The presence of pamidronate in bone cement affects serum biochemical markers in the rat

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Abstract

The main aim of the study was to assess whether the presence of biphosphate pamidronate (PA) in the cement implanted into the tibial bones had any effect on the chosen biochemical markers in rat's serum characterising homeostasis. Forty adult male Wistar rats were divided into two control groups and two experimental groups. Tibial bone of rats in the experimental groups was implanted with PA-enriched cement, whereas the bone in control-group’s rats was implanted with cement without PA. Serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) were determined three and six weeks after the surgery. Statistically significant differences in the activities of AST and CK of the rats after implantation with non-enriched cement when compared to rats given PA-enriched cement implantation, were found. Six weeks after treatment, AST levels decreased significantly in rats with PA-enriched implants, whereas rats in the control group (implanted with non-enriched cement) demonstrated a significant increase in AST activity in comparison to the same values determined after three weeks and values of PA-enriched cement rats determined after six weeks. The activities of CK were higher in rats with PA-enriched implants than in the control group three weeks after surgery, but six weeks after the treatment, rats implanted with enriched cement reached lower values than animals implanted with non-enriched cement. The use of PA in the cement had also some positive effect on the homeostasis of the rats after the surgery and a positive influence on the post operative muscle regeneration process.

Key words: rats, pamidronate, bone cement, alanine aminotransferase, aspartate aminotransferase, creatinine kinase.

Introduction

Bisphosphonates (BPs) are antiresorptive drugs, with a high affinity for hydroxyapatite (calcium) (12). Bisphosphonates are primary agents in the current pharmacological arsenal against osteoclast-mediated bone loss due to osteoporosis, Paget’s disease of the bones, malignancies which metastasise to bones, multiple myeloma, and hypocalcaemia of malignancy. In addition to their currently approved uses, bisphosphonates are commonly prescribed for prevention and treatment of a variety of other skeletal conditions, such as low bone density, osteogenesis imperfecta, or fibrous dysplasia of bones (5, 10, 14). The BPs are classified into two groups, N-containing BPs (NBPs) and non-N-containing BPs (non-NBPs), each with different mechanisms of action. The NBPs, such as alendronate, residronate, ibandronate, and pamidronate (PA), act on the cholesterol pathway by inhibiting diphosphate synthase in the mevalonate pathway (8, 10). The non-N-BPs, such as clodronate and etidronate, are transformed metabolically into cytotoxic ATP analogs that inhibit ATP-dependent intracellular enzymes (14). Because BPs have an affinity to bone minerals, they act specifically on bone. Despite the fact that BPs act specifically on osteoblasts and osteocytes, the complete mechanism of BPs’ action is still unknown. Pamidronate, with its
specific binding affinities, appears to absorb to
calcium phosphate crystals in bone, thus blocking
dissolution by inhibition of osteoclast-mediated
bone resorption. During resorption of bone by
osteoclasts, the ingestion of BPs interferes with
specific intracellular processes, which impairs
osteoclast function and eventually causes apoptosis or
cell death (7, 15). BPs regulate osteoblastic functions,
such as proliferation and differentiation, prevent from
apoptosis of osteoblasts, modulate osteoblastic
production of extracellular matrix proteins, and
regulate osteoblastic expression and secretion of
various growth factors and cytokines (16, 30). Many
recent studies, both preclinical and clinical, also
focused on further potential applications of BPs in
orthopedics (15, 16, 18).

Our previous study demonstrated that local
treatment with BPs can affect the level of bone
turnover markers in rats’ serum (23). Our further
results obtained by Ion Pair HPLC and CE methods
showed that pamidronate is eluted from the BP-
enriched cement after three and six weeks of
incubation in 0.9% NaCl solution (20). These results
may explain the changes in the level of cytokines in
rats’ serum. The release of pamidronate with BP-
enriched cement also explains enhancement of the
microstructure of bones in rats treated with BP-
enriched cement (21, 22). The surgical procedure is
usually connected with several stress symptoms in the
animal organism. The level of damage and the rate of
tissue regeneration can be monitored by the
determination of specific muscle and liver
biochemical markers commonly used in clinical
practice.

