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COMPARATIVE HISTOLOGICAL AND IMMUNO-HISTOCHEMICAL ANALYSIS OF THREE ROOT CANAL SEALERS: AN IN VIVO STUDY

Khaled A. Behery *, Alaa A. El-Baz **, Sara Mohamed Ali*** *and* Ali Youssef Elgendy ****

ABSTRACT

Aim: To evaluate the biocompatibility of GuttaFlow Bioseal and CeraSeal compared with AH Plus by histological and immunohistochemical analysis.

Materials and methods: Twenty-four male Wistar albino rats were randomly assigned to three groups according to the evaluation periods, each group contained 8 rats, group (A): for evaluation on day 7, group (B): for evaluation on day 14 and group (C): for evaluation on day 30. Each rat received 3 implants of polyethylene tubes containing the tested sealers and kept in place for 7,14 and 30 days. The polyethylene tubes were excised with the surrounding tissues to be prepared for histological evaluation by hematoxylin and eosin stain to confirm the inflammatory response. Also, immunohistochemical analysis was performed by assessment of the prevalence of immunopositivity of Interleukin 6 (IL-6) and assessment of the immunostaining intensity. All the obtained data from the computer image analysis were statistically evaluated. The ANOVA (analysis of variance) test was used to compare the mean values between different groups. Student t-test was used to compare mean % values between 2 groups to evaluate the significance (if present) between all groups.

Results: Regarding the biocompatibility evaluation after 7 days of implantation, AH Plus showed a severe inflammatory response histologically along with elevated immune-expression of IL-6, followed by CeraSeal and then GuttaFlow Bioseal. Inflammation decreased in the 14 and 30-day time intervals in all tested groups.

Conclusion: CeraSeal is the most biocompatible endodontic sealer in comparison with GuttaFlow Bioseal and AH Plus.

KEYWORDS: GuttaFlow Bioseal; CeraSeal; AH Plus; Inflammatory; Immunostaining.

* MSc student, Faculty of Dentistry, October University for Modern Sciences and Arts.
** Professor of Endodontics, Faculty of Dentistry, Cairo University.
*** Lecturer of Oral Pathology, Faculty of Dentistry, October University for Modern Sciences and Arts.
**** Lecturer of Endodontics, Faculty of Dentistry, October University for Modern Sciences and Arts.

INTRODUCTION

Endodontic treatment is a sequential series of treatments including microbial control by removing the infected pulp tissues and shaping the root canals, followed by filling the empty pulp space. Root canal sealers offer several essential functions including anti-microbial action, acting as a lubricant to facilitate obturation and finally providing adhesiveness to improve the sealing and stability of the root canal filling [1]. They are classified according to their composition into zinc-oxide-eugenol sealers, calcium hydroxide sealers, glass ionomer sealers, resin-based sealers, silicon-based sealers and calcium silicate-based sealers. The availability of bio-ceramic-based sealers in the field of endodontics has been limited to the past three decades, coinciding with the growing use of bio-ceramic technology in the medical and dental fields [2].

CeraSeal (Meta Biomed Co., Cheongju, Korea) is a calcium silicate-based bio-ceramic root canal sealer characterized by anti-microbial activity, unique stability and high pH level [3, 4]. CeraSeal consists of tricalcium silicates, dicalcium silicates, calcium aluminates, zirconium oxides, and thickening agents [5]. Another recently introduced bio-ceramic silicone-based sealer is GuttaFlow Bioseal (Coltène/Whaledent AG, Altstatten, Switzerland) which is a reliable cold-filling system for root canals containing gutta-percha and sealer in a single application. GuttaFlow Bioseal differs from other GuttaFlow sealers by incorporating bioactive glass, comprising silica, calcium oxide, sodium oxide, and phosphorus oxide [6]. Furthermore, it has exceptional flow characteristics, very low solubility, and good radiopacity [7]. AH Plus (Dentsply, DeTrey, Konstanz, Germany) is the epoxy resin sealer that has undergone the most extensive research and evaluation in the literature [8]. It is widely regarded as the benchmark for comparison and is commonly employed as a control sealer in research [9].

