An animal study - underutilized vista of research in dentistry with special reference to biocompatibility of root canal sealer

Varsha S. Pandit, Sanjay Pandit[1], Nitin Shah[2]
Department of Conservative dentistry and Endodontics, Bharati Vidyapeeth Deemed University's Dental College and Hospital, [1]Department of Sharir Rachana (Anatomy) at Bharati Vidyapeeth Deemed University's College of Ayurved, [2]Department of Conservative dentistry and Endodontics, Bharati Vidyapeeth Deemed University's Dental College and Hospital, Pune, India

ABSTRACT

Background: Endodontic sealers are designed to be used only within the root canal but are frequently extruded through the apical constriction and often placed in intimate contact with periapical tissues for extended periods of time. Hence, assessment of biocompatibility of endodontic sealers is critical to the clinical success of endodontic therapy. **Materials and Methods:** Eighteen Wistar rats were divided into three groups of 6 each for observation after completion of 14, 30 and 90 days following implantation, respectively. Polyethylene tubes filled with new sealer, and tube without sealer [control] were implanted subcutaneously. The sample subcutaneous tissues from sacrificed rats were analyzed histologically for inflammatory response and were graded with FDI criteria as minimal, moderate and severe. Results were analyzed statistically with Student's t-test and ANOVA tests. **Results**: Inflammatory reaction to the polyethylene tube was minimal at 14 and 90 days period and to the new sealer it was severe at 14 days and moderate at 30 and 90 days period. **Conclusions:** 1. Cytotoxicity of the individual ingredient of the new sealer should be investigated to find out its chemical reaction occurring at tissue interface resulting in persistence of inflammation. 2. This subcutaneous implantation method is a practical method for qualitative evaluation of endodontic material and can yield exact detailed information about tissue reaction of material on a cellular level. 3. Hence, animal study is positive, efficient and valuable method to carry out research successfully in dentistry.

Key words: Animal study, biocompatibility, sealers, subcutaneous implantation

Access this article online
Website: www.jicdro.org
DOI: 10.4103/2231-0754.121862
Quick Response Code:

INTRODUCTION

Several studies have been conducted to assess the biocompatibility of sealers[1-4] which is essential for ensuring their good performance and success of endodontic treatment. To evaluate the biological response of new endodontic material introduced into the market, preliminary studies with *in vivo* experimental material, such as implanting these materials in the connective tissue of laboratory animals are commonly performed.[4]

It is now appreciated that the sealer has a primary role in sealing the canal.[5] A number of sealers have been formulated in the last several decades.[6] Amongst the characteristics of the sealers used in obturation portrayed by Grossman,[7] the most important is that it should be biocompatible i.e. non-irritating to periapical tissue.

Although, endodontic sealers are designed to be used only within the root canal, they are frequently extruded through the apical constriction [8] and often placed in intimate contact with periapical tissues for extended periods of time. (Thus, their biological compatibility is of special importance in clinical practice.) It is generally accepted that the biocompatibility of endodontic sealers is critical to the clinical success of endodontic therapy.[9]

The large variation in the toxicological and tissue-irritating properties of the materials studied by Brown and Friend, 1968; Spangberg, 1981,[10] seems to be not related with whether the tissue is irritated when it comes in contact with the sealer, but rather related with what degree and how long it is irritated

Address for correspondence:
Dr. Varsha S. Pandit,
Plot No. 13, Shri Hari Housing Society, Shridharnagar, Dhankawadi, Pune - 411 043, Maharashtra, India.
E-mail: drpanvar@yahoo.co.in

and hence, it is necessary to evaluate the biocompatibility of these materials for a stipulated period of time.

The methodology to evaluate the biocompatibility parameters comprises of initial tests, secondary tests and usage studies. Subcutaneous implantation of an endodontic material into the connective tissue of rats has been recommended for evaluation of the biocompatibility and the tissue reaction of the material.[11] Friend and Browne [12] concluded that the use of Teflon or polyethylene tubes filled with freshly mixed materials and implanted subcutaneously has greater resemblance to the clinical situation than any other methods.

Resin based sealers have steadily gained popularity. The search for a biocompatible root canal sealer is constant. We have taken a new resin based sealer which has been manufactured by a Dental Company, India and has not yet been marketed. This sealer has not undergone any type of biocompatibility tests, which is necessary before its clinical use.

The purpose of this study, hence, (?) is to evaluate the biological tissue response of the sealers and to highlight the role of animal studies in dentistry by assessing the biocompatibility of sealers using subcutaneous implantation method in rats.

