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SUBCUTANEOUS REACTIONS TO IMPLANTATION OF TUBES FILLED WITH AH PLUS AND NEW SEALER

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ABSTRACT

Introduction: *The purpose of this study was to evaluate and compare the subcutaneous reactions of New sealer with AH Plus by subcutaneous implantation in rats as a part of assessment of its biocompatibility.* ***Methods:*** *Twenty seven Wistar rats were divided into three groups of 9 each for observation after completion of 14, 30 and 90 days following implantation respectively. Polyethylene tubes filled with New sealer, AH Plus and tube without sealer (control) were implanted subcutaneously. The sample tissues from sacrificed rats were analyzed histologically.* ***Results:*** *Inflammatory response was graded with FDI criteria as minimal, moderate and severe. Results scrutinized with Student's 't' and ANOVA statistical tests. Inflammatory reaction to AH Plus was moderate at 14 days and minimal at 30 and 90 days, on the contrary, to New sealer it was severe at 14 days and moderate at 30 and 90 days.* ***Conclusions:*** *Inflammatory reaction to AH Plus, in the present study, was moderate at 14 days and minimal at 30 and 90 days. On the contrary, inflammatory reaction to New sealer was severe at 14 days and moderate at 30 and 90 days. The above observations suggests that AH Plus had better biocompatibility at 90 days observation period than New sealer.*

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[I] INTRODUCTION

Several studies have been conducted to assess the biocompatibility of sealers [1-4] essential for ensuring their good performance and success of endodontic treatment. To evaluate the biological response of new endodontic material introduced in to 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, 6]. A number of sealers have been formulated in the last several decades [7]. Amongst the characteristics of the sealers used in obturation portrayed by Grossman [8], 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 [9] and often placed in intimate contact with periapical tissues for extended periods of time. Thus, it is generally accepted that the biocompatibility of endodontic sealers is critical to the clinical success of endodontic therapy [10].

The large variation in the toxicological and tissue-irritating properties of the materials [11], 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 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 [12]. Friend and Browne [13] 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 e.g. AH Plus is a well established resin sealer. The search for a biocompatible root canal sealer is constant. We have taken new resin based sealer which has been manufactured by Prime Dental Company, India and has not yet been marketed. This

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sealer has not undergone any type of biocompatibility test, which is necessary before its clinical use.

The purpose of this study, hence, is to evaluate the biocompatibility of the New sealer and compare the biological tissue response of the newly developed resin sealer with well established resin sealer AH Plus and to gauge the efficacy and utility of the New sealer in the future.

[II] MATERIALS AND METHODS

Twenty Seven Wistar rats weighing 150-200gm were divided into three groups of 9 each.

Group I – 14 days observation period
Group II - 30 days observation period
Group III – 90 days observation period

In each animal two different materials were implanted at both sides.

Sterilized polyethylene tubes, 10mm in length with1.4mm inner and 1.6mm outer diameters, heat sealed at one end and the opposite end kept open so as to simulate the root canal were used. The New sealer and AH Plus, were mixed according to the manufacturer's instructions and filled in the tubes **[Table–1]**. Material smeared outside the tube was wiped off with the sterile gauze. Empty polyethylene tubes (EPT) were used as control.
The rats were anaesthetized by intra-peritoneal injection of Pento-barbitone sodium (30mg per kg of body weight). With aseptic precautions two pre-prepared polyethylene tubes with different sealers or control tubes were implanted in 15 mm long subcutaneous pockets prepared at two different sites at the inter-scapular area. The two sites of implantation were separated from each other by 20mm to prevent the interference of one sealer from the other [13].

The animals were sacrificed on termination of the experimental periods viz. 14, 30 and 90 days. The skin overlying the implant area was shaved and then the skin including subcutaneous tissue containing the implant was removed along with the surrounding tissue.

The specimen was fixed with 10% formalin and 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 haematoxylen and eosin.

The slides prepared were thoroughly examined by the two senior pathologists under a light microscope, (Nikon; 40 X), 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 **Table-2**) [14].

Under 40X microscopic field, cell count was carried out on each section in ten grid fields by using an occulometer 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.

