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A new concept of gentamicin loaded HAP/TCP bone substitute for prophylactic action: in vivo pharmacokinetic study

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Abstract Despite systemic prophylaxis, infection rates after orthopedic surgery can reach more than 1%. A new HAP/TCP bone substitute loaded with 125 mg of gentamicin was designed for prophylactic use. Its aim was to enhance the efficacy of systemic prophylactic treatments by increasing the local antibiotic concentration. The release rate of gentamicin from the bone substitute was investigated after implantation in the femoral condyle of five sheep. In order to investigate the local and systemic gentamicin concentrations, synovial fluids and blood samples were assessed over a 5-day period. The mean gentamicin concentration peak in blood was 4.2 μg/ml and the mean local concentration in synovial fluids during the first 8 h was 305 μg/ml. After 48 h, the concentrations in blood and synovial fluids were less than 0.5 μg/ml. No remaining gentamicin was detected in bone substitutes explanted after 8 days of implantation. The gentamicin release rate from the bone substitutes assessed corresponds to the recommendations for the prophylactic use of antibiotics: high local concentration but limited in time (less than 48 h) not to select antibiotic-resistant bacterial strains. Our results indicated that this implant should be an effective prophylactic tool in orthopedic surgery in combination with systemic prophylaxis.

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1 Introduction

Chronic osteomyelitis and infections on prostheses remain a considerable problem in orthopedic surgery because of the severe functional disabilities endured by the patients. In order to reduce the risks of infection, prophylaxis is carried out by intravenous injection of an antibiotic 1 or 2 h before surgery [1]. The aim is to saturate the patient tissues with antibiotic during the entire time of the surgery. However, as bone is poorly vascularized and as surgery disturbs the vascularization of the operated site, the local concentrations are low and bacteria should proliferate. Antibiotics must be administrated in high concentrations over prolonged periods of time to yield adequate local concentrations. But such high drug concentrations in blood over long periods may induce toxicity [2]. Furthermore, for long treatments, antibiotic-resistant microbial strains can emerge because of the selective pressure induced by the antibiotic [3]. Such resistant strains are a major problem in hospitals, and treatments must take into account the preservation of the efficiency of the available antibiotics.

Local delivery of antibiotics would have the advantage of achieving high local drug concentrations with low risks of systemic toxicity. Antibiotic loaded polymethylmethacrylate (PMMA) bone cements [3] introduced by Buchholz and Engelbrecht in 1970 have been shown to enhance the efficiency of intravenous prophylactic treatments for total hip replacements [4]. However, less than 10% of the load is released during the first 5–10 days of implantation [3, 5]. The remaining antibiotic is released at low levels over many months [2, 6] and could select antibiotic-resistant strains [3, 7, 8]. For procedures different from cemented prosthesis setting, no local antibiotic delivery system is now available for prophylaxis.

For bone repair surgery, a new gentamicin loaded hydroxyapatite and tricalcium phosphate (HAP/TCP) bone substitute was developed for prophylactic use and was assessed in vitro in a previous study [9]. The release rate obtained in physiological serum was correlated to the bone substitute volume: the larger the bone substitute, the longer the release duration. For all the bone substitute shapes assessed, the entire gentamicin load (125 mg) was released in less than 48 h. The purpose of the present study was to assess in vivo the systemic and local gentamicin concentrations obtained. The aim was to obtain (i) local concentrations higher than the minimal bactericidal concentration (MBC) of the majority of the bacteria responsible for infection in orthopedic surgery, (ii) systemic concentrations lower than the toxicity threshold of gentamicin, (iii) a release duration less than 48 h not to select antibiotic-resistant microbial strains.

The gentamicin loaded bone substitutes were implanted at a bone site in the left condyle of the femur of 5 sheep. Synovial fluid and blood were sampled over a period of 5 days in order to investigate the local and systemic concentrations of gentamicin released by the bone substitute. Other gentamicin loaded bone substitutes were implanted in the muscle of 5 other sheep and blood was sampled over a 3-day period in order to investigate the influence of the contact surface between the implant and muscle tissues on the gentamicin systemic concentrations. After 8 days of implantation, the implants placed in muscle were explanted in order to quantify the dose of gentamicin remaining in the biomaterial.