MATERIALS AND METHODS

The protocol of this study was approved and permission was taken from the Ethics Committee of Bharati Vidyapeeth University Medical College, Pune, formed as per rules and regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (i.e. CPCSEA).

Eighteen Wistar rats weighing 150-200gm were divided into three groups of 6 each [Table 1]. In each animal, polyethylene tube was implanted subcutaneously at one side and the new sealer was implanted on the other side.

Sterilized polyethylene tubes of 10 mm length with 1.4 mm inner and 1.6 mm outer diameters were used. These tubes were heat sealed at one end and the opposite end kept open so as to simulate the root canal, were used. The new sealer was mixed according to the manufacturer's instructions and filled in the tubes. Material smeared outside the tube was wiped off with sterile gauze. Empty polyethylene tubes (EPT) were used as a control.

The rats were anesthetized [Figure 1] by an intra-peritoneal injection of Pentobarbitone sodium [30 mg per kg of body weight]. With aseptic precautions, an incision was made or incision was given [Figure 2] and two pre-prepared polyethylene tubes with the sealer or control tubes were implanted [Figure 3] in 15 mm long subcutaneous pockets [Figure 4]. These subcutaneous pockets were prepared at two different sites at the inter-scapular area. The two implantation sites were separated from each other by 20 mm to prevent interference from the other site.

The animals were sacrificed on termination of the experimental periods viz. 14, 30 and 90 days. The skin including the

Table 1: Groups for observation

Groups	Observation period	Weight	No. of Wistar rats
Group I	14 days	150-200 gm	6
Group II	30 days	150-200 gm	6
Group III	90 days	150-200 gm	6

Figure 1: Anesthesia-Intraperitoneal

Figure 2: Incision

subcutaneous tissues containing the implant was removed along with the surrounding tissue.

The specimen was fixed with 10% formalin. After fixing, tissue was processed for paraffin embedding. A paraffin block was oriented in such a way that it was parallel to the long axis of the tube and serial sections of 5 - 6 µm were obtained. These were then stained with hematoxylin and eosin stains.

The slides prepared were thoroughly examined by two senior pathologists under a light microscope, (Nikon 40X magnifications), to check the inflammatory reaction. This was a blind assessment without the observer knowing either the length of the observation period or the material tested. The inflammatory response was graded by observing necrosis, inflammatory cell response, vascularity, fibroblastic proliferation and epithelial proliferation (Based on F.D.I. Criteria).[9] Under 40X microscopic field, cell count was carried out on each section in ten grid fields by using an oculometer grid and results were expressed as average number of cells per grid field.

Tissue response scores were subjected to statistical analysis. To verify its significance, Student's t-test and ANOVA test were applied.

RESULTS

At the end of the14 day observation period, there was an infiltration of neutrophils, lymphocytes, few macrophages around the EPT. Scattered inflammatory cells were present along the side of the tube. New blood vessels and fibroblastic proliferation was observed which indicates formation of granulation tissue. The presence of few inflammatory cells, new blood vessels and fibroblastic proliferation indicates a mild inflammatory reaction [Table 2].

The presence of inflammatory cells i.e. neutrophils, eosinophils, lymphocytes, macrophages and foreign body

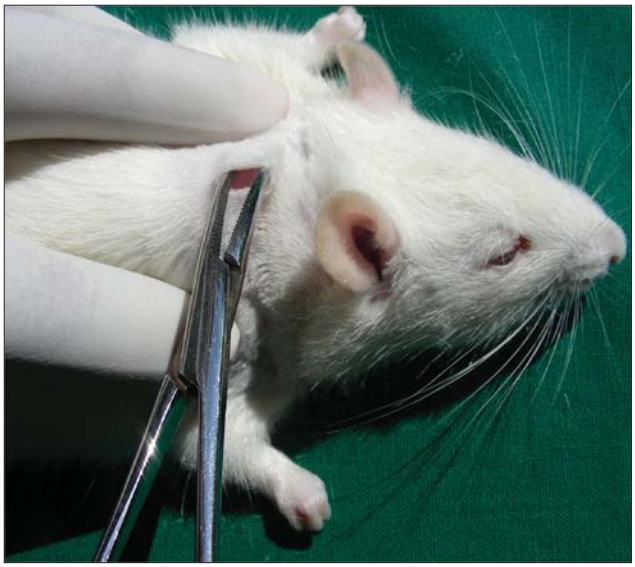

Figure 3: Pocket formation

Figure 4: Tube implantation

Table 2: The criteria for assessment of tissue response or reactions (Federation Dentaire Internationa Subcutaneous Implantation Test -assessment criteria)

