In vivo reduction of biofilm seeded on orthopaedic implants

safety and efficacy of portable electromagnetic induction heating

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Aims

Electromagnetic induction heating has demonstrated in vitro antibacterial efficacy over biofilms on metallic biomaterials, although no in vivo studies have been published. Assessment of side effects, including thermal necrosis of adjacent tissue, would determine transferability into clinical practice. Our goal was to assess bone necrosis and antibacterial efficacy of induction heating on biofilm-infected implants in an in vivo setting.

Methods

Titanium-aluminium-vanadium (Ti6Al4V) screws were implanted in medial condyle of New Zealand giant rabbit knee. Study intervention consisted of induction heating of the screw head up to 70°C for 3.5 minutes after implantation using a portable device. Both knees were implanted, and induction heating was applied unilaterally keeping contralateral knee as paired control. Sterile screws were implanted in six rabbits, while the other six received screws coated with *Staphylococcus aureus* biofilm. Sacrifice and sample collection were performed 24, 48, or 96 hours postoperatively. Retrieved screws were sonicated, and adhered bacteria were estimated via drop-plate. Width of bone necrosis in retrieved femora was assessed through microscopic examination. Analysis was performed using non-parametric tests with significance fixed at $p \leq 0.05$.

Results

The width of necrosis margin in induction heating-treated knees ranged from 0 to 650 μm in the sterile-screw group, and 0 to 517 μm in the biofilm-infected group. No significant differences were found between paired knees. In rabbits implanted with sterile screws, no bacteria were detected. In rabbits implanted with infected screws, a significant bacterial load reduction with median 0.75 Log10 colony-forming units/ml was observed ($p = 0.016$).

Conclusion

Induction heating was not associated with any demonstrable thermal bone necrosis in our rabbit knee model, and might reduce bacterial load in *S. aureus* biofilms on Ti6Al4V implants.

Article focus

- Assessment of thermal necrosis margins in adjacent bone after induction heating.
- Assessment of antibacterial efficacy of induction heating disinfection in an in vivo setting.
- Feasibility of the technique in a surgical environment.

Key messages

- Thermal bone necrosis amounted to less than 1 mm, and no differences were found with regard to untreated knees.
- A statistically significant bacterial load reduction was achieved after induction heating.

Strengths and limitations

- Induction heating was tested in an in vivo setting, with all the hindrances and difficulties of a realistic surgical environment.
- This study used a limited number of animals: 24 knees in 12 rabbits.
- Contactless temperature monitoring via thermographic imaging is less accurate within the complexity of the surgical environment.
- Combined effects with other disinfection techniques, as well as over different bacterial strains, are yet to be studied.

Introduction

Implant infection is a major complication in orthopaedic surgery, with an overall incidence of 2%.[1,2] It encompasses high morbidity and mortality rates, a decline in life quality, and repeated surgeries.[3] Debridement, antibiotics and implant retention (DAIR) is the least aggressive curative option, but healing rates following DAIR remain modest, currently accepted to be around 50% to 80%.[2-4] One of the main concerns is the ability of infecting bacteria to develop biofilm, which entails a decreased metabolic activity, biofilm-specific expression of antibiotic resistance genes, enhanced horizontal gene transmission, and structural properties that hinder antibiotic diffusion and phagocytosis.[2,4,5] These features determine their resistance to most current therapies.