2 Materials and methods

2.1 Gentamicin loaded HAP/TCP bone substitutes

A new commercial bone substitute composed of 70% hydroxyapatite and 30% β-tricalcium phosphate containing 125 mg of gentamicin[1] was used in this study. This bone substitute had a high ratio of porosity (70%) composed of macroporosities (300–600 μm in size) and microporosities (1–2 μm in size). The porosities were interconnected with macro-interconnections of 10–15 μm [9, 10]. Gentamicin was incorporated in the porosities by impregnation with a solution of gentamicin sulphate and sterile water (sterilized water for injection, Aguettant, France) after machining and cleaning of the HAP/TCP matrix. After impregnation, the implants were dried, packaged and sterilized[2] Gentamicin sulphate and sterile water conforming to the European pharmacopoeia [11, 12] were used.

The bone substitutes were machined in shape of cylinders 10 mm diameter and 15 mm height. The 125 mg of gentamicin were equally distributed in two cylinders. For implantation in the muscle, the bone substitutes were machined in shape of parallelepipeds 10 × 5 × 5 mm and the 125 mg of gentamicin were equally distributed in 4 parallelepipeds.

2.2 In vivo essays

The local committee for animal studies of the Veterinary School of Lyon (France) had previously approved this animal experiment.

Implantation in bone The experiment was conducted on 5 female Charolais sheep (Group B1–B5, weight 39–55 kg, age 16–22 months). After anesthesia with Halothane and Oxygen, the left leg was clipped and soaped seven times and an antiseptic solution has been applied.[3] The sheep were placed in dorsal recumbency for a classical medial approach. After arthrotomy, the patella was luxated. 15 mm long and 10 mm diameter holes were drilled in both condyles without effraction of the intercondylar notch. The gentamicin loaded bone substitutes (two cylinders Ø10H15 mm with a total amount of 125 mg of gentamicin) were placed in the medial part of the medial femoral condyle and in the lateral part of the lateral condyle. The two implants were totally embedded in the holes (see Fig. 1). The patella was reduced and the arthrotomy closed with three layers: capsular, sub-cutaneous and cutaneous tissues. The skin sutures were removed at 10 days. Pain was prevented with morphine chlorhydrate during the surgical procedure and 8 h post operative and ketophene was administered for 2 days after surgery. After recovering from anesthesia the animals were allowed free movement in their boxes.

To evaluate the position of the implants in the condyles, craniocaudal and latero-medial radiographies of the stifle were performed with a N800 HF radiology equipment[4] at the end of the surgery.

[1] ATLANTIK *Genta*, Medical Biomat, France.

[2] Gamma sterilization between 25 and 40 kGy, Ionisos, France.

[3] Betadine, 10% solution, Pharmaceuticals S.A. Merignac, France.

[4] Trophy, Marne-la-vallée, France.

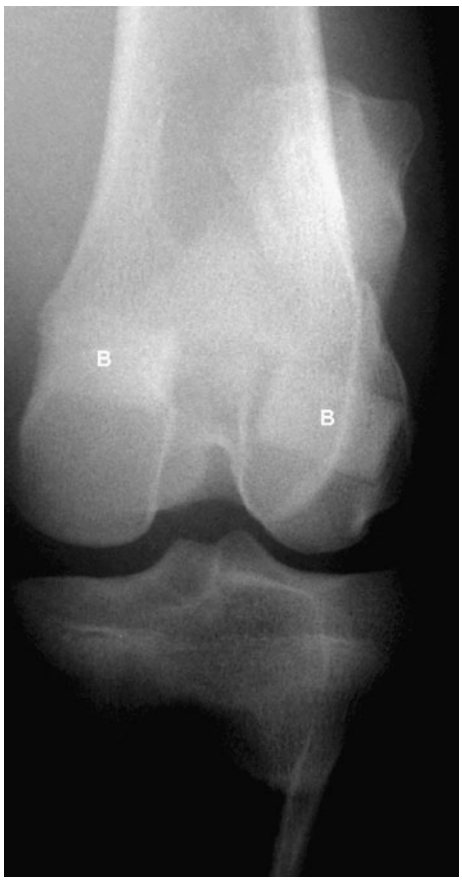

Fig. 1 Post-implantation left stifle X-ray: Two cylindrical loaded bone substitutes (**B**) containing on the whole 125 mg of gentamicin

Synovial liquid and blood samples collected 0.5, 1, 2, 4, 6, 8, 24, 48, 72, 96, and 168 h after the closure of the articulation. Synovial liquid samples were collected by intra-articular punctures aseptically performed alternatively in the lateral or medial articular capsule. Synovial liquid and blood samples were centrifuged and frozen at −20°C for conservation before analysis.

Muscular implantation The experiment was conducted on five female Charolais sheep (Group M1–M5, weight 45–55 kg, age 16–22 months). After intravenous anesthesia with ketamine and xylazine, the left shoulder and scapula were clipped and disinfected three times. Two 7 cm distant parallel incisions were made in the skin and muscular fascia of the supra spinatus muscle. An intramuscular tunnel was prepared from the skin approach to gently introduce the gentamicin loaded bone substitutes (4 parallelepipeds 10 × 5 × 5 mm with a total amount of 125 mg of gentamicin). After verification of the intramuscular position of the four implants, the surgical wound was closed with fascia, subcutaneous and cutaneous stitches. Blood samples were collected 1, 2, 4, 6, 8, 24, 48, and 72 h after the closure of the wound. The blood samples were centrifuged and the serum separated. After 8 days, implants were explanted under general aesthesia in order to measure the remaining quantity of gentamicin. Blood samples and implants were frozen at −20°C for conservation before analysis.

2.3 Immunoassay of gentamicin

The gentamicin concentration in blood and synovial fluid samples was determined by using the COBAS INTEGRA fluorescence polarization system (Roche) [13]. The samples were incubated with a mouse monoclonal antibody, and then a tracer reagent was added. The light emission, which was proportional to the gentamicin concentration, was measured at 515 nm. The detection level for gentamicin was 0.14–10 μg/ml.

The gentamicin amount remaining in the explanted implants after 8 days of implantation in muscular tissue was determined by the same method after elution of the remaining gentamicin in a salt solution. To measure the remaining gentamicin in the bone substitutes, elution was carried out by introducing the implant in 10 ml of 0.9% sodium chloride solution,[5] crushing the implant and agitating it on a plate agitator at 80 rpm at 37 ± 1°C for 72 h. The concentration in supernatant was measured by immunoassay as described above.

3 Results

3.1 Implantation in bone: local and systemic concentrations

Gentamicin immunoassay results of blood and synovial liquid sampled over a 5-day period after implantation in bone have been summarized in Table 1; Figs. 2 and 3.

Gentamicin blood concentrations The concentration increased to reach a maximum and then decreased. For the sheep B1–B5, the peak of concentration was observed at 4 h. The extreme values for this peak were 3.02 and 6.50 μg/ml for an average of 4.22 μg/ml and a standard deviation of 1.58 μg/ml. These values are far below the toxic concentration of 12 μg/ml. Given the small number of animals in the study, a statistical analysis and an extrapolation to larger series is difficult. However, considering a normal law with a mean value of 4.22 μg/ml and a standard deviation of 1.58 μg/ml measured on five animals, the probability that the mean value of the general population is comprised between 2.02 and 6.4 μg/ml is of 95%. In the most unfavorable hypothesis of a mean value of 6.4 μg/ml, the probability that the toxic concentration of 12 μg/ml could be reached is only of 7%. Therefore, if a

[5] Aguettant, Lyon, France.