	Mild	Moderate	Severe
2 weeks	The tissue is well organized and no more inflammatory reaction where tissue is exposed to the materials at the end of the tube.	Some inflammatory cells at the open end of the tube. The tissue adjacent to the test material has retained its structure but contains leukocytes [not in remarkable accumulation], lymphocytes, plasma cells, macrophages, occasional Foreign Body Giant Cells.	Distinct tissue reaction at the open end of the tube, fibrous un inflamed tissue along its midsection. The tissue at the open ends of the tube has lost its structure and contains an accumulation of neutrophilic leukocytes and lymphocytes
12 weeks	same as above	Some chronic inflammatory cells like lymphocytes, plasma cells, macrophages, occasional F.B.G. cells at the open end of the tube, with fibrous tissue along the mid section of the tube.	Severe tissue reaction at the open end of the tube with fibrous un inflamed tissue along its midsection. The tissue at the ends of the tube may regained some of its structure but contains some accumulation of – lymphocyte, plasma cells, macrophages, occasional foreign body giant cells [Chronic inflammation]

Note be – Continued presence of neutrophilic leukocyte indicates continued tissue disintegration caused by the material

giant cells were noted with the new sealer and the fibroblastic proliferation was not seen. Foreign body giant cells (F.B.G.) were observed with engulfed material.

On comparing the control and new group, the average number of neutrophils, eosinophils, lymphocytes and macrophages differs significantly ($P < 0.001$) at open end, as well as at the sides of the tube and were on higher sides in new sealer. F.B.G. cells were present only around the new sealer.

At the end of the 30 day observation period, the inflammatory reaction subsided in the EPT. Formation of a fibrous capsule had started and granulation tissue was becoming avascular. At the open end, some scattered lymphocytes and few macrophages were observed, but no foreign body giant cells were present.

In the new sealer group, moderate inflammatory reaction persisted. Formation of avascular granulation tissue was not seen and there was persistence of F. B. G. Cells (in lower case), but neutrophils were absent.

In comparison of the control and new sealer groups, the average number of lymphocytes and macrophages differ significantly and were more in the new sealer group at the open end and on the sides of the tube. Eosinophils and F.B.G. cells were present only around the new sealer.

After the 90 day observation period, healing was complete in EPT with formation of a fibrous capsule. But in the new sealer group, persistence of chronic inflammatory cells infiltration was noted. Macrophages and F. B. G. cells were seen along with few lymphocytes at the open end, as well as at the sides of the tube. The formation of avascular granulation tissue and fibrous capsule was not seen.

In comparison of control and new groups, the number of macrophages differs significantly ($P < 0.001$) at the open end and more so in the new sealer. The histopathological results are summarized in [Table 3].

DISCUSSION

The biocompatibility of a dental material is an important requirement because the toxic components present in the material could produce irritation or even degradation of surrounding tissues, especially when accidentally extruded into the periradicular tissues.[13] Hence, when a new material is introduced into the market its properties should be investigated. Most endodontic sealers are highly toxic when freshly prepared. Their irritating effect increases as the material-tissue contact surface area increases.[14] Several studies have evaluated sealer cytotoxicity using *in vitro* cell culture assays,[15,16] implantation into muscle and periradicular response.[17] *In vivo* tests are based on clinical and histological evaluation of tissue responses. The implant test in subcutaneous tissue as recommended by FDI [13] allows the testing of the material as it is utilized in the clinical setup. To assess biocompatibility by preliminary *in vivo* studies, the most commonly used test is the subcutaneous implantation of the material to be studied in small animals.[18] Among these animals, the rat is most frequently used because, in addition to being an experimental model that satisfactorily represents the body of a mammal, it has adequate dimensions to allow easier and safer management and a more accelerated metabolism when compared to other animals. This allows one to obtain relevant results in a short period of time.[18]

Table 3: Results at a glance

No.	Observation period	Control [EPT]	New sealer
1	14 days	Minimal	Severe
2	30 days	Minimal	Moderate
3	90 days	Complete healing	Moderate

The implantation of materials into the subcutaneous connective tissue of rats is considered a suitable secondary test for evaluation of biocompatibility properties of restorative and endodontic materials. This standard practice for biological evaluation of dental materials and their components are recommended before usage test.[12,13] This method allows for the standardization of the tissue/ material contact area providing the opportunity to compare the biocompatibility of freshly manipulated materials.[12]

In the present study, polyethylene tubes were used because of their suitability for maintaining the test materials in contact with the tissue in a controlled manner.[19,20] A small inner diameter of the tube was selected to minimize the flow of material out of the tube and yet allow loading of the sealer. The 10 mm of tube length was sufficiently long to have a control surface on the sides of the tube and the experimental surfaces of the sealer at the open end of tube.[8] The study was done over an observation period of 14, 30 and 90 days. The 14 and 30 day periods were necessary to observe the initial response of the sealers and the 90 day period showed the presence of ongoing inflammation or the resolution of inflammation.

