The influence of foreign body surface area on the outcome of chronic osteomyelitis

Carmen Cristina Surdu-Bob, Cristin Coman, Florica Barbuceanu, Danut Turcu, Nicolae Bercaru, Marius Badulescu

ABSTRACT

Reproducible animal models of osteomyelitis close to the clinical scenario are difficult to obtain as the animals either die shortly after inoculation of bacteria or the bone cures itself of infection. Additional materials used as foreign bodies offer increased chances for localized infection due to bacterial attachment and are closer to clinical pathology.

Through in vivo experimentation we investigated here the influence of surface area of a series of foreign bodies on the final outcome of the animal model, in terms of reproducibility, survival rate and time necessary for onset of chronic disease. Stainless steel Kirschner wire segments, stainless steel balls and cotton meshes were employed for this purpose.

The clinical, microbiological, radiological and histological results obtained were compared with the simple case where no foreign body was used. The follow-up period was 57 days. The cotton meshes, which had the highest surface area, were observed to present the best outcome, with the lowest disease onset time interval (of 1 week earlier than the others), the highest survival (of 90%) and disease reproduction rate (90%). The only clinical pattern of the mesh group rabbits was short lived inflammation while the other rabbits presented also some other clinical signs such as rhinorheas, abscesses, rush and/or dyspnea. Moreover, this model is the most suitable for further treatment studies, as the cotton meshes could be easily removed after disease onset, without any intervention on the bone. This is important, as the treatment would address the bacteria present within the bone parts (marrow, cortex, periosteum etc.) not those forming the biofilm.

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1. Introduction

Osteomyelitis is a bone disease caused by bacterial or fungal infection involving parts of the bone. Due to the poor vascular system specific to the bone, the defensive system is not able to cure the disease and antibiotics have limited access, also. In the acute form, the infection is supplicative and the blood supplies of the medulla and the periost are compromised. An avalanche of bone destruction mechanisms leading to bone death follow and the chronic form is thus installed [1].

Treatment of staphylococcal infection is difficult because bacteria develop resistance to antibiotics [2]. Current treatment of osteomyelitis includes high dose antibiotics (like Nafcillin, Ceftriaxone, Cefazolin, Ciprofloxacin, Cefazidime, Clindamycin, Vancomycin etc.) for 6 weeks to 6 months, pain medications and surgery [3–6]. Therefore, research on finding better therapies is still in progress.

The need to obtain improved therapies has triggered the necessity of finding reproducible experimental models of this disease.

It is known that chronic osteomyelitis is difficult to induce in animals. This is due either to the fact that the animals die shortly after inoculation of the pathogen, or the bone does not develop the disease.

Several animal models were tried and reviews were made by different authors [2,7]. A large range of species of animals, from mice and rats to chicken, rabbits, dogs, pigs and goats were involved until now. A trade-off between the size of bone/animal and
the costs associated with the use of larger animals is considered when choosing the animal species. Different bones were used for induction of osteomyelitis: tibia, femur, mandible, radius. As observed by the above mentioned authors, the most used model is the rabbit tibial model. While being a relatively small animal, the rabbit has sufficiently large bones for complex surgical intervention involving insertion of foreign bodies and also for analysis.

Among the bacterial species usually involved for inducing osteomyelitis experimentally, Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa are the most frequently used species [1–3,7].

Regarding infection promoter and route of inoculation, some models use fracture or bone hole creation together with insertion of a foreign body. Some authors injected sclerosing agents such as sodium morrhuate in the medullary cavity to better mimic the clinical scenario [8–10]. The benefit is the fast progression of infection by induction of aseptic bone necrosis, vascular congestion, and thrombosis, but there are drawbacks such as non-natural evolutionary pattern of infection and possible interferences with treatment.

The main rationale for involving foreign bodies in animal models of osteomyelitis is to make surfaces available for bacterial adherence and proliferation which localize and increase infection [7]. It is well known that bacteria attach to surfaces and develop biofilms provided that the chemical and physical environment is suitable. By surface attachment, they protect themselves from hostile environments (such as antibiotics). The composition and porosity of surfaces are very important in biofilm formation. Therefore, the foreign bodies employed in animal models of osteomyelitis need to be biocompatible. Common such materials are: PMMA rods, PVA sponges, bone cement, metal rods/wires, plates or bone wax [2,11–14].

In terms of surface porosity, the higher the better for biofilm formation. Bacteria synthesize and deposit polysaccharide substances on surfaces and create a protective environment (matrix) for further colonization [15]. Direct correlations of surface area and amount of biofilm formed was observed since a long time ago and was reported by many authors [16–18].

Apart from the high surface area needed for increased bacterial adherence, the foreign bodies to be used in animal models of osteomyelitis should ideally be easily removable, a requirement necessary for further treatment studies where the material has to be removed before treatment administration. They should also be small for an increased chance of localization of infection, and also induce minimal invasion and less suffering for the animal.