Table 1 Blood and synovial liquid gentamicin concentrations measured after implantation of the gentamicin loaded bone substitutes in the condyle of femur of 5 sheep (concentrations after implantation in bone identified B1–B5 for sheep 1–5)

Time (h)	Gentamicin concentration (μg/ml)									
	Blood					Synovial liquid				
	B1	B2	B3	B4	B5	B1	B2	B3	B4	B5
0.5	0.43	0.36	/	0.93	1.85	/	/	/	/	/
1	1.13	1.01	0.71	0.96	3.28	**980**	**2565**	35	26	32
	/	1.81	2.03	2.22	4.15	/	/	32	61	137
4	**3.12**	**3.19**	**3.02**	**5.25**	**6.50**	784	104	47	**78**	**180**
	2.03	/	2.54	3.98	5.08	/	/	/	/	/
B	1.31	2.27	0.40	3.46	2.56	154	108	**55**	73	35.5
24	0.48	0.29	0.40	0.35	0.35	20.6	2.7	7.2	133	6.4
48	0.20	0.17	0.15	0.16	0.25	2.6	0.82	0.79	0.87	0.55
72	0.38	0.14	0.17	0.15	0.18	0.49	0.24	0.19	0.33	0.22
96	0.23	/	0.19	0.14	0.20	0.2	0.26	0.15	0.2	0.25
168	0.21	0.15	0.14	0.14	0.22	0.16	0.15	0.28	0.15	0.22

Concentration peaks are in bold. "/" means that the value has not been recorded

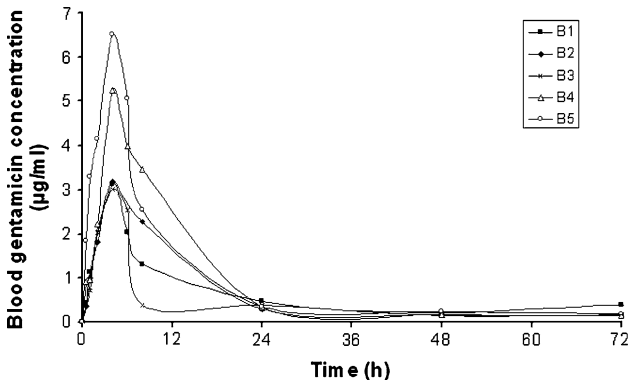

Fig. 2 Blood gentamicin concentrations measured after implantation of the gentamicin loaded bone substitutes in the condyle of femur of 5 sheep (concentrations after implantation in bone identified B1–B5 for sheep 1–5)

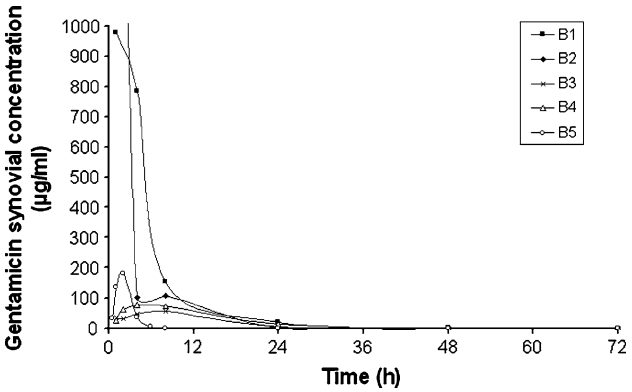

Fig. 3 Synovial liquid gentamicin concentrations measured after implantation of the gentamicin loaded bone substitutes in the condyle of femur of 5 sheep (concentrations after implantation in bone identified B1–B5 for sheep 1–5)

larger series of animals would be required to ensure that the risk of reaching the toxic concentration is close to zero, the results obtained in this small-scale animal study tend to show that the concentration in blood should remain sufficiently low.

After 48 h, all the values were lower than 0.4 μg/ml.

