1. Introduction

For partially and fully edentulous patients, endosseous dental implants is the most popular treatment option. Most endosseous implants must have a secure interface with the adjacent bone before functional prostheses can be inserted. Osseointegration is a critical factor for implant fixation.

Osseointegration, a concept developed by Brånemark in 1965[1], is defined by direct contact between bone tissue and a biomaterial without the interposition of fibrosis. Titanium is an excellent biomaterial: its integration is through plastic deformation of the bone-implant interface. The metal is permanently incorporated into the bone[1,2]. When Brånemark first used a titanium implant embedded in the maxilla in 1965, no one suspected that the implant would still be there 40 years later[3,4]. Nowadays, osseointegration is widely used in dental, maxillofacial and oncology surgery, where bone defects pose major functional and aesthetic problems.

Several factors play a preponderant role in this osseointegration. A rapid and successful osseointegration may guarantee a quality implant fixation[5,6]. Some studies have evaluated the surface and shape of the implant. Rough surfaces allow a higher percentage of implant contact compared to implants with smooth surfaces[7–9]. Implant surface changes, drug delivery systems and biophysical stimulation may achieve better osseointegration[5,6,9].

Several means have been devised to stimulate osteogenesis and bone turnover. Among these different means, ultrasounds and more particularly low-intensity pulsed ultrasounds or LIPUS have proved their interests. LIPUS is a nonthermal and noninvasive technique that utilizes physical stimulation by acoustic pulsed energy[10].

The interest in LIPUS appeared about two decades ago with research on their use in biological media. Many studies have shown...
the ability of LIPUS to stimulate bone, cartilage, tendon and mucosal regeneration[11–15]. At the cellular level, ultrasound promotes the synthesis of collagen by fibroblasts, the formation of bone matrix by osteoblasts, the differentiation of bone mesenchymal stem cells (BMSC) in osteoblasts on titanium surfaces and the synthesis of aggrecan in chondrocytes. LIPUS stimulates the increase in the number of mineralization nodules of microfilaments, pseudopods and extracellular matrix[9].

Several studies have evaluated the interest of LIPUS in the osseo-integration of dental implants in murine or rabbit models[5,16–21]. However, the carrying capacity of dental implants is limited because of thin bones and it is difficult to confine LIPUS energy because of bone narrowness[12]. The energy is often transmitted to the control side. Thus, porcine model may be a more suitable model because the bones are wider and longer compared to murine and rabbit models. Moreover, this model is closer to the human model.

To our knowledge, this article is the first in vivo preliminary study which evaluates the ability of LIPUS to stimulate bone tissue regeneration in contact with a titanium dental implant in a porcine model.

2. Material and Methods

The study was carried out in the experimental surgical laboratory at Leon Berard Center in Lyon, France. And it was approved by the Ethics Committee of Claude Bernard Lyon 1 University and the animal’s care was in accordance with institution guidelines.

2.1 Animals

8 Landrace female adult mini-pigs aged 2 months were used in this study. They were taken care of in the animal laboratory in agreement with the local ethical and scientific committee of the Institute of Experimental Surgery at the Léon Bérard Center in Lyon (France). The animals were in a temperature-controlled room (17-24°C) with mixed type food ad libitum and water. The hygrometry is regulated between 45 and 55%. A 12h / 12h lighting cycle is set up.

The mean weight was 12.8 kg at the start of the study (Day 0) and 32.3 kg at euthanasia (Day 42). The animals benefited from a 7-day acclimatization period at the pet store of the Institute of Experimental Surgery before any intervention. They were fasted (food and water) 24 hours before each intervention.

2.2 Implants

The implants used are titanium implants, rough with osteoconductive surface (Ra=1μ), of cylindrical biconical type, with turns on the whole body of the implant, with self-tapping effect, with a diameter of 3.5 mm and a length of 9 mm (Bio-Xellent, Drive, France) (Fig. 1).

2.3 Surgical technique

Implants are placed in the operating room under general anesthesia.

The intramuscular premedication of the animals is carried out a quarter of an hour before the general anesthesia with a mixture of 3 to 5 mL of Ketamine (15 mg/kg, Imalgene 1000”), 1.5 to 2.5 mL of Azaperone (2.2 mg/kg, Stresnil”) and 1 mL of Atropine (0.5 mg/ml). A 20G catheter (11/10) is placed in the ear vein.

