IN VITRO GENOTOXICITY OF CALCIUM PHOSPHATE-BASED BONE SUBSTITUTE MATERIAL ON HUMAN PERIPHERAL MONONUCLEAR LEUKOCYTES

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ABSTRACT

Background and aim: Calcium phosphate-based bone substitute materials, also called calcium phosphate cements (CPCs), are widely used in orthopedic surgery. However, the reported results regarding the genotoxicity using these materials are inadequate in literature. We evaluated the DNA damaging effects of a CPC on peripheral mononuclear leukocyte cultures.

Material and methods: Mononuclear leukocytes from nine healthy donors blood samples were separated, and five culture plates were created from each donor’s cells. Three were used to evaluate concentration-related effects (10, 100, and 200 µg/mL) of CPC and the others were used as negative and positive controls. DNA damage was assessed using the comet assay, and changes in oxidative status parameters, including total oxidant status (TOS) and total antioxidant capacity (TAC), were measured using the remaining culture supernatant.

Results: We found that mononuclear leukocyte DNA damage increased with the concentration of CPC (p < 0.05). Relative to the negative control group, the highest mononuclear leukocyte DNA damage was found with 200 µg/mL CPC (p = 0.001). In addition, TOS and TAC values for the culture supernatants were not significantly different among the groups (p > 0.05). DNA damage was positively correlated with increasing concentration of CPC (r = 0.824; p < 0.001), but there was no significant correlation between DNA damage and oxidative status parameters within each group.

Conclusions: This study indicates that the DNA-damaging effects of CPC may be due to a concentration-dependent direct chemical effect. Thus, when using CPCs in clinical practice, their potential to cause DNA damage should be considered.

Key words: Calcium phosphate-based bone substitute materials, calcium phosphate cements, DNA damage, comet assay, oxidative stress.

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Introduction

Calcium phosphate-based bone substitute materials, also called calcium phosphate cements (CPCs), are used for bone replacement, bone repair, and augmentation in orthopedic surgery due to the unique combination of osteoconductivity, bio-compatibility, and moldability, CPCs are excellent alloplastic materials for osseous augmentation. Non-exothermic setting, negligible shrinkage, excellent in vivo resorption, and regeneration are the most important properties of these materials. Because of donor side morbidities associated with autogenous bone grafts, including pain, blood loss, increased operative time, risk of infection, and nerve injury, CPCs are used increasingly in a broad range of orthopedic surgeries.

Although several CPCs with different chemical compositions are used in orthopedic surgery, some of them has been shown to have a relatively high resorption rate. In addition, with increasing time in vivo, this CPC is penetrated by small blood vessels that become surrounded by circumferential lamellae of bone, a situation closely resembling the evolving haversian system; moreover, this process occurs more rapidly in the cortex than in the medulla.

There is a generally accepted correlation between genotoxic and carcinogenic effects of different chemical components. Genotoxicity can be evaluated in in vitro tests designed to detect DNA damage. Single cell gel electrophoresis, or the comet assay, is a reliable method for evaluating DNA damage in CPC-exposed cells in vitro. Because of its simplicity and sensitivity, the comet
assay has gained wide acceptance for assaying genotoxicity. Furthermore, it shows high sensitivity for detecting carcinogens, and the fact that observations are made at the level of peripheral blood mononuclear leukocytes.

Total oxidant status (TOS), total antioxidant capacity (TAC), and the oxidative stress index (OSI) are useful tools for evaluating the oxidative-antioxidative balance in organisms. The relationship between oxidative stress and DNA damage has been evaluated in several studies. These studies have indicated that elevated DNA damage is related to increased oxidative stress.

To the best of our knowledge, there are only a few reports on peripheral mononuclear leukocyte DNA damage caused by CPCs of different chemical composition, but there is no report on any such damaging effects of high resorption featured CPC or whether oxidative stress causes DNA damage. Thus, in this study, we assessed the DNA damaging effects of a high resorption featured CPC, in vitro at different concentrations (10, 100, and 200 µg/mL) on peripheral mononuclear leukocytes cultures. We also measured the TOS and TAC values of the culture supernatant to evaluate whether oxidative stress caused DNA damage.

Materials and methods

Subjects

In total, samples were taken from nine healthy donors, five females and four males. Anyone with a systemic infection, use of vitamin supplements or therapeutic drugs, diabetes mellitus, a history of smoking or cancer, previous radio-or chemotherapy, exposure to diagnostic X-rays during the previous 6 weeks, malignancy, intensive sport activities during the last week, or high alcohol consumption was excluded.

The study was approved by the local ethics committee. Informed consent was obtained from all blood donors.

