

Inflammatory Response to Injectable Biomaterials for Stabilisation of Vertebral Compression Fractures

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Biomaterials used for stabilisation of compressed vertebrae due to osteoporosis are currently mainly based on conventional bone cement, polymethyl methacrylate (PMMA). New material alternatives based on fully ceramic materials, e.g. calcium aluminates cements (CAC), are under development. In this in vivo study the early inflammatory response elicited by both cured and uncured CAC and PMMA cement was investigated in a rat model. Titanium (Ti) and sham sites were used as controls. Cell viability, cell proliferation, inflammatory cell recruitment and pro inflammatory cytokine secretion of tumour necrosis factor alpha (TNF- α) and monocyte chemoattractant protein-1 (MCP-1) was evaluated. The experimental observation period was 1 day and the results showed that both of the cured cements were more biocompatible and revealed a smaller inflammatory response than Ti, contrary to the uncured cements which provoked a larger inflammatory response and higher cell death than Ti. The inflammatory responses revealed by cured CAC and PMMA had no significant difference. © Society for Biomaterials and Artificial Organs (India), 20090622-45.

Introduction

Vertebral compression fractures (VCF) are the hallmark of osteoporosis and the incidence shows an exponential increase with age [1]. VCF is a leading cause of disability and pain in the elderly and this injury will become even more frequent in the future, because of the increasing amount of elderly people [2].

Over the last decade VCF has been treated by vertebroplasty, which includes injection of cement into the affected vertebrae through a needle [3]. The injectable cement sets and hardens in situ, which results in an immediate stabilisation of the vertebrae which in turn result in significant reduction of pain for the patient [4]. The materials used in this treatment has until now been based on conventional bone cement, polymethyl methacrylate (PMMA). More recently new cements have been evaluated,

based on ceramics, such as calcium phosphate cements and calcium aluminate cements (CAC). A recent study has shown that CAC has similar mechanical properties as PMMA [5].

The early inflammatory response following implantation of a biomaterial into the body is of great interest, since this can generate information about the healing process, and thus the biocompatibility of the material. When a biomaterial has been introduced into the body, a haematoma is formed due to the trauma induced by surgery [6]. This is followed by migration of inflammatory cells from the microcirculation to the interface between the biomaterial surface and the injured tissue. The role of the inflammatory phase, (which lasts about 3 days in rodents, a week in rabbits, and

a couple of weeks in man) is to remove the debris due to trauma and to provide the appropriate signals for the shift from inflammation to repair and regeneration of the tissue [7].

The size, shape, and chemical and physical properties of a biomaterial may be responsible for variations in the intensity and duration of the inflammatory or wound-healing process. Thus, intensity and/or time duration of the inflammatory reaction may characterize the biocompatibility of a biomaterial [8]. The predominant cell type present in the inflammatory response varies with the different phases. Neutrophils predominate during the first phase, acute inflammation, and are then replaced by monocytes during the later phase, chronic inflammation. The acute inflammation lasts for minutes to days. It is characterised by the exudation of fluid and plasma proteins and the emigration of leukocytes, mainly neutrophils (polymorphonuclear neutrophils, PMN), from the vascularisation to the injury/implant site. Neutrophils are short-lived and disappear after 24-48 hours. An exchange of neutrophils to mononuclear cells occur in the exudate around Ti implants during the first 3-48 hours of implantation in the rat s.c. tissue [9]. The neutrophil emigration is assisted by cell adhesion molecules, i.e. the cytokines tumour necrosis factor alpha (TNF- α) and interleukin-1 (IL-1). The major role of PMN cells in acute inflammation is to release lysosomal enzymes and phagocytose microorganisms and foreign materials.

