FINAL REPORT

Biocompatibility of four experimental formulations in subcutaneous connective tissue of rats.

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INTRODUCTION

The objective of this study was to determine by means of subcutaneous implantation, the biocompatibility of four experimental formulations. Although the materials are not intended to be in direct contact with the pulp, inadvertent pulp exposure or contact with intraoral soft tissues may occur. Therefore, initial subcutaneous implantation tests to determine their safety for use in humans was completed according to the ADA/ANSI Guidelines-Specification 41 and ISO 10991. The ANSI/ADA Guidelines-Specification 41 and ISO 10991 provide guidance with respect to recommended tests.

The test materials were denoted as follows:

Group 1. A control material, a zinc oxide and eugenol cement (ZOE)

Group 2. Pulpdent Resin Modified Glass Ionomer (RMGI) cement, self-curing

Group 3. Pulpdent RMGI light-cured (40s)


Group 5. Pulpdent Provisional cement self-cured.

1. IMPLANTATION TEST

See surgical methods

2. METHODS

For implantation tests, materials were placed into tissues appropriate for the evaluation of the biological safety of the material. The implant was not intended to be loaded mechanically or for testing its function.

After a period of time, the local effects on the tissue surrounding the implants were evaluated. These implantation tests were used to assess sub-chronic effects in the short-term, up to 90 days. Tissue responses were compared to the control material (ZOE Cement), which clinical acceptability has been established.

ISO 10993-1 & 2 documents specify test methods for the assessment of the local effects of an implant material on living tissue, at a macroscopic and microscopic level. These standards were used as references for this testing.

PREPARATION OF TEST MATERIAL SPECIMENS
Pulpdent provided the materials to be tested together with detailed instructions for handling and mixing.

Pulpdent delivered the experimental materials for testing in sterile condition or they were prepared under aseptic conditions.

PREPARATION OF CONTROL MATERIAL SPECIMENS
The control material was a ZOE cement, which was purchased commercially.

ANIMALS & TISSUES
Animal husbandry was in accordance with ISO 10993-2 and national regulatory requirements for laboratory animals. Male Wistar rats were selected for implantation into subcutaneous connective tissues, which had the appropriate size for the implant test specimens and the intended duration of the test in relation to the expected life span of the animals.

For this experiment, 36 rats were used. Post-operative time intervals were as follows:
- 12 rats – 10 days
- 12 rats – 30 days
- 12 rats – 90 days

For each time period and for each experimental material, three rats were used. The control material was implanted in the left side of the dorsal portion of the animal while an experimental material was implanted on the right side. The test and control materials were implanted under the same conditions in the same animal.

TEST PERIODS
The local tissue response to implanted materials was assessed in short-term tests of duration 10, 30 and 90 days.

3. SURGERY
72 autoclaved silicone tubes measuring 10 mm long with an outer diameter of 2 mm and a lumen of 1 mm were filled flush at both ends with the test materials. The samples for all Groups were prepared prior to implantation. The materials were mixed according to the instructions provided by the manufacturer and introduced into the lumen of the silicone tube at both ends. The openings were then covered with a mylar matrix strip. All materials of Groups 1, 2, 4 and 5 were allowed to bench set for 20 minutes, while in Group 3 a halogen light curing unit was used that had an output of at least 450 mw/cm² for 40s. Sample preparation and all implantation procedures were done under aseptic and sterile condition.

White male Wistar rats weighing approximately 200 g each were used for subcutaneous implantation. An incision in the dorsal area was made and widened with blunt dissection. The silicone tubes with the test materials were placed subcutaneously followed by suturing the incision. (See surgical procedures for more information). The animals were killed in groups of 12 each after 10, 30, and 90 days.

The implants together with their surrounding tissues were carefully dissected in blocks and fixed in 10% neutral buffered formalin. After fixation for 48 hours, the tissues were processed for paraffin embedding. The paraffin blocks were oriented parallel to the long axis of the tubes and then longitudinal serial sections approximately 7-µm thick were obtained from the implants. They were stained with hematoxylin and eosin and protected with cover slips and balsam. To estimate the tissue response in the areas adjacent to the end of the tubes three sections belonging to the
central areas of each specimen were analyzed at different magnifications under a light microscope equipped with an ocular micrometer. These sections were observed for the occurrence and thickness of a fibrous capsule, the vascular changes and the various types of inflammatory cells. The observer was blinded to treatment allocation. A four-category evaluation system was used for measuring and recording the microscopic observations. The tissue reactions were scored according to the following criteria:

0. **No reaction:** fibrous capsule formation and absence of inflammatory cells;
1. **mild reaction:** presence of a fibrous capsule formation with a few lymphocytes and plasmocytes;
2. **moderate reaction:** fibrous capsule formation with the presence of polymorphonuclear leukocytes, lymphocytes, plasmocytes and macrophages;
3. **Severe reaction:** Presence of large accumulations of polymorphonuclear leukocytes, lymphocytes, plasmocytes, macrophages, foreign-body giant cells and congested capillaries.

Data were statistically analyzed to determine significant differences between materials at each observation period.