Biocompatibility is regarded as a crucial feature of root canal sealers, among other desirable biological properties [8, 10]. Biocompatibility of materials pertains to their ability to fulfil their intended purpose without inducing hazardous or detrimental effects on biological systems while eliciting a suitable reaction from the host [11]. During endodontic therapy, the materials utilized for filling the root canal have the possibility to come in contact with the periapical tissue. These materials should ideally aid in the healing process [8, 10].

Various techniques have been employed to assess the biocompatibility of endodontic sealers. An effective and commonly employed technique involves the implantation of the material into the subcutaneous connective tissue of rats. The irritative impact of the materials can be assessed through histological analysis of the tissue reaction surrounding the implants by using hematoxylin and eosin stain [12].

Another approach for assessing the biocompatibility of a material is the immunohistochemical analysis. Immunohistochemistry (IHC) is a widely used technique for selectively detecting and identifying proteins (antigens) in cells of a tissue slice. Overall, the immunohistochemical analysis provides a comprehensive perspective that aids in comprehending the data obtained from other methodologies [13].

Therefore, this study aimed to evaluate the biocompatibility of GuttaFlow Bioseal and CeraSeal compared with AH Plus by histological and immunohistochemical analysis. The null hypothesis stated that there would be no significant differences between the three tested endodontic sealers (CeraSeal, GuttaFlow Bioseal and AH Plus) regarding their biocompatibility with the surrounding tissues.

MATERIALS AND METHODS

Ethical Approval:

The research ethical committee (Faculty of Dentistry - MSA University) approved the method employed in this study and the research was granted confirmation of conductance number (ETH37).

Sample Size Calculation:

The sample size was determined based on the results of (**Alves Silva et al 2020**) [14]. An effect size that indicates the difference between bio-ceramic sealer versus AH Plus sealer regarding inflammatory cell count at 7 days was found to be 1.87. Using independent t-test and assuming an effect size of 1.87, a minimum sample of 18 rats (6 for each of the three study groups) was required to detect a significant difference in the same outcome and the sample size was increased to 24 rats (8 rats for each group). The type I error probability associated with this test was set at 0.05 and power was set at 0.8. The sample size was calculated using the G-Power software version 3.1.9.2.

Selection of animal model:

All procedures followed the standards of national institutes of health as outlined in the guide for the care and use of laboratory animals [15] and were carried out under strict aseptic conditions. Twenty-four male Wistar albino rats of an average age of 2-3 months and an average weight of 150-200 grams were selected for this study. Animals were kept in plastic cages in a climate-controlled room and fed with ad libitum in addition to water. The room's photoperiod (12 hours of light/dark), temperature (23°C±2°C), and humidity (55%±10%) were all standardized [16].

Classification of samples:

The twenty-four rats were randomly assigned to 3 groups according to the evaluation periods and each group contained 8 rats as follows:

1. Group (A): for evaluation on day 7 (first period).
2. Group (B): for evaluation on day 14 (second period).
3. Group (C): for evaluation on day 30 (third period).

Subcutaneous implants:

Animals were anesthetized using ether intraperitoneally [16]. Each animal's back was shaved and surgical sites were disinfected. Three incisions were made with a #15 Bard-Parker blade in upper right and left arm areas and lower right limb area [17].

The implantation technique was carried out under aseptic conditions with the use of sterile polyethylene tubes. These tubes had an internal diameter of 1.5 mm, an external diameter of 2.2 mm, and a length of 10.0 mm. AH Plus, CeraSeal and GuttaFlow Bioseal were prepared according to the manufacturer's instructions and inserted into the polyethylene tubes.

A subcutaneous pouch with a mean depth of 20 mm was bluntly dissected, to accommodate the implants. Each rat received three implants, one near the upper right arm which had the AH Plus sealer, another near the upper left arm which contained the CeraSeal sealer, the last one near the lower right limb which contained the GuttaFlow Bioseal sealer, and these implants were kept in place for 7,14 and 30 days. Wound sites were cleaned with 10% iodine solution and sutured. On day 7 (first period), day 14 (second period), and day 30 (third period), eight rats were sacrificed using an anesthetic overdose of ether [17].