Chronic inflammation is characterised by the predominance of mononuclear cells (macrophages, monocytes and lymphocytes), and tissue destruction, neovascularisation and fibrosis. Following emigration from the vasculature, monocytes differentiate into macrophages, which may live up to months. Monocyte migration may continue for days to weeks, depending on the injury and the implanted biomaterial. Macrophages are the most important cells in chronic inflammation because of the great number of biologically active products it can release, e.g. chemotactic factors, cytokines (e.g. TNF- α), arachidonic acid metabolites, reactive oxygen metabolites, complement components, coagulation factors, growth-promoting factors and neutral proteases. Monocyte chemo attractant protein-

1 (MCP-1) is an essential chemokine involved in monocyte migration across endo- and epithelial barriers to sites of injury and infection [10]. While TNF- α promotes acute inflammation, MCP-1 promotes chronic inflammation.

To date, not much information about the inflammatory response elicited by CAC and PMMA has been reported. This in vivo study aims to evaluate the early inflammatory response provoked by both cured and uncured CAC and PMMA cement in a rat soft tissue model previously used for other implant materials [11]. Ti and sham operation sites serve as controls. Ti represents a material with low magnitude of the inflammatory response and very good clinical performance, while sham operation sites serve as inflammatory control elicited by the operational procedure.

Materials and Methods

Implants: The injectable vertebroplasty cements used in this study were PMMA (Vertebroplastic, LOT 1927216, LOT 2231254, DePuy Spine, Johnson & Johnson, Sollentuna, Sweden) or CAC (Xeraspine, LOT HPC-03-0702001, Doxa, Uppsala, Sweden). In order to reduce the form factor of the injectable materials, a system of titanium cups holding the material was manufactured with the following dimensions (height 4 mm, \varnothing 10 mm and basin wall thickness 1 mm). The cups were filled with cements (Figure 1) leaving one surface with the material tested. The materials were let curing prior to surgery, either 7 minutes for handling reasons during surgery (non-cured) or during 14 days to obtain a fully cured material (cured). All implants were grinded to down to 1000 grit silica paper, representing a surface roughness of well below 1 μ m. The two groups represent the inflammatory response from the setting reaction (uncured) and to the material (cured). Solid titanium disks (height 4 mm, \varnothing 10 mm) and sham operations sites were used as controls. All implants were sterilized by gamma radiation.

Animal model: The operation procedure is described in detail in an earlier study [12]. In brief, 24 Female Sprague-Dawley rats, weighing 225-275 g, fed on standard pellet diet and water were used. The rats were anaesthetized with a mixture of 2.7 % isofluran and air (Univentor 400 Anaesthesia Unit, Univentor, Malta) and

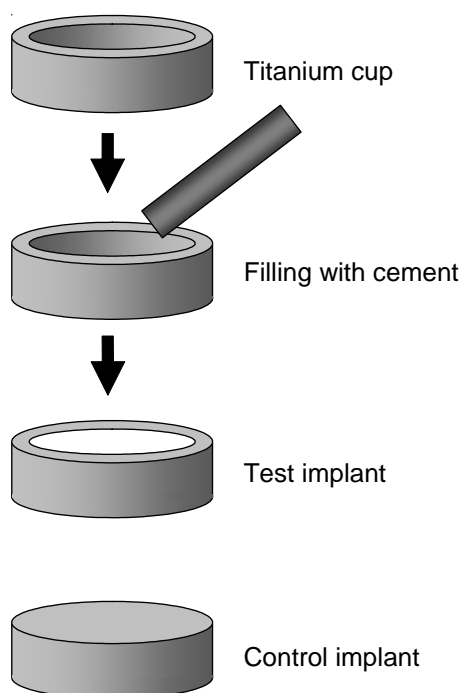


Figure 1: Schematic presentation of the implants used. For the cements titanium cups were filled with the cement in order to keep a uniform shape. One side of the test implants possessed the cement either PMMA or CAC, either as fully cured (cured) or as minimally cured (uncured). The control implants were solid titanium disks with the same dimensions

0.01 mg Temgesic (Schering-Plough AB, Stockholm, Sweden) was given as analgesic s.c. pre-operatively. They were shaved on the dorsum and the skin cleaned with 5 % chlorohexidine (5 mg/ml Pharmacia AB, Stockholm, Sweden) and about 10 mm long incisions were made through the skin about 15 mm lateral to the midline, followed by the creation of subcutaneous pockets by careful blunt dissection.