Among the biomarkers, aspartate aminotransferase (AST) (EC 2.6.1.1) and L-alanine: 2-oxoglutarate aminotransferase (ALT) (EC 2.6.1.2) are commonly determined. It is a known fact that increased levels of ALT and AST are generally a result of liver disease associated with some degree of hepatic necrosis such as cirrhosis, carcinoma, viral or toxic hepatitis, and obstructive jaundice. Elevated ALT and AST activities have been also found in extensive trauma and muscle disease, circulatory failure with shock, hypoxia, myocardial infarction, and haemolytic disease (17). The third biomarker commonly used in clinical practice is creatine kinase (CK) (EC 2.7.3.2.), that CK is a central controller of cellular energy homeostasis. By reversible interconversion of creatine into phosphocreatine, CK builds up a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels. Thus, CK plays particularly important role in tissues with large and fluctuating energy demands like muscle and brain, with the mitochondrial isoenzyme of CK (MiCK) being important for the energetics of oxidative tissue (9, 4).

The aim of the presented study was to assess
whether the presence of pamidronate, in the cement
implanted into the tibial bones has any effect on
the chosen biochemical markers characterising
homeostasis in rat serum. The regeneration rate in the
group of rats which were implanted with unaltered
cement with the group implanted with PA–enriched
cement, was compared. The changes in the activities
of AST, ALT, and CK in the serum were determined
three and six weeks after the surgical procedure.

Material and Methods

Animal model. Forty adult Wistar CRL(W1)WU
BR rats, weighing approximately 240 g, were used in
the experiment. The experimental procedures were
reviewed and approved by the Bioethical Committee
of Animal Experimentation of the Medical University
of Lublin.

The rats were divided into two control and two
experimental groups. Rats in the experimental groups
were implanted with PA-enriched cement into the
tibia, while rats in the control groups were implanted
with bone cement without PA. Each experimental
group was associated with its own dedicated control
group (three or six weeks after surgery).

Rats were anaesthetised using thiopental with
depth of anaesthesia being assessed by pedal reflex.
After each animal was anaesthetised, it was placed in
a supine position and the skin over the tibia was
shaved and disinfected. A skin incision was made
over proximal part of the tibia. After exposing the
proximal tibia, freshly mixed bone cement was
pressurised into the bone canal through a pre-drilled
hole using a syringe with a modified needle. Twenty
rats were given cement mixed with PA, while the
remaining 20 rats were given cement without PA
(control groups). The wound was closed using Vicryl
sutures and staples.

Assays procedure. Twice, three and six weeks
after the surgery, the rats were anaesthetised using
thiopental (30 mg/kg) and blood samples were
collected from the left ventricle. Then, the blood
samples were introduced into pyrogen-free tubes
containing heparin (Endo Tube ET; Chromogenix, Mo
Indal, Sweden). Plasma was immediately separated at
4°C by centrifugation at 180 g for 10 min and stored
in pyrogen-free tubes (N201 Test Tubes; Biowittaker,
USA) at -70°C. Serum activities of alanine
aminotransferase (ALT), aspartate aminotransferase
(AST), and creatine kinase (CK) were determined
using a standard kit test (Thermo Scientific, USA).

Statistical analysis. Statistical analyses were
performed using one-way analyses of variance
(ANOVA). Post-hoc comparison of means was
conducted with the Tukey’s test for multiple
comparisons, when appropriate. All data is presented
as means ± SEM. For cases with rejected hypothesis
of normal distribution or homogeneity of variance,
nonparametric ANOVA rank Kruskal-Wallis multiple
comparisons was used. The data is shown as the median with the first and third quartiles. The data was considered statistically significant at confidence level of $P < 0.05$. All statistical calculations were performed using 10.0 STATISTICA software (StatSoft, Poland).