Besides disinfection methods included in DAIR (debridement, antiseptic solutions, mechanical scrubbing, dilution, antibiotics), new techniques include extrinsic physical agents such as photodynamic therapy, sonication, high-energy plasma, electric pulses, or heat.[6] In particular, heat is among the most employed disinfection methods in the food manufacturing and health industries to sanitize surfaces and equipment. Hydrated biofilms are proven to be susceptible to temperatures as low as 60°C.[7]

Disinfection using electromagnetic induction selectively administers heat to metallic surfaces in a contactless fashion.[8,9] In 2017, Pijls et al[9] demonstrated the efficacy of this technique over planktonic forms of several bacterial species. Later, the same group published their results over mature biofilm of *Staphylococcus epidermidis,*[10] where a 6.7-Log reduction was achieved after induction heating up to 60°C for 3.5 minutes. Further published results explored the combined effect with different antibiotics and mechanical cleaning.[11,12]

One possible drawback of induction heating would be thermal necrosis of adjacent bone, which could induce implant loosening and thus limit its application on well-integrated prostheses. In a study by Berman et al,[13] bone response to heat was characterized using rabbit tibiae, showing the first signs of necrosis over 70°C in cortical bone and over 55°C in cancellous bone. Histopathological changes were already present after one week. However, the relationship between acute thermal bone necrosis and implant loosening is not yet established.

To the best of our knowledge, no studies have been published assessing induction heating disinfection of metallic implants in an in vivo setting. This would allow to further validate the efficacy of this technique, and could also provide us with some data regarding its effect on host tissues.

The objective of this study was to assess the safety in terms of adjacent bone necrosis and antibacterial efficacy after induction heating disinfection of mature biofilms on infected metallic orthopaedic implants in the knee joint.

Methods

The selected implant infection model was a rabbit knee receiving a Ti6Al4V screw in the medial femoral condyle, where *Staphylococcus aureus* biofilm had been previously grown. The study intervention consisted of heating the exposed metallic surface up to 70°C for 210 seconds via electromagnetic induction, and it was administered during surgery, right after implantation and before layered closure, using a portable device. After a fixed number of days, animals were returned to the operating theatre for sacrifice and sample collection. Screws were sonicated for bacterial quantification, while distal femur segments were microscopically examined for necrosis assessment.

Study design

Each rabbit received implants in both knees. One knee was subjected to experimental treatment (induction heating), while the other served as a paired control, alternating side. Six rabbits (12 knees) were included in each group, since smaller numbers cannot show statistical significance in non-parametric matched-sample analysis.

First, a six-rabbit series (rabbits A to F) was implanted with sterile screws. The animals were killed and samples were obtained 24, 48, and 96 hours after implantation and treatment (two rabbits for each timepoint). This group served to confirm the sterility of the technique and to isolate the thermal effect of induction heating on underlying bone, as well as to determine the timing of pathological manifestations of acute thermal bone necrosis.

After the results from the sterile screw group showed no differences between 24 and 96 hours in terms of bone necrosis, a second six-rabbit series (rabbits G to L) was implanted with screws incubated with *S. aureus* biofilm as described below. This group served to further characterize thermal bone necrosis, as well as to assess antibacterial efficacy. The animals were killed and samples were obtained 24 hours after implantation and treatment.

Joint implant model

The selected implants were Ti6Al4V 18 mm × 2.7 mm non-locking screws, with round head and star-shaped socket cap (Acumed Small Fragments, USA), implanted in the medial femoral condyle.

Specific pathogen-free New Zealand white male rabbits ranging from 2.550 to 3.200 kg were used. All rabbits were housed in individual cages in an air-conditioned room at 22°C (± 2°C) and on 12-hour light-darkness cycles. This study was approved by the Animal Care and Use Committee of our institution. Animal care and maintenance were performed following institutional guidelines defined by national and international legislation (Real-Decreto 53/2013, Council of Europe 2010/63/EU-Directive). ARRIVE Checklist criteria were followed.

Biofilm development

To assess bactericidal efficacy on a species frequently involved in arthroplasty infections, *S. aureus* ATCC 29213 strain was selected. This strain was kept frozen at -80°C until the experiments were performed. It was cultured for 24 hours on tryptic soy agar with 5% sheep blood (bioMérieux, France) at 37°C and with 5% CO_2. Posteriorly, we obtained an inoculum of approximately 10^6 colony-forming units per millilitre (CFU/ml) in brain heart infusion with 2% glucose as a biofilm inducer. In order to generate the biofilm, 3 ml of this inoculum were dispensed over each screw in an Eppendorf tube. Screws were incubated for 24 hours at 37°C with 5% CO_2. After incubation, screws were washed three times with sterile saline solution (SS) with 9% NaCl (B Braun, Germany) to eliminate planktonic forms, and were refrigerated until implantation, which took place in the following one to four hours.

