Original

In Vivo Tissue Response of Endodontic Bio-ceramic Materials

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Abstract: In order to evaluate the biocompatibility and mineral repair capacity, mineral trioxide aggregate (MTA), portland cement normal setting (PCn) and fast setting (PCf) and calcium hydroxide-based paste (Calen) filled silicone tube were implanted into the dorsal subcutaneous connective tissues of 25 Wistar rats. Animals were euthanized at 24, 72 hours, 7, 15 and 30 days. Implants with surrounding tissues were fixed with 10% buffered formaldehyde and processed for histological routine techniques. Slides (5 μm serial cuts) were stained with H&E and Von Kossa stains for morphological, qualitative and quantitative analysis by light microscopy. Calen showed severe and moderate inflammatory response and granulomatous reaction with psammoma body-like formation. PCn and MTA have similar behavior, with mild inflammatory reaction from 8% and 4%, respectively. Even though, PCn and MTA demonstrated analogous biological reaction, MTA developing thick artificial mineral precipitation (p = 0.007). All sealers demonstrated a similar inflammatory response at all time periods studied (p = 0.678).

Key words: Biocompatibility, Calcium hydroxide, Mineral trioxide aggregate, Portland cement.

Introduction

In the last thirty years, bio-ceramic materials were introduced in Medical and Dental fields. They are classified as I-Bioinert: non interactive with biological systems (Alumina, Zirconia), II-Bioactive: when produce interfacial interactions with surrounding tissue (Bioactive glasses, Bioactive glass ceramics, Hydroxyapatite, Calcium silicates) and III-Biodegradable: soluble or resorbable, eventually replaced or incorporated into tissue (Tricalcium phosphate, Bioactive glass1). Bio-ceramics have ability to either functions as natural tissues or to be resorbed and stimulate natural regeneration2-5. Recently, bio-ceramic based sealers were introduced in endodontic, categorized as I Calcium silicate based-cements with mineral trioxide aggregate (MTA) and portland cement; II Calcium phosphate, Tricalcium phosphate, Hydroxyapatite, Calcium Hydroxide based sealers. And III Mixture of Calcium Silicates and Calcium Phosphates based sealers2-3. Portland Cement used in the construction industry as a binder in concrete, lately have been introduced in dentistry. The first-generation material was mineral trioxide aggregate (MTA) composed of Portland cement and bismuth oxide6. The Portland cement component when mixed with water results in the formation of calcium silicate hydrate, calcium hydroxide and ettringite7.

To detect chemical elements, samples of MTA and Portland cement were analyzed by inductively coupled plasma emission spectrometry (ICP-ES)8. Comparative analysis for fifteen different elements revealed a significant similarity between them, with the exception that there was no detectable quantity of bismuth in Portland cement1. There are many studies suggesting that MTA is a suitable material to be used as retrograde filling cement, perforations and pulpotomies, due to its good sealability, biocompatibility and deposit of hard tissue at dental apices and dental pulp stump1,4. Related to PC, it was demonstrated its similarity to MTA2,3, it has high alkalinity (pH 10), which inhibits bacterial proliferation4, good sealing and some studies consider PC a substitute for MTA5. However, there is scarce research concerning the biological tissue response from Portland Cement materials in vivo5,10. From Group II called Calcium phosphate-based sealers, Calcium Hydroxide was selected for its ability to react with the surrounding tissues. The Calcium hydroxide Ca(OH)2 is widely used based on its biological, physic-chemical, antimicrobial, among other characteristics15. CaO materials have been used for root canal sealers, retrograde root fillings, intra-canal medicament between appointments, apexitogenesis, arrest of root resorption defects, and direct or indirect pulp-capping procedures15. Although calcium hydroxide has been widely accepted in clinical practice, some studies show it may be cytotoxic due to high alkalinity16,20. This characteristic is due to the release of hydroxyl ions responsible for antimicrobial actions, and for promoting mineralized tissue formation. Our research was focalized to evaluate by ISO international standards (10993-6 and 740521,22), the local effects from Portland Cement (normal and fast
setting) and Mineral Trioxide Aggregated (MTA) as experimental groups, and Calcium Hydroxide Paste as positive control group; after their implantation into silicone tubes in rat subcutaneous connective tissues to compared biocompatibility and mineral tissue repair; at short periods of time (24, 72 hours and 7, 15 and 30 days).