Implant installation was performed according to a randomization scheme, made for 3 different groups of animals (8 rats per group) in order to avoid systemic effects of the uncured materials (Table 1). After implant installation the skin was closed with 3 sutures of non-resorbable Suturamid® 5-0 Fs-2 (Ethicon Inc, Sommerville, N.J., USA). There were no implant pockets in

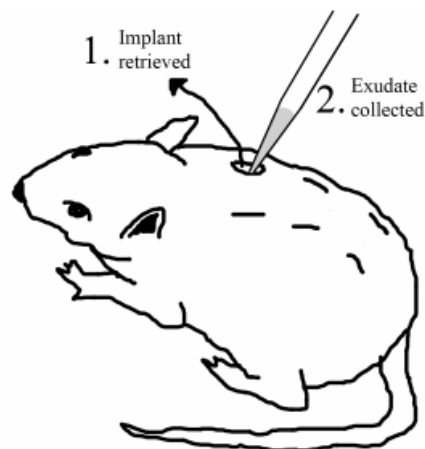


Figure 2: Schematic image of the retrieval of the implant and the collection of the exudates from the surgical pockets

contact with each other. All surgical procedures were performed in an aseptic way with sterilised instruments. Experiments were approved by the Local Ethics Committee, Göteborg University.

The animals were harvest after 1 day post-operative, where the rats were anaesthetized, cleaned with 5 % chlorohexidine and sacrificed by an overdose of pentobarbital i.p. The sutures were taken away and the wound surfaces were gently drawn apart with tweezers to retrieve the implant. Each implant was gently retrieved with tweezers and placed in separate polystyrene wells with 400 µl lysis buffer (Reagent A100, Nucleocounter™ system, ChemoMetec A/S, Denmark) and 400 µl stabilising buffer (Reagent B, Nucleocounter™ system, ChemoMetec A/S, Denmark) and the number of attached cells was calculated by counting cells

Table 1: Summary of the study groups. The uncured materials were separated due to possible systemic effects, while the pre-cured materials could be evaluated in the same animals saving an additional group

Implants	Group 1	Group 2	Group 3
	Cured material	Uncured PMMA	Uncured CAC
PMMA (Test)	1	2	
CAC (Test)	1		2
Ti (Control)	1	2	2
Sham (Control)	1	2	2
Number of rats	8	8	8

with the NucleoCounter™ system (ChemoMetec A/S, Denmark). After removal of the implant, the exudate in respective site were separately collected (Figure 2) by repeated aspirations (x5) with total 300 µl sterile HBSS (with Ca²⁺ and Mg²⁺, Gibco, UK) and were kept on ice. A small amount from each exudate (10 µl exudate/sample) was stained with for calculation of concentration and number of PMN and Mono cells. The exudates were centrifuged 5 min at 400 g and the supernatants were saved for the analysis of lactate dehydrogenase (LD), TNF- α and MCP-1 and the cell pellets were prepared with 50 µl lysis buffer (Reagent A100, Nucleocounter™ system, ChemoMetec A/S, Denmark) and 50 µl stabilising buffer (Reagent B, Nucleocounter™ system, (ChemoMetec A/S, Denmark) for counting of total number of exudates cells. The amount of cells was counted by the NucleoCounter™ system (ChemoMetec A/S, Denmark).

Inflammatory cells analysis: The total cell concentration and percentages of PMN and Mono cells in the exudates were calculated by staining the cells with the nucleostaining Gentain violet and number of PMN and mononuclear cells were counted by light microscopy using burker chamber.

Cell viability analysis: The lactate dehydrogenase (LD) concentrations (µkatal/L, Sahlgrenska University Hospital, Göteborg University) in the exudates were determined and expressed as total amount.