Surgical technique

The control side was the first to be implanted, and the implant procedure on the treatment side began only after skin closure of the contralateral knee was completed.

The implantation procedure and study intervention were performed under general anaesthesia (induction with ketamine, buprenorphine, and medetomidine, maintenance with inhaled sevoflurane). In a supine position, both legs were shaved and the skin was prepared with povidone-iodine. Sterile surgical draping was placed to allow the exposure of both knees. The skin was incised 3 to 4 cm along the anterior midline, and medial condyle exposure was achieved through a medial parapatellar approach and lateral dislocation of the patella. A 4 mm area on the articular surface of medial femoral condyle was countersunk using a high-speed ballpoint milling cutter (Dremel; Robert Bosch Tool Corporation, USA) to accommodate the screw head, and a 1 mm tunnel aligned with the femoral diaphysis was drilled. The corresponding screw was then implanted, either sterile or incubated with biofilm depending on the experimental group. The study intervention, when indicated, was applied at this stage, immediately after implantation.

Layered closure (extensor apparatus, skin) was performed with 2/0 width suture silk. Local anaesthesia with bupivacaine was injected in extensor apparatus and subcutaneous tissue. The rabbits were returned to their individual cages for the postoperative period, and analgesia with meloxicam was administered.

Study intervention

Our group has developed a Portable Disinfection System based on Induction Heating (PDSIH, patent number 2207005-EPP0), designed for its use to be feasible in an operating theatre (Figure 1). This system consists of a coil implemented using a PQ32/30 half core (core material: 3C90). The winding consists of 30 turns, equally distributed in five layers of six turns per layer. Litz wire (360 strands, 44 AWG) is selected to reduce high-frequency losses. The resulting inductance value is 62 µH. A 50 kHz switching frequency was selected to achieve a good trade-off between size, enabling a compact and portable implementation, and switching losses. The resulting skin depth is 295 µm. A full bridge inverter was selected, yielding approximately $2{\cdot}V_{ac,rms}$ maximum applied voltage in the worst-case scenario (maximum output power). The inherent contactless characteristics of the proposed induction heating system inherently protect both the patient and the surgical setting. Additionally, the system is fully wrapped in a plastic cover, and reinforced insulated wire is selected to enhance safety. The system is designed to deliver up to 500 W of maximum output power. The estimated electrical efficiency is close to 97%. The power can be controlled by using a slider, from approximately 0 to the maximum power.

The experimental treatment consisted of electromagnetic induction heating using the PDSIH, in order to maintain a 70°C surface temperature of the screw head for 210 seconds. To this end, the PDSIH was held stationary 1 to 2 cm over the metallic surface and aligned with longitudinal axis of the screw with the potentiometer knob fixed at 100% power. An initial 20-second pulse was needed to attain 70°C. Surface temperature was monitored between pulses with a thermographic camera (Fluke TiS75+; Fluke Corporation, USA) with emissivity fixed as 0.63. Further pulses were administered with variable durations to maintain surface temperature between 70°C and 80°C. This procedure was executed without further manipulation of the metallic surface, to avoid mechanical damage to biofilm (Figure 2). With this induction heating method, heat is mostly administered to the exposed screw head, while the screw tip, located deep inside the bone, should remain underheated. Figure 3 shows the thermal distribution in an ex vivo heated Ti6Al4V screw as seen in thermographic images.