Histological evaluation of inflammation:

Sections of 4μm thickness were cut from paraffin-embedded specimens and stained with hematoxylin and eosin for histological evaluation to confirm the inflammatory response. A light microscope (Leica ICC50 HD Microscope, Leica Microsystems,

Switzerland Ltd) was used to examine these sections qualitatively and quantitatively at magnifications of x100 and x200. A scoring system was used to confirm and evaluate the inflammatory response. Scores were defined as follows: 0: none or few inflammatory cells, no reaction; 1: <25 cells, mild reaction; 2: 25 to 125 cells, moderate reaction; 3: ≥125 cells, severe reaction. The fibrous capsule was categorized as "thin" when the thickness was < 150 μm and "thick" at > 150 μm [18].

Immunohistochemical evaluation of the inflammatory effect:

Paraffin-embedded tissue sections of 4 microns thick were cut from groups A, B, and C. These tissue sections were mounted on positively charged slides to be immune-stained with supersensitive biotin–streptavidin staining technique. Tissue sections were deparaffinized, rehydrated and treated with 3% H_2O_2 for 10 minutes to block the endogenous peroxidase activity. For antigen retrieval, the slides were boiled in 10 mm citrate buffer, pH 6.0 in an autoclave where its temperature was adjusted to 120°C and maintained stable for 15 minutes followed by cooling at room temperature for 30 minutes.

Background staining was blocked by putting 2–3 drops of 10% goat non-immune serum blocker on each slide and incubating them in a humidity chamber for ten minutes. Without washing, excess serum was drained from each slide. The slides were incubated with the primary antibody (Interlukin-6 antibody IL-6 (E-4), Santa Cruz Biotechnology, USA) for 30 minutes at room temperature in a humified chamber. After washing with phosphate buffer solution (PBS), the slides were treated with the biotin-labeled link antibody; then, the streptavidin conjugated to horseradish peroxidase was used. The diaminobenzidine (DAB) chromogen was applied to visualize the antigen-antibody reaction. All the slides were immersed in Mayer's hematoxylin for counterstaining. Finally, the sections were covered by coverslips using an aqueous mounting medium. Then, all the sections were examined by an image analyzer computer system using the software Leica ICC50 HD Microscope (Leica Microsystems, Switzerland Ltd).

The results were evaluated semi-quantitatively according to the percentage of positive cells in five randomly selected fields under a high-power microscope (200-fold magnification) for each sample. The immunohistochemically stained sections were examined using:

A) Ordinary light microscope to assess the prevalence of immunopositivity of Interleukin 6 (IL-6).

B) Image analyzer computer system to assess the intensity of immunostaining. The image analysis was performed using a computer system (Leica Qwin processing and analysis software, version V3.5.1) consisting of color video camera, color monitor, and CPU of IBM personal computer connected to the microscope. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units.

The intensity of the reactions within the stroma was measured by the optical density in 10 small measuring fields in each specimen using a magnification of 200. After grey calibration, the image was transformed into a grey delineated image to choose the areas exhibiting positive reactivity with accumulation of all grades of reactivity (i.e., minimum, maximum and median grey). Areas of positivity were masked by blue, red and green binary colors and mean values were obtained.

Statistical Analysis

All the obtained data from the computer image analysis were statistically evaluated. This data represented the difference in mean inflammatory cell count, the difference in mean capsule thickness, the value of interleukin-6 immuno-expression and immunostaining intensity. They were given as mean values ± SD (standard deviation).

The ANOVA (analysis of variance) test was used to compare the mean values between different groups. Student t-test was used to compare mean % values between 2 groups to evaluate the significance (if present) between all groups. The p-values were considered as $p > 0.05$ = not significant (NS) and $p \leq 0.05$ = significant.

RESULTS

1. Histological evaluation of inflammation:

Control group (AH Plus sealer):

After 7 days, the AH Plus control group showed a severe inflammatory response with the presence of neutrophils, macrophages, lymphocytes and a few eosinophils with a thin fibrous capsule **(Figures 1 a and 1 b)**. At 14 days, the AH Plus control group showed moderate inflammatory reaction with a more organized thin fibrous capsule **(Figures 1 c and 1 d)**. At 30 days, histological examination of the AH Plus control group also showed moderate inflammatory reaction with an organized thin fibrous capsule **(Figures 1 e and 1 f)**.