Pro-inflammatory analysis: For determination of the content of secreted TNF- α and MCP-1 the Quantikine® rat TNF- α Immunoassay (R&D

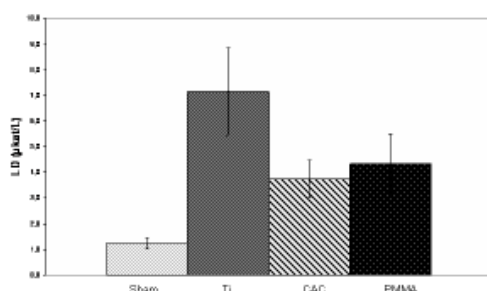


Figure 3: LD-values representing the viability of the cells on sham sites (n=8), Ti (n=7), CAC (n=8) and PMMA (n=8). A significant difference was found between sham and the implants

systems, UK) and [(r MCP-1] Biotrak ELISA system (Amersham Biosciences, UK) were used. All exudate supernatant samples were frozen at -70°C until analysis. The samples were placed in the pre-coated wells and processed according to the suppliers of the assays. The optical density was detected in an ELISA reader (SpectraMAX plus, Molecular Devices, Crawley, UK) by subtracting readings at 540 nm from readings at 450 nm. HBSS (with Ca²⁺ and Mg²⁺, Gibco, UK) were analysed as a negative control to normalize the possible negative interactions from buffer salt in the exudates samples. Standard curves run in parallel with the samples were used for the determination of the cytokine concentrations. Assay range for MCP-1 sensitivity < 5pg/ml: TNF- α sensitivity < 5pg/ml.

Statistical analysis: Statistical analysis was performed with the software StatView 4.5. The Friedman Test was used in order to evaluate if there were any significant ($p < 0.05$) differences in-between the different groups. For further analysis non-parametric Wilcoxon Signed Rank Test was used to compare individual groups by paired observations.

Results

Cured materials

The cured materials, PMMA and CAC, were hard and no signs of uncured material were noticed during surgery. There were no complications during surgery and no adverse effects on the rats after 24 hours. However, one Ti implant had fallen out from the surgical pocket due to loosen sutures and could not be analyzed. The error bars in each of the histogram below represent the standard error. Number of implants, n=8 for sham, CAC and PMMA, and n=7 for Ti.

Cell viability: The viability of the cells was significantly lower at the sites with implants compared to the sham operation (high LD-concentration corresponds to low cell viability). The trend among the implants was higher viability for cells around CAC and PMMA compared to Ti, however not statistically significant (Figure 3).

Cell attachment: The total amount of cells was measured both in the exudates retrieved from the surgical pocket at explantation and cells which had attached to the implant materials. Ti

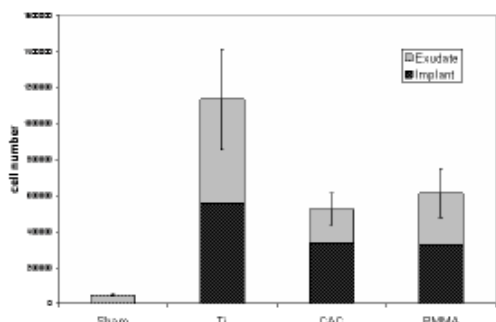


Figure 4: Total amount of cells found on the implant (light) and in the exudate (dark) for sham sites (n=8), Ti (n=7), CAC (n=8) and PMMA (n=8). A significant difference was found between sham and the implants

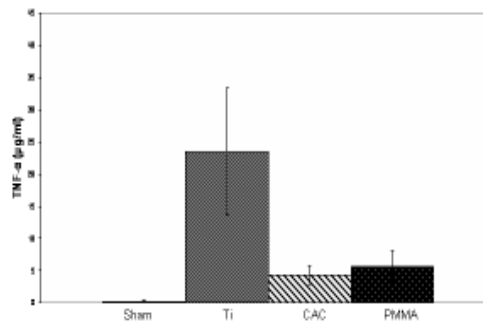


Figure 6: Amounts of TNF-α found in the surgical pockets of explantation for sham sites (n=8), Ti (n=7), CAC (n=8) and PMMA (n=8). A significant difference was found between sham and the implants respectively between Ti and the other groups