**Results**

A statistically significant difference in the activities of AST and CK were found between control and experimental groups three and six weeks after the surgery. Three weeks after the surgery, an increase in AST activity was observed in rats implanted with PA-enriched cement, when compared to rats implanted with non-enriched cement ($P = 0.000160$). Six weeks after treatment, AST levels decreased significantly in rats with PA-enriched cement, whereas rats in the control group (implanted with non-enriched cement) showed a significant increase in AST activity in comparison to the same values determined after three weeks ($P = 0.000159$) and values of PA-enriched cement rats determined after six weeks ($P = 0.000175$) (Fig. 1).

![Fig. 1. Median activity of AST (U/L) in rats’ serum after bone implantation with clean cement in comparison with rats implanted with bisphosphonate (BP)-enriched cement. Statistical analyses were performed using one-way analyses of variance (ANOVA). Post-hoc comparison of means was conducted with the Tukey’s test for multiple comparisons, when appropriate](image1)

No statistically significant differences in the activity of ALT were detected when the rats of control group were compared with rats with implanted PA-enriched cement (Table 1).

![Fig. 2. Median activity of CK (U/L) in rats’ serum after bone implantation with clean cement in comparison with rats implanted with bisphosphonate (BP)-enriched cement. Statistical analyses were performed using one-way analyses of variance (ANOVA). Post-hoc comparison of means was conducted with the Tukey’s test for multiple comparisons, when appropriate](image2)

Highly statistically significant differences ($P = 0.00$) in the activity of CK were detected in the treatment groups.

<table>
<thead>
<tr>
<th>Time after implantation</th>
<th>ALT activity (U/L) in rats implanted with clean cement</th>
<th>ALT activity (U/L) in rats implanted with PA-enriched cement</th>
</tr>
</thead>
<tbody>
<tr>
<td>three weeks</td>
<td>41.5 ± 3.4</td>
<td>44.3 ± 3.7</td>
</tr>
<tr>
<td>six weeks</td>
<td>39.5 ± 2.9</td>
<td>46.3 ± 4.2</td>
</tr>
</tbody>
</table>

Data is presented as arithmetical means ± standard errors of a mean. Statistical analyses performed using one-way analyses of variance (ANOVA) showed no statistically significant differences between investigated groups of rats ($P = 0.44$).

A significant increase in CK values in the group implanted with clean cement was observed six weeks after the implantation compared to levels three weeks after the surgery ($P = 0.000162$) (Fig. 2). The activities of CK were higher in rats with PA-enriched implants than in the control group three weeks after surgery, but six weeks after the treatment, rats implanted with the enriched cement reached lower values than animals implanted with non-enriched cement. The activities of CK after PA-cement implantation increased after six weeks when compared to the same group after three weeks. The activity continued to increase in both control groups (Fig. 2).

**Discussion**

The common biomarkers of tissue injury are enzymes which are released into the plasma as a consequence of injury, and are typically present at low concentration in the blood and body fluids. Serum enzymes, such as ALT, AST or CK are used as efficient indicators of organism homeostasis. The activity of ALT...
is generally higher than that of AST in acute viral or toxic hepatitis. AST is highly active in the heart, liver, skeletal muscles, kidneys, and erythrocytes. Destruction of these tissues by pathologies such as myocardial infarction, viral hepatitis, liver necrosis, cirrhosis, and muscular dystrophy may result in raised serum levels of AST. The AST and CK are known as a muscle-specific proteins used for the detection of increased permeability of the muscle membrane. Creatine kinase, also known as creatine phosphokinase (CPK), is a cytoplasmic enzyme that converts creatine to creatine phosphate using energy provided by the conversion of ATP to ADP. The reverse reaction, donation of a phosphate from creatine phosphate to ADP to form ATP, provides energy for contraction of muscle, among other actions (1, 29).