CeraSeal group:

At 7 days, a severe inflammatory infiltrate was detected in the form of macrophages, neutrophils and lymphocytes. Dilated blood vessels were prominent along with a thin fibrous capsule formation **(Figures 2 a and 2 b)**. At 14 days, the inflammatory reaction was moderate with decreased inflammatory and vascular reaction, and the fibrous capsule was thin **(Figures 2 c and 2 d)**. At 30 days, the inflammatory reaction was mild with an organized connective tissue and fibrous capsule **(Figures 2 e and 2 f)**.

GuttaFlow Bioseal group:

At 7 days, histological examination revealed severe inflammatory infiltrate in the form of macrophages, neutrophils and lymphocytes, with a well-formed fibrous capsule **(Figures 3 a and 3 b)**. At 14 days, the inflammatory reaction was moderate with decreased inflammatory and vascular reaction **(Figures 3 c and 3 d)**. At 30 days, the inflammatory reaction was moderate with decreased inflammatory and vascular reaction **(Figures 3 e and 3 f)**.

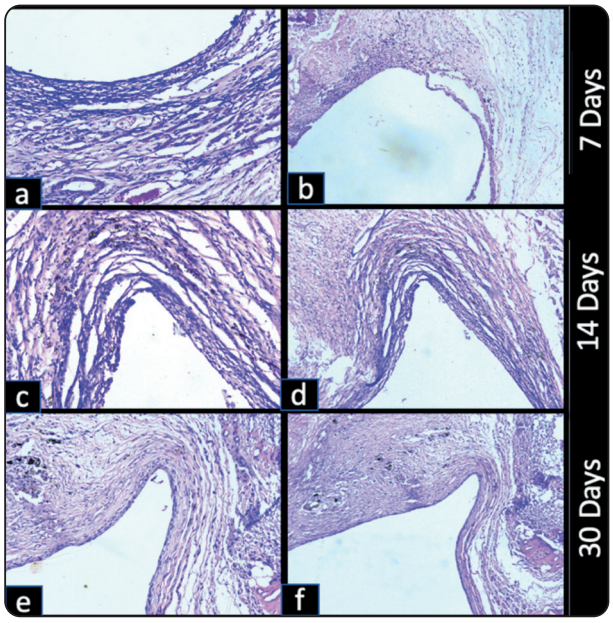

Fig. (1 a-f): Photomicrographs of AH Plus group at 7, 14 and 30 days at x200 magnification showing inflammatory cell infiltrate (a, c and e) and at x100 magnification (b, d and f) showing the fibrous capsule around the tube site (H&E section).

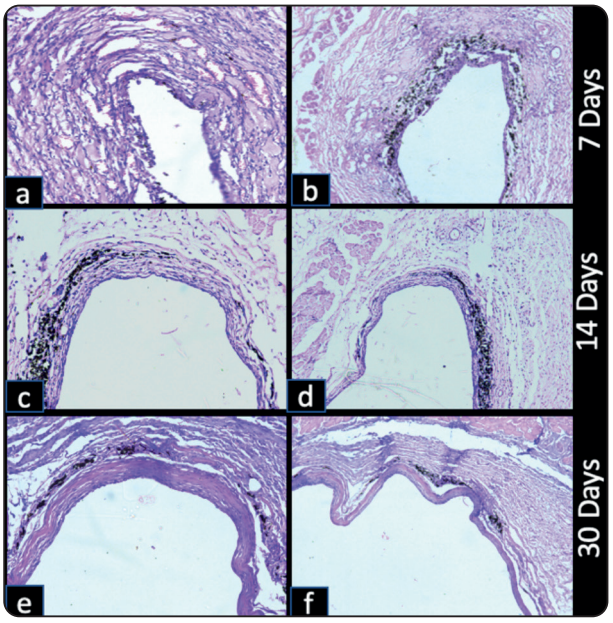

Fig. (2 a-f): Photomicrographs of CeraSeal group at 7, 14 and 30 days at x200 magnification showing inflammatory cell infiltrate (a, c and e) and at x100 magnification (b, d and f) showing the fibrous capsule around the tube site (H&E section).