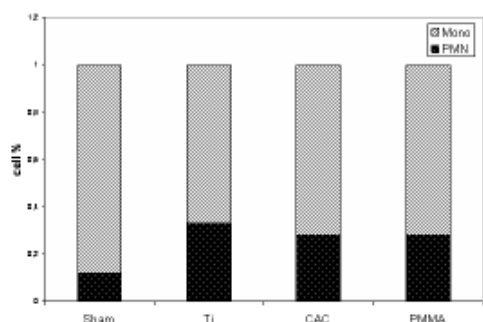


Figure 5: Relative quantity of PMN and Mono cells among the total number of cells in the surgical pocket at the time of explantation for sham sites (n=8), Ti (n=7), CAC (n=8) and PMMA (n=8). No significant difference was found

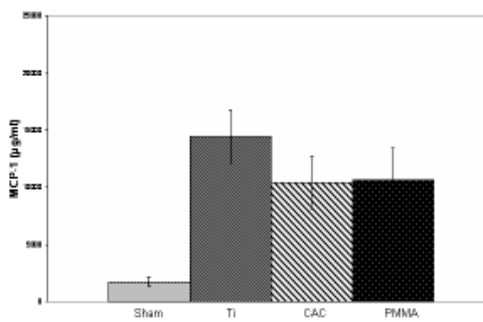


Figure 7: Amounts of MCP-1 found in the surgical pockets of explantation for sham sites (n=8), Ti (n=7), CAC (n=8) and PMMA (n=8). A significant difference was found between sham and the implants

attracted most cells, however not significant, compared to the other implants. The amount of cells was lower at the sham operation site compared to all material sites (Figure 4).

Inflammatory cells: The cell type in the surgical pocket was investigated in terms of PMN and Mono cells. The amount of PMN cells was around 30% for the implant materials while the sham site had 12%. No significant difference was found between the groups (Figure 5).

Pro-inflammatory response: The pro-inflammatory response, TNF-α and MCP-1 were measured in from the supernatants of the centrifuged exudates from the surgical pocket. Ti showed significantly higher concentrations

of TNF-α compared to the others and all the implants showed higher concentrations than the sham site (Figure 6). The MCP-1 concentrations were significantly higher in the exudates from the implant materials compared to the sham operation site. Both CAC and PMMA showed less inflammatory response, ie. TNF-α, than Ti (Figure 6). CAC and PMMA showed significantly lower levels of MCP-1 than Ti (Figure 7).

Uncured materials

The uncured materials, PMMA and CAC, were implanted in different rats and compared with sham sites and Ti. The error bars in each of the histogram below represent the standard error

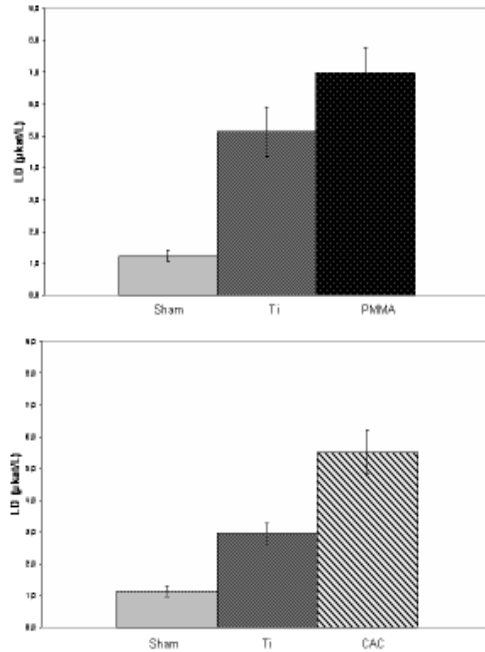


Figure 8: LD-values representing the viability of the cells. A) Sham sites, Ti and uncured PMMA. (n=16). A significant difference was found between sham and the implants. B) Sham sites, Ti and uncured CAC (n=16). A significant difference was found between sham and the implants respectively between Ti and CAC

of the mean and based on n=16 (8 rats x2/rat) for all groups.

Cell viability: The cell viability was significantly lower for the implants sites than for the sham sites. Contrary to the cured materials, both PMMA and CAC provided lower cell viability than Ti. The cells on CAC were significantly less viable than on the Ti-implants, while there were no significant difference between PMMA and Ti (Figure 8).