Blood sample collection and preparation of cells

Blood samples (6 mL) from donors were taken from the antecubital vein into tubes with heparin, stored at 2-4°C in the dark to prevent further DNA damage, and processed within 2 h. Mononuclear leukocyte isolation for the cell culture was performed by centrifugation using Histopaque 1077 (Sigma, USA). Heparinized blood (3 mL) was carefully layered over 3 mL Histopaque and centrifuged (35 min, 500xg, 25°C). The interface band containing mononuclear leukocytes was washed with phosphate-buffered saline (PBS) and then collected by centrifugation (400xg, 15 min). The resulting pellets were re-suspended in PBS and the cells were counted with an automatic cell counter (Abbott 3700, USA). Membrane integrity was assessed by means of the trypan blue exclusion method and revealed membrane integrity in 95% of cells.

Cell culture and treatment

Briefly, 10 µL cell suspension containing 2×10⁹ cell/µL was seeded onto culture plates containing 10 ml cell culture medium (RPMI 1440). Dry form of the high resorption featured CPC (Cementek®; Teknimed LC Vicen Bigorre, France), used to assess potential DNA damage, is made of a mixture of 49% tetracalcium phosphate, 38% α-tricalcium phosphate, 12% sodium glycerophosphate, and 1% polydimethylsiloxane. First, five culture plates were set up for each donor’s cells, three for different concentrations and others as controls. To assess concentration-related effects, stock cement suspensions were added to each culture plate to obtain final concentrations of 10, 100, and 200 µg/ml culture medium. The negative control group was treated with the vehicle (PBS). For a positive control, mononuclear leukocytes were exposed to hydrogen peroxide (H₂O₂) at 100 µM for 5 min on ice. All materials tested were dissolved in PBS.

Alkaline single-cell gel electrophoresis assay (comet assay)

The cultures were incubated at 37°C for 3 h. After the exposure periods, culture medium was centrifuged (500xg, 5 min) to separate the cells and, cell viability was re-assessed. Membrane integrity was found more than 90% in all incubated cells. Mononuclear leukocyte DNA damage was analyzed using the comet assay according to Singh et al. with some modifications. Cell suspension (10 µL, around 20,000 cells) was mixed with 80 µl 0.7% low-melting agarose in PBS at 37°C. Then, 80 µl of this cellular suspension was spread onto slides that had previously been coated with 1.0% normal melting point agarose in PBS at 37°C. Then, 80 µl of this cellular suspension was spread onto slides that had previously been coated with 1.0% normal melting point agarose (60°C; NMA) and covered with a cover slip at 4°C for at least 5 min to allow the agarose to solidify. After removing the cover slips, the slides were submerged in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM Na₂ETDA, 10 mM Tris-HCl pH 10-10.5,
1% Triton X-100, and 10% DMSO, added just before use) for at least 1 h. Slides were then placed side by side in a horizontal gel electrophoresis tank containing freshly prepared alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na2ETDA, pH > 13). The slides were left in this solution for 25 min to allow DNA unwinding and expression of alkaline-labile sites as DNA strand breaks. Electrophoresis was conducted at a current of 300 mA for 25 min at 4°C. All procedures were conducted under minimal illumination and the electrophoresis tank was covered with black paper to avoid additional DNA damage due to light. Then the slides were washed with a neutralized solution of 0.4 M Tris-HCl, pH 7.5, for 5 min. The dried microscope slides were stained with ethidium bromide (2 µg/mL in distilled H2O; 70 µL/slide), covered with a cover slip, and analyzed using a fluorescence microscope (Olympus BX51, Japan) at 20x magnification provided with epi-fluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm; barrier 580 nm). A hundred cells were randomly scored by eye in each sample (Fig. 1), on a scale of 0 to 4, based on fluorescence outside the nucleus. The scale used was: 0, no tail; 1, comet tail less than half the width of the nucleus; 2, comet tail equal to the width of the nucleus; 3, comet tail longer than the nucleus; and 4, comet tail more than twice the width of the nucleus (Fig. 2).

Scoring cells in this manner has been shown to be as accurate and precise as using computerized image analysis. The individual scoring of the slides was blinded, using coded slides. The visual score for each class was calculated by multiplying the percentage of cells in the appropriate comet class by the value of the class. The total visual comet score characterizing the degree of DNA damage in the entire study group was the sum of the scores in the five comet classes. Thus, the total visual score could range from 0 (all undamaged) to 400 (all maximally damaged) arbitrary units (AU), as reported by Collins et al.\(^\text{14}\). The comets were scored independently by two investigators.