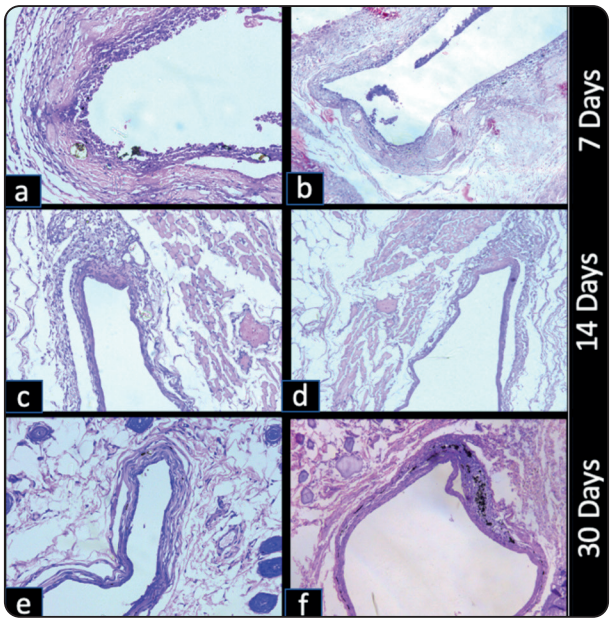

Fig. (3 a-f): Photomicrographs of GuttaFlow Bioseal group at 7, 14 and 30 days at x200 magnification showing inflammatory cell infiltrate (a, c and e) and at x100 magnification (b, d and f) showing the fibrous capsule around the tube site (H&E section).

ANOVA statistical test showed that there was no significant difference in the mean inflammatory cell count between different groups after 7 or 14 days with ($p>0.05$). A comparison of the mean inflammatory cell counts after 30 days between each two groups using paired student t-test, revealed a significant difference between AH Plus (66.3± 50.5) and CeraSeal (16.3± 9.1), with ($p<0.05$). No significant difference was found in the mean inflammatory cell count between AH Plus (66.3± 50.5) and GuttaFlow Bioseal (70.1± 27.8), with ($p>0.05$). A significant difference was found in the mean inflammatory cell count between CeraSeal (16.3± 9.1) and GuttaFlow Bioseal (70.1± 27.8), with ($p<0.05$) **(Table 1) (Figure 4)**.

TABLE (1) Difference in mean inflammatory cell count between different groups after 30 days using Paired Student's t-test.

Group	Mean inflammatory cell count		
	M±SD	t-Value	p-Value
AH Plus	66.3± 50.5	2.420	0. 016*
CeraSeal	16.3± 9.1		
AH Plus	66.3± 50.5	0.162	0. 436
GuttaFlow Bioseal	70.1± 27.8		
CeraSeal	16.3± 9.1	4.474	0. .0004*
GuttaFlow Bioseal	70.1± 27.8		

M: ***Mean inflammatory cell count***
SD: ***Standard deviation***
**** p ≤ 0.05 = significant.***

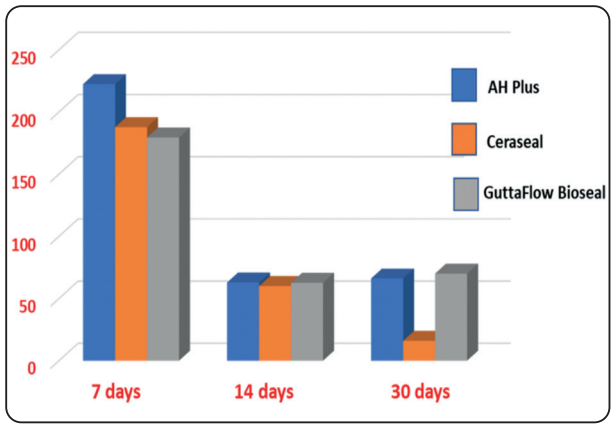

Fig. (4) A bar chart representing the mean inflammatory cell count between different groups among different periods.

