A comparative histological, histochemical and immunohistochemical study of the biocompatibility of three different nano-restorative materials implanted in rats’ connective tissue

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ABSTRACT

This study was conducted to evaluate the subcutaneous connective tissue reaction to the newly-developed nano-restorative materials: Filtek Supreme XT, Ceram X and Ketac N100. These materials were placed in polyethylene tubes and implanted into the dorsal connective tissue of albino rats. Tissue biopsies were collected and histologically examined 1, 4 and 8 weeks after the implantation procedure. The presence of inflammation, type and location of inflammatory cells, calcification and fibrous tissue formation were recorded. Mast cells and macrophages were quantitatively assessed using toluidine blue stain and CD68 immunoexpression respectively. Microscopic analysis revealed that Ceram X and Filtek supreme XT produced an irritating effect only during the initial post implant period. Ketac N100 caused the severest irritation, producing an inflammatory reaction which ranged from moderate to intense over the entire experimental period. Comparing mast cell number and CD 68 area % between the control and the three tested materials, there was a significant difference throughout the experiment (p=0.000). The highest mast cell number was recorded in the Ketac N group after one week, while the greatest CD 68 immunoexpression was noted after 4 weeks in the same group. It can be concluded that the tested materials induce different and time dependent inflammatory reactions and that Ketac N 100 is less biocompatible compared to Filtek supreme NT and Ceram X.

INTRODUCTION

Biomaterials science is in the midst of the largest transition in its history in terms of refocusing and embracing new and exciting technologies (Bayne, 2005). Nanotechnology, also known as molecular nanotechnology or molecular engineering, is the production of functional materials and structures in the range of 0.1 to 100 nanometers—the nanoscale—by various physical or chemical methods (Kirk et al, 1991). In the current decade, the revolutionary development of nanotechnology has become the most highly energized discipline in science and technology (Schultz, 2000). The intense interest in using nanomaterials stems from the idea that they may be used to manipulate the structure of materials to provide dramatic improvements in electrical, chemical, mechanical and optical properties (Whitesides and Christopher, 2001).

One of the most significant contributions to dentistry has been the development of resin-based composite technology. A dental composite can be defined as a three dimensional combination of at least two chemically different materials. Basically, they are composed of an organic matrix, load particles (glass, quartz, melted silica) and a bonding agent, usually an organic silane, with a dual characteristic enabling chemical bonding with the load particle and co-polymerization with the monomers of the organic matrix (Peutzfeldt , 1997). Adhesively bonded composites have the advantage of conserving sound tooth structure with the potential for tooth reinforcement, while at the same time providing a cosmetically acceptable restoration (Fortin and Vargas, 2000).
2003, Mitra and his associates developed a new dental nanocomposite, Filtek Supreme Universal Restorative (3M ESPE Dental Products, St. Paul, Minn.) that has two types of nanofillers: nanomeric particles and nanoclusters. They used optimal combinations of these nanofillers in a proprietary resin matrix to prepare the nanocomposite system with a wide range of shades and opacities. Nanofillers permit substantially smaller interparticle distances and thus improve the strength and esthetic properties (Mitra et al, 2003).

The term ‘glass-ionomer’ has traditionally been applied to that group of materials which undergo setting through an acid-base reaction between an ion-leachable glass powder and a water-soluble polymeric acid such as poly-acrylic acid. The traditional glass-ionomers are characterized by properties such as brittleness, adhesion and fluoride release (McCabe, 1998). Resin-modified glass-ionomers are dental restorative materials of the glass-ionomer family. In addition to the aforementioned components, they contain organic monomers, typically 2-hydroxyethyl methacrylate (HEMA) and an associated initiator system (McLean et al, 1994).

Ceramic materials were used as pulp capping material (Hayashi et al, 1999) and as root canal filling materials with varying degrees of success (White and Goodis 1991). On the other hand, Glass-ionomer cement (GIC) was frequently used as a restorative material for both permanent and deciduous dentitions (ten Cate and van Duinen, 1995). However, biocompatibility studies of GIC (Brook and Hatton, 1998; Callis and Santini, 1987) exhibited evidence of initial cytotoxicity with freshly prepared samples, with decreasing toxicity as setting occurred. Recently, Ceram X a light curable nano-ceramic restorative and Ketac™ N100, a light-curing nano-ionomer restorative paste were developed with nanotechnology.

A material is considered biocompatible if it does not produce harmful or toxic reactions in the tissues it contacts or adverse systemic reactions as a result of elements, ions, and/or compounds it release (O’Brien, 2009). Moreover, it should be free of potentially sensitizing agents that could lead to an allergic response, and should have no carcinogenic potential (Phillips, 1991). An adverse reaction may be due to the toxicity of a dental material. Therefore toxicity may be regarded as one reason of non-biocompatibility of a dental material. The toxicity of the dental material can be evaluated by in vitro tests, animal experiments and clinical trials (Polyzois, 1994).

The foreign body reaction composed of macrophages and foreign body giant cells is the end-stage response of the inflammatory and wound healing responses following implantation of a medical device, prosthesis, or biomaterial (Anderson et al, 2008). Nevertheless, little data is available about tissue reaction to restorative materials recently developed with nanotechnology. Therefore, the purpose of the present study was to histopathologically examine the biocompatibility of the restorative materials: Filtek™ Supreme XT, ceram X and Ketac™ N100, by implanting them into rat subcutaneous connective tissue. In addition, mast cells and macrophages were assessed using toluidine blue stain and CD68 immunoexpression respectively.
MATERIALS AND METHODS

Experimental animals and implantation procedure

Twenty-four male albino rats weighing 200-250 mg were used in this study. The animals were housed in plastic cages in a room with a 12-h day–night cycle, temperature of 24 ± 2°C and humidity of 45–64%. All animals were fed with semi purified diet and water ad libitum. Animal care and experimental procedure were performed in accordance with guidelines of the review board of Kasr-El-Ainy animal and experimental lab, Faculty of Medicine, Cairo University.

The commercial names, the manufacturer and the composition of the used materials are listed in table 1. The material to be tested was freshly prepared as advised by the manufacturer and placed in a polyethylene tube (10mm long x 15 mm internal diameter), just before the operation.

For materials implantation, the dorsal skins of the animals were shaved under xylazine (10 mg/kg) and ketamine (25 mg/kg) anesthesia and disinfected with 5% iodine solution. Four incisions were made on the back of each animal over a length of 2 cm using a no. 15 blade in a head-to-tail alignment orientation. These incisions were made on the dorsal surface of the front limbs (right and left) and on the dorsal pelvic area (right and left). The skin was reflected and a space about 2 cm was created in the subcutaneous tissue by blunt dissection with a hemostat to accommodate the polyethylene tube. Each animal received one tube filled by Ketac N 100 (in the dorsal surface of the right front arm), a tube filled with Filtek Supreme XT (in the dorsal surface of the left front arm) and a tube filled with ceramX (in the dorsal surface of the right back limb). For control purposes, empty polyethylene tubes closed from both sides by heat were implanted on the dorsal surface of the left back limb. The skin was closed with 3/0 silk suture. Sterile instruments and aseptic techniques were used throughout the entire experiment. The histological evaluations were performed 1 week, 4 weeks and 8 weeks post implantation. At each period, 8 rats were sacrificed by anesthetic overdose; the tubes and surrounding tissues were removed by tissue dissection technique and fixed in 10% buffered formalin at pH 7.0.

Specimen preparation and criteria of histological response evaluation

Following formalin fixation, specimens were routinely processed and embedded in paraffin wax, serially sectioned at a setting of 5 µm, and stained with hematoxylin-eosin. From each tissue sample, 5 sections presenting the greatest inflammatory reaction were examined with a light microscope. The areas of inflammatory reaction were evaluated quantitatively and the number of inflammatory cells was scored as: 0, no inflammation (no or few inflammatory cells); 1, mild inflammation (<25 inflammatory; 2, moderate inflammation (increased reaction zone, 25–125 inflammatory cells; or 3, severe inflammation (focal areas of necrosis, <125 inflammatory cells) (Yaltirik et al, 2004). The type of inflammatory cells (neutrophils, lymphocytes, macrophages, mast cells and giant cells) was determined. Fibrous capsule, necrosis and formation of calcification were recorded as present or absent.

Histochemical and immunohistochemical procedures

Toluidine blue stain (Drury and Wallington, 1980) was used to observe the occurrence of mast cells, their distribution and degranulation.
For CD 68 immunostaining, endogenous peroxidase was blocked by immersing sections in 3% H$_2$O$_2$ (in PB) for 20 min followed by washing with PBS (2-5 min) and blocking with 10% normal horse serum for 30 min at room temperature. For retrieval of antigens masked by formalin-fixation and paraffin-embedding, tissue sections were pretreated by microwaving in Tris EDTA buffer (pH 9.0) at 95-100°C for 20 minutes. Sections were incubated by mouse anti-human CD68 (Dako, Cat.# M0814) primary antibody for 1 h at room temperature. After triple washing steps with PBS, sections were transferred to the secondary antibody (biotinylated, affinity-purified, goat anti-rabbit IgG (Vector, Burlingame, USA) at 1:200 dilution in PBS for 45 min at room temperature. The sections were washed with PBS (3-5min) and were then incubated with ABC (avidin–biotin horseradish peroxidase complex) according to the manufacturer’s instructions for 45 min at room temperature. After intensive washing steps (2-5min with PBS, 1-5min with PB) the sections were incubated for 8 min with diaminobenzidine (DAB). Slides were rinsed in PBS and then dehydrated in graded alcohol solutions. Each slide was counter stained with haematoxylin. A negative control without primary antibody was prepared for each specimen.

**Image analysis and statistical evaluation**

To standardize the results, the mast cells were counted in 10 fields (x 400 magnification) using specific software Leica Qwin V and the mean number was calculated. Furthermore, CD68 immunostaining was examined by the image analyzer computer system with the software Leica Quin 500. The CD68 immunoreactivity was measured in the form of an area and area percent in a standard measuring frame per 10 fields using the magnification x 400 by light microscopy transferred to the monitor. The areas of the most intense staining were masked by a blue binary colour that could be measured by the computer system. Results were statistically analyzed using one-way ANOVA analysis at 5% significance level.
### Table (1): The commercial name, the manufacturer and the composition of the used materials

<table>
<thead>
<tr>
<th>Materials</th>
<th>Manufacturer</th>
<th>Composition</th>
</tr>
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<tbody>
<tr>
<td>Filtek™ Supreme XT Universal</td>
<td>3M ESPE, Dental St. Paul, MN USA</td>
<td>- Bis-GMA, bis-EMA, - UDMA and TEGDMA - 59.5% in volume (clusters of 0.6–1.4µm, individual particle size of 5–20 nm) - Zirconia and silica</td>
</tr>
<tr>
<td>Ceram X</td>
<td>DENTSPLY DETREY GmbH De-Trey-Str.1 78467 Konstanz GERMANY</td>
<td>- Methacrylate modified polysiloxane - dimethacrylate resin - Fluorescence pigment - UV stabilizer - Camphorquinone (photo-initiator) - Ethyl-4(dimethylamino)benzoate - Barium-aluminium-borosilicate glass - Methacrylate functionalised silicon dioxide nano filler - Iron oxide pigments, titanium oxide pigments and aluminium sulfo silicate pigments</td>
</tr>
<tr>
<td>Ketac™ N100 Light-Curing Nano-Ionomer Restorative</td>
<td>3M ESPE, Dental St. Paul, MN USA</td>
<td>- De-ionized water - Methacrylate component (Blend including HEMA) - Vitrebond Copolymer (a methacrylate modified polyalkenoic acid) - Filler components (Fluoroaluminosilicate glass Nanomers, and Nanoclusters)</td>
</tr>
</tbody>
</table>

### RESULTS

#### Histopathological findings

**Control group (empty polyethylene tube)**

One week post implantation of an empty polyethylene tube, microscopic examination revealed a vascular response consisting of mild to moderately dilated blood vessels. Mild chronic inflammatory cell infiltration (mainly lymphocytes) and delicate fibrosis were noted. The inflammatory reaction subsided 4 weeks after tube implantation, while the vascular and fibrous responses remained unchanged. At the 8th week observation period, the tissue surrounding the implanted tube consisted of loose collagen fibers, with localized area of fiber condensation. Few normal-sized or mildly dilated blood vessels were detected, while inflammatory cells were almost absent. A delicate fibrous capsule surrounded the tissue reaction. No evidence of calcification or necrosis could be detected in the control group throughout the experiment (Fig.1).
Fig (1) Photomicrograph of control group, A: After one week, mild to moderate dilatation of blood vessels (arrows) and delicate fibrosis (F). B: At the end of the experiment, the vascular response (B) subsides, localized area of fiber condensation (F) is observed. (S) skin surface, (C) subcutaneous tissue (H&E x40).