The surgical procedures were as follows.

- The animals were anesthetized.
- The hair was removed from the surgical area by clipping and shaving.
- The area was washed with an antiseptic solution.
- The remaining hair did not come in contact with the implants or the wound surfaces.
- Since the surgical technique can profoundly influence the result of any implantation procedure, it was carried out under aseptic conditions and in a manner that minimized trauma at the implant site. The implants were placed as follows.
  - After incision and blunt dissection the subcutaneous connective tissue was exposed.
  - The area was rinsed with sterile saline
  - The test and control materials prepared according to the instructions provided by the manufacturer and placed in the autoclaved silicone tubes were introduced into the subcutaneous tissue.

- After surgery, the wound was closed using gut sutures, taking precautions to maintain aseptic conditions.

4. **EUTHANASIA**

At the termination of the experimental period, the animals were killed with an overdose of anesthetic (see ISO 10993-2).

**EVALUATION OF BIOLOGICAL RESPONSE**
The biological response was graded and documented by observing macroscopic and histopathological test responses as a function of each post-operative time period. The responses of the test materials were compared to the control.

Materials are acceptable for a usage test that cause no or slight reaction. Materials causing moderate reactions need further testing to establish it’s irritant component before usage tests. Materials causing severe and very severe reactions are unacceptable.

**Results:**

Macroscopic examination at the sites of implantation revealed that wound healing was satisfactory at all observation periods. Microscopic observation showed that all implants were surrounded by fibrous connective encapsulation of irregular thickness. It could be easily distinguished from the tissue reaction at the open end of the tubes in which the test materials were in direct contact with the tissues.

**10-day observation**

**Group 1.** The areas in contact with the test material showed a severe (grade 4) granulomatous tissue reaction with many polymorphonuclear neutrophils (PMN), lymphocytes, fibroblasts and newly formed vessels.

**In Groups 2 and 3,** a granulomatous (Grade 4) tissue reaction with inclusions of the tested materials was observed. Higher magnifications revealed that the materials into the tissues are mainly composed by many crystal-like structures.

**Groups 4 and 5** revealed similar features but some amount of the granulomatous tissue was detected inside of the tubes. In Group 5, a concentration of crystal-like structures surrounded by foreign-body giant cells was observed to be into the surrounding tissues.

**30-day observation**

**Group 1.** In direct contact with the test material there are areas of tissue necrosis with included particles of the material. Below, a severe granulomatous tissue reaction (Grade 4) containing many Polymorphonuclear (PMN) leucocytes, lymphocytes, plasmocytes and newly formed vessels was observed. Macrophages and foreign-body giant cells with engulfed particles of the material were also observed.

**Group 2.** At the end of the tubes, a well-localized fibrogranulomatous (Grade 3) tissue containing many randomly distributed crystal-like particles was observed. In direct contact with the test material, a juvenile fibrous capsule was present.
Group 3 and 4. Both groups showed similar features as Group 2 but also some localized accumulation of crystal-like particles within the fibrogranulomatous tissue. The mass of material was seen to be surrounded by a well-defined fibrous encapsulation.

Group 5. At the end of the tubes there were well defined (Grade 3) fibrogranulomatous tissues containing various accumulations of dark particles of the test material. Minor amounts of crystal-like particles were also observed and randomly distributed within the granulomatous tissue. These particles were surrounded by a well-defined dense fibrous encapsulation. In direct contact with the material a thicker fibrous capsule without inflammatory cells was seen.

90-day observation

Group 1. In contact with the test material there was a persistent granulomatous tissue slightly invaginated within the lumen of the tubes. Higher magnifications showed a mixed inflammatory cell population with PMN leucocytes, lymphocytes, plasmocytes, macrophages and newly formed vessels.

Group 2. In contact with the test material a persistent fibrogranulomatous tissue (Grade 3) was seen. In some areas a considerable concentration of inflammatory cells composed of PMN leucocytes, lymphocytes and fibroblasts was seen. Within these areas, many crystal-like particles were detected.

Group 3. A thick fibrogranulomatous capsule was seen in direct contact with the test material. Below, there are many concentrations of crystal-like particles surrounded by granulomatous tissues containing macrophages and multinucleated giant cells.

Group 4. Samples showed similar features as Group 3. Many of the crystal-like particles were clearly distinguished by using polarized light.

Group 5. This group showed similar features as was seen in the 30-day observation. Many particles of the material are surrounded by a dense and persistent fibrogranulomatous tissue, which appeared to be slightly invaginated within the lumen of the tubes.

Comments
All tested experimental materials showed similar adverse reactions as the control one (Group 1), which persisted after 90 days. Groups 2 to 5 showed many crystal-like or dark particles randomly distributed within the tissues, which contributed to a continuation of the foreign-body, as well as the inflammatory, reaction. Although all these materials were originally manufactured for restorative purposes and intended not to be in direct contact with the pulp, accidental pulp exposure can always occur in clinical operative dentistry and when using the above described materials tissue reactions has to be prevented by using adequate pulp capping materials.
REFERENCES:


