A method for evaluation of initial tissue response to biomaterials

A. WENNBERG, G. HASSELGREN & L. TRONSTAD

Department of Endodontics, School of Dentistry, University of Lund, Malmö, Sweden

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In the present paper an implantation technique is described whereby the effect of the surgical operation is eliminated and initial tissue reactions to materials may be studied. A teflon body was implanted intramuscularly in rabbits. After six weeks the overlaying tissue was excised and the implant removed. An intact, nonepithelialized tissue surface was exposed, which due to the shape of the implant showed three indentations. Materials were placed in the indentations for 15 minutes and the tissue reaction was registered by enzyme histochemical methods. Silicate cement, zinc phosphate cement and a 4% phenol solution caused an inhibition in the dehydrogenase enzyme activity in the tissue subjacent to the indentations. The severity of the tissue reaction, indicated by the width of the inhibition zone, varied among the test materials. Silicate cement caused the widest inhibition zone and the phenol solution the narrowest one. These results correlate well with previous tissue compatibility studies and indicate that the method is applicable for in vivo screening of initial tissue response to biomaterials.

Key-words: Material testing; implantation; enzyme histochemistry

Alf Wennberg, Department of Endodontics, School of Dentistry, S-214 21 Malmö, Sweden

Implantation tests in small animals have been widely used in evaluation of biocompatibility of dental materials (7, 16, 23, 31). However, a drawback with all implantation methods have been that the surgical operation required in order to place the test material in contact with the selected tissue, causes an inflammatory reaction in the tissue. This reaction persists for several days (9, 29), thus complicating an assessment of the initial tissue response to an implanted material.

In vitro evaluation of setting materials has revealed that some materials show a high initial toxic effect which then rapidly decreases after the setting is completed (10, 11, 28). These findings emphasize the need for an *in vivo* test method whereby the initial tissue response to a material may be evaluated.

It is known that inert, solid materials that are implanted into a tissue, in time will heal in and be surrounded by nonepithelialized and virtually noninflammatory connective tissue (3, 22, 27). If the healed-in implant is carefully removed, a connective tissue surface may be exposed that is undamaged by the surgical procedures and conceivably is suitable as test surface for assessing the initial tissue response to materials.

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Enzyme histochemical methods have supplied valuable data in the evaluation of tissue response to materials (14, 15, 18, 20).

Since changes in enzyme activity precede histologically observable changes, evaluation methods using enzyme histochemical technique should be particularly useful for assessment of early tissue damage.

The purpose of the present study was to develop a test method whereby the initial tissue response to a material may be determined. The desired test surface was obtained by implanting specially designed teflon bodies in the muscle tissue of rabbits. The tissue response was assessed by enzyme histochemical methods.

MATERIALS AND METHODS

Implantation procedures

Rabbits were anesthetized by intramuscular injection of a mixture of fentanyl and fluanison (Hypnorm Vet®, Leo, Helsingborg, Sweden), 0.75 ml/kg body weight. The exterior side of both thighs was depilated and washed with a solution of 5 per cent iodine in alcohol. An incision was made through the skin and the thigh muscles exposed. Using blunt dissection two pockets were made in the muscle tissues, one in the biceps muscle of thigh and one in the lateral great muscle. A teflon implant was then inserted into each pocket. It consisted of a semicylindrical base (10 x 25 mm) from which three slightly tapered rods (diameter 3 mm) protruded 4 mm. Before implantation the teflon body was degreased, cleaned, and autoclaved. Care was excercised to place the implant with the protruding rods facing the interior side of the muscle. The muscle tissues were sutured with catgut, and agraffes were used to close the skin incision. After surgery each rabbit received an intramuscular injection of 20000 IU/kg body weight of benzylpenicilliumprocain (Penicillinprokain Vet®, Novo, Bagsvaerd, Denmark). A total of 12 teflon bodies were implanted in three rabbits.

Test materials

Silicate cement (Super Syntrex®, de Trey, Zürich, Switzerland), zinc phosphate cement (Pharmacent®, Pharmacia, Uppsala, Sweden), and a solution of 4 per cent phenol, obtained by mixing crystalline phenol with glass distilled water, were used as the test materials. In the controls isotonic saline was substituted for the test materials.

Test procedures

Eight weeks after the implantation of the teflon bodies the thighs of the rabbits were again depilated. Twenty-four hours later the animals were anesthetized and the depilated areas thorougly washed with isotonic saline. An incision was made through the skin and the muscle tissue covering the implant was removed to expose the base of the teflon body. Bleeding was carefully controlled. Then the teflon body was gently lifted away, exposing a tissue surface with three indentations corresponding to the three protruding rods of the implant (Fig. 1). The tissue surface was flushed with isotonic saline, prewarmed to 37 °C, and the indentations dried with absorbent paper points. Freshly mixed, the test materials were placed into the indentations and left in contact with the tissue for 15 minutes. Each group of two implants (six indentations) included one control indentation. In addition, two control experiments were carried out where the tissue contact time was increased to 45 min. At the end of the tissue-material contact time a block of muscle tissue surrounding the three indentations was dissected out and immediately frozen in hexane cooled to -75 °C with solid CO_2.

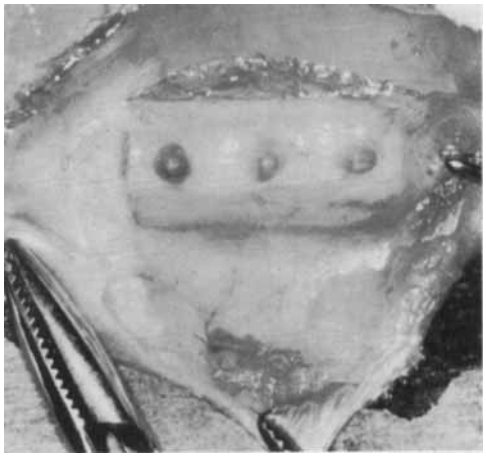

Fig. 1. Part of rabbit thigh muscle. A teflon body has been removed after an implantation period of 6 weeks. A nonepithelialized tissue surface has been exposed with 3 indentations suitable for placement of test materials. x 2.

Evaluation procedures

The frozen specimens were embedded in an aqueous solution of carboxymethylcellulose on a microtome stage and immersed in CO_2-cooled hexane. According to the method of Ullberg (30) 10–20 μm thick sections, stabilized with Scotch tape No. 688 (3M, St. Paul, USA) were taken through the three indentations of the specimens. Sectioning was carried out at $-20\,^{\circ}C$, and after freeze-drying for 48 hours, the sections were brought to room temperature under an air-tight hood to prevent moisture condensation.

Still attached to the tape, the sections were incubated for histochemical demonstration of succinate dehydrogenase (SDH, E.C. 1.3.99.1) and lactate dehydrogenase (LDH, E.C. 1.1.1.27) as markers of the citric acid (Krebs) cycle and glycolysis. The incubations were carried out according to the methods described by Barka and Anderson (1). After washing in distilled water, the sections were mounted in glycerin jelly and examined under the light microscope. As an indicator of the degree of tissue damage or tissue reaction the inhibition of enzyme activity was registered. In the tissue subjacent to each indentation the width of the inhibition zone was measured 40 times magnified.

For the purpose of control, some sections were heated to 100 °C in distilled water and incubated together with the other sections.

In two instances the excised tissue blocks were fixed in 10% neutral-buffered formalin, embedded in paraffin and serially sectioned at 5 μm. The sections were stained with hematoxylin and eosin.

RESULTS

Histological examination

A thin uniform layer of fibrous connective tissue had formed next to the implants. A few inflammatory cells were seen in this area. The subjacent muscular tissue was free of inflammatory cells (Fig. 2). No changes were seen in the areas of the tissue contacting the test substances or the isotonic saline.

Histochemical examination

The results are summarized in Table 1.

Staining for dehydrogenase activity was noted in all cells of the muscle and the connective tissue when the indentations had been filled with isotonic saline (Figs. 3, 4). When the indentations had been filled with silicate cement, zinc phosphate cement or the phenol solution there was a zone of inhibited dehydrogenase activity in the tissue contacting the test materials. Freshly prepared silicate cement caused a zone of inhibition that was wider than 1 mm in 6 of 7 tests (Figs. 5, 6). Freshly prepared zinc phosphate cement caused a zone of inhibited enzyme activity ranging from 0.5 to 1 mm in

Table 1. *Inhibition of enzyme activity after 15 minutes material-tissue contact time*

Test substances	No. of tests	Width of inhibition zone (mm)			
		0	0.5	< 0.5–1.0	> 1.0
Silicate cement.	7			1	6
Zinc phosphate cement.	7			7	
Phenol solution 4% . . .	7		5	2	
Saline	6	6			
Saline (45 min.)	2	2			

Fig. 2. Contact area between teflon body and tissue after an implantation period of 6 weeks. A thin layer of connective tissue with scattered inflammatory cells has formed adjacent to the implant. The subjacent muscle tissue is free of inflammatory cells. Htx-eosin. x 80.

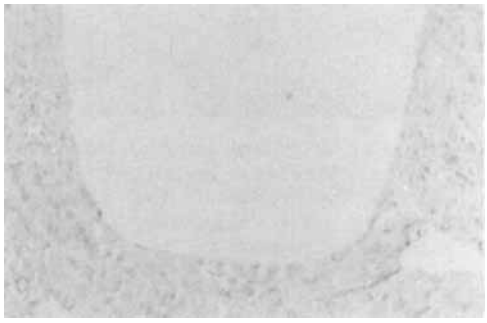

Fig. 3. Frozen section incubated for histochemical demonstration of succinate dehydrogenase activity. Indentation was filled with isotonic saline which was left in place for 45 minutes. Staining for enzyme activity is seen throughout the tissue. x 20.

all tests. The inhibition zone caused by the 4% phenol solution was less than 0.5 mm in 5 tests and 0.5 to 1 mm in 2 tests. In most instances the inhibition zone engaged both the layer of connective tissue and the subjacent muscle tissue. No differences were found in the inhibition pattern of the two enzymes tested.

The control incubations of sections pretreated with heat showed no staining.

DISCUSSION

The initial tissue response to biomaterials has been evaluated by methods utilizing a nonkeratinized, epithelialized tissue as test surface i.e. the conjunctiva of rabbits (8, 17, 21) or the oral mucosa of hamsters and dogs (2, 12, 13). Brånemark (2) used the oral mucosa in the hamster to evaluate local tissue effects of sodium fluoride. No microvascular disturbances were observed after application of the test substances onto an intact mucosa. However, when sodium fluoride was applied onto a deepithelialized mucosa, the microvascular damage, caused by the surgical procedure, was aggravated. Similar findings have been reported by Lindhe *et al.* (13). A chlorhexidine gluconate solution applied onto the dekeratinized oral mucosa of hamsters had no effect on the subjacent connective tissue, whereas applica-

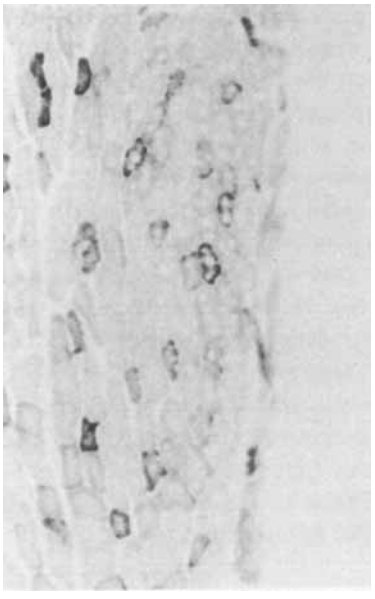

Fig. 4. Enlargement from Fig. 3 showing staining for enzyme activity in the tissue subjacent to the indentation surface. x 80.