In the present study, the inflammatory reaction was observed microscopically. In the present study, results were interpreted by preparing histological slides and grading was done based on F.D.I. Criteria[13] by counting neutrophils, lymphocytes, macrophages, foreign body giant cells, epithelial proliferation, vascularity and collagen fiber deposition.

The present study demonstrated quick healing around the implanted polyethylene tubes by thin fibrous capsules. The reaction was minimal at 14 days, as well as at 30 days and showed complete healing at 90 days. Absence of any

inflammatory reaction at 90 days confirms the findings of many previous studies that polyethylene tubes can be considered as a good model for animal studies. Torneck [21] has shown similar fibrous tissue repair with no lasting inflammation surrounding the polyethylene tubes.

The new sealer used in this study was aggressive on the subcutaneous tissue in the beginning. The inflammatory reaction, however, reduced by 30 and 90 days. A stronger action of the sealer in the beginning and attenuation of the inflammatory response over time have been reported in other studies.[12,22-25]

After14 days observation in the new sealer group, neutrophils, lymphocytes, macrophages, and F.B.G. cells were more at the open end as compared to the sides of the tube. The fibroblastic proliferation was not seen. Lymphocytic infiltration was highly significant as compared toempty polyethylene tubes.

At 30 days, inflammatory reaction was reduced and neutrophils were absent in both groups. The formation of avascular granulation tissue was not seen with the new sealer. This shows that the inflammatory response was reduced to moderate with respect to the new sealer.

At 90 days, neutrophils were absent and F.B.G. cells were present only in the new sealer. The persistence of chronic inflammatory cell infiltration was noted in the new sealer and the formation of avascular granulation tissue and fibrous capsule were not seen.

In our study, the foreign body giant cells were observed with the engulfed material inside the cells in 30 day and 90 day samples of the new sealer. Whereas, the foreign body response was maintained throughout the study period for new sealer. This indicates irritating components of the new sealer which should be analyzed and should be studied further to know the exact chemical reaction in the tissues.

CONCLUSIONS

1. Cytotoxicity of the individual ingredients of the new sealer should be investigated to find out its chemical reaction occurring at the tissue interface resulting in persistence of inflammation.
2. This subcutaneous implantation method is a practical method for qualitative evaluation of endodontic materials and can yield exact detailed information about tissue reaction of materials on a cellular level.
3. Hence, animal studies are positive, efficient and valuable methods to successfully carry out research in dentistry.

RECOMMENDATIONS

Methodological problems may occur during the procedure should be avoided carefully.

1. If the material sticks along the side of the tube it should be wiped off absolutely otherwise those tubes should not be used.
2. To overcome the problem of inability to achieve standardized material-tissue contact faced by some investigators due to use of both end open tube one should use only one end open tube by sealing the other end.
3. Do not remove tubes from the specimens despite the difficulty in sectioning of the tube. One should cut the tissue along with the tubes in specimens to avoid removal of the most important extratubal tissue interface.
4. To avoid deformation of the sections, the sections of specimens in the long axis of the tube should be made by using new and sharp microtome blades.
5. Sections that may get torn due to hardness of the material should be discarded.

ACKNOWLEDGMENT

1. The Department of Conservative Dentistry and Endodontics, Bharati Vidyapeeth University's Dental College and Hospital, Pune, India.
2. The Department of Pharmacology and Central Animal house, Bharati Vidyapeeth University's Medical College, Pune.
3. The Department of oral pathology and Microbiology, Bharati Vidyapeeth University's Dental College and Hospital, Pune.
4. Dr. Thakkar Paresh, Director of Prime Dental, India.
5. Dr. Deshmukh Sarojini, Professor and HOD, Department of oral pathology and Microbiology, Bharati Vidyapeeth University's Dental College and Hospital, Pune.
6. Dr. Indurkar S.V. Professor, Department of General pathology and Microbiology, Bharati Vidyapeeth University's Dental College and Hospital, Pune.

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Cite this article as: Pandit VS, Pandit S, Shah N. An animal study - underutilized vista of research in dentistry with special reference to biocompatibility of root canal sealer. J Int Clin Dent Res Organ 2011;3:58-63.

Source of Support: Nil. **Conflict of Interest:** None.