Materials and Methods

Animals

Twenty-five male Wistar rats weighing 250 g ± 100 g were obtained from the Animal Research Center (Medical School, Tucumán National University, Argentina). Animals were housed in pairs, in a specific pathogen-free environment, with a temperature of 22.4°C to 23.8°C, relative humidity of 45% to 62%, and a 12-hour light-dark cycle. A standard commercial diet and tap water were available ad libitum. The protocol was approved by the local Ethical Committee for Animal Research (Tucumán National University and CONICET). All procedures were developed in accordance with the Guide for the Care and Use of Laboratory Animals 8th edition.

Materials

The materials were divided into a) Control Groups: Silicone: the response of the side wall of silicone tubes acting as a non-toxic, inert material; Calcium Hydroxide: Calen (SS White Dental Articles, Rio de Janeiro, Brazil), as positive control; and b) Experimental Groups: Mineral trioxide aggregate (MTA). ProRoot (Dentsply Tulsa Dental, Tulsa, OK, USA). Portland cement with normal setting (PCn).Cemento Portland with filler calcáreo. (LOMA NEGRA, Buenos Aires, Argentina). Portland cement fast setting (PCf). Practik (KLAUKOL, San Justo, Argentina).

They were prepared according to the manufacturer’s instructions. MTA and PC (fast and normal setting) were prepared in the same way. Three parts of powder and one part of sterile water were mixed until a creamy consistency (30") was obtained.

Surgery

The skin of the animals was shaved and disinfected. An antero-posterior incision was made, creating four pockets. The sterilized silicone tubes (10 mm x 1 mm) filled with the assay materials: Calen, MTA, PCn and PCf, were implanted into each subcutaneous tissue pocket, perpendicular to the incision. The study period was short-term testing and the animals were euthanized by overdose of anesthetic solution (five animals per period) at 24, 72 hours, 7, 15 and 30 days. The tubes and surrounding tissues were removed and fixed in 10% formaldehyde buffer solution, pH 7.

Histological Procedures

Skin samples with implanted tubes and surrounding tissues were included in paraffin blocks, cut into 4-6 μm thick sections. They were stained with Hematoxylin-Eosin (H&E) and Von Kossa. Observations were made with a light microscope at 4X, 10X, 40X, 60X and 100X, and polarized light microscope studies. A single pathologist evaluated all tissues. Subsequently, another pathologist (certified by Argentine Health Ministry License Nº 31455) performed an independent review to verify microscopic observations. The results reported reflect the mutually-agreed-upon diagnoses by both pathologists.

The histological parameters were necrosis, acute (polymorphous neutrophils) and chronic (lymphocytes-macrophages) inflammation, abscess, granulation and fibrous tissues, giant foreign body reaction and mineral precipitation, to obtain qualitative scores at the tube end. Grade A. Mild or no inflammation: no local inflammatory reaction appeared either on tissue exposed to experimental material or along the tube. Grade B. Moderate inflammation: Some inflammatory cells, lymphocytes, plasma cells, macrophages, and occasionally foreign body cells at
Histological distribution (%)

- Crystal Mineral Precipitation
- Congestion
- Granulation Tissue

Figure 2. At 30 days shows percentage distribution of crystal precipitation, congestion and granulation tissue compartments from experimental endodontic sealers.

