Abstract: This study evaluated the biocompatibility of a new silicone-based sealer (GuttaFlow Bioseal) in rat subcutaneous tissue and compared the results with those for GuttaFlow2 and AH Plus. Each of 16 Wistar rats received four subcutaneous tissue implants, namely, GuttaFlow Bioseal, GuttaFlow2, AH Plus, and one empty polyethylene tube. Eight rats were euthanized at day 8 and the remaining eight at day 30. Histological sections were stained with haematoxylin and eosin and analysed with a light microscope. Scores were established for inflammatory reaction, macrophage infiltrate, thickness of the fibrous capsule, and vascular changes. Differences between groups were assessed by using the Friedman test with Bonferroni correction. Histological analysis showed that GuttaFlow Bioseal had the lowest inflammatory reaction of all tested sealers at day 8. At day 30, the silicone-based sealers had similar inflammation profiles, but inflammation scores were nonsignificantly higher for AH Plus than for the negative control. The inflammatory reaction decreased from day 8 to day 30 in all sealers. GuttaFlow Bioseal had the most macrophage infiltrate. Under the present experimental conditions, GuttaFlow Bioseal induced limited inflammatory reactions at days 8 and 30, and initial inflammatory reactions to GuttaFlow2 and AH Plus subsided within 30 days. All tested sealers exhibited satisfactory biocompatibility at day 30 after subcutaneous implantation.

Keywords: biocompatibility; root canal sealer; subcutaneous tissue; bioactive materials; bioceramic; bioceramic-based sealers.

Introduction

Endodontic treatment aims to prevent or cure apical periodontitis. To achieve that, residual pulp must be removed, along with its breakdown products and any microorganisms present inside the root canal system (1). Cleaning and shaping need to be followed by three-dimensional obturation of the endodontic space, to prevent coronal microleakage and entomb any potential remaining microorganisms or irritants (2). The root canal filling material must have suitable marginal sealing properties, adequate setting time, low solubility, and acceptable biocompatibility. Because of the risk of extrusion over the apical constriction and direct contact with periapical tissues, biocompatibility is important for the performance of filling material in root canal treatment (3,4). Further, tissue response to materials might influence healing of periapical tissues and endodontic treatment outcomes.
Therefore, sealers must have good biocompatibility and be well tolerated by periapical tissues (6). Detailed evaluation of biocompatibility comprises initial assessment of the toxicity profile of sealers, secondary in vivo tests in laboratory animals (2,7-11), and usage studies in primates or humans (12).

Endodontic sealers currently used in clinical practice utilize a variety of materials, such as epoxy resin, zinc oxide-eugenol, glass ionomer, calcium hydroxide, tricalcium phosphate, and silicone. The silicone-based sealer GuttaFlow (Coltène/Whaledent, Altstätten, Switzerland) contains a mixture of gutta-percha powder and polydimethylsiloxane, with nanometer-sized silver particles added as preservative. GuttaFlow2 (Coltène/Whaledent), an evolution of GuttaFlow, is another cold-flowable system that combines gutta-percha powder (particle size, <30 μm) and sealer. Both GuttaFlow and GuttaFlow2 are silicone-based sealers that differ in the form of the silver particles used: the former contains nanosilver and the latter microsilver (13). GuttaFlow Bioseal (Coltène/Whaledent), a novel formulation of polydimethylsiloxane combined with calcium silicate and silver embedded in a glass matrix, was launched in 2015. Calcium silicate-based materials are recognized as bioactive materials because they can induce hard-tissue formation in dental pulp and bone (14,15).

In previous biocompatibility assays of cell cultures, both silicone-based sealers had good biological properties on human gingival fibroblasts (13) and human periodontal ligament stem cells (14); nevertheless, no in vivo study has confirmed the safety and effectiveness of these sealers for clinical applications.

This in vivo study therefore evaluated the biocompatibility of a new silicone-based sealer (GuttaFlow Bioseal) after subcutaneous implantation in rats and compared the results with those of GuttaFlow2 and AH Plus (Dentsply De Trey, Konstanz, Germany). The null hypothesis was that there would be no difference in histological characteristics between sealers.