Cell attachment: The amount of cells was measured in the same manner for the uncured materials as for the pre-cured ones, i.e. both in the surgical pocket (exudat) at explantation and cells attached to the implants. The implants attracted significantly more cells than the sham sites, while there were no significant difference between PMMA and Ti respectively between CAC and Ti. There were more cells attached to the implants, than cells in the exudat. There

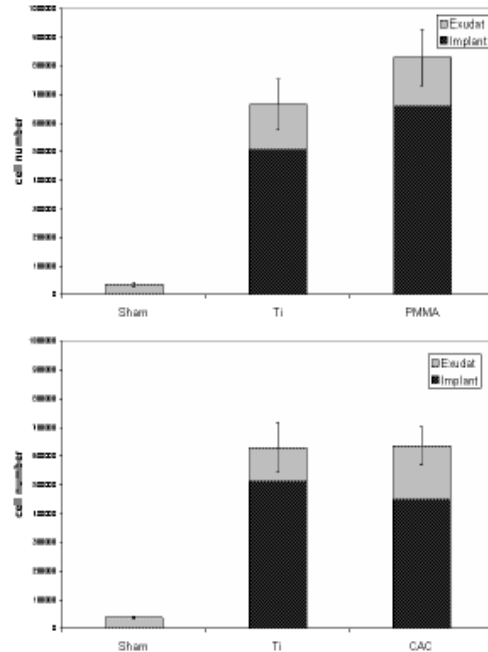


Figure 9: Total amount of cells found on the implant (light) and in the exudate (dark). A) Sham sites, Ti and uncured PMMA (n=16). A significant difference was found between sham and the implants. B) Sham sites, Ti and uncured CAC (n=16). A significant difference was found between sham and the implants

were more cells on the uncured PMMA and CAC compared to Ti, contrary to the cured ones, which attracted fewer cells than Ti (Figure 9)

Inflammatory cells: The cell types, i.e. PMN and Mono cells, in the surgical pocket were investigated in the same manner as for the cured materials. The trend of the amount of PMN cells was the same for the uncured materials as for the cured materials, i.e. that the implants recruited more PMN cells than the sham sites. There were significantly more PMN cells in the exudates around PMMA and CAC respectively, than in the sham sites. The PMN cell number was greater in the exudate around the uncured PMMA and CAC than on the Ti, however not significantly, contrary to the cured ones, which had fewer PMN cells than Ti (Figure 10)

Pro-inflammatory response: The pro-inflammatory cytokines TNF- α and MCP-1 were

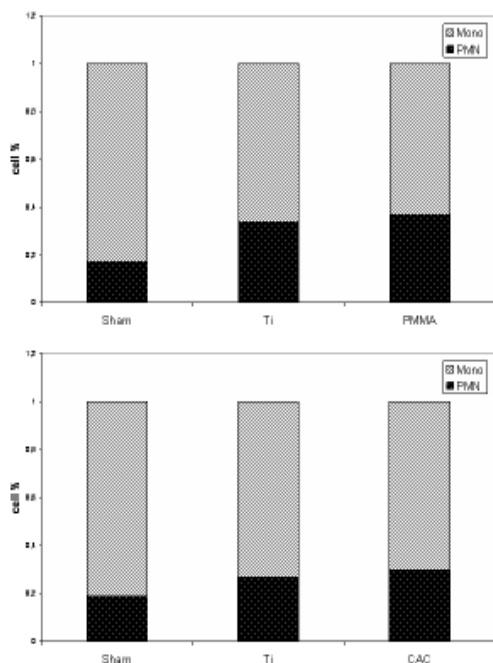


Figure 10: Relative quantity of PMN and Mono cells among the total number of cells in the surgical pocket at the time of explantation. A) Sham sites, Ti and uncured PMMA (n=16). A significant difference was found between sham and PMMA. B) Sham sites, Ti and uncured CAC (n=16). A significant difference was found between sham and CAC