Table 1. Tissue response distribution in subcutaneous connective tissue by cements expressed as A, B and C scores in percentages. (A) Mild or no reaction: no local inflammatory reaction on tissue exposed to experimental material. Or along the tube, presence of fibrous capsule. (B) Moderate inflammation: A few inflammatory cells, lymphocytes, plasma cells, macrophages, and occasionally foreign body cells at the tube end (analysis area), and fibrous tissue along the tube. (C) Severe inflammation: necrosis and polymorph neutrophil leukocyte (PMN) collection and lymphocytes, plasma cells, macrophages, fibrous tissue, occasionally foreign body giant cells. MTA and PCn setting shows 8% and 4% without inflammation at 30 days.

| Material  | A    | B    | C    | Total |
|-----------|------|------|------|-------|
| Silicon   | 100% | 0%   | 0%   | 100%  |
| CaHO      | 0%   | 22%  | 78%  | 100%  |
| PCn       | 8%   | 22%  | 69%  | 100%  |
| MTA       | 4%   | 39%  | 56%  | 100%  |
| PCfr      | 0%   | 35%  | 65%  | 100%  |

Statistical Analysis

Qualitative scores were analysed with Fisher’s Exact Test (p < 0.05). The percentage data from the quantitative study were presented as mean and ± standard deviation. analysed using Kruskal Wallis Test (p < 0.05).

Results

Histopathological Observations and Qualitative Scores

Negative Control Group: Silicone

At 24 and 72 hours inflammatory cells were observed. At 7 days, thin fibrous connective tissue with few inflammatory cells and fibroblasts was detected. At 30 days thick fibrous connective tissue was found (Fig. 1A). All samples (100%) showed Grade I, no or mild inflammatory reaction (Table 1).

Positive Control Group: Calcium Hydroxide with polyethylene glycol (Calen)

At all time periods analyzed, numerous extensive necrosis areas were observed at the end of tube, where Calen contacts tissues. However, their amount and extension decreased at 30 days. A severe acute inflammatory reaction was observed since 24 h, 72h, 7 days and 15 days. From 15 days to 30 days, granulation tissue was replaced by fibrosis and hyalinization. Calen developed an inflammatory reaction grade B in five samples (22%) and C in 18 samples (78%) (Table 1). Also, at the tube end, numerous crystalized-like structures were deposited in two patterns: 1. cuboid crystals palisade lined and 2. multiple rounded, oval or amorphous formations with discrete, laminated, concentrical calcareous precipitation resembling psammoma bodies, surrounded by giant foreign body cells in 15 samples (Fig. 1B, 1C and 1D).

Experimental Groups

MTA

At 24 and 72 hours, acute inflammatory reaction, congestion, necrosis and hemorrhage were found. At 7 and 15 days a chronic inflammatory reaction, granulation tissue and hyaline deposits were observed. At 30 days, a mature granulation tissue and fibrosis were observed. In addition, crystallized mineral precipitation (Von Kossa positive structure) was distinguished. At the tube end a crystallized precipitation was observed at the beginning of the experiment, although at 30 days the deposit was arranged in randomly scattered layers. The black, brown or white crystals were irregular, globular, rounded or laminated and loosely agglomerated, surrounded by macrophages and foreign body giant cells, at 7, 15 and 30 days. (Fig. 1G). Qualitative response from MTA showed inflammatory reaction B (moderate) in 9 samples (39%) and C (severe) in 13 samples (56%). However, at 30 days, only one sample (4%) was A (mild or no inflammatory reaction) (Table 1).

PCn(normal setting)

At 24 and 72 hours, vascular congestion, hemorrhage and acute inflammatory reaction were observed. Necrosis and abscess were also found. At 7 days, chronic inflammatory cells prevailed and granulation tissue and fibrous connective tissue were deposited. At 15 days granulation tissue and fibrosis with lymphocytic-mononuclear cells infiltrate were observed. A mineral crystal precipitation was found since 24h throughout all study periods. At 15 and 30 days macrophages with phagocytosed particles inside; and foreign body giant cells were observed around those particles (Von Kossa-positive). This disordered amorphous granular mineral precipitation, more scattered than MTA, was deposited on the tube end, surrounded by a hyalinized fibrous for-
mation (Fig. 1E). The qualitative results from PC normal setting showed two samples (8%) with no or mild reaction (A), five samples (22%) with moderate reaction (B) and sixteen samples (69%) with severe reaction (C) (Table 1).