### Materials and Methods

Sixteen young adult Wistar rats (age, 8-10 weeks; body weight, 120-260 g) were selected. All procedures were in accordance with the standards of the National Institutes of Health, as set forth in the Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering, reduce the number of animals used, and use alternative in vivo techniques. Approval for this study was obtained from the Institutional Ethics Committee on the Use of Animals of the Faculty of Medicine of the University of Coimbra (ORBEA 17/2015). The animals were housed in temperature-controlled rooms. After administration of isoflurane anaesthesia (Vetfluorane, Virbac, Sintra, Portugal), the animals underwent dorsal hair removal. Disinfection was achieved with a povidone-iodine solution (Egrema, Paracélsia, Porto, Portugal). An incision (length, 1 cm) was made in each quadrant of the dorsal region with a No. 15 scalpel blade, in head-tail orientation, to create two scapular and two caudal pockets, equidistant from the spine. To receive the implants, blunt-tipped scissors were used to bluntly dissect the subcutaneous cellular tissue and create surgical recesses with a mean depth of 20 mm, parallel to the spine. Four subcutaneous polyethylene tubes (length, 8 mm; internal diameter, 0.9 mm) (Abbot, São Paulo, Brazil) were implanted in each animal. One empty polyethylene tube served as the negative control, and the other three were filled with one of the following sealers: GuttaFlow Bioseal, GuttaFlow2, and AH Plus (Table 1). The sealers were mixed according to the manufacturers’ instructions and prepared under aseptic conditions just before implantation. The incisions were later closed with 3-0 silk suture (Silkam HR26, B. Braun Surgical, Rubí, Spain). The animals were maintained in individual boxes with standard food and water ad libitum. The animals were euthanized at the end of the experimental periods.

### Table 1 Composition of the tested sealers, as specified by the manufacturers

| Sealer/Manufacturer | Composition |
|---------------------|-------------|
| GuttaFlow Bioseal    | Gutta-percha powder, Platinum catalyst, Silicates, Polydimethylsiloxane, Silicone oils, Silver, Zinc oxide, Zirconium dioxide, Bioactive glass, Colour pigments |
| Coltène/Whaledent AG, Altstätten, Switzerland | Gutta-percha powder, Platinum catalyst, Silicates, Polydimethylsiloxane, Silicone oils, Silver, Zinc oxide, Zirconium dioxide, Colour pigments |
| Dentsply De Trey, Konstanz, Germany | Bisphenol-A and F epoxy resin, Calcium tungstate, Zirconium oxide, Silica, Iron oxide dibenzyl diamine, Aminoadamantane, Tricyclodecane diamine, Silicone oil |

(4,5). Therefore, sealers must have good biocompatibility and be well tolerated by periapical tissues (6). Detailed evaluation of biocompatibility comprises initial assessment of the toxicity profile of sealers, secondary in vivo tests in laboratory animals (2,7-11), and usage studies in primates or humans (12).
(8 and 30 days after implantation). Biopsy samples containing the tubes and surrounding tissues (with 1-cm safety margins) were collected and fixed in 10% buffered formaldehyde (Sigma-Aldrich, Steinheim, Germany). After fixation, tissues were sequentially dehydrated, cleared, impregnated, and embedded in Paraplast (Sigma-Aldrich). Serial 5 μm thick sections were cut, mounted on slides, and stained.

Histological sections prepared from skin specimens were stained with haematoxylin and eosin and analysed at magnifications up to 200× with a light microscope (Nikon Eclipse E600, Tokyo, Japan) coupled to a digital camera (Nikon ACT-IC). Tissue reaction was evaluated by an investigator blinded to the material type and implantation interval, in accordance with a modification of criteria described by Parirokh et al. (16). Tissue samples were assessed according to a scoring system for inflammatory reaction (magnification field 40×: 0, absent; 1, mild; 2, moderate; 3, severe); macrophage infiltrate (magnification field 200×: 0, <10 cells; 1, 10 to <30 cells; 2, ≥30 cells); thickness of the fibrous capsule (magnification field 100×: 0, no fibrous capsule; 1, thin capsule; 2, thick capsule), and vascular changes (magnification field 100×: 0, none; 1, mild; 2, moderate; 3, severe). Differences between groups were assessed by using the Friedman test with pairwise comparisons only and application of Bonferroni correction. For each endodontic sealer, differences in relation to observation interval were evaluated with the Mann-Whitney U test. Significance was defined as $P < 0.05$.