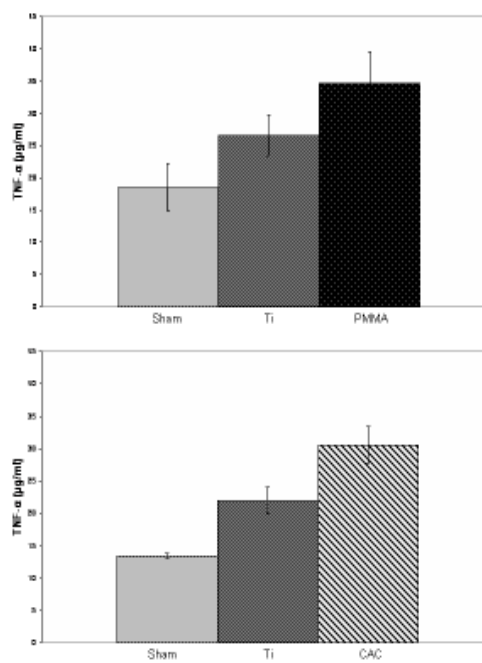


Figure 11: Amounts of TNF- α found in the surgical pockets of explantation. A) Sham sites, Ti and uncured PMMA (n=16). A significant difference was found between sham and the implants. B) Sham sites, Ti and uncured CAC (n=16). A significant difference was found between sham and the implants respectively between Ti and CAC

measured in the supernatants of the centrifuged exudates from the surgical pocket. Contrary to the cured materials, the uncured materials showed higher concentrations of TNF- α in the exudates around the PMMA and CAC than around Ti, however only significantly higher on CAC. There were higher concentrations of TNF- α in the exudates around PMMA and CAC respectively, than in the sham sites (Figure 11).

The trend of the MCP-1 on the uncured materials was that PMMA and Ti provoked almost the same amount of these cytokines (no significant difference), while CAC had significantly higher amount of MCP-1 than Ti. All the implants had significantly more MCP-1 than the sham sites (Figure 12).

Discussion

Cell viability: All the implants elicited higher LD-values in the exudate indicating less viable cells

than the sham operation sites. While the uncured cement materials, PMMA and CAC, provoked lower cell viability than the Ti-implants, the cured cements elicited higher cell viability than Ti. The uncured materials have a higher chemical activity compared to the cured materials and Ti. This results in increase in temperature and possible monomer leakage (i.e. PMMA) and increase in pH of the surrounding tissue (i.e. CAC). This can be the cause to the lower cell viability [3].

Cell attachment: All the implants attracted more inflammatory cells than the sham sites. The difference between the uncured and cured materials was similar regarding attraction of inflammatory cells as for the LD-values, i.e. the cured cement materials attracted less inflammatory cells than Ti, while the uncured cements attracted almost the same amount of cells (CAC) as, or more (PMMA) than Ti. Another

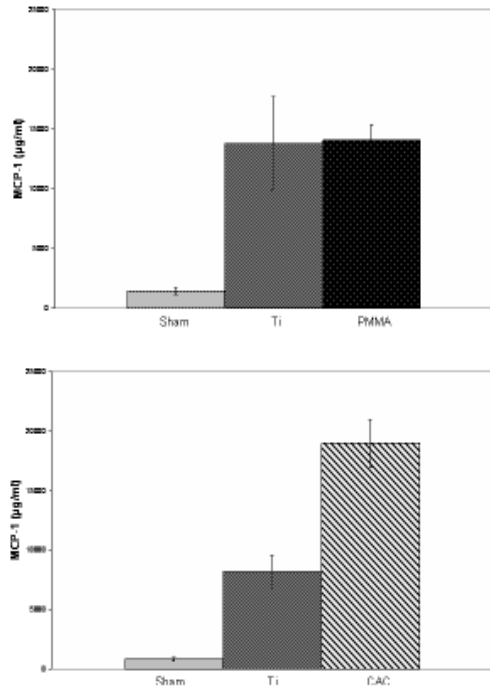


Figure 12: Amounts of MCP-1 found in the surgical pockets of explantation. A) Sham sites, Ti and uncured PMMA (n=16). A significant difference was found between sham and the implants. B) Sham sites, Ti and uncured CAC (n=16). A significant difference was found between sham and the implants respectively between Ti and CAC

tendency was that the uncured implants attracted more cells than were found in the surgical pocket (exudate), while the cured implants attracted almost the same amount of cells as the exudates. These results may be explained by a different surface structure obtained when curing *in vivo* rather than *ex vivo*.