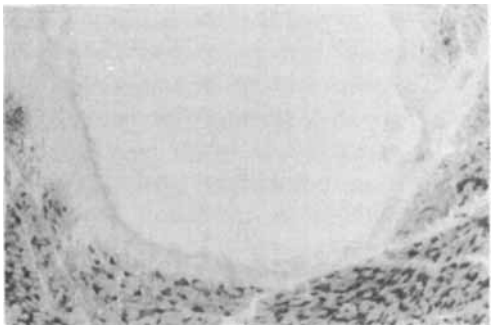

Fig. 5. Frozen section incubated for histochemical demonstration of succinate dehydrogenase activity. Indentation was filled with silicate cement and the tissue-material contact time was 15 min. In the tissue adjacent to the indentation a zone without staining for enzyme activity is seen. x 20.

Fig. 6. Enlargement from Fig. 5 showing the well defined zone without staining for enzyme activity in the tissue subjacent to the indentation surface. x 80.

tion onto a surgically exposed connective tissue produced vascular disturbances. These findings indicate that the initial tissue response to biomaterials by preference should be assessed using a nonepithelialized tissue surface. However, since removal of the epithelial layer per se damages the subjacent connective tissue (2) an intact, nonepithelialized connective tissue surface is preferable as the test surface.

As mentioned in the introduction, previous findings have established that a teflon body implanted into a tissue heals in without complications and that the surrounding tissue adapts well to the surface of the implant. It has also been shown that a thin layer of connective tissue forms adjacent to the material irrespective of whether the implantation has been carried out intraperitoneally (3), in bone (22), or in subcutaneous tissue (33). These findings were again confirmed in the present study. The teflon bodies were always surrounded by a thin layer of fibrous connective tissue. After removal of the implant, the exposed nonepithelialized tissue surface was influenced by the test substances, and the initial tissue response could be evaluated without interference of the surgical procedures. Due to the special shape of the implant, indentations had formed in the tissue. The purpose of the indentations was to facilitate the application of the test substances to the tissue.

In enzyme histochemical evaluation of tissue response to implants a number of enzyme systems have been studied (19). Changes in dehydrogenase activity appear to be a particularly valid criterion for evaluation of tissue damage, due to the involvement of the dehydrogenases in basal metabolic activities of the cell (14, 15, 20). It

has been observed that changes in succinate dehydrogenase activity correspond closely to changes in the activities of other dehydrogenases (19). These findings were corroborated in the present study where no differences were seen in the inhibition patterns of succinate dehydrogenase and lactate dehydrogenase activity. It seems to be sufficient, therefore, to work with only one of the dehydrogenases in routine material testing and succinate dehydrogenase appears to be the practical alternative because the presence of coenzymes is not required for its demonstration. This view has also recently been advanced by Salthouse (19).

In order to study the validity of the test method presently suggested, substances which have been thoroughly tested in previous *in vitro* and/or *in vivo* experiments were chosen as test materials (5, 6, 10, 24, 32).

As was expected, the histological evaluation did not reveal any sign of tissue damage after 15 minutes influence of the test substances. With regard to the histochemical evaluation, normal enzyme activity was found in all specimens influenced by isotonic saline. However, in the specimens influenced by the cements or the phenol solution the dehydrogenase activity was clearly affected after 15 minutes material-tissue contact time. This is in keeping with the findings reported by Rölling *et al.* (18) that inhibition of dehydrogenase activity in pulp tissue could be demonstrated as early as 5 minutes after application of formocresol.

The test substances influenced the enzyme activity of the tissues to varying degrees. Freshly prepared silicate cement caused a wider zone of inhibition than zinc phosphate cement, a finding which correlates well with observations from both *in vitro* and *in vivo* experiments (5, 6). The phenol solution caused an unexpectedly restricted tissue response in view of the results of earlier *in vitro* tests (24, 32). However, it may seem that the ability of phenol to coagulate proteins to a certain degree, impedes the diffusion of the drug into a tissue (4, 26).

It appears that, in conjunction with the present implantation method changes in dehydrogenase activity may be utilized as a valid criterion of initial tissue response to materials. Also, the method apparently permits a reasonable grading of the response. Liquids as well as freshly prepared, unset materials can be evaluated. Materials may also be introduced into a mold with the same size and form as the indentations, allowed to set, and then be transferred to the tissue indentations for testing at selected time intervals. Conceivably, the method may also be of value in the evaluation of tissue recovery after influence of irritants.

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