### Results

Macroscopic clinical evaluation showed that wound healing was satisfactory after both observation periods. All implants remained in situ, and surgical wounds healed without signs of infection or rejection. Table 2 shows the score distributions for all studied variables in both observation periods and the results of statistical analysis. No foreign body reaction was observed in any experimental group.

|                          | Inflammatory reaction (0/1/2/3) | Macrophage infiltrate (0/1/2/3) | Thickness of fibrous capsule (0/1) | Vascular alterations (0/1/2/3) |
|--------------------------|--------------------------------|---------------------------------|----------------------------------|-------------------------------|
|                          | P-value day 8                   | P-value day 30                   | P-value day 8                     | P-value day 30                 |
| Negative control         | 0/75/25/0                      | 100/0/0/0                       | 0.002*                           | 75/25/0/0                     |
| GuttaFlow Bioseal        | 0/85.7/14.3/0                  | 50/50/0/0                       | 0.026*                           | 10/14.3/0/0                   |
| GuttaFlow2               | 0/28.6/57.1/14.3               | 62.5/37.5/0/0                   | 0.002*                           | 42.9/14.3/0/0                 |
| AH Plus                  | 0/25/25/50                     | 25/62.5/12.5/0                  | 0.008*                           | 25/12.5/37.5/0                |

$P$-value columns correspond to comparisons of values for the two observation periods for each group (Mann-Whitney test), and $P$-values in the bottom row correspond to comparisons of values during a single observation period (Friedman test). * indicate statistically significant differences.

### Table 2 Evaluation results for all groups, at days 8 and 30

|                          | Inflammatory reaction (0/1/2/3) | Macrophage infiltrate (0/1/2/3) | Thickness of fibrous capsule (0/1) | Vascular alterations (0/1/2/3) |
|--------------------------|--------------------------------|---------------------------------|----------------------------------|-------------------------------|
|                          | P-value day 8                   | P-value day 30                   | P-value day 8                     | P-value day 30                 |
| Negative control         | 0/75/25/0                      | 100/0/0/0                       | 0.002*                           | 75/25/0/0                     |
| GuttaFlow Bioseal        | 0/85.7/14.3/0                  | 50/50/0/0                       | 0.026*                           | 10/14.3/0/0                   |
| GuttaFlow2               | 0/28.6/57.1/14.3               | 62.5/37.5/0/0                   | 0.002*                           | 42.9/14.3/0/0                 |
| AH Plus                  | 0/25/25/50                     | 25/62.5/12.5/0                  | 0.008*                           | 25/12.5/37.5/0                |

$P$-value 0.072 0.010* 0.032* <0.001* 0.392 0.010* 0.044* 0.347

Values are reported as relative frequencies (percentages) of each score. The $P$-value columns correspond to comparisons of values for the two observation periods for each group (Mann-Whitney test), and $P$-values in the bottom row correspond to comparisons of values during a single observation period (Friedman test). * indicate statistically significant differences.

### Negative control

At day 8 (Fig. 1A, B), a fibrous capsule with immature connective tissue collagen fibers and a mild acute inflammatory response to the tube were observed. This cell population had the typical appearance of polymorphonuclear leukocytes (PMNs; Fig. 1B), as it mainly comprised neutrophils; vascular changes were scored as none to mild. Some macrophages (score, 1) were observed. Images at day 30 (Fig. 2A, B) show resolution of the inflammatory reaction and a mature, thin, dense capsule of connective tissue (Fig. 2B). No vascular changes or macrophage infiltration were observed.

### GuttaFlow Bioseal

At day 8, a mild inflammatory reaction and moderate vascular response were observed (Fig. 1C, D); a thin, well-defined capsule was seen at the implant-tissue interface; macrophage infiltration was scored as severe, and cells showed signs of active phagocytosis. Thirty days after implantation (Fig. 2C, D), chronic inflammatory reaction was absent or mild, and a thin capsule of connective tissue was observed (Fig. 2D). The macrophage infiltrate score decreased from severe to moderate, and the vasculature was normal in most samples.