Filtek Supreme XT

Microscopic examination of the tissue reaction to Filtek Supreme XT after one week revealed a vascular response consisting of moderately dilated blood vessels and extravasated red blood cells. Moderate chronic inflammatory cell infiltration (mainly lymphocytes), sparse neutrophils and wavy collagen fiber deposits were noted (Fig. 2).

Four weeks after material implantation, the inflammatory reaction became mild, while moderate to severe blood vessel dilatation was still observed. Wavy collagen fiber deposition was noted in the vicinity of the implanted tube and an incomplete fibrous capsule surrounded the tissue reaction (Fig. 3).

At the 8th week observation period, loose to wavy collagen fibers and a delicate fibrous capsule surrounded the implanted tube. Moderately dilated blood vessels were detected. Sparse chronic inflammatory cells were noted. There was no evidence of calcification or necrosis throughout the experiment (Fig. 4).

Fig (2) Photomicrograph of Filtek supreme XT group after one week revealing wavy collagen deposits (F) and moderate chronic inflammatory cell infiltration (I) surrounding the implanted tube (T), (H&E x100).
Fig (3) Photomicrograph of Filtek supreme XT group after 4 weeks revealing moderate blood vessel dilatation and wavy collagen fiber deposition in the vicinity of the implanted tube (T) and an incomplete fibrous capsule (F) surrounding the tissue reaction (H&E x40). The higher magnification in the inset illustrates the chronic inflammatory reaction, consisting of lymphocytes and numerous macrophages (M), (H&E x400).

Fig (4) Photomicrograph of Filtek supreme XT group after 8 weeks showing loose to wavy collagen fibers (F) and moderately dilated blood vessels (arrows) in the subcutaneous tissue beneath the skin appendages (S), (H&E x40)

**Ceram X**

Microscopic examination of the tissue reaction to Ceram X after one week revealed a vascular response consisting of numerous normal or moderately dilated blood vessels. Delicate collagen fibers surrounded the implanted tube. Moderate chronic inflammatory cells (mainly lymphocytes) were detected infiltrating the muscles and subcutaneous tissue (Fig.5).

Four weeks after material implantation, the inflammatory reaction became milder and was mainly composed of lymphocytes with few giant cells and macrophages.
Angioblastic proliferation consisting of numerous normal-sized or mildly dilated blood vessels was observed. Wavy collagen fiber deposition was noted in the vicinity of the implanted tube and a well-organized fibrous capsule surrounded the tissue reaction (Fig. 6).

At the 8th week observation period, the granulation tissue surrounding the implanted tube consisted of wavy collagen fibers, numerous normal-sized blood vessels and sparse dispersed chronic inflammatory cells (mainly lymphocytes, with few giant cells). A well-formed fibrous capsule surrounded the tissue reaction. There was no evidence of calcification or necrosis throughout the experiment (Fig. 7).

Fig (5) Photomicrograph of Ceram x group after one week revealing numerous normal or moderately dilated blood vessels (arrows). Chronic inflammatory cells (I) and delicate collagen fibers (F) are detected surrounding the site of the implanted tube (T), (H&E x40)

Fig (6) Photomicrograph of Ceram x group after 4 weeks revealing numerous normal or mildly dilated blood vessels (arrows). Chronic inflammatory cells (I), wavy collagen fibers and a well-organized fibrous capsule (F) surrounded the implanted tube (H&E x100)
Fig (7) Photomicrograph of Ceram x group after 8 weeks revealing a dense aggregation of granulation tissue infiltrated by chronic inflammatory cells (I) and a well-organized fibrous capsule (F) surrounding the implanted tube site (T), (H&E x40). A higher magnification of the granulation tissue and a giant cell are seen on the right (H&E x100).

**Ketac N 100**

A severe inflammatory infiltration of both the acute and chronic type was seen in response to Ketac N 100 after 1 week. Angioblastic and fibroblastic proliferations were encountered. Hyperemic blood vessels, extravasated red blood cells and dense aggregation of granulation tissue surrounded the implanted tube (Fig. 8).

In the intermediate observation period, marked differentiation of cells of the mononucleate phagocytic system into macrophages and multinucleated giant cells was observed. The fibrous response manifested as wavy collagen fiber deposition. Moderate blood vessel dilatation and extravasated blood cells were noted (Fig. 9).