General anesthesia is performed by mixing in a 5 ml syringe: 2.5 mL of Xylazine 2% (Rompun”) and 2.5 mL of Ketamine (Imalgene 1000”), the whole is injected into a vial of Zoletil 100° powder. The mixture thus obtained is then injected intravenously using the catheter previously placed at a dose of 0.015 mL/kg for induction (duration approximately 30 min). The anesthesia can be prolonged by injecting half the dose used for induction.

An implant is placed on each tibial crest in the region of tibial metaphysis, 2 cm below condyles (Fig. 2). The surgical technique is the same as in human oral surgery:

The implant sites were prepared using standard surgical technique. The bone surface was exposed after a skin incision. The periosteum was incised and a peristeal flap was raised. One dental implant with a diameter of 3.5 mm and a length of 9.0 mm (Bio-Xellent, Drive, France) was placed in each tibial metaphysis region of the pig, forming a right angle to the major axis of the bone. The implant was entirely threaded into the path with its end placed at the level of the

Fig. 1. Titanium implants of cylindrical biconical type, with turns on the whole body of the implant, diameter 3.5 mm and length 9 mm (Drive, France).

Fig. 2. Illustration of the placement of the implant in the tibial crest (red point), 2 cm below condyles.
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All drilling procedures were performed under continuous sterile saline irrigation. The tightening was performed to 35N cm⁻¹. The closure screws were placed after the implantation. The flap and the skin were closed with the resorbable sutures (Vicryl®, Johnson & Johnson, Brussels, Belgium). Prophylactic antibiotics were administered intra-muscularly daily for 3 postoperative days.

2.4 Insonification

A signal generator (Hewlett-Packard 8116A, Palo Alto, California, U.S.A.) and a power amplifier (Kalmus model 150CF power amplifier, Engineering International, Woodinville, WA, USA) were used to generate ultrasounds. The electrical power sent to the transducer is continuously monitored using a power reflection meter (Rohde & Schwarz NAP, München, Germany). The ultrasonic transducer is a 12 mm diameter flat disc mounted in a PVC body and covered with a latex membrane filled with degassed water. The membrane is covered with gel (Uni’Gel US, Asept), to ensure acoustic coupling with the skin of the animal. To minimize temperature increase during the sonication and ensure that the temperature remains stable and doesn’t bias the results, a cooling system has been fixed to the insonification device. The coolant velocity was 170 ml / min and the cooling bath represented by a water tank was at room temperature (22 °C).

The sensor has been mounted on a rigid support. This stabilizes it and facilitates its placement on the animal’s skin (Fig. 3).

The LIPUS parameters used were: 1 MHz frequency, pulsed 1:4, 2 ms signal duration with an acoustic intensity of 300 mW/cm² measured in water. Parameters was chosen according the habits of the research department and previous studies [14,22,23].

LIPUS was applied to the implant on the right tibia the day after implant insertion. The probe was applied to the implant forming a right angle to the major axis of the bone (Fig. 3). LIPUS was applied during 15 minutes per day, on 5 consecutive days and during 3 weeks. The left tibia was not treated. Therefore, each pig served as its own control. The animals were anesthetized during the insonification.

2.5 Sampling

The animals are euthanized on day 42 or at 6 weeks, date on which ossification is well started or even almost complete, according to the protocol decreed by the Ethics Committee. Osteotomies are performed 2 cm on each side of the implant with a back-and-forth saw. Thus, samples with a length of 4 cm were harvested.

The samples are then immersed in formalin and transferred to the imaging department for histomorphometric analysis.

2.6 Sample imaging

The animals are analyzed within the small animal imaging platform, Animage (CERMEP - living imaging, Bron, France). The samples are passed through an X-ray microscanner (SkyScan 1076 microcomputerized X-ray tomograph, SkyScan, Aartselaar, Belgium). The scanner is used in isotropic mode providing pixels of 17.6 microns per side. From the raw data, the reconstruction of images makes it possible to obtain isometric images in XYZ mode. The data thus reconstructed is composed of a series of slices of plane images separated by the same distance of 17.6 microns.

In the bone, the presence of titanium implants required special precautions with the parameters of the scanner. These were determined to minimize the disturbance of the images by the presence of the implant. Other precautions have been taken in the reconstruction process, making it possible to obtain from the raw images, images reconstructed in the XYZ plane: smoothing of order 2, “ring artefact reduction” of 20% and “beam hardening” of 10%; the defect pixel mask function has also been used to further reduce the noise caused by the presence of the implant.