### GuttaFlow2

At day 8 after implantation (Fig. 1E, F), most specimens exhibited a moderate inflammatory response associated with mild to severe macrophage infiltration (Fig. 1F). Blood vessels near the implants exhibited mild to moderate congestion. At day 30 (Fig. 2E, F), most samples had no or a mild chronic inflammatory reaction, a thin capsule of connective tissue (Fig. 2F), and normal vasculature. Macrophage infiltration was moderate.

### AH Plus

At day 8 (Fig. 1G, H), a severe inflammatory reaction with high densities of PMNs and macrophage cells - scored as mild - and moderate to severe blood vessel congestion
Fig. 1 Histological sections of the interface between host tissue and implant at day 8 after subcutaneous implantation (hematoxylin and eosin staining). (A) Negative control showing a mild inflammatory response to the polyethylene tube (T) and a fibrous capsule (FC); (B) A cellular population comprising PMN (yellow arrows) and fibroblast cells with limited vascular changes. (C) GuttaFlow Bioseal sample showing optimal biocompatibility with surrounding tissue and a very thin, well-defined capsule (FC) at the implant-tissue interface and a mild inflammatory reaction; (D) Development of new blood vessels (blue arrow) around the implant, as well as some macrophage cells (green arrow). (E) GuttaFlow2 sample showing a thin capsule of connective tissue (FC) with a mild inflammatory reaction; (F) New vascular structures (blue arrows) can be seen near the implant, as well as a substantial number of macrophages (green arrows) full of granular material in their cytoplasm. (G) AH plus group sample exhibiting a thick fibrocellular capsule (FC) and high densities of PMN (yellow arrows) and macrophage cells (green arrows) in the connective tissue matrix; (H) Several macrophages (green arrows) and degranulation are present near and some distance from the implant site, usually in close proximity to vascular structures.

Fig. 2 Histological sections of the interface between host tissue and implant at day 30 after subcutaneous implantation (hematoxylin and eosin staining). (A) Negative control showing a mature, thin, dense capsule of connective tissue (FC) around the polyethylene tube; (B) A few macrophages (green arrows) can be seen near and at some distance from the implant site. (C) GuttaFlow Bioseal sample showing a thin capsule of connective tissue (FC) perfectly integrated with surrounding tissues; (D) Higher magnification view of the interface implant-host tissue showing absence of inflammatory reaction and a few macrophages (green arrows). (E) GuttaFlow2 sample showing implant integration with surrounding tissue, a thin layer of connective tissue around the implant (FC), and no inflammatory reaction; (F) A higher magnification view of (E) confirming the absence of inflammatory cells at the interface of host connective tissue and implant. (G) AH Plus group sample showing a moderate inflammatory reaction associated with fibrocellular encapsulation (FC); (H) High densities of PMN (yellow arrows) and macrophages (green arrows), as well as newly formed blood vessels (blue arrows), are present at the interface of the implant and host tissue, which indicates the intensity of the inflammatory reaction.
were observed at the implant/host tissue interface (Fig. 1H). A thick layer of connective tissue (Fig. 1G) was present around the tube. At day 30 after implantation (Fig. 2G, H), a mild chronic inflammatory reaction was observed, and a mature, well-defined fibrocellular capsule was present.

**Group comparison**

At day 8, the inflammatory reaction for the GuttaFlow Bioseal was less than that for GuttaFlow2 and AH Plus (Fig. 3), although the difference was not significant \( P = 0.072 \). At day 30, inflammation scores were nonsignificantly higher for AH Plus than for the negative control (Table 3). GuttaFlow Bioseal and GuttaFlow2 performed similarly. All groups exhibited a significant reduction in inflammatory reaction from day 8 to day 30.

GuttaFlow Bioseal had the greatest amount of macrophage infiltrate at day 8 \( (P = 0.027 \text{ vs negative control, } P = 0.683 \text{ vs GuttaFlow2, and } P > 0.05 \text{ vs AH Plus}) \). Although macrophage infiltrate decreased over time in all groups, the differences were not statistically significant. At day 30, macrophage infiltrate scores significantly differed between groups \( (P < 0.001) \), and the amount of infiltrate was significantly higher for GuttaFlow Bioseal and GuttaFlow2 than for the negative control \( (P = 0.001 \text{ and } P = 0.016, \text{ respectively}) \).