**Measurement of total oxidant status (TOS) and total antioxidant capacity (TAC)**

TOS and TAC levels in the remaining culture supernatant were determined using an automated measurement method developed by Erel\(^\text{15,16}\). Oxidative stress index (OSI) The ratio of TOS to TAC yielded the OSI, an indicator of the degree of oxidative stress\(^\text{16}\). For calculations, the resulting unit of TAC was changed to mmol/L, and the OSI value was calculated according to the following formula: OSI (arbitrary unit), TOS (µmol H₂O₂ equiv./L)/ TAC (mmol Trolox equiv./L).

**Statistical analysis**

Statistical analyses were performed using the SPSS software (ver. 16 for Windows, SPSS, Chicago, IL). Continuous variables were expressed as medians ± interquartile range (IQR). Distributions of data were tested with the one-sample Kolmogorov-Smirnov test and were found not to be normally distributed. For multiple comparisons, the non-parametric Kruskal-Wallis H test was used for comparisons within the groups and the Bonferroni test was used if statistical significance was found. The correlation between DNA damage and concentrations of CPC (10, 100, or 200 µg/mL) was assessed using the Spearman correlation test. In addition, parameters within each group were assessed using Pearson’s correlation test. A two-tailed p value ≤ 0.05 was considered to indicate statistical significance.

**Results**

There were five female (mean age 29.60±2.96 years) and four male donors (mean age 30.25±4.03 years).
There was no statistically significant difference between the donors with regard to age or sex (p > 0.05). As can be seen in Table 1, TOS, TAC, and OSI values of the culture supernatant did not differ among the groups (p > 0.05). Cell viability was found to be more than 90% in all groups, allowing the comet assay to be performed.

![Figure 3: DNA damage in peripheral mononuclear leukocytes, assessed using the comet assay.](image)

Table 1: Oxidative stress parameters and DNA damage at different concentrations of CPC, and in positive control and negative control groups. Values are expressed as median ± interquartile range (IQR).

<table>
<thead>
<tr>
<th>Different concentration groups</th>
<th>10 μg/ml (n=9)</th>
<th>100 μg/ml (n=9)</th>
<th>200 μg/ml (n=9)</th>
<th>Positive control* (n=9)</th>
<th>Negative control† (n=9)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOS (μmol H₂O₂ Eqv./L)</td>
<td>5.01±0.43</td>
<td>4.98±0.36</td>
<td>5.03±0.31</td>
<td>5.08±0.39</td>
<td>4.96±0.30</td>
<td>0.966</td>
</tr>
<tr>
<td>TAC (mmol Trolox Eqv./L)</td>
<td>0.11±0.02</td>
<td>0.10±0.02</td>
<td>0.10±0.01</td>
<td>0.11±0.02</td>
<td>0.11±0.02</td>
<td>0.519</td>
</tr>
<tr>
<td>OSI (Arbitrary Unit)</td>
<td>4.74±1.29</td>
<td>4.90±0.96</td>
<td>4.60±0.33</td>
<td>4.88±1.23</td>
<td>4.49±0.83</td>
<td>0.638</td>
</tr>
<tr>
<td>DNA damage (Arbitrary Unit)</td>
<td>4.00±3.00</td>
<td>8.00±10.00</td>
<td>26.00±13.00ab</td>
<td>142±43d</td>
<td>2±5c</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Although there was no statistically significant difference among the groups in terms of culture supernatant TOS, TAC, and OSI levels, our results showed that when the CPC concentration increased, DNA damage increased significantly. We saw the highest mononuclear leukocyte DNA damage with 200 µg/mL CPC for all donors relative to the negative control. These results demonstrate that the genotoxicity of CPC could be due to a direct, concentration-dependent chemical effect without causing cytotoxicity. Moreover, there was a positive correlation between DNA damage and increasing concentration of CPC (10, 100, or 200 µg). Also,
the lack of any correlation between DNA damage and oxidative status parameters within the groups shows, in this in vitro study, the ineffectiveness of oxidative status on DNA damage.