2.7 Image analysis

The analysis is carried out using CtAn™ software from SkyScan™ associated with the microscanner, from images in XYZ mode. The bone area surrounding the first third of the implant was studied. This area is considered the region of interest (ROI), which corresponds to an average length of 3.2 mm (Fig. 4). The analysis is performed on a
1 mm circular crown around the implant. Thus, for a diameter of 3.46 mm of the threaded part of the implant defined from the images, the ossification is studied inside a bone tube with an internal diameter of 3.46 mm, an external diameter of 5.46 mm and a length of 3.2 mm.

The bone in this area around the implant is then analyzed after setting two thresholds: a low threshold and a high threshold. The low threshold is set to eliminate all that is too transparent to X-rays to be bony, the high threshold to eliminate all that is too opaque to be bony, essentially the implant. These thresholds are used to define the areas of the image considered to be bony, ie binarized bone.

Among the many parameters that can be obtained with the analysis software, three are retained:
- The Bone Volume / Total Volume ratio (BV/TV) which represents the volume represented by the bone compared to the total volume of interest.
- The Intersection Surface (IS) which is the intersection surface of the volume of interest by the binarized bone. It was analyzed according the distance from the implant surface (at 200 μm, 400 μm and 600 μm).
- The trabecular bone thickness (TbTh) around the implant.

2.8 Statistical analysis

The first species risk was set to 5%.

The Wilcoxon signed-rank test was performed for the statistical analysis.

The study was used statistical software SAS 9.4.

3. Results

Data is presented in Table 1, Table 2 and Table 3.

BV/TV ratio is presented in Table 1. At 42 days, BV/TV ratio is significantly higher on the treated side than the untreated side (42.1 +/- 8.76% versus 32.31 +/- 10.11%, p < 0.02).

Trabecular bone thickness is represented in Table 2. At 42 days, TbTh is significantly higher on the treated side than the untreated side (0.13 +/- 0.01 mm versus 0.10 +/- 0.01 mm, p < 0.01).

The intersection surface is represented in Table 3 according to the distance from the implant surface. At 42 days, it is also significant.

Table 1. Results of Bone Volume / Total Volume (%)

| Pig | Treated side | No treated side | p* |<0.02 |
|-----|--------------|-----------------|----|------|
| 1   | 29.2         | 19.3            |    |      |
| 2   | 49.6         | 41.3            |    |      |
| 3   | 57.8         | 49.8            |    |      |
| 4   | 35.9         | 38.8            |    |      |
| 5   | 42.2         | 27.3            |    |      |
| 6   | 37.7         | 28.9            |    |      |
| 7   | 44.5         | 29.2            |    |      |
| 8   | 39.9         | 23.9            |    |      |

Mean 42.1 (+/-8.76) 32.31 (+/-10.11) p* < 0.02

* Wilcoxon Signed-Rank test

Table 2. Results of the Trabecular Thickness (mm)

| Pig | Treated side | No treated side | p* |<0.01 |
|-----|--------------|-----------------|----|------|
| 1   | 0.104        | 0.088           |    |      |
| 2   | 0.126        | 0.101           |    |      |
| 3   | 0.143        | 0.122           |    |      |
| 4   | 0.112        | 0.098           |    |      |
| 5   | 0.144        | 0.108           |    |      |
| 6   | 0.139        | 0.079           |    |      |
| 7   | 0.134        | 0.117           |    |      |
| 8   | 0.122        | 0.101           |    |      |

Mean 0.13 (+/-0.01) 0.10 (+/-0.01) p* < 0.01

* Wilcoxon Signed-Rank test
from the implant surface

Table 3. Results of the Intersection surface (mm²) according to the distance from the implant surface

| Pig | Treated side | Untreated side |
|-----|--------------|----------------|
|     |              | At 200 micrometers from implant surface |              |
| 1   | 31.9         | 29.5           |
| 2   | 45.7         | 38.2           |
| 3   | 52.9         | 40.9           |
| 4   | 23.3         | 19.7           |
| 5   | 56.7         | 43.7           |
| 6   | 49.8         | 44.5           |
| 7   | 25.5         | 23.5           |
| 8   | 39.8         | 29.4           |
| Mean | 40.7 (±12.68) | 33.68 (±9.63) | p* <0.01 |