Sample collection and processing

Animal sacrifice and sample collection were performed 24, 48, or 96 hours after implantation depending on the experimental group. Euthanasia was performed by potassium chloride overdose under general anaesthesia. Skin preparation with povidone-iodine and surgical draping were identical to the implantation procedure. A medial parapatellar approach was performed through previous incision. The screw was extracted with a sterile technique, avoiding manipulation of the metallic surface except for screwdriver insertion, and kept in a refrigerated sterile canister for microbiological samples. Posteriorly, surgical incision was extended; muscle, tendon, and ligament insertions in the distal femur were released, and the distal third of the femur was resected with an electric rotating saw and stored in a recipient with 40% formaldehyde with phosphate buffer.

All 24 distal femur segments retrieved from 12 rabbits were fixated for 48 hours in 40% formaldehyde, phosphate-buffered solution. Their length and condylar width were measured, and photographs of anterior and posterior views

Fig. 1
Portable Disinfection System based on Induction Heating (PDSIH, patent number 2207005-EPP0).

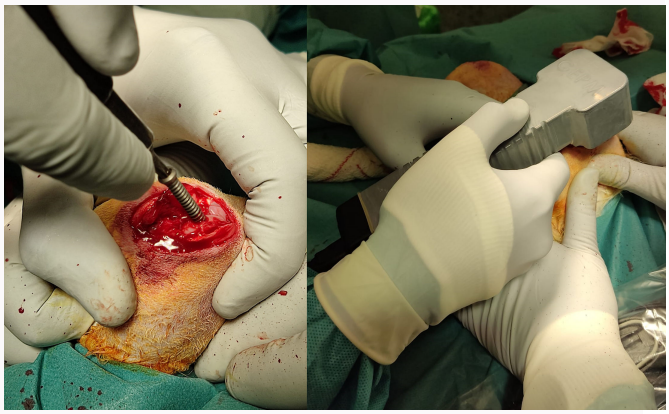

Fig. 2
Left: Screws were implanted in the medial femoral condyle, avoiding manipulation of the surface. Right: Experimental treatment was applied immediately after implantation.

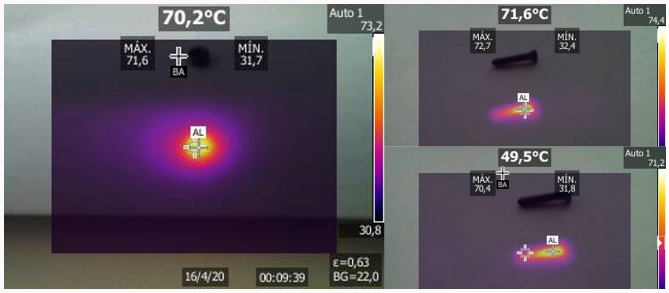

Fig. 3
Thermographic images of ex vivo heated screws. Left: Front view of the screw head. Right: Lateral view of heated screw immediately after removal. Note that the temperature at the tip of the screw (bottom right) is significantly lower than that of the head (upper right).

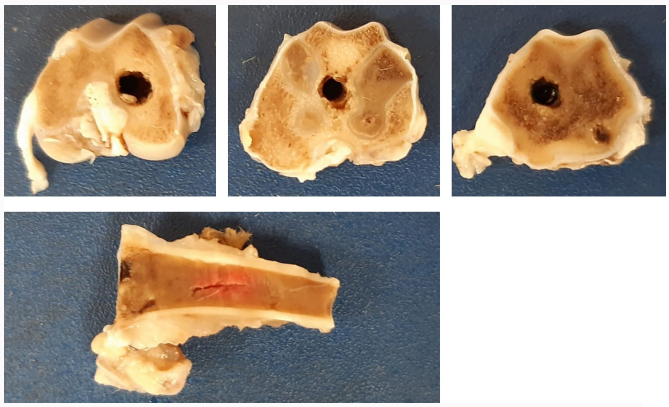

Fig. 4
Distal femur sections were cut transversally 4, 7, and 14 mm from distal apex, and sagitally containing the screw track.