After 8 weeks, the vascular response was less intense, whereas moderate chronic inflammatory cell infiltration and dense granulation tissue were still observed. No evidence of necrosis could be detected throughout the experiment, while encapsulation and calcification were observed in the third observation period (Fig. 10).

Fig (8) Photomicrograph of Ketac N 100 group after one week revealing dilated blood vessels engorged with red blood cells (B), severe chronic inflammatory cell infiltration (I) and dense aggregation of granulation tissue surrounding the implanted tube (T), (H&E x100).
Histochemical findings

Using the toluidine blue stain, mast cells were identified by their purple-colored cytoplasmic granules and their oval or round nucleus. In the control group, the number of mast cells remained negligible throughout the experiment. In the experimental groups, mast cells count was maximum 7 days after tube implantation, but slightly declined after 4 weeks, with subsequent decrease after 8 weeks. The mast cells were located within the fibrous and inflammatory tissue surrounding the implanted tubes. Mast cell degranulation was observed in most specimens (Fig. 11-12). The greatest number of mast cells was observed in Ketac N 100 group after one week. The difference between mast cells
number associated with the control and three experimental groups was statistically significant (p= 0.000), (Table 2, Fig. 15).

Fig. (11) Photomicrograph of Filtek supreme XT group after one week revealing mast cells (arrows) in the subcutaneous tissue at the vicinity of the implanted tube, (Toluidine blue x100). A higher magnification illustrating the purple granules of the mast cells is seen on the right (Toluidine blue x400)

Fig. (12) Photomicrograph of Ceram X group (A) and Ketac N100 group (B) after one week revealing mast cells (arrows) in the subcutaneous tissue at the vicinity of the implanted tube. Note the mast cell degranulation in the Ketac N100 group (Toluidine blue x400).

**Immunohistochemical findings**

CD 68 positive cells were observed in groups or singly dispersed within the tissue surrounding the implanted tubes in all experimental groups. These cells had the morphological features of macrophages and were identified by brown cytoplasmic staining (Fig. 13-14). Macrophages immunostaining reached its maximum level in the intermediate observation period with subsequent decline after 8 weeks. The greatest area % of immunoreactivity was detected in the Ketac N100 group. There was a statistically significant difference in CD 68 immunoreactivity between the control group and the three experimental groups (p=0.000), (Table 2, Fig. 15).
Fig. (13) Photomicrograph of Filtex Supreme XT (right) and Ceram X (left) after 4 weeks illustrating CD68 positive macrophages within the tissue reaction surrounding the implanted material (CD 68 x400).

Fig. (14) Photomicrograph of Ketac N100 after 4 weeks (left) illustrating CD68 positive macrophages within the chronic inflammatory reaction surrounding the implanted material. The macrophages count decreased after 8 weeks (right), (CD 68x400).

Table 2. Inflammatory scores, mast cells number and CD68 area % in the control and experimental groups and their statistical significance.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Inflammatory scores</th>
<th>Mast cell number</th>
<th>CD 68 area %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>One week 4 weeks 8 weeks</td>
<td>One 4 weeks 8 weeks</td>
<td>One 4 weeks 8 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>1 0 0</td>
<td>5.81±0.79 3.97±1.71 3.6±1.18</td>
<td>0.26±0.11 0.51±0.06 0.28±0.09</td>
</tr>
<tr>
<td>Filtex</td>
<td>2 1 0</td>
<td>16.72±3.26 12.33±2.44 7.88±2.25</td>
<td>0.67±0.14 2.48±0.87 1.98±0.64</td>
</tr>
<tr>
<td>Ceram X</td>
<td>2 1 0</td>
<td>11.84±4.56 7.99±2.56 4.91±2.59</td>
<td>0.54±0.15 1.58±0.66 1.16±0.43</td>
</tr>
<tr>
<td>KetacN100</td>
<td>3 2 2</td>
<td>23.37±6.33 15.74±5.78 9.76±2.25</td>
<td>0.83±0.26 4.89±1.31 3.67±1.09</td>
</tr>
<tr>
<td>F value</td>
<td>- - -</td>
<td>24.5803 17.2051 13.7328</td>
<td>15.2819 38.2615 37.2072</td>
</tr>
<tr>
<td>P value</td>
<td>- - -</td>
<td>0.000** 0.000** 0.000**</td>
<td>0.000** 0.000** 0.000**</td>
</tr>
</tbody>
</table>
Inflammatory scores, mast cells number and CD68 area%