IL-6 Immunostaining intensity (Optical Density)

The expression of IL-6 in the subcutaneous tissue is shown in **(Figure 5 a-i)**. IL-6 was found mostly clustered around the tube periphery site. The AH Plus control group and GuttaFlow Bioseal group had greater IL-6 expression compared to the CeraSeal group in 14 and 30-day time intervals. Regarding CeraSeal group, IL-6 expression peak was at the 7-day interval, which clearly decreased till the 30-day interval unlike the AH Plus control group and GuttaFlow Bioseal group, which showed no decrease in the immunostaining.

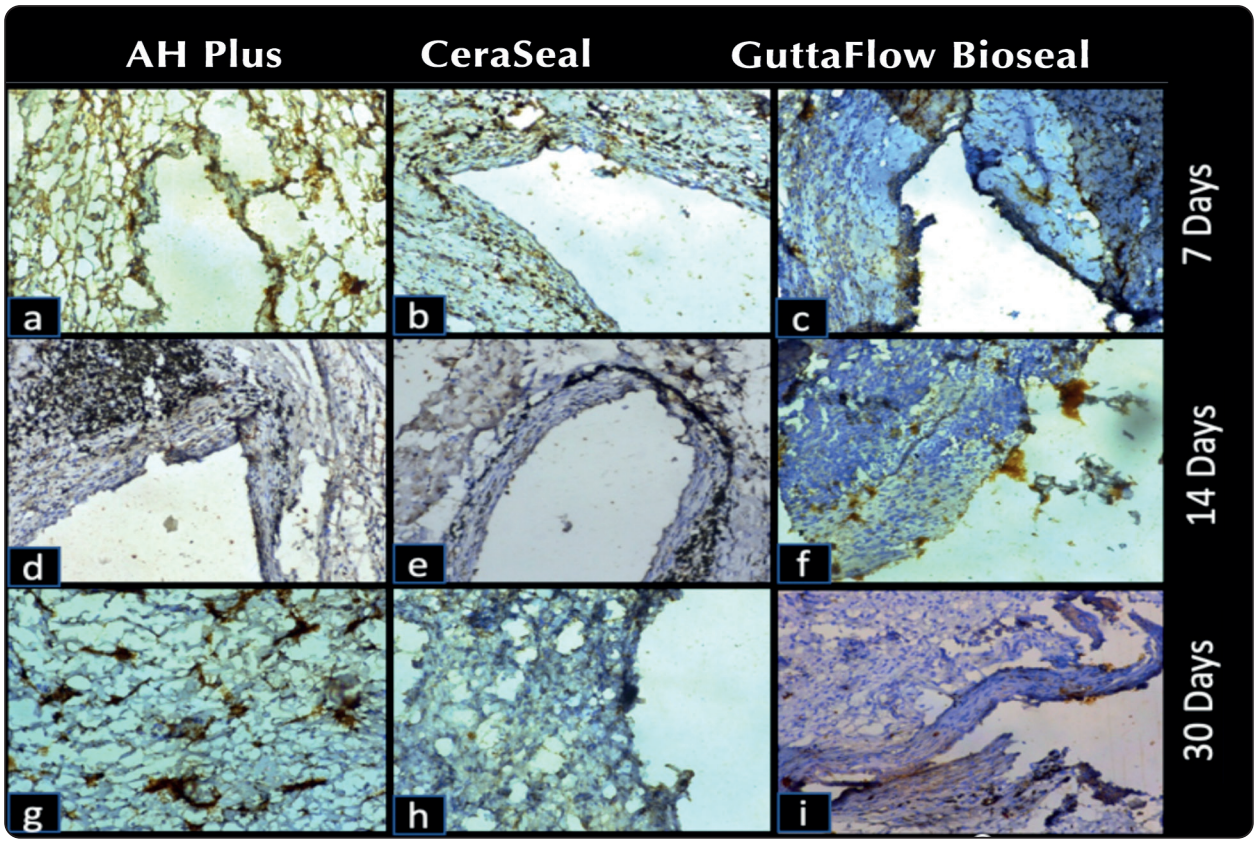

Fig. (5 a-i): Photomicrographs showing immunohistochemical stained sections for IL-6 in the subcutaneous tissues after 7, 14 and 30 days in different groups. IL-6 expression in AH Plus (a, d and g), CeraSeal (b, e and h) and GuttaFlow Bioseal (c, f and i) in x200 magnification.