were taken. All specimens were cut using a 1,000 m/min mechanic saw (Exakt Pathology Saw; Exakt Tools, Germany) in three transversal sections 2, 7, and 14 mm from the distal articular surface, and a sagittal section centred at the apex of the screw cavity (Figure 4). These sections were decalcified in 7% nitric acid for 12 hours, rinsed with water, and paraffin-embedded. Histological preparations were obtained, cutting 4 µm sections with a microtome and stained with haematoxylin-eosin. Microscopic examination assessed the presence and width of bone necrosis, as well as the presence of an acute inflammatory component, using the European Bone and Joint Infection Society (EBJIS) criterion of ≥ five polymorphonuclear neutrophils (PMN) per high power field (HPF).[14]

Retrieved screws were transported in sterile refrigerated recipients to the Microbiology laboratory. They were sonicated with 3 ml saline solution (SS) (B Braun, Germany), in sterile 6.5 ml tubes (Sarsted, Germany), with a low-power ultrasonic bath sonicator Ultrasons-H 3000840 (J P Selecta, Spain) at 22°C for five minutes.[15] This sonicated fluid was diluted 1:10 serially with SS, and adhered bacteria were estimated with the drop plate technique.[16]

Statistical analysis

Statistical analysis was performed with GraphPad Prism 8.0.1 software (GraphPad 2018, USA). Data were evaluated by means of non-parametric unilateral Wilcoxon signed-rank test for matched samples. Statistical significance was fixed at $p \leq 0.05$.

Results

A total of 24 knees were implanted in 12 rabbits: six rabbits (12 knees) with sterile screws and killed after 24 hours (2 rabbits), 48 hours (2 rabbits), and 96 hours (2 rabbits); and six rabbits (12 knees) with biofilm-coated screws and killed after 24 hours.

Bone necrosis

The width of the bone necrosis margins throughout all distal femur samples ranged from 0 to 1,000 µm. This 1,000 µm maximum corresponded to a non-treated, sterile screw knee. Necrosis margins in treated knees ranged from 0 to 650 µm in the sterile screw group and from 0 to 517 µm in the biofilm-infected group. No significant differences were found in terms of bone necrosis margins when comparing matching treated versus untreated knees. Results are summarized in Table I and plotted in Figure 5.

These differences were analyzed using Wilcoxon signed-rank test for matched samples. Significance levels in

Table I. Bone necrosis, µm.

Rabbit	Treated knee	Evolution, hrs	Section 1 (2 mm)		Section 2 (7 mm)		Section 3 (14 mm)		Section 4 (Sagittal)	
			Treated	Control	Treated	Control	Treated	Control	Treated	Control
A	Right	24	105	0	275	104	570	0	0	0
B	Right	96	0	250	570	515	650	0	540	690
C	Left	96	0	0	195	330	520	220	0	0
D	Left	24	100	0	530	255	560	330	0	140
E	Right	48	0	420	500	1,000	0	403	0	0
F	Left	48	430	0	445	785	0	0	0	0
Median (IQR)			50 (0 to 186)	0 (0 to 293)	473 (255 to 540)	423 (217 to 839)	540 (0 to 590)	110 (0 to 348)	0 (0 to 135)	0 (0 to 278)
G	Left	24	0	140	0	450	0	305	0	0
H	Right	24	517	165	200	362	344	250	166	380
I	Left	24	0	150	150	350	380	430	0	65
J	Right	24	113	675	293	219	65	215	0	210
K	Left	24	0	397	102	380	507	450	132	137
L	Right	24	0	200	157	120	278	585	135	140
Median (IQR)			0 (0 to 214)	183 (148 to 467)	154 (77 to 223)	356 (194 to 398)	311 (49 to 412)	368 (241 to 484)	66 (0 to 143)	139 (49 to 253)