![Bar chart showing inflammatory scores, mast cells number, and CD68 area% for different groups.](chart)

**Fig. (15) Inflammatory scores, mast cells number and CD68 area % in the control and experimental groups**

**DISCUSSION**

A dental restorative material may influence the health of oral soft tissues in several ways, especially by delivering water-soluble components into the saliva and the oral cavity as well as by interacting directly with the gingiva and periodontal ligament (Leyhausen, 1998). Therefore, the selection and evaluation of any material or device intended for use in humans requires a structured assessment into four phases; general toxicity (cell culture), local tissue irritation (animal implantation), pre-clinical (animal usage assays), and clinical evaluation (patient trials) to protect patients from possible hazards presented by dental materials and new biological restorative treatments (Murray et al, 2007).

The present study was conducted to access the biocompatibility of newly developed restorative materials. The tested materials were placed in 10 mm long, 15 mm inner diameter polyethylene tubes. Whilst materials to be tested were directly applied subcutaneously in some studies (Yesilsoy et al, 1988), the implantation of the materials in tubes is advocated in others (Zmener et al, 1990, Maher et al, 1992 and Holland et al, 1999). When compared to the direct application of the material, this method helps to provide stabilization of the material in place (Yaltirik et al, 2004) and to eliminate possible effects caused by different surface structures of individual specimens, thus
achieving the standardization of the material-tissue interface (Geurtsen, 2000; Yaltirik et al, 2004).

The reactions to the empty tubes in this study were similar to those of Yaltirik et al, (2004) and Zmener (2004), who reported an initial slight concentration of inflammatory cells in the subcutaneous tissues adjacent to the controls. This reaction rapidly subsided by time and a thin fibrous capsule surrounded the tube (Yaltirik et al, 2004 and Zmener, 2004). This reaction is probably the result of surgical trauma of tube implantation. However, microscopic analysis done by Batista et al, 2007 showed that the empty polyethylene tubes caused no inflammatory reaction.

In the present study, a moderate inflammatory reaction was noted in association with the nanocomposite restorative material Filtek supreme XT. The persistence of a chronic inflammatory response to the composite resin may be due to the continued breakdown or release of irritant products from the restoration. It has been found that, due to degradation and/or corrosion, several components including the triethylene glycol-dimetaacrylate (TEGDMA) are leached out from composite resin in considerable amounts in the first 24 hr after polymerization. This, in turn may cause adverse local and/or systemic effects (Geurtsen, 1998).

The constituents of Filtek-supreme XT: BisGMA, BisEMA, UDMA and TEGDMA have been linked to various adverse biological reactions. The higher cytotoxicity of TEGDMA compared with HEMA could further be explained by its ability to interact with the lipid bilayer of cell membranes in a surfactant-like manner (Schuster et al. 1996) and/or by its ability to cause lipid peroxidation in vitro as it was already described by Fujisawa et al. (1984). Both membrane effects can cause cell death (Raffray and Cohen 1997). Moreover, it has been demonstrated that the (co)monomer TEGDMA may also significantly interfere with intracellular glutathione (Freidig et al. 2001; Engelmann et al. 2002) and that early and extensive depletion of the intracellular GSH level in tissues may significantly contribute to cell death (Engelmann et al. 2002). Moreover, the nearly complete exhaustion of this molecule significantly reduces its cellular detoxifying potency (Geurtsen and Leyhausen, 2001). On the other hand, the cytotoxicity of BisGMA might depend on its high liposolubility (Issa et al. 2004).

TEGDMA has been reported to be toxic in different cell lines [Geurtsen et al, 1998, Theilig et al, 2000, Kehe et al, 2001]. Al-Hiyasat et al (2005) stated that the cytotoxicity could be related to the amount of TEGDMA that was leached from the flowable composites compared with their non-flowable traditional composite. The term ‘cytotoxicity’ is used to describe the cascade of molecular events that interfere with macromolecular synthesis, causing unequivocal cellular and functional and structural damage (Aldridge, 1993). Although in the present study necrosis was not detected in the Filtek supreme XT group, the recorded vascular, inflammatory and fibrous responses could be attributed to the cytotoxicity of the components of this material. This conclusion accords with the notion of Geurtsen (2000) that the high cytotoxic potency of TEGDMA may significantly contribute to adverse local and systemic effects.
Furthermore, the inflammatory responses observed in the Filtek group in this study accords with the results of Schmaltz et al (2000) who observed that the treatment of oral epithelial cells with non-toxic TEGDMA concentrations elevated the IL-6 expression five folds. This cytokine participate in tissue inflammation through the modulation of B-cell differentiation and T cell proliferation. Recently, Chang (2009) suggested that BisGMA released from composite resin may potentially induce pulpal inflammation via stimulation of reactive oxygen species (ROS) production, subsequent cyclo-oxygenase (COX-2) gene expression and prostaglandin (PGE2) production. Furthermore, the tendency of TEGDMA to promote microbial growth, may contribute to tissue injury (Spahl et al, 1998). The tissue reactions noted in the present study are consistent with Ozbas et al. (2003).