The current work is aimed at finding a simple and reliable animal model of orthopedic chronic osteomyelitis based on foreign body materials which would comply with most requirements and could, hopefully, be used in therapy studies.

2. Experimental design

2.1. Foreign bodies

Three kinds of foreign bodies were used: 1 mm diameter and 10 mm length stainless steel Kirschner wire, 0.8 mm diameter spherical stainless steel particles and 1 mm diameter cotton fiber balls. The metallic materials were chemically corroded for 15 min in 3:1 HCl:HNO₃ solution to increase surface area.

The cotton balls were made using two 10 cm cotton threads (detached from standard medicinal cotton mesh) rolled together into a small ball.

2.2. Inoculum preparation

Being a common microorganism in human osteomyelitis, Staphylococcus aureus has been used in many animal models of this disease [1]. In this work, the microbial strain used for induction of bone infection was ATCC 6538.

In order to revitalize the bacterial culture preserved in our collection, after rehydration of the strain in nutritive broth (Biokar Diagnostics), three passages were made in the same media at 18–24 h interval. The bacterial culture was then incubated at 35–37 °C and a concentration of 5 × 10⁶ CFU/ml was obtained from the last passage.

2.3. Animal groups and surgical procedure

Four groups of ten male and female white New Zealand rabbits each with different foreign bodies (Table 1) were contained in individual cages with computer controlled humidity (45–65%) and temperature (16–21 °C).

The animals were 6 months old with an average weight of 2.7 kg. Water and food were available ad libitum. The bacterial culture was inoculated in the tibia of the left leg, while the right leg was used as reference, in all animal groups.

The in vivo experiments were undertaken under national and international regulations concerning animal testing, using a protocol approved by the ethics committee of our institute.

The surgical procedure was undertaken as follows. Under total anesthesia and aseptic conditions, the skin on the antero-medial shaft of the tibia was incised directly down to the bone, exposing 3–4 cm. Three bone defects were then drilled perpendicularly at 3–5 mm distance one from the other, starting at 25 mm distance from the femoral-tibial-patellar joint using a 1.1 mm drilling pin. This procedure was applied to all rabbits in all groups. A quantity of 0.2 ml pathogen culture was evenly distributed among the three holes and inoculated using a 27G needle. Insertion of foreign bodies followed. Relative positions of drilled holes and foreign bodies can be observed in the images presented in Fig. 1. Before insertion, the foreign bodies were sterilized under UV radiation.

The meshes were introduced into the holes leaving out of the hole one end of the thread. This setup is of great importance for further treatment studies, as it allows easy removal of the mesh by pulling out the end of the thread.

Table 1

Animal grouping and infection promoter.

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Infection promoter</th>
<th>Number of foreign bodies per drilled hole in the tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>Kirschner wire</td>
<td>1 wire</td>
</tr>
<tr>
<td>III</td>
<td>Stainless steel spheres</td>
<td>4 spheres</td>
</tr>
<tr>
<td>IV</td>
<td>Cotton meshes</td>
<td>1 mesh</td>
</tr>
</tbody>
</table>

Fig. 1. Radiological images of rabbit tibia showing positions of foreign bodies, just after inoculation.

The costs associated with the use of larger animals is considered when choosing the animal species. Different bones were used for induction of osteomyelitis: tibia, femur, mandible, radius. As observed by the above mentioned authors, the most used model is the rabbit tibial model. While being a relatively small animal, the rabbit has sufficiently large bones for complex surgical intervention involving insertion of foreign bodies and also for analysis.

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The wound was closed using skin adhesive and Aluminum spray was used for added protection. After the surgical intervention was completed, the animal received analgesics (Ketofen) over a period of 3 days.

2.4. Animal monitoring

Progression of infection was observed over 57 days and the following examinations were undertaken: general and local clinical, microbiological, histological, radiological examination.

General and local clinical examinations were undertaken daily. Body weight and temperature were monitored every 2 days in the first week after bacterial inoculation and on a weekly basis thereafter, up to the end of the experiment (57 days). Visual observation and palpation of the inoculation site for signs of inflammation and abscesses was also undertaken.

Hematological tests of blood taken in EDTA microcontainers from the marginal vein of the ear were performed in all animals, on the 23rd, 43rd and 50th day, using a Pro CYTE 1D-DEX instrument. The following blood counts were monitored: RBC, PCV, hemoglobin, MCV, MCH, MCHC, RDW, RETIC, RETIC, WBC, Neutrophils, Lymphocytes, Monocyte, Eosinophil, Basophil, Blood platelets, MPV and PDW. The data obtained were compared to normal values taken from literature [19].

Microbiological examination consisted on performing biopsies of the hematogenous bone marrow from the inoculation site (posterior leg) as well as from the front leg.