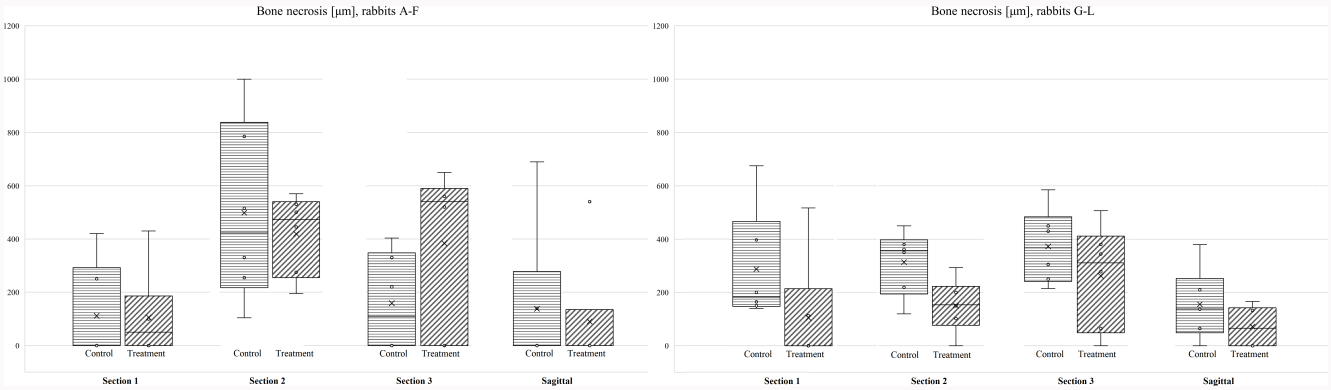

Fig. 5
Bone necrosis (µm) differences between matched knees. Left: Sterile screws. Right: Biofilm-infected screws.

the sterile-screw group were p = 0.500 (Section 1), p = 0.344 (Section 2), p = 0.156 (Section 3), and p = 0.250 (Sagittal section). In the biofilm-incubated screw group, these were p = 0.109 (Section 1), p = 0.078 (Section 2), p = 0.156 (Section 3), and p = 0.031 (Sagital section), all of them pointing towards a lesser necrosis margin in the treated knee.

When studying the possible delay in the onset of histopathological manifestations of thermal acute bone necrosis, no correlation could be observed between necrosis margins and time elapsed between intervention and sample collection: Spearman Rho correlation coefficients were randomly scattered around 0 for the different sections studied, and varied inconsistently between treated knees, control knees, and their differences, ranging between -0.47 and +0.67.

Bacterial load reduction

In the first six-rabbit series (rabbits A to F), which were implanted with sterile screws, no presence of CFUs on sonication fluid was revealed, thus corroborating the sterility of the technique.

In rabbits G to L, which were implanted with screws incubated with mature *S. aureus* biofilm, a bacterial load reduction was observed in all animals with a median of 0.75 (IQR 0.52 to 1.08) Log10 CFU/ml when comparing treated versus untreated knees. This reduction was statistically significant (p = 0.016). The results are summarized in Table II and plotted in Figure 6.

Acute inflammatory response, as well as intraosseous or subsynovial abscessification, were found in 40 out of 48 femur

Table II. Rabbits implanted with screws incubated with *Staphylococcus aureus* biofilm.

		Control		Treated		Difference	Reduction percentage
Rabbit	Treated knee	CFU/ml	Log10 CFU/ml	CFU/ml	Log10 CFU/ml	Log10 CFU/ml	
G	Left	148,000	5,170	2,020	3,305	1.865	98.635
H	Right	580,000	5,763	172,000	5,236	0.528	70.345
I	Left	380,000	5,580	126,000	5,100	0.479	66.842
J	Right	4,560,000	6,659	900,000	5,954	0.705	80.263
K	Left	4,500,000	6,653	720,000	5,857	0.796	84.000
L	Right	3,420,000	6,534	516,000	5,713	0.821	84.912
Median (IQR)		6,149 (5,477 to 6,655)		5,474 (4,652 to 5,882)		0.750 (0.516 to 1.082)	82.230

CFU, colony-forming units.