In clinical practice, in the reconstruction of bone defects, use of autogenous bone grafts has been considered the gold standard but it does have several disadvantages, such as donor site pain, blood loss at the donor site, increased operative time, and risks of infection and nerve injury\(^4\). In addition, cancellous bone grafts have very low initial mechanical strength, and cortical bone grafts are difficult to shape and to place in bone defects. Using allografts in orthopedic surgery also has drawbacks, such as limited supply, high cost, and risks of viral transmission and immunogenicity\(^1\). Because of these problems with autografts and allografts, the development of synthetic alternative biomaterials for bone augmentation, repair, and replacement has gained importance in recent years. However, although polymethyl methacrylate (PMMA) bone cements can be molded to fill bone defects, they cause exothermic and toxic reactions and can jeopardize local bone healing\(^17\). Calcium phosphate-based biomaterials have similarities to bone, including biodegradability, bioactivity, and osteoconductivity. These materials can have compressive strength in clinical practice, and they can be resorbed in vivo\(^6\). Like other materials that are used for bone repair, CPCs show several advantages, such as being easy to shape and to place in the surgical site. Thus, for filling bone defects with irregular shapes, CPCs can be used very effectively\(^18\). Because the first CPCs were introduced for use in orthopedic surgery, various CPCs with different chemical compositions, based on calcium and phosphate compounds, have been formulated, and their characteristics and in vivo and in vitro properties have been investigated\(^8\). Several studies have shown that CPCs can be used to fill bone defects in unstable tibial plateau fractures\(^17\), to improve the procedure for arthrodesis of the wrist\(^22\), for treating calcaneal bone cysts and pathological fractures\(^19\), for the restoration of traumatic thoracolumbar vertebral fractures\(^20\), to improve stability in femoral neck fractures fixed with cannulated screws\(^21\), and to fill the subchondral space in proximal humerus fractures\(^22\).

In an experimental study comparing the biocompatibility and rate of resorption of different CPCs, Spies et al.\(^5\) indicated that some of the CPCs had the highest resorption rate after 52 weeks, and that this continued over time. During resorption, CPC was penetrated by small blood vessels, and osteoclasts degraded the materials in a layer-by-layer fashion, starting at the bone-cement interface; macrophages and giant cells were the major cell types involved in the resorption\(^23\). Because of that we used high resorption featured CPC to evaluate DNA-damaging effects. Because genotoxicity can be evaluated using in vitro tests designed to detect DNA damage, several in vitro studies have been conducted to evaluate the genotoxic effects of PMMA bone cements in recent years\(^24,25\). On the other hand, to the best of our knowledge, only one report\(^7\) has indicated that CPC containing hydroxyapatite is not cytotoxic, but that at concentrations ≥ 100 µg/mL, it induces DNA damage. Accumulation of DNA damage with time can lead to gene modification in cells that may be mutagenic or carcinogenic\(^8\). In addition, relationships between the pathogenesis of many cancer types and DNA damage as well as DNA damage repair defects have been shown in several studies\(^26,27\). In the present study, to evaluate concentration-related effects of CPC on DNA damage, we used 10, 100, and 200 µg/mL CPC, and negative and positive groups as controls.

Single-cell gel electrophoresis (the comet assay) is a rapid and well-established fluorescent test to quantify DNA damage in individual cells. It has advantages of speed, simplicity, and the fact that observations are made at the level of peripheral blood mononuclear leukocytes\(^9\). This simple, rapid, and sensitive technique is extremely useful and has been used to assess the extent of endogenous DNA damage\(^28\). In addition, in vitro studies are simple, inexpensive to perform, provide a significant amount of information, can be conducted under...
controlled conditions, and may help to determine the mechanism(s) of cellular toxicity. Cell culture studies are commonly used to evaluate genotoxicity. The results obtained from these assays observed in vitro may be indicative of effects observed in vivo(30). Thus, we used the comet assay to evaluate DNA damage in peripheral mononuclear leukocyte cultures.

Several recent studies have contributed to the body of research in this area. Selel et al.(32) mentioned that increased oxidative stress may lead to DNA damage and this might be involved in the pathogenesis of lung cancer. Likewise, Gulum et al.(32) indicated that decreased antioxidant capacity and increased oxidative stress might be associated with increased DNA damage. Thus, in this study, to eliminate the effect of oxidative stress on DNA damage, an equal distribution of the mononuclear leukocytes of each donors in all groups was provided. In this way, a direct chemical effect of CPC on DNA damage without the effect of oxidative stress was obtained.

The present study has some limitations. First, it has an in vitro design. However, it was a pilot study and was designed to evaluate the DNA-damaging effect of CPC and whether the effects of oxidative stress mediate DNA damage. Second, the number of samples used in this study was small.

In conclusion, this is the first report to evaluate CPC-induced DNA damage in cultures of peripheral mononuclear leukocytes and oxidative status in culture supernatants. Although all groups showed a similar oxidative status, the highest DNA damage was found in the 200 µg/mL CPC concentration group. Although there was a positively correlation between DNA damage and increased concentration of CPC, we found no correlation between DNA damage and oxidative status parameters within each group. According to these results, without changing the oxidative status parameters in the culture supernatants and causing cytotoxicity, the genotoxicity of CPC may be due to a direct, concentration-dependent chemical effect. While the conditions in this in vitro study may differ from the clinical situation, the genotoxic effects of CPC raise concerns about the potential to cause adverse effects in vivo and should be considered when using CPCs in clinical practice. Further prospective clinical investigations are needed to evaluate cellular responses to CPCs in orthopedic surgery over the long-term.

References

13) Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of


