 hours (H&E) x100.
Figure 8

Boiled beef bone at 1 week (von Kossa) x100.
Figure 9

Anorganic bone at 1 week (von Kossa) x100.
Figure 10

Boiled beef bone at 1 month (H&E) x 400, showing Howship lacunae like structures.
Figure 11

Autogenous bone at 1 month (H&E) x 100, at margin of host bone.
Figure 12

Anorganic bone at 1 month (von Kossa) x100.
Figure 13

Anorganic bone at 3 months (H&E) x100.
Figure 14

Anorganic bone at 3 months (von Kossa) x100.
Figure 15

Anorganic bone at 6 months (H&E) x100.
Figure 16

Anorganic bone at 6 months (von Kossa) x100.
APPROVAL SHEET

The thesis submitted by Dr. John M. Sachs has been read and approved by four members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

June 8, 1961

Date

Signature of Advisor