Table: 1. Composition of sealers

No	Sealer	Composition	Manufacturer
1	AH Plus	Paste A - Epoxy resin, Calcium tungstate, ZrO_2, Aerosil, Iron oxide Paste B - Adamontone amine, N.N – dibenzyl, 5 – oxananedi amine, 1, 9 – TCD diamine, Calcium tungstate, Zirconium oxide, Aerosil , Silicone oil	Dentsply / Mailleffer, Okla., USA
2	New sealer	Paste A - Epoxy resin bisphenol A Paste B - Aminoethyl ethanolamine, Cocamine ethoxylated	Prime Dental, India

[III] RESULTS

At 14 day observation period, at EPT there was an infiltration of neutrophils, lymphocytes, few macrophages. New blood vessels and fibroblastic proliferation was observed which indicates formation of granulation tissue. This few inflammatory cells, presence of new blood vessels and fibroblastic proliferation indicates mild inflammatory reaction. The presence of inflammatory cells i.e. neutrophils, eosinophils, lymphocytes, macrophages and foreign body giant cells were noted with the New sealer and AH Plus. The fibroblastic proliferation was not seen. The foreign body giant cells (F.B.G.) were observed with engulfed material.

In comparison of Control and New sealer group average number of neutrophils, eosinophils, lymphocytes and macrophages differs significantly ($p < 0.001$) were on higher sides in New sealer. F.B.G.Cells present only in New sealer. In comparison of New sealer and AH Plus, AH Plus showed abundant granulation tissue and was not seen with New sealer. Highly significant number of cells with the New sealer as compared to AH Plus. Lymphocytic infiltration was more with the New sealer. Fibrous

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capsule formation was not seen with New sealer. All above showed a statistical significant difference ($p<0.001$) in neutrophils, lymphocytes and foreign body giant cells and were more in New sealer, these findings are suggestive of severe inflammatory reaction with the New sealer, on other hand, AH Plus showed moderate inflammatory response. **[Figure–1A and -B]**.

Table: 2.The criteria for assessment of tissue response or reactions (Federation Dentaire International 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 & 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.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-Continued presence of neutrophilic leukocyte indicates continued tissue disintegration caused by the material

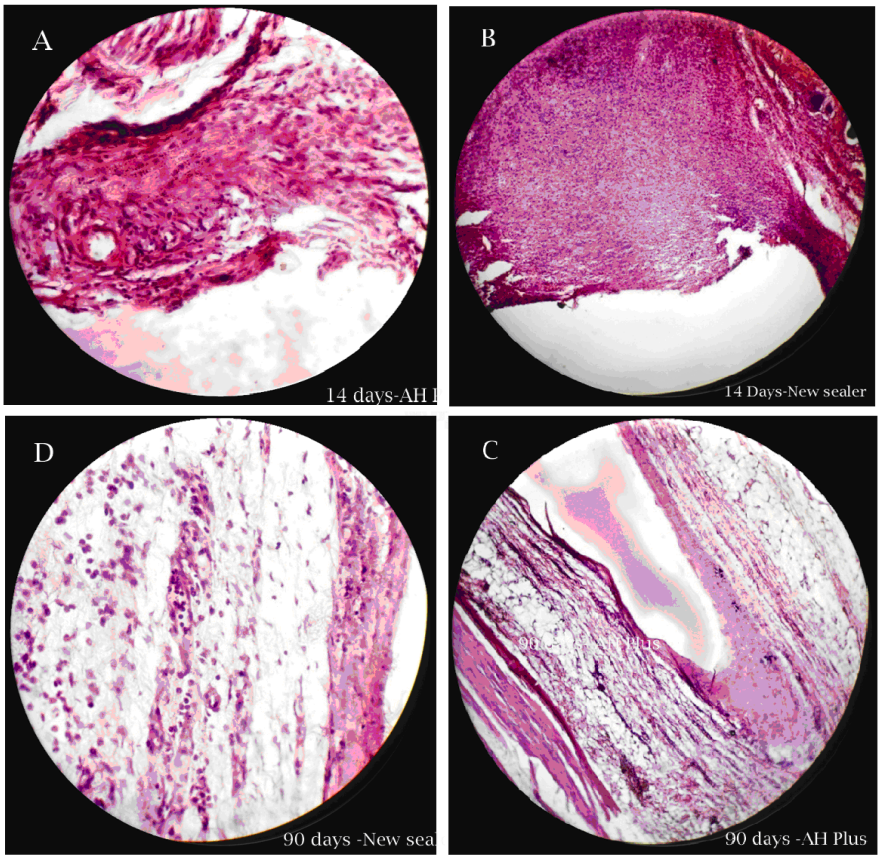

Fig: 1. A) AH Plus, 14 days - Moderate tissue reaction. **B)** New sealer, 14 days - Severe tissue reaction. **C)** AH Plus, 90 days – Mild tissue reaction. **D)** New sealer, 90 Days - Moderate tissue reaction.