Standard characterization and identification techniques involving biochemical and immunological assays were undertaken.

Histological and microbiological examinations were undertaken 36, 50 and 57 days after inoculation. Cross sectional segments from the tibial bone (six sections per tibial bone, two from the sides of each of the three holes) were fixed in neutral buffer formalin, and then in 5% trichloroacetic acid for 3 days. The samples were dehydrated in alcohol and toluene for 24 h. Next, the specimens, were embedded in Paraplast Plus (Sigma) embedding media, at 60°C. Paraffin sections (5 μm), were de-waxed and stained with the trichromic Masson method. The presence of specific acute and chronic osteomyelitis lesions was observed using an optical microscope. An acute form was diagnosed when the following lesions were observed: areas of agglutinated bacterial cells, microabscesses, deformation of Haversian canals, multilamellar periosteal reaction, predominant polymorphonuclear cells. Chronic osteomyelitis was differentiated from the acute form by the presence of endosteal resorption, subendosteal and medullar fibrosis, necrosis, bone repairing processes (osteoblasts, osteoclasts), calcification and presence of mononuclear cells.

Radiological images were taken every 2 weeks, starting on the day before inoculation. Both legs were imaged simultaneously, the right leg being used as reference. Their analytical reading was done independently by three radiologists and consisted of observing the presence of the following lesions: periosteal elevation, architectural deformation, widening of the bone shaft, formation of new bone and deformation of soft tissue.

2.5. Statistical analysis

Statistical significance of differences in weight gain and blood counts between rabbit groups was analyzed using one way ANOVA followed by a post-hoc analysis with the Tukey–Kramer variant due to the differing number of samples in the groups.

3. Results

The surface area of the materials used as foreign bodies, available for bacterial attachment, was different for the three types of implants employed. The total area of the Kirschner wire segment was smaller than that of the six steel spheres and much smaller than that of the cotton ball. Images of the surface of a chemically corroded stainless steel ball and of a cotton mesh wire are presented in Fig. 2.

3.1. Clinical observation

Of the 40 rabbits employed in this experiment, seven died of septicaemia within the first 11 days after surgery, as evidenced on necropsy examination (survival rates by rabbit group presented in Table 2). They have all presented inaptness and adynamia starting with the second day after surgery, in contrast with all the other rabbits.

The surviving rabbits mainly had inflammation and abscesses, but also rhinorrhoea (two rabbits), dyspnea (one rabbit) or rash (one rabbit) was observed, while others presented no infection sign throughout the observation time, although they finally developed osteomyelitis. Two rabbits did not develop osteomyelitis, one in Group II and one in Group III.

3.1.1. Weight loss

Similar changes in body weight were observed in all groups, with weight loss in the first week after surgery. Nevertheless, a statistically significant difference in weight gain (Fcritical = 2.947, F = 0.787) between Groups III and IV was observed in the first 2 weeks after inoculation, as shown in Fig. 3.

Body temperature was within normal values (38.5–39.5°C) throughout the experiment, with only a couple of readings of 40°C.

3.1.2. Blood counts

Normal values of most blood counts were found in all rabbits. Abnormal values were observed in leukocyte count (higher values) and neutrophils count and rate (lower values).

The presence of inoculated Staphylococcus aureus bacteria in the bone marrow of infected legs was detected in less than half of the number of investigated rabbits.

All rabbits in the experiment except for two (one in Group II and one in Group III) developed osteomyelitis. An overview of acute/chronic diagnosis given by histological investigation of the rabbits is presented in Table 2. Day 50 marked the presence of chronic osteomyelitis in Groups I and IV only. Nevertheless, all rabbits tested had chronic osteomyelitis on day 57.

3.2. Histological

Images of affected bone parts (medullary canal, cortex and periost) are presented in Fig. 4. In the acute phase, laminar periosteal reactions limited to 2–3 thin eccentric foils and periosteal thickening were typical. Acute form features of the medullary canal found were: leukocyte infiltration, hemorrhagic microcenters and the presence of coccoïd bacterial cells. The cortex was found heavily affected since the acute form, presenting multiple microabscesses and fragmentary polymorphonuclear leukocytes. A highly damaged medulla was observed histologically in the chronic phase of the disease (osteomyelobiosis). The chronic phase showed necrosis, rarefaction and polymorphonuclear cellular reaction at the periosteal level. Modifications of the cortex continued in the chronic phase and consisted on enlargement of Haversian canals filled with granulation tissue, bone mineralization, presence of polymorphonuclear leukocytes and microabscesses.

3.3. Radiological

Bone neoformation was the main lesion observed. Typical radiological images of the inoculated tibia showing bone neoformation and deformation of soft tissue are presented in Fig. 5.
Fig. 2. Microscopic image of a) chemically corroded stainless steel ball and b) cotton wire.