Gentamicin synovial liquid concentrations: for the sheep B1 and B2, the peak was observed at first hour with very high concentrations (respectively 980 and 2565 μg/ml); for the sheep B4, B5 and B6, the peak was observed between the fourth and the eight hour with lower values (55–180 μg/ml). Between the first and the eight hour, the mean concentration was 305 μg/ml with a standard deviation of 597 μg/ml and all the recorded values were higher than 26 μg/ml. After 48 h, all the recorded values were lower than 0.5 μg/ml. The concentrations measured are far above the minimal bactericidal concentration of 12 μg/ml. However, due to the large standard deviation of the results, no statistical analysis was possible. Therefore, more experiments would be required on a larger series of animals, to fully insure that the amount of Gentamicin synovial liquid concentration is always larger than the required 12 μg/ml.

3.2 Implantation in muscle tissues: systemic concentrations and residual amount in the implant

Gentamicin immunoassay results of blood sampled during the 3 days after implantation of the bone substitutes in a muscular site have been summarized in Table 2 and Fig. 4. The peak of concentration was observed between the second and the fourth hour depending on the individual.

Table 2 Blood gentamicin concentrations measured after implantation of the gentamicin loaded bone substitutes in the muscle of 5 sheep (concentrations after implantation in muscle identified M1–M5 for sheep 1–5)

Time (h)	Gentamicin concentration (μg/ml)				
	Blood				
	M1	M2	M3	M4	M5
1	2.03	2.58	3.62	0.94	0.82
2	**4.33**	3.63	**4.95**	4.92	2.85
3	/	**3.65**	4.38	**6.95**	3.82
4	3.56	3.17	4.15	6.12	**4.20**
6	2.19	/	/	/	
8	1.15	1.30	1.55	2.97	2.48
24	0.17	/	0.14	0.17	0.19
48	0.14	/	0.14	0.14	0.14
72	0.14	/	0.14	0.14	0.14

Concentration peaks are in bold. "/" means that the value has not been recorded

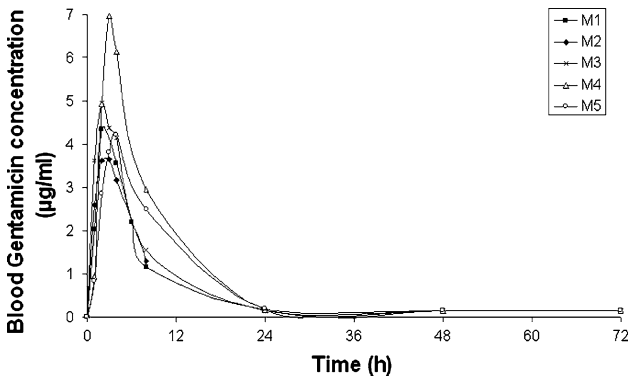

Fig. 4 Blood gentamicin concentrations measured after implantation of the gentamicin loaded bone substitutes in the muscle of 5 sheep (concentrations after implantation in muscle identified M1–M5 for sheep 1–5)

The extreme values for this peak were 3.65 and 6.95 μg/ml for an average of 4.82 μg/ml and a standard deviation of 1.28 μg/ml. After 24 h, all the values were lower than 0.2 μg/ml. These results appeared to be very close to concentrations in blood obtained after implantation in bone.

After explantation of the bone substitutes after 8 days of implantation, a mean gentamicin dose of 3.6 ± 0.3 μg was eluted from the HAP/TCP matrix, which represented less than 0,003% of the initial amount.

4 Discussion

On the basis of these results, five main key points can be discussed:

4.1 With regard to the choice of prophylactic action versus therapeutic action

Many studies have been reported on bone substitutes loaded with antibiotics but often the aim of these studies is to treat chronic osteomyelitis [14, 15]. Such therapeutic applications need a release rate which is very different than that required for prophylactic action: the release duration should be longer than 10 days with high concentrations during the entire duration of the treatment. Most of the publications present technological solutions to slow the release of the antibiotic from the bone substitute: embedding of the antibiotic in a polymer [16–18] (but if the polymer is not osteoconductive, this can have a negative impact on the main function of the bone substitute which remains bone healing) or modifying the porosity of the ceramic matrix [19, 20] (but to slow the release rate, porosity should be closed whereas for osteoconduction the porosity should be interconnected [21]). If the bone substitute is not modified, the release rate is too rapid [17]. The final difficulty is related to the nature of the antibiotic incorporated in the bone substitute. To treat bone infections, microbiologists have to identify the bacteria responsible for the infection and then choose the most efficient antibiotic: a "ready to use" antibiotic loaded bone substitute will only be useful if the antibiotic loaded fits the conclusions of the microbiologists.