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At 30 day observation period the inflammatory reaction was subsided in EPT. Formation of fibrous capsule had started, granulation tissue was becoming avascular. Inflammatory reaction was reduced and neutrophils were absent with both sealers. The AH Plus showed statistically significant difference ($p<0.001$) in cell count as compared to control for macrophages but not ($p>0.001$) for lymphocytes and foreign body giant cells. The New sealer showed a statistically significant difference ($p<0.001$) in cell count for macrophages and lymphocytes but not ($p>0.001$) for foreign body giant cells. The comparison between New and AH Plus sealer showed a statistical significant difference ($p<0.001$) in lymphocytes, macrophages which was more in New sealer.Formation of avascular granulation tissue was more in AH Plus and not seen with New sealer. This shows that inflammatory response was reduced to moderate in New sealer and minimal in AH Plus.

At 90 days observation period, neutrophils were absent in both the sealers. F.B.G.cells were present in New sealer but absent in AH Plus. The comparison between New and AH Plus showed statistical significance ($p<0.001$) for macrophages and lymphocytes which was more in New sealer. AH Plus revealed minimal inflammatory reaction with fibrous tissue formation and F.B.G.Cells were absent. The persistence of chronic inflammatory cell infiltration was noted in New sealer and the formation of avascular granulation tissue and fibrous capsule were not seen. The New sealer had statistically significant response for macrophages, lymphocytes and foreign body giant cells as compared to AH Plus. This indicated persistent irritation of the tissue by the New sealer **[Figure–1C and -D].** Results are summarized in **Table-3 and -4.**

[IV] DISCUSSION

Before introducing a new material in the market, it is fundamental that its properties must be tested. From a biological point of view, its biocompatibility must be evaluated because eventual toxic components present might cause irritation, degeneration, or even necrosis of the tissues adjacent to the material [15, 16]. 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 [18].

Table: 3. Results at a glance

NO. Observation Period		Control(EPT)	AH Plus	New sealer
1	14 days	Minimal	Moderate	Severe
2	30 days	Minimal	Minimal	Moderate
3	90 days	Complete healing	Minimal	Moderate

Table: 4. Histological tissue response, Differential cell count, 14 days, 30 days and 90 days after implantation

Cells	AH Plus			New sealer			Control		
	Day 14	Day 30	Day 90	Day 14	Day 30	Day 90	Day 14	Day 30	Day 90
Neutrophils	38.33±2.3	0.00±00	0.00±00	48.50±1.3	0.00±0.0	0.00±0.0	16.00±1.4	0.00±0.0	0.00±0.0
Eosinophils	11.67±2.3	0.00±0.0	0.00±0.0	13.00±1.8	6.17±1.3	3.33±0.6	9.33±0.9	0.00±0.0	0.00±0.0
Lymphocytes	20.67±1.7	24.17±3.4	13.33±2.3	29.50±0.9	33.33±1.3	16.83±1.7	13.50±0.9	23.67±1.7	10.33±0.7
Macrophages	6.83±0.6	7.17±0.6	4.33±1.1	8.00±0.8	10.67±1.3	7.50±0.7	4.50±0.5	5.67±0.7	1.83±0.8
F.B.G.Cs.	5.00±0.0	1.00±1.4	0.00±0.0	6.17±0.3	3.67±0.4	3.17±0.8	0.00±0.0	0.00±0.0	0.00±0.0

*The results of the cell counts were expressed as the mean value obtained from the total number of cells which were counted in all specimens of each material (±SD)

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To assess biocompatibility by preliminary 'in vivo' studies, most commonly used is subcutaneous implantation of the material to be studied in small animals [17]. Among these animals, the rats 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 animal, which allows one to obtain relevant results in a short period of time [27, 17].