ANOVA statistical test showed that the mean optical density of IL-6 between different groups after 7 and 14 days showed no significant difference ($p>0.05$). The mean optical density of IL-6 showed a significant decrease in immunostaining between different groups after 30 days ($p<0.05$), with CeraSeal showing the least immunostaining followed by GuttaFlow Bioseal, then AH Plus group (control group), as shown in **(Figure 6)**.

A comparison of IL-6 immunostaining intensity after 30 days between each two groups using paired student t-test, revealed a significant difference in mean optical density of IL-6 immunostaining between AH Plus (90.6± 3.6) and CeraSeal (51.2± 1.1), with ($p<0.05$). A significant difference in the mean optical density of IL-6 immunostaining between AH Plus (90.6± 3.6) and GuttaFlow Bioseal (74.8± 10.4), with ($p<0.05$). A significant difference in the mean optical density of IL-6 immunostaining between the CeraSeal group (51.2± 1.1) and the GuttaFlow Bioseal group (74.8± 10.4), with ($p<0.05$) **(Table 2)**.

TABLE (2) Difference in mean optical density of IL-6 between AH Plus and CeraSeal after 30 days using Paired Student's t-test.

Group	Mean optical density of IL-6		
	M±SD	t-Value	p-Value
AH Plus	90.6± 3.6	23.032	0.0001*
CeraSeal	51.2± 1.1		
AH Plus	90.6± 3.6	3.203	0.0125*
GuttaFlow Bioseal	74.8± 10.4		
CeraSeal	51.2± 1.1	5.032	0.0010*
GuttaFlow Bioseal	74.8± 10.4		

M: Mean inflammatory cell count
SD: Standard deviation
**** p ≤ 0.05 = significant.***

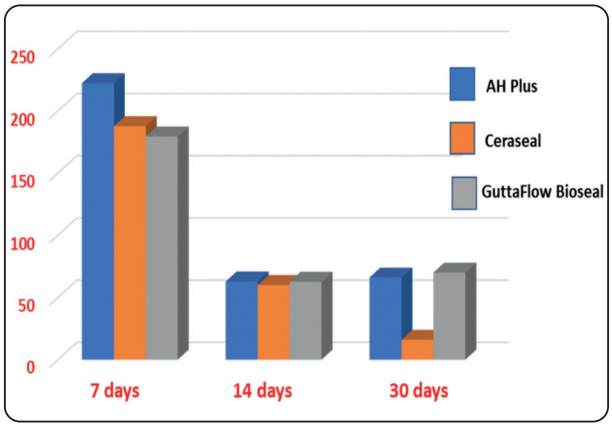

Fig. (6) A bar chart representing the mean optical density of IL-6 between different groups among different periods.

DISCUSSION

For a wide range of clinical applications, there is currently a vast selection of root canal filling materials in the market. Endodontic sealers with biocompatibility, sufficient physiochemical qualities, bioactivity, and antibacterial activity are considered to be suitable [19]. Although there are widely available different categories of endodontic sealers, none of them match all of these criteria [20]. The majority of endodontic sealers are known to be toxic or may demonstrate varying degrees of cytotoxicity on tissues, which would cause sluggish wound healing, and inflammatory reactions [21].

A material's biocompatibility is its ability to carry out particular tasks when inserted into a living tissue without causing harm to this tissue [11, 22]. Dental materials must be biocompatible to avoid damaging the surrounding tissues, especially when toxic components are unintentionally extruded into the periradicular tissues. Nearly all endodontic sealers are toxic when they are first mixed; as a result, these sealers should be examined in ways that disclose their safety profile in real-world clinical settings [23]. A biomaterial's biocompatibility is evaluated by looking at how long and how intense the inflammatory reaction is. Therefore, the duration of the reaction in tissues should be determined by histological study of the response to materials.

In the present study, the biocompatibility was evaluated by implanting polyethylene tubes filled with AH plus, CeraSeal and GuttaFlow Bioseal sealer materials in subcutaneous tissues of 24 Wistar albino rats. The most dependable and simple procedure that can be used in animal model studies is tissue implantation. In earlier studies, implants in subcutaneous tissues were most frequently employed [24, 25, 26]. The subcutaneous tissue-tube interface resembles the reactions that take place following root canal obturation, making subcutaneous tissue implantation one of the most plausible approaches for evaluating the in vivo biocompatibility of the root canal sealers. For a material to be judged biocompatible, the tissue reaction to the tested material in animal models must be comparable to the response of the control. The resin epoxy-based sealer, AH Plus is considered the gold standard of endodontic sealers due to its remarkable physiochemical properties despite their known liability to show a degree of cytotoxicity [27, 28].