In the present study, the aim of the antibiotic loaded bone substitute is prophylactic. The release duration should be less than 48 h; the dose to be incorporated is 150 mg for gentamicin and a large spectrum antibiotic should be efficient in most orthopedic indications.

4.2 With regard to the experimental model

A sheep model has been chosen for this study because the release rate depends on the volume of the bone substitute [9] and the toxicity of gentamicin depends on the dose implanted compared to the weight of the animal. The size of sheep allows the implanting of the same volume of bone substitute and the same dose of gentamicin as for humans. In humans, the local concentration of antibiotic is evaluated by testing the draining liquids [5], but this method can not be applied to animals because of the related infection risks. To investigate the local antibiotic concentration in vivo, a traditional method is to test bone samples around the implant. However, individual physiological reactions could have an influence on the results if many bone samples are collected from the same animal at different times. If only one sample is collected per individual, many animals would have to be euthanized. The experiment has been conducted on growing sheep because the vascularization of the distal epiphysis and the synovial membrane is

the same and comes from the femoral epiphysial artery. This characteristic disappears in adult sheep. In growing sheep, the epiphysial bone is very lacunal and trabecular with many vessels. The diffusion of an antibiotic through the blood capillaries of the synovial membrane is very rapid and the antibiotic concentration in the synovial liquid is close to the concentration in the epiphysial bone. In the present study, each animal was punctured twelve times with no local physiological reaction and the number of animals used was greatly decreased.

4.3 With regard to the concentrations in blood and risks of toxicity

The dose of gentamicin contained in the bone substitute was chosen to be non-toxic. The concentrations in blood confirm that the serum peak is less than the half of the toxicity threshold of gentamicin (10–12 μg/ml in blood) and is limited in time. The statistical analysis of the results shows that the probability to overpass the toxicity threshold is less than 7%. The peak of concentration in blood has been reached 4 h after implantation in bone site and a little earlier after implantation in muscular site (between the second and the fourth hour). It has been demonstrated previously in vitro [9] that the release rate is related to the bone substitute volume. The smaller size and higher aspect ratio of the blocks implanted in the muscular site can explain the faster releasing of gentamicin. However, the high vascularization of the muscular tissues should also participate in a faster passing into blood.

For a patient with normal renal function, after intramuscular injection (IM) of gentamicin, the serum peak is generally reached within 1 h and the half-life is approximately 2.5 h [22, 23]. The serum rate is greatly decreased within 8 h and elimination continues for 24–48 h. In the present in vivo study, the intensity of the serum peak (4–5 μg/ml on average for a dose of gentamicin ranging from 2.3 to 3.2 mg/kg) is significantly lower than that which would have been obtained after intramuscular injection of the same amount of gentamicin. For humans, the same peak intensity (4 μg/ml) is generally reached with an intramuscular injection of 1–1.5 mg/kg [22]. It has been demonstrated previously in vitro [9] that for the blocks Ø10H15 mm and 10 × 5 × 5 mm, respectively, 85 and 100% of the gentamicin amount diffuse out of the bone substitute within 8 h. Compared to an IM injection, the progressive release of gentamicin through the porosities of the ceramic matrix delays the serum peak and limits its intensity.

However, the gentamicin serum peak should be higher with bone substitutes than with gentamicin loaded acrylic bone cements. According to the Wahlig et al. clinical study [5] the serum peak is 2 μg/ml after setting of a cement containing 1000 mg of gentamicin, but only 67 mg of gentamicin (6.7% of the total amount) is released from the cement during the first 24 h. In the present study, the whole of the gentamicin amount contained by the bone substitute (125 mg) is released within 24 h. This explains why the serum peak is 4 μg/ml.