In the present study, one week results showed that moderate inflammatory responses developed in the subcutaneous tissues of the Ceram X group. The intensity of this reaction subsided in the subsequent observation periods. An increasingly thickening fibrous capsule surrounded the implanted material. These findings indicate that the material is well tolerated by the tissues. These findings accord with (Sjogren, 2000) who reported no evidence of cytotoxicity in the dental ceramics studied, indicating good biocompatibility in vitro. Reports on biocompatibility problems with dental ceramics in vivo have been rare. Breakdown products of dental ceramics have not been reported to have known toxic effects, and several of the ions in dental ceramics are considered nontoxic (Schuster, 1996). However, the tissue responses observed in the Ceram X group in the present study are consistent with the notion of Messer et al (2003) that even ceramic materials show biological effects to varying degrees.

The inflammatory reaction associated with Ceram X could be partially attributed to its content of titanium oxide, which was reported to cause irritation in the periapical area (Erausquin, 1970). Moreover, the photo-initiator camphoroquinone present in Ceram X was found in significant amounts in aqueous extracts from resin-based materials and revealed moderate cytotoxic effects. This was confirmed by Atsumi et al. (1998) with permanent human submandibular-duct cells.

In the current study, it was noticed that the tissue responses elicited by Ketac were consistently more intense than those provoked by Filtek supreme XT and Ceram X. Hume and Gerzina, 1996 considered that the nature of the tissue responses to subcutaneously implanted materials was either allergic or toxic.

The findings of the present study confirm those of Souza et al, who detected an inflammatory response that varied from moderate to intense at 7 days after subcutaneous implantation of resin modified glass-ionomer samples in rats. These authors linked these tissue responses to the cytotoxicity of the released HEMA (Souza et al, 2006). Ribeiro et al (2006) and Schmid-Schwan et al (2009) reported that some components of glass–ionomer cements show both genotoxic and cytotoxic effects in higher concentrations. Similarly, Nicholson and Czarnecka (2006) considered that resin-modified glass-ionomers cannot be considered biocompatible to nearly the same extent as conventional
glass-ionomers because 2-hydroxyethyl methacrylate (HEMA) which has a variety of damaging biological properties is known to be released from these materials.

Several studies revealed various adverse biological actions caused by HEMA which enters in the composition of Ketac N100. The study of Buollaguet et al, (2000) demonstrated clearly that extremely small amounts of HEMA are capable of causing major disruption to functioning cells, inhibiting proliferation and decreasing mitochondrial activity by 60–80%. Moreover, HEMA has been shown to elicit an immunological response in mice, as it is able to bind to endogenous proteins, which leads to the possibility of auto-antibody production in vivo [Sandberg et al, 2002]. Experiments showed that HEMA induced apoptotic death in peripheral blood mononuclear cells (Becher et al, 2006). Furthermore, it has been shown that micronuclei develop in cells affected by HEMA and TEGDMA [Schweikl et al, 2006]. This indicates that HEMA causes damage to the chromosomes and breaks the DNA strands. The tissue responses provoked by Ketac N100 in the present study can be attributed to the above mentioned biological effects.

Furthermore, the present study demonstrated numerous calcific deposits in association with Ketac N 100, thus denoting a significant remineralization potential exerted by this fluoride-releasing restorative material. This finding is in line with ten Cate and van Duinen, (1995) who reported hypermineralization of dentinal lesions adjacent to glass-ionomer cement restorations.

In the present study, collagen fiber deposition was noted in the Ketac N 100 group. This is consistent with Leyhausen et al, 1998, who reported no or minimal growth inhibition of human primary fibroblasts of the attached gingiva and permanent mouse fibroblasts exposed to Ketac Fil Applicap™ extract for 48 hours. The presence and disposition of fibrous tissue [Economides et al, 1995, Kolokouris et al, 1998], around implants of dental materials, are indicative to tissue response [Costa et al, 2000].