The local treatment with BPs may regulate important mediators involved in osteoclastogenesis, such as receptor activator of nuclear factor - xB ligand (RANKL), synthesised by osteoblasts, and may modulate osteoprotegerin (OPG), a decoy receptor of RANKL, absorbing and preventing receptor activator of nuclear factor xB (RANK) activation. Viereck et al. (30) demonstrated that the primary effect of BPs in differentiated osteoblasts on the RANKL/OPG system is the enhancement of OPG production. They also showed that the BPs pamidronate and zoledronic acid act directly on human osteoblastic cells to increase the secretion of OPG, a potent inhibitor of bone resorption. The results obtained from our earlier study clearly support the hypothesis that cement enriched with BPs may regulate important mediators involved in osteoclastogenesis such as RANKL synthesised by osteoblasts, and may modulate OPG (23). Our research shows that PA-enriched cement affects not only the markers of bone turnover, but also the level of other enzymes in serum. The results presented in this work indicate statistically important differences in the activity of AST and CK, when the rats after implantation with non-enriched cement and PA-enriched cement are compared three and six weeks after the surgery. The activities of ALT were not significantly different. It suggests the lack of liver damage in the presence of the non-enriched cement and PA-enriched cement in the rat organisms. The changes of AST and CK values in the rat serum indicate the mechanical muscle damage that appears as a result of implant surgery. Our study demonstrates that local treatment with BPs can affect the level of enzymes in the serum. High levels of AST in serum of rats six weeks after implantation of cement without PA into the bone, when compared to three weeks after the surgery, were found. A reduction in serum levels of AST, six weeks after implantation of PA when compared with that recorded three weeks after the surgery, was noticed. The same dependence was obtained by comparing the level of CK in the rats’ serum. There was also a high level of CK in the rats’ serum three weeks after the implantation of the bone cement enriched with PA, while in the controls CK concentration was maintained at a much lower level. The level of CK in the serum six weeks after the surgery significantly decreased relatively to the level observed three weeks after the operation, while the levels significantly increased in the control group.

In our earlier report, it was proved that the use of bisphosphonate enriched-cement had a positive effect on bone turnover by acting directly on some important markers in the rats’ serum (23). Our next micro-CT study showed some beneficial effects on the bone’s microarchitecture (21). Implanted BP-enriched cement simply changes the bone turnover in normal rats’ bone toward a positive balance between bone formation and bone resorption. All these factors lead to positive effect on bone formation together with enhanced growth. Some experimental in vitro and in vivo research showed a great potential of using BPs in healing of fractured bones, reducing osteoarthritis, or enhancing ingrowth of bone allografts (5, 25, 27). It was earlier proposed that the introduction of bisphosphonate compounds can be a very promising procedure connected with the treatment of musculoskeletal disorders in clinical practice (19, 27, 28). Furthermore, the data obtained in the present study demonstrates that the use of pamidronate as a component of cement also has some positive effect on muscle homeostasis in rats after the surgery. Therefore, the use of PA-enriched cement implants has positive effects on bone turnover and may also have some positive influence on post-operative regeneration processes. These results are in agreement with the study presented by Osterman et al. (25), Francis et al. (13), and Barbier et al. (6), which demonstrate the anti-inflammatory efficacy of both clodronate and aminobisphosphonate (pamidronate), risedronate, in established rat adjuvant induced arthritis. It was also showed that free pamidronate can suppress the pro-inflammatory cytokine generation more than non-aminobisphosphonates such as clodronate (26).

In conclusion, the local use of pamidronate affects not only typical bone turnover markers but also significantly changes the activities of general enzymatic biomarkers. A statistically significant decrease in the activity of AST and CK in rats six weeks after implantation with PA-enriched cement, in comparison to the control group, suggests that the potential release of some amounts of PA into the rat serum can accelerate muscle regeneration and anti-inflammatory processes.
The values obtained for the ALT/AST activities suggest no post-surgical liver damage in experimental animals.

Beneficial response to the systemic introduction of BP-enriched bone cement suggests that this material can be used in animals for filling bone defects that arise as a result of diseases or injuries, as well as in joint replacements. The role of BP-enriched bone cement in the treatment of fractures in animals, and the prevention of complications seems to be promising.

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References