4.4 With regard to the local concentrations and efficacy in prophylaxis

The majority of the gentamicin susceptible bacteria have a minimal inhibitory concentration (MIC) of approximately 3 μg/ml and one estimates that the minimal bactericidal concentration (MBC) is 2 or 4 times higher than the MIC, i.e. 6–12 μg/ml [23]. As gentamicin is a dose-dependant antibiotic, the higher the amount, the more effective it is. The bactericidal effect appears after only a few minutes of contact with bacteria. In this study, the local concentrations measured in the synovial liquid are very high. The lowest registered peak value is 55 μg/ml, the highest is 2565 μg/ml and during the first 8 h of implantation, the mean local concentration is 305 μg/ml. These concentrations are far above the MBC and at 24 h the majority of the concentrations are still effective. It can be assumed that these strong concentrations should make gentamicin effective on bacteria usually of intermediate sensitivity.

However, the local concentrations of gentamicin are heterogeneous depending on the individuals and no statistical analysis can be done on the results obtained. Two groups can be noticed: for the sheep B1 and B2, the local concentrations registered at 1 h are very high (respectively 980 and 2565 μg/ml) and decrease slowly, while for the sheep B3, B4 and B5 they are lower (but always above the effective concentrations) and form a peak with a maximum between the fourth and the eight hour (respectively 55, 78 and 180 μg/ml). In this second group, the earlier the peak is, the higher it is. The differences between the two groups could be explained by differences in mobility of the sheep after surgery. Sheep B1 and B2 stand 20–24 h after waking up, while sheep B3, B4 and B5 recovered their mobility just 2 h after the end of surgery. As the articular capsule has been opened during surgery, the renewing of the synovial liquid and the elimination of gentamicin should be accelerated by the movement of the sheep.

According to the results of the present in vivo study, it can be assumed that the local concentrations of gentamicin released from the bone substitute should be higher than the local concentrations released from gentamicin-loaded bone cements: The dose released from the bone substitute during the first 24 h is higher than the dose released by a 1000 mg gentamicin-loaded cement and the release is more rapid [5].

4.5 With regard to the release duration and bacterial resistances

To avoid selection of resistant strains, it is generally recommended that the duration of antibiotic prophylactic treatments should be lower than 48 h. In this study, the release duration is 24 and at 48 h the mean gentamicin concentrations are 0.19 μg/ml in blood and 1.13 μg/ml in the synovial liquid. The assays carried out on the bone substitutes implanted in the muscle and explanted at 8 days confirm that gentamicin is entirely released and is not encapsulated in the HAP/TCP matrix. Thus, there is no risk of selecting resistant strains by a slow release at low concentration as it is has been observed with antibiotic loaded acrylic cements [2].

5 Conclusion

In this in vivo study of the release rate of gentamicin from porous HAP/TCP bone substitutes, very high local concentrations were measured (305 μg/ml on average over the first 8 h), while the mean serum peak was 4–5 μg/ml. The lowest local gentamicin concentration peak observed was more than 4 times higher than the effective threshold (MBC) of gentamicin and the concentration in blood was less than half of the toxicity threshold. The release duration was 24 h and it has been verified on bone substitutes explanted at 8 days that no gentamicin remains in the HAP/TCP matrix. These characteristics (high dose but limited in time) were compatible with the usual recommendations for antibiotic prophylaxis.

Gentamicin is a broad spectrum antibiotic which is efficient on most of the bacteria responsible for infections in orthopedic surgery. Moreover, this antibiotic shows synergetic effects in association with other appropriate antibiotics. Gentamicin loaded bone substitutes should be a useful prophylactic tool if combined with an intravenous (IV) antibiotic prophylactic injection. IV injections allow a protection of the patient vascularized soft tissues from the beginning of the surgical procedure and the gentamicin loaded bone substitute increase the efficacy of the protection in the operated site and bone tissues.

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