Inflammatory cells: The implants provoked a higher amount of acute inflammatory cells, PMN cells, than the sham sites. While the cured cements attracted less PMN cells than Ti, the uncured cements attracted slightly more PMN cells than Ti. This inflammatory response is similar to those mentioned in the cell viability and proliferation results. Since PMN cells are characteristic for acute inflammation, while Mono cells are characteristic for chronic inflammation, the amount of PMN cells can give an indication of the inflammatory response and

healing process around the biomaterial. Correlations between cell toxicity and acute inflammatory response have been shown in a study. The more toxic a material was the more prolonged was the recruitment of acute inflammatory cells, PMN cells [9]. The present study also indicates a possible correlation between the cell viability and the PMN cell amount. The cured cements provoked higher cell viability response and lower recruitment of PMN cells than Ti and the uncured cements. Since there was only one measurement point in this study, one day after the implantation, no kinetic information about the inflammatory response can be given.

Proinflammatory cytokines: The secretion of the proinflammatory cytokine TNF- α was much lower in the exudates around the cured cements than on Ti, compared to the uncured cements which provoked a higher cell-secretion of this cytokine than Ti. This trend is similar to the previous results.

The secretion of the chemokine MCP-1 had a different pattern compared to the secretion of TNF- α . The amounts of MCP-1 on the cured cements were closer to that on Ti, although in a lesser extent. For the uncured cements the MCP-1 was almost the same for PMMA as for Ti, while the MCP-1 amount on CAC was significantly higher than on Ti. This might be an indication of a faster inflammatory response and healing process for CAC than for PMMA, since MCP-1 attracts monocytes, the characteristic cells in chronic inflammation.

According to Andersson et al, there might be a correlation between the surface flexibility (high molecular mobility) and the chronic inflammation response. Surfaces with more flexibility caused slightly more chronic inflammation than more rigid ones. The flexibility of a surface might also have a correlation with the thickness of fibrous tissue formed in the biomaterial-biological tissue-interface [13]. This might lead to a possible correlation of the thickness of the fibrous tissue and the chronic inflammation response. The PMMA bone cement interface has a non-bone bonding character since a fibrous tissue is formed, while the interface between CAC and the biological tissue is very intimate [14, 15].

Since both of the injectable cements PMMA and CAC harden inside the affected vertebrae within

a few minutes, their state as fluid cement is not persistent. The inflammatory cell response elicited by the uncured cements shows the relevant information regarding the cell-tissue response around the cement during the implantation, while the inflammatory response elicited by the hardened cements shows how the material will transient into a more biocompatible form. Both the material chemistry and the curing process seem to affect the inflammatory response. The precipitated hydrates formed during the curing process of CAC, leads to a more biocompatible form of the cement, while the exothermic reaction in the PMMA curing process causes a greater inflammatory reaction elicited by the uncured PMMA cement.

The present study only gives information about the inflammatory cell state one day after the implantation. Thus, this study does not give any kinetic information about the inflammatory response, but only indicates the main differences in the early inflammatory response after insertion of the implants.

Although there were some differences between the PMMA and CAC inflammatory responses, they were not significantly different. Since the uncured cements were placed in different rats, the difference between them could not be

detected directly, but only compared with the control materials. As Ti represents a material with low magnitude of the inflammatory response and good clinical performance, both PMMA and CAC showed very low inflammatory response, particularly in their cured form.

Taken together, this experimental study provides evidence that the cements elicit a similar acute cellular response in soft tissues as titanium.

Conclusions

All the implants promoted higher inflammatory response than the sham operation sites. The cured CAC and PMMA cements were more biocompatible and revealed a smaller inflammatory response than Ti, contrary to the uncured cements which provoked a greater inflammatory response and higher cell death than Ti. Cured CAC provided a slightly lower inflammatory response than cured PMMA.

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