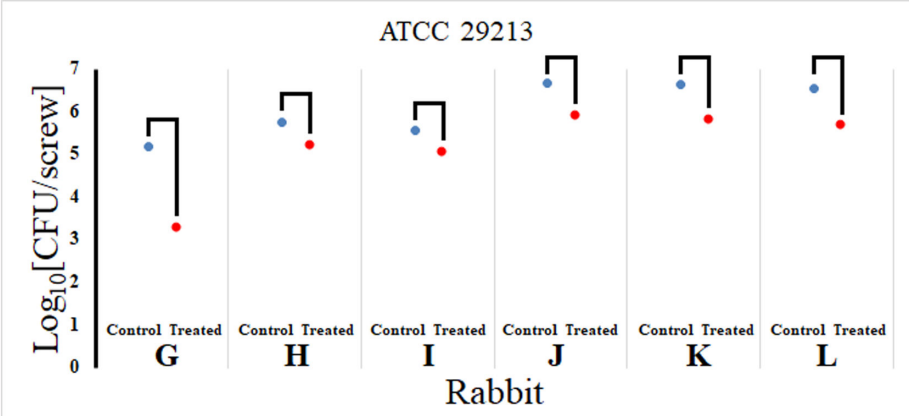

Fig. 6
Bacterial load (Log10 colony-forming units (CFU)/ml) in screws retrieved from rabbits J to L (biofilm-infected).

sections studied in the infected screw group. No differences were observed between treated and non-treated femora.

Discussion

Necrosis margins were less than 650 μm wide in all induction heating-treated femora. No significant differences were found between paired knees. In rabbits implanted with infected screws, a significant bacterial load reduction with median 0.75 Log10 CFU/ml was observed. These results allow us to persist with the hypothesis that induction heating using PDSIH could grow into an effective therapy for periprosthetic joint infection (PJI). To our best knowledge, these are the first published results involving electromagnetic induction heating on an in vivo model.

Regarding the safety of the technique, none of the treated femora showed more than a 650 μm wide necrosis margin. This is an even more encouraging result than the mere absence of differences with matched controls: bone necrosis was within the range of other familiar effects, such as surgical cement exothermal hardening.[17,18] Other sources of thermal aggression in our experiment were high-velocity reaming and drilling prior to screw introduction. These procedures were identical in both treated and untreated knees, and thus the similarity between groups further suggests that necrosis due to induction heating is not substantially greater than that caused by conventional instrumentation.

The onset time of acute thermal bone necrosis is not well established. As stated above, in the study by Berman et al[13] bone thermal necrosis was assessed after one, two, and three weeks, and no differences were observed regarding osteocyte cell absence and lamellar matrix disruption. To take this uncertainty into account, we first analyzed a six-rabbit series with logarithmically distributed evolution periods of 24, 48, and 96 hours, and did not find a correlation between observed necrosis and time elapsed. According to our results, osteocyte absence due to acute thermal bone necrosis would develop in rabbits within the first 24 hours after heating. The current literature appears to be insufficient to confirm this fact.

One possible hindrance in the development of a clinical application of induction heating is size scaling: while thermal energy scales with volume, heat flux scales with surface area. Furthermore, thermal diffusion into the adjacent bone may depend on blood supply characteristics among other factors. These effects may drastically affect the temperature reached in underlying bone when applied to human-sized implants.

Regarding microbiological analysis, bacterial load reduction was achieved in all the rabbits. The magnitude of this reduction, with a median of 0.75 Log10 CFU/ml, was modest compared to a previous induction heating study by Pijls et al,[10] who reported reductions greater than 6 Log10 CFU/ml on other Gram-positive bacteria. However, precise quantitative results should be taken with caution at this stage because neither concomitant therapies with possible synergistic effects were applied, nor were time evolution or involvement of immune response being evaluated. Moreover, no bacterial load threshold is actually defined to determine which clinical situations will evolve into healing.