The duration of this effect on tissues as well as the tissue features to irritating potential are evaluated through histopathological examination of the

subcutaneous response following various experimental periods[29]. Additionally, the development of a fibrous tissue capsule around the material shows that the tissues can tolerate it. In this regard, it is advised that preliminary tests (such as subcutaneous, muscular, and osseous implant tests) be carried out first, followed by other preliminary tests and only then should tests on humans be carried out [30].

In the inflammatory process, there is an elevated release of proinflammatory cytokines, specifically interleukins (ILs), along with the presence of inflammatory cells such as polymorphonuclear leukocytes and macrophages [31]. After tissue injury, the production of interleukin 6 (IL-6) occurs promptly and temporarily, aiding in host defense by promoting inflammation and immunologic reactions [32]. Therefore, immunohistochemical evaluation was carried out by assessing the prevalence of IL-6 immunopositivity.

Regarding the results of the evaluation of biocompatibility of the three tested sealers, after 7 days of implantation, AH plus showed a severe inflammatory response histologically along with elevated immune expression of IL-6. The strong initial inflammatory response might be due to its high amines content, which are incorporated to accelerate the setting time. Moreover, the release of bisphenol A diglycidyl ether, which is a mutagenic component found in resin-based materials, might also lead to cytotoxicity and could possibly participate in the inflammatory reaction [30]. CeraSeal and GuttaFlow Bioseal also expressed severe inflammatory responses in the same time interval. However, both sealers showed a lower degree of inflammation than AH Plus.

The initial severe immune response triggered by the CeraSeal sealer could be explained as the freshly mixed sealer releases significant amounts of calcium and hydroxyl ions that affect calcium homeostasis around cells which negatively affects cell metabolism and ultimately causes cell death with a decline in the rate of cell viability [33]. Furthermore, the heat generated during the setting process of the calcium silicate sealers promotes the recruitment of inflammatory cells, which subsequently release cytokines [29]. This could explain the severe inflammatory reaction and the high IL-6 expression at the 7-day time interval.

In the present study, inflammation gradually decreased throughout the 14-day time interval and eventually reached a moderate level for all groups. CeraSeal was found to be significantly more biocompatible than AH Plus and GuttaFlow Bioseal at the 30-day evaluation period, showing both mild inflammation on histological analysis and a decline in the mean optical density of IL-6. CeraSeal's biocompatibility could be linked to the calcium release and alkaline pH [34].

GuttaFlow Bioseal displayed severe inflammatory reaction at 7-day evaluation time interval followed by moderate inflammatory reactions at 14 and 30-day evaluation time intervals. This was also accompanied by greater immunoexpression for IL-6 compared to CeraSeal. Because GuttaFlow Bioseal contains bioglass particles, it facilitates the exchange of bioactive ions and other cement components with the surrounding media [30, 35]. While bioactive ions such as silicon, calcium, and phosphorous enhance the migration, growth, and adhesion of cells, other soluble components that are released may trigger the immune system's response [30,36]. This could partially explain the inflammatory reaction observed in the GuttaFlow Bioseal group along with enhanced IL-6 immunoexpression. These findings are not in line with **Collado-González et al 2017** [37], who stated that GuttaFlow Bioseal exhibited better biological performance in cell cultures of mesenchymal stem cells from the periodontal ligament. Another previous study also referred to the alkalinizing effect of GuttaFlow Bioseal which promotes a favorable tissue reaction and expedites tissue healing [30].

CONCLUSION

Based on the findings of this study, it can be concluded that CeraSeal is the most biocompatible endodontic sealer in comparison with GuttaFlow Bioseal and AH Plus sealers. Further studies are required to evaluate different properties of CeraSeal and GuttaFlow Bioseal to confirm their reliability in clinical endodontic treatment.

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