**PCf (fast setting)**

At 24 and 72 hours, vascular congestion, acute inflammatory reaction with polymorphonuclear neutrophils (PMN), necrosis areas and micro abscesses were observed. At 7 and 15 days chronic inflammatory cells (lymphocytes and macrophages) with granulation and fibrous connective tissue appeared. The mineral crystal precipitation was present since 24hs throughout all study periods. The particles were brown, black or translucent, rounded, larger and less agglomerated compared to MTA (Fig. 1F). At 15 and 30 days, giant foreign body cells and numerous macrophages with phagocytic particles appeared, generating foreign body granuloma in three cases. At 30 days a mineral granular Von Kossa-positive structure was surrounded by hyaline deposit and fibrous tissue. The qualitative results from PC fast setting showed eight samples (35%) with moderate reaction and fifteen samples (65%) with severe reaction (Table 1).

**Quantitative Observations**

The morphometrical results from experimental groups observed at 30 days after implantation were expressed in bar Fig. 2. The percentages from congestive areas were Calen 3.44% ± 2.87, PCn 1.98% ± 1.88, PCF 0.08% ± 0.18 and MTA 0.7% ± 1.24. No statistical differences were observed (p = 0.12). The percentages for granulation tissue area were Calen 49.15% ± 19.76, PCn 62.95 ± 11.42, PCF 77.74 ± 13.31 and MTA 57.74 ± 15.8. There were no statistical differences between groups (p = 0.50). Crystallized mineral precipitation percentages values were 16.25% ± 6.9 for Calen, 9.93% ± 3.94 for PCn, 6.32% ± 5.38 for PCF and 29.64% ± 6.96 for MTA. MTA showed statistically significant differences (p = 0.007) compared to the other groups. There was counted per 40X field foreign body giant cells (FBGC), by Image Pro Plus analysis system. The media (μ) of FBG Cells found were in Calen 10, PCn, 1, PCF 0,5 and 0 for MTA.

**Discussion**

In relation to biocompatibility, our results could be considered as non compatible according to traditional concepts; or compatible according to the present concept, based on the ability of a biomaterial, to induce an appropriate and advantageous host response during its clinical usage. Our results showed an A score at 30 days with PCn (8%) and MTA (4%) respectively, in agreement with authors that reported no inflammation. We also found moderate or severe inflammation in 22% inflammatory reaction obtained without mild reaction did not agree with a previous popular notion called calcium hydroxide-based sealers as biological sealers with a persistent chronic inflammation and foreign body granulomatous reaction observed. With respect to mineral precipitation, the formation of a calcareous palisade at the tube end was appropriate, but not the psammosa body structures found in the granulomatous reaction, associated with either benign or malignant diseases. These structures were described here for first time after subcutaneous tissue implantation with OHCa (Calen).

Calcium hydroxide is a strong base. It is slightly soluble in water, releasing Ca and OH ions through ionic dissociation. High pH causes irritation and necrosis, stimulating and forming mineralization, in a process similar to dystrophic calcifications. The dystrophic calcifications observed with Calen in our results could be explained by the slow disaggregation of its components and the development of a foreign body granulomatous reaction. We concluded that OHCa (Calen) was not bio-compatible, while highest biocompatibility was observed with PCn (8%) and MTA (4%). MTA developed a thick crystal precipitation compared with PC; however, both mineral formations were distinctive, disaggregated, quartered, irregular, disordered and inconsistent.

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**Conflict of Interest**

The Authors declares no conflict of interest related to this study.

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