It is clear that mast cells play a variety of roles as an important part of the innate immune system, in a variety of circumstances including infections (Malaviya et al, 1996-Jippo et al, 2003), angiogenesis (Crivellato and Ribatti, 2005), and autoimmune diseases (Eklund, 2007). In the present study, an increase in mast cell numbers was noted in the experimental groups throughout the experiment. Moreover, mast cell degranulation was detected by the toluidine blue stain. According to Shoshana et al (2008), mast cells are defined by the presence of granules containing a variety of effector molecules, most notably serine proteases of several subtypes. Mast cells originate from the bone marrow, yet their final differentiation takes place in tissues and is influenced greatly by their interactions with those tissues.

The increase in mast cell number noted in the present study indicates that the test materials provoked an immune response. This theory is emphasized by the fact that IgE- and IgG antibodies are among the major stimulators of mast cells (Shoshana et al, 2008). Moreover, the current study revealed an increased fibroblastic response in association with the increased numbers of mast cells. This can be explained by the fact that mast cells
can affect the fibroblast functional behavior and, consequently, the fibrosis process, by releasing pre-formed mediators—such as histamine, proteoglycans, proteolytic enzymes and cytokines (Kupietzky and Levi-Schaffer, 1996).

Moreover, the present experiment detected prominent angioblastic proliferation in relation to the mast cells, consistent with the notion that mast cells release pro-angiogenic factors such as heparanase and VEGF (Shoshana et al, 2008). Furthermore, inflammatory reactions were detected in association with the degranulated mast cells in the present study. Mast cell degranulation with histamine release and fibrinogen adsorption is known to mediate acute inflammatory responses to implanted biomaterials (Zdolsek et al, 2007, Tang et al, 1998). Interleukin-4 (IL-4) and interleukin-13 (IL-13) also are released from mast cells in a degranulation process and can play significant roles in determining the extent and degree of the subsequent development of the foreign body reaction (Keegan 2001, McKenzie and Matthews, 2001).

The mononuclear phagocyte system consists of closely related cells of bone marrow origin, including blood monocytes and tissue macrophages. From the blood, monocytes migrate into various tissues and transform into macrophages. The journey from bone marrow stem cell to tissue macrophage is regulated by a variety of growth and differentiation factors, cytokine adhesion molecules and cellular interactions (Collins, 1999).

Biomaterials may initiate several and complex biological reactions in host tissues, and the cell biomaterial interactions can determine the release of mediators including monocytes and lymphocytes chemotactic factors (Bosetti et al, 1999). In the present study, macrophages were observed within the tissue reaction surrounding the implanted materials. The progression of events in inflammation and the foreign body response requires the extravasation and migration of monocytes/macrophages to the implant site. The guided movement of monocytes/macrophages occurs in response to chemokines and other chemoattractants (Anderson et al, 2008). Cellular interactions which occur at the tissue implant interface represent an important determinant of biomedical polymer biocompatibility (Remes and Williams, 1991). Macrophage activation induce the cells to further differentiate, phagocytose or secrete products to the surrounding environment (Trevor, 1990). In the current study, angioblastic and fibroblastic proliferations were detected in association with macrophages, supporting the notion that macrophages are capable of secreting growth and angiogenic factors that are important in the regulation of fibro-proliferation and angiogenesis. [Martin P and Leibovich, 2005]. On the other hand, multinucleated giant cells were demonstrated within the tissue reaction surrounding the implanted materials in the present study, thus indicating that biomaterial surface adherent macrophages fuse to form foreign body giant cells. This notion is supported by Athanasou and Quinn (1990) who detected that foreign body giant cells display an antigenic phenotype similar to monocytes and macrophages, reflecting the fact that giant cells are formed from the fusion of monocyte-derived macrophages.
CONCLUSION

Although the reactions analyzed in the present study do not closely reflect those in the teeth and oral tissues, they are valid in that they constitute a preliminary stage in evaluating the irritation potential of these materials. The implanted materials induced different and time dependent inflammatory reactions, mast cells and microphages migration, in addition to distinct fibrosis development. Ketac N 100 revealed a less biocompatible tissue reaction compared to Filtek supreme NT and Ceram X.

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