Table 2
Diagnosis data. Number of rabbits and percentages.

<table>
<thead>
<tr>
<th>Group</th>
<th>Before Day 11</th>
<th>Day 36</th>
<th>Day 50</th>
<th>Day 57</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Investigated</td>
<td>ACUTE</td>
<td>CHRONIC</td>
<td>No infection</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 3. Evolution of weight gain.

The other lesions observed were bone architecture deformation and enlargement of bone shaft, but fewer animals developed them.

4. Discussion

It is known that chronic osteomyelitis is difficult to induce in animals [11]. This is due either to the fact that the animals die shortly after inoculation of the pathogen, or the bone does not develop the disease. Optimal bacterial culture concentrations and/or standard protocols using biofilm promoting implants are not established.

This work was devoted to a comparative in vivo study to observe the influence of surface area of foreign bodies on inducing osteomyelitis. Other material-related characteristics of the animal model included size and easiness of removal after installation of disease. The outcome in terms of survival rate, disease reproduction rate and time necessary for onset of chronic disease were evaluated and compared to the case where only bacterial culture was inoculated.

All animals were administered the same amount of bacterial culture (0.2 ml) for comparison purposes.

An important rationale of the infection protocols chosen was to avoid the use of sclerosing agents which may interfere with normal progression of the disease. Instead, foreign bodies with different surface areas were employed for increasing the chance for biofilm formation and localization of infection. This is a critical pathogenic mechanism of osteomyelitis [8].

Increased surface area provides higher chances for bacteria to attach [20–22]. This explains the results of our in vivo investigation where the foreign body with the higher surface area was observed to be the best in the most important aspects: survival rate and
disease reproducibility rate (as shown in Table 2). There were rabbits in the other groups who did not present the disease up to the end of experiment. Most probably, their immune system cleared the bacteria before it got the chance for surface attachment.

Distinctive pathological signs of septicemia such as inappetence and adynamia were observed in rabbits which died shortly after surgery. Similar clinical signs were observed by Weisbroth et al. [23] and also by Abdel-Gwad who used a very high concentration bacterial culture to study specific lesions of septicemia [24].

Weight loss in the first week after inoculation followed by weight gain up to the end of the experiment was observed in all surviving rabbits. Although weight loss was more pronounced in...
Group IV in the first week, these rabbits gained weight fast and reached the weight of the others in the following weeks, showing that the overall health of Group IV rabbits was not fatally affected within the 8 weeks interval of study. This weight pattern makes Group IV model important to guarantee disease reproducibility without trading-off the survival rate. Weight loss was also found in humans [1] developing osteomyelitis.

It was interesting to observe also that short lived or even no clinical signs of infection were registered throughout the experiment in most animals, regardless of type of infection promoter used. This is also the characteristic feature in human chronic osteomyelitis [1]. The only clinical sign in the cotton mesh ball model rabbits (Group IV) was short lived local inflammation, making the group the most reproducible.

Most hematological results were within normal values. Reported work in current literature shows no specific blood tests to confirm osteomyelitis, also [1].

Microbiological investigation using bone biopsy can only confirm the presence of bacteria but negative microbiological results cannot guarantee that the bone is not infected. These tests are always accompanied by imaging and histology for a precise diagnostic [25].

In this experiment, usual histology signs of acute and chronic osteomyelitis such as the presence of inflammatory cells, periosteal and cortex modification, necrosis, new bone formation and the presence of bacterial colonies were observed. Nevertheless, the timescale of chronic disease onset differed between groups (see Table 2), with a distinct behavior in Group IV. Here, the chronic form was found a week earlier than in the other groups. Early installment of chronic disease is a benefit in animal models, experiment costs and animal suffering being thus lowered.

Of note, the mesh does not induce artifacts in imaging by MRI or microtomography due to its non-metallic nature. Another advantage is the fact that the mesh can be easily removed for further treatment studies of the osteomyelitic bone. This is possible by pulling out the end of the thread forming the mesh ball which was left outside the bone at the time of inoculation (as seen in Fig. 6). Removal of the foreign body is important, as the treatment would address the bacteria present within the bone parts (marrow, cortex, periosteum etc.) not those forming the biofilm.

5. Conclusions

All but two investigated animals expressed osteomyelitis at time intervals from 30 days—acute to 60 days—chronic. The animals in the cotton mesh ball group developed the chronic disease 1 week earlier.

The results of our comparative study have revealed that the cotton mesh ball model outperformed the others in terms of survival rate, disease reproduction rate, disease on-set time interval and reproducibility of clinical evolutionary pattern. Other important advantages of the cotton mesh ball model are: superior localization of infection, easiness of removal, non-interference with MRI or micro-tomography imaging, minimal invasion and less suffering for the animal.

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References