At day 8, fibrous capsules did not significantly differ between endodontic sealers \( (P = 0.392) \). At day 30, although the groups tested showed significant differences, no significant differences were seen in pairwise comparisons of sealers.

The GuttaFlow Bioseal and AH Plus samples exhibited significant decreases in vascular changes from day 8 to day 30 \( (P = 0.017 \text{ and } P = 0.012, \text{ respectively}) \), and normal vascular structure findings were observed in most samples at the end of the observation period.

**Discussion**

Biocompatibility is defined as the ability of a material to perform specific functions when implanted in living tissue, while not damaging the tissue. Implantation into rat subcutaneous tissue is one of the most useful tests for determining the type and development of local reactions induced by endodontic sealers (8-11). Biocompatibility of dental materials is an important requirement, as toxic components can induce irritation or even destruction of surrounding tissues, especially when accidentally extruded into peri-radicular tissues (2,4,7,17). Almost all endodontic sealers are toxic when freshly prepared; thus, such sealers should be tested in conditions that reveal their safety profile under clinical conditions (17-19).

Biocompatibility of biomaterials is assessed by examining the intensity and duration of inflammatory response. Hence, histopathological analysis of the response to materials should determine reaction duration in tissues (11,20). In this study, early (8 days) and late (30 days) responses were assessed. The initial mild reaction in control samples was probably caused by surgical trauma, since the sterile polyethylene tube is an inert device and does not cause inflammation (21). In fact, at

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**Table 3** Results of pairwise comparison of inflammatory reaction values at day 30 (Bonferroni correction)

|              | GuttaFlow Bioseal | GuttaFlow2 | AH Plus |
|--------------|-------------------|------------|---------|
| Negative control | >0.05             | >0.05      | 0.058 |
| GuttaFlow Bioseal   | >0.05             | 0.884      |         |
| GuttaFlow2            | 0.471             |           |         |

All data are \( P \)-values
day 30 reactions around control tubes had resolved, and a healthy connective tissue capsule was present around the implants.

All polyethylene tubes were implanted before material setting, during which the possibility of dissolution and biological risk are increased (19,22). The tubes were filled with freshly mixed sealers, thus promoting extrusion of materials to surrounding tissues. The contact area corresponded to a circle with a minimum diameter of 0.9 mm (the inner diameter of the tubes), which is less favourable than usual clinical conditions in which the foramen of a prepared canal is smaller (around 0.3-0.6 mm). Hence, sealer particles directly interact with tissues surrounding the open ends of the tubes and induce reactions that might be worse than those observed clinically.

To the authors’ knowledge, this is the first in vivo study of GuttaFlow Bioseal after the in vitro study by Collado-Gonzalez et al. (14). Under the present experimental conditions, GuttaFlow Bioseal displayed excellent biocompatibility, as indicated by the low inflammatory reaction scores observed after both evaluation intervals and the improvement in vascular changes in the late assessment. Although these results cannot be directly extrapolated to human beings, the standardized test conditions allowed for accurate comparisons of sealers (23).

AH Plus, which was selected as the reference because its biological properties are well known (7,24), had the highest inflammatory reaction scores. Its strong initial toxicity might be attributable to its high content of amines, which are used to accelerate setting time (7,23). Release of bisphenol A diglycidyl ether, a mutagenic component of resin-based materials, might also cause cytotoxicity (13) and could contribute to the stronger initial inflammatory reaction. In contrast, recovery from the initial inflammatory response was faster for both silicone-based sealers. These findings confirm previous observations and reflect the fact that the chronic inflammatory response to epoxy resin-based materials is longer (23).

The inflammatory reaction in AH Plus samples considerably differed regarding the observation period, with high spread of results. Nevertheless, the overall severity of the inflammatory reaction decreased with time, which indicates that all the tested sealers had satisfactory biocompatibility (10,11,24,29,30).

Under the present experimental conditions, GuttaFlow Bioseal induced limited inflammatory reactions at both observation periods. Initial inflammatory reactions to GuttaFlow2 and AH Plus subsided by day 30, and all the tested sealers exhibited satisfactory biocompatibility at day 30 after subcutaneous implantation.

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Conflict of interest
Prof. Santos reports grants from Coltène/Whaledent, during the conduct of the study.

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