Bacterial quantification differed significantly between different animals: in rabbits G to I, viable bacteria retrieved from the control knee ranged between 5 and 6 Log10 CFU/ml. These values fell inside what would be the domain of treated knees for rabbits J to L, in which control-knee bacterial load amounted to more than 6.5 Log10 CFU/ml. However, as stated above, bacterial load was always reduced in the treated knee within the same rabbit. As far as we understand, matched sample analysis is the most sensible approach, since knees of the same animal share biological features and immune response, and screws were implanted and retrieved during the same surgical procedures.

One limitation of this study is that given the reduced number of animals studied, statistical power may not have been sufficient to show existing differences in bone necrosis. However, the fact that necrosis margins were under 650 µm strongly supports our hypothesis regarding the safety of the technique. Another limitation is the inaccuracy of thermal monitoring of the screw surfaces. Thermographic imaging was used, similarly to previous studies in the field.[8-12] This method allows for a contactless monitoring and thus avoids mechanical disruption of the biofilm and prevents biological contamination; it measures instantaneous temperature in the surface, does not depend on thermal diffusion, and keeps the measuring instrument distanced from the electromagnetic induction source. Nevertheless, the low thermal emissivity of highly reflective polished metallic surfaces hinders its accuracy. Also, in this in vivo setting, the small size of screw caps, the presence of biological fluids in the surgical field, as well as the reflection of thermal radiation from higher-emissivity surrounding tissues at 37°C, add to the difficulty of accurate monitoring. This fact, together with the heat-sink effect, may have led to underheating of the screw tip, located deep inside the bone. However, while actual temperatures may have oscillated around the 70°C target, our results still showed a bacterial load reduction in the absence of a demonstrable increment in bone necrosis. Also, the underheating of the bone-implant interface, where no bacteria should be found in a well-integrated implant, is desirable to reduce bone necrosis. Validating this technique in the presence of these difficulties, which are inherent to more realistic clinical settings, was one of the main purposes of this study. One further limitation would be the short evolution time before sample collection, which may have been insufficient for histological signs to develop: our results suggest that osteocyte cell absence is already present 24 hours after the thermal injury; this is why we studied the first rabbit series with increasing evolution times, without any evidence of time correlation. However, it is possible that some osteocytes further away from the heat source without any noticeable signs after the first 24 hours may eventually undergo cellular necrosis, hence underestimating our measured necrosis margins. Finally, the ageing of the biofilm can also influence the heating effect because of the changes in the metabolism in the sessile cells and increase of persister cells in the biofilm.[19] However, our objective is not to sterilize the implant, but to use the system as an aid together with the DAIR protocols in order to improve the outcome of patients who were treated with this methodology.

In order to determine the clinical feasibility of induction heating disinfection, the isolated and combined effects of induction heating and other techniques, such as mechanical cleaning and antibiotic therapy, should be assessed in future studies. Additionally, this study only assessed the effect of induction heating on a *S. aureus* strain, while the effects on other species involved in PJI cannot be extrapolated yet. Future studies will be needed to assess the efficacy of this technique on a wider range of microorganisms.

Supplementary material

ARRIVE checklist.

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Data sharing

All data generated or analyzed during this study are included in the published article and/or in the supplementary material.

Ethical review statement

This study was approved by the Ethical Committee in our institution (Órgano Encargado del Bienestar Animal, Hospital Universitario La Paz, OEBA 04-2020) and local administration (Dirección General de Agricultura, Ganadería y Alimentación, Consejería de Medio Ambiente, Ordenación del Territorio y Sostenibilidad, Comunidad Autónoma de Madrid, Spain. Ref.: PROEX 277.7/20). Animal care and maintenance were performed following the guidelines defined by national and international regulations (Real-Decreto 53/2013, Council of Europe 2010/63/EU-Directive).

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