| Pig | Treated side | Untreated side |
|-----|--------------|----------------|
|     |              | At 400 micrometers from implant surface |              |
| 1   | 18.6         | 15.3           |
| 2   | 32.7         | 25.8           |
| 3   | 39.2         | 25.7           |
| 4   | 13.8         | 11.3           |
| 5   | 27.4         | 20.4           |
| 6   | 33.1         | 27.1           |
| 7   | 12.7         | 10.4           |
| 8   | 24.7         | 19.7           |
| Mean | 25.28 (±9.63) | 19.46 (±6.59) | p* <0.01 |

| Pig | Treated side | Untreated side |
|-----|--------------|----------------|
|     |              | At 600 micrometers from implant surface |              |
| 1   | 12.9         | 10.4           |
| 2   | 17.5         | 15.3           |
| 3   | 28.5         | 19.4           |
| 4   | 10.1         | 9.6            |
| 5   | 22.2         | 18.9           |
| 6   | 19.4         | 17.2           |
| 7   | 11.5         | 9.8            |
| 8   | 22.1         | 18.3           |
| Mean | 18.03 (±6.3) | 14.86 (±4.27) | p* <0.01 |

* Wilcoxon Signed-Rank test

To our knowledge, this is the first preliminary study evaluating the ability of LIPUS in a porcine model to stimulate bone formation around the dental implant. A significant and positive response of LIPUS treatment on osseointegration was found in this study.

4. Discussion

Biologically, when the implant is inserted into the bone cavity, an inflammatory phase is created. This is the first phase of osseointegration. A blood clot between the implant and the alveolar bone sets in and is replaced by osteoid tissue and new trabecular bone. This is replaced by lamellar bone in direct contact with the surface of the implant[24]. At cellular level, during this process, osteoblasts and osteocytes secrete extracellular organic matrix, rich in collagen, for mineralization[24–26].

Vascularity is important for osteogenesis process and the healing of bone and the healing of peri-implant bone. LIPUS had significant effects on bone repair processes through many mechanisms. It has been shown to increase blood flow around a fracture site, suggesting that it stimulates vascularity[27]. Angiogenesis, vascular permeability, the secretion of growth factors[22,23,28–31], and nutrient delivery can be increased by the thermal, fluid and vibration effect. Furthermore, it can increase the differentiation of the fibroblasts, chondroblasts, and osteoblasts with the opening of membrane channels[28,32–35].

LIPUS can also increase activity of osteoblasts and promote the differentiation of BMSC for osteoblasts[9]. It could positively regulate the expression of ossification biomarkers and matrix protein in bone mesenchymal stem cells BMSCs, further increasing the number of osteoblasts[9].

In the literature, many studies have evaluated implant osseointegration using the rabbit tibial model[5,16–20] or rat tibial model[21]. Tibial metaphysis and diaphysis have been used but the bones are thin. Recently, increasing studies have established the mice model of maxillary implant placement to better imitate the microenvironment of oral implantation[36–38]. Jiang et al. have studied the osteointegration of dental implants in the maxillary first molars extraction sockets in mice and proved better osseointegration of the implant with LIPUS[39]. However, regardless of the rabbit or mouse model, the carrying capacity of dental implants is limited and it is difficult to contain LIPUS energy due to the narrowness of the bones[12].

Our study is the first to evaluate implant osseointegration using a porcine model. This may be more suitable as the bones are wider and longer than murine and rabbit models. The metaphysis region was chosen to avoid bone fracture compared to the diaphysis region.

These results suggest that LIPUS may have a stimulating effect on the osseointegration of dental implants since the BV / TV ratios, the intersection surfaces and the trabecular thicknesses are significantly in favor of the insonified group. LIPUS significantly promotes trabecular bone thickness and bone remodeling by increasing new bone tissue and improving the healing process at the bone-implant interface.

These results agree with those of the study by Jiang et al[39].
They also study the role of aCGRP which can be one of the mechanisms of osseointegration and bone regeneration.

These results agree also with the histological examination of Liu et al.[20]. They showed that osteoinduction of dental implants progressed and started earlier with LIPUS treatment. The calcified fibrous layer and trabecular bone around the dental implant, visible on the surface of the implant, appeared earlier with LIPUS treatment. And later, the trabecular bone was gradually replaced by the lamellar bone, which matured earlier with LIPUS.

5. Conclusion

Our results confirmed that LIPUS can significantly increase bone formation and accelerate the healing process at the bone-implant interface. Its low toxicity, low immunogenicity and non-invasion make it an complementary treatment of choice in implantology.

Conflicts of interest

The authors have declared that no conflict of interest exists.

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