Most endodontic sealers are highly toxic when freshly prepared. Their irritating effect increases as material-tissue contact surface area increases [19]. Several studies have evaluated sealer cytotoxicity using in vitro cell culture assays [20, 21], implantation into muscle and peri-radicular response [22]. In vivo tests are based on clinical and histological evaluation of tissue responses. The present study is confined to an 'in vivo' test for evaluating tissue reaction to AH Plus and New sealer by implanting the materials subcutaneously in Wistar rats, the effect of empty polyethylene tube was also studied and compared to the response produced by sealers. The implant test in subcutaneous tissue as recommended by FDI [14] allows the testing of the material as it is utilized in the clinical setup. The implantation of material into 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 is recommended before usage test [13, 14]. This method allows for the standardization of the tissue/ material contact area providing the opportunity to compare the biocompatibility of freshly manipulated materials [13].

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 [23, 24]. Friend and Browne [13] 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. 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 10mm of tube length was sufficiently long to have a control surface of side of the tube and the experimental surfaces of the sealer at the open end of tube [13]. 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.

Results were interpreted by preparing histological slides and grading was done, based on F.D.I. Criteria [14], by counting neutrophils, lymphocytes, macrophages, foreign body giant cells and epithelial proliferation, vascularity and collagen fiber deposition.It 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 [25] has shown similar fibrous tissue repair with no lasting inflammation surrounding the polyethylene tubes.

Microscopically, the inflammatory reaction was observed in AH plus and New sealer. These two sealers were aggressive on the subcutaneous tissue in the beginning. However, though the difference in inflammatory reaction between both the sealers is significant, inflammatory reaction was reduced by 30 and 90 days. Similar responses have been reported in previous studies [26, 13, 27, 22, 28].

The statistical analysis showed the comparison between control and New sealer at 14 days which appeared to be statistically significant for neutrophillic, eosinophilic, lymphocytic and macrophagic response. The New sealer continued to irritate the tissue and hence formation of avascular granulation tissue or fibroblastic collagen synthesis resulting into fibrous capsule formation was not seen, which was observed in the control. This response was less with AH Plus. Comparing control with New material at 30 days, showed statistically significant difference in lymphocyte, macrophage response. The New sealer also showed statistically significant difference, with the presence of foreign body giant cells, whereas this response was not significant with AH Plus as compared to control. At 90 days, only the New sealer had statistically significant response for lymphocytic infiltration and presence of macrophages and foreign body giant cells. The New sealer containing amino ethyl ethanolamine showed lymphocytic infiltration at open end as well as sides of the tube, this response when statistically compared was more in New sealer as compared to AH Plus. The macrophagic response was marked with New sealer as compared with AH Plus.

The foreign body giant cells were observed with the engulfed sealer inside the cells in 30 days and 90 days samples of the New sealer. This indicates persistent irritation of the tissues by the sealer. But on the other hand these cells were seen only in 14 days sample of AH Plus suggestive of gradual decrease in inflammatory reaction. The initial inflammatory reaction may be due to epoxy resin content of the New sealer and AH Plus as well, since, many studies found that several composite resins liberate formaldehyde in amounts sufficient to cause local allergic reaction [17]. This foreign body response was maintained throughout the study period for New sealer unlike AH Plus, suggestive of irritating components in the New sealer, and AH Plus may release formaldehyde from its components in decreasing amounts in aged specimens [29, 30] therefore reducing the inflammatory reaction in later period. The New sealer contains cocamine which is a derivative of coca shrub (Eruthroxylon coca), it has got a local tissue reaction in the form

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of vasoconstriction. This vasoconstriction may be the cause of tissue necrosis in an inflamed tissue, resulting in the exacerbation of inflammation due to New sealer [31]. It also has been demonstrated in one study that water diffusion leads to erosion of composite resin material causing release of unreacted monomers [32]. Hence, the exact cause of persistence of inflammatory reaction due to the New sealer should be investigated, analyzed by further studies to know the exact chemical reaction in the tissues.

In brief, inflammatory reaction to AH Plus, in the present study, was moderate at 14 days and minimal at 30 and 90 days **[Table–3]**. The above observations suggests that AH Plus had better biocompatibility at 90 days observation period than New sealer.

[V] CONCLUSIONS

1) Poor biocompatibility of New sealer was established
2) Severe irritation at 14 days and moderate at 30 and 90 days by New sealer as compared to AH Plus sealer.
3) 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.

CONFLICT OF INTERESTS

Authors declare no conflict of interests

FINANCIAL DISCLOSURE

The work was carried out without any financial support

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3) The histological analysis was accomplished in the Department of oral pathology and Microbiology, Bharati Vidyapeeth University's Dental College and Hospital, Pune.
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