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Original Article

Stimulation of oral mucosal regeneration by low intensity pulsed ultrasound: an in vivo study in a porcine model

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Abstract

Purpose: Many studies have shown the ability of low intensity pulsed ultrasound (LIPUS) to stimulate the bone, cartilage and tendon regeneration but only a few studied LIPUS interest in the regeneration of the oral mucosa. The purpose of this study is to assess the ability of LIPUS to stimulate the regeneration of the palatal mucosa in a porcine model.

Methods: Ten adults mini-pigs were used. Two mucosal wounds were realised on the left and right side of the palate of each pig. The right side was treated with LIPUS at 1 MHz of frequency and 300 mW/cm2 of acoustic intensity. The left side was not treated. The morphology of the wound was evaluated using a polymer silicone molding.

Results: The difference between two sides was significant from day 7 with a p value <0.0001. At day 21, the wound is completely healed on all pigs with LIPUS. The control soft tissue defect exposed a healing of 80%.

Conclusions: The present study showed that the use of LIPUS on the oral mucosa accelerates the healing of the masticatory mucosa.

Keywords: *LIPUS (low intensity pulsed ultrasound), Oral mucosa, Tissue engineering, Soft tissue regeneration*

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1. Introduction

The maxillofacial surgeon has sometimes to deal with mucosal defect. Wound healing is a complex process to obtain a re-epithelialization. It involves many cell types, inter-cellular messengers (growth factors), molecules implied in anabolism, cytokines and cell cycle regulators [1]. Tissue repair is mainly performed by connective tissue wich is composed of two basic elements: an extracellular matrix (ECM) and cells. The ECM consists of a fundamental substance composed of glycosaminoglycans and especially fibers, such as collagen. Cells are mostly fibroblasts, which secrete ECM components: glycosaminoglycans, fibronectin, and collagen [2].

Low-intensity pulsed ultrasound (LIPUS) is a nonthermal and noninvasive technique that utilizes physical stimulation by acoustic pulsed energy [3]. This is frequently used for the treatment of fresh fractures or delayed healing of fractures because it shortens the time required for biological wound healing.

Low-intensity ultrasound therapy is usually defined as a mean sound intensity between 100 mW/cm2 and 3 W/cm2. When the mean sound intensity is above 5 W/cm2, ultrasounds are considered as high intensity. The current low-intensity ultrasound applications are activation of normal physiological response of an injury or acceleration of transportation of drugs through the skin [4]. The term LIPUS emerged about two decades ago, when research showed the effects on collagen synthesis and fibroblast growth in vitro [5].

Many studies have shown the effectiveness of LIPUS in stimulating the regeneration of bones, cartilage and tendons and in shortening the healing time of biological wounds [6–9]. LIPUS is also effective in dental applications like implantology by enhancing bone formation around miniscrew implants [6][10]. The positive effect of low-intensity pulsed ultrasound stimulation on hard tissue regeneration has been demonstrated. However, only a few studies have focused on the effects of LIPUS in soft tissue regeneration and particularly of oral mucosa

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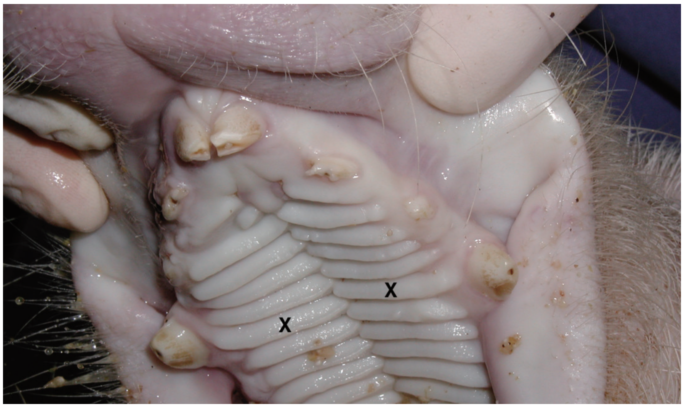

Fig. 1. Photographs of the palate of a pig with localisation of future mucosal pellets (X).

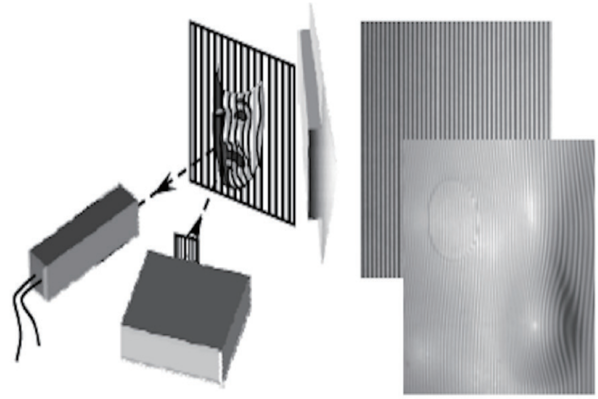

Fig. 2. System used to analyze soft tissue defect by using fringe projection techniques. This system consisted in a charge coupled device camera (CDD camera) and a fringe projection. These were projected onto the surface to be analyzed and images of fringes were acquired by the camera at an angle of 45 ° to the fringe projector.

healing. The effects remain unclear [11][12]. Respectively, Maeda et al. [1] and Ikai et al. [13] demonstrated that LIPUS enhances healing of rats' palatal mucosa and accelerates healing of periodontal wounds after flap surgery. Moreover, expression of connective tissue growth factor (CTGF) in gingival cells (GE1) has enhanced after LIPUS exposure [1,14]. Also, the proliferation and migration of gingival epithelial cells could be promoted by LIPUS exposure and secondary, the wound closure was accelerated [1,14].

To our knowledge, this article is the first in vivo study which evaluates the ability of LIPUS to stimulate the regeneration of palatal mucosa defect in a porcine model. The aim of this present study was to compare healing time between control and LIPUS treated wounds after 21 days.

2. Materials and methods

The study was carried out in the experimental surgical laboratory at Leon Berard Center in Lyon, France.

2.1. Animals

10 adult mini-pigs aged 2 months were used for this study. They were supported in the animal laboratory in agreement with the local ethical committee. The animals were housed in a temperature-controlled room with mixed type food ad libitum and water. Each mini-pig was its own control.

2.2. Surgical technique

The procedure was performed under general anaesthesia using Kétamine (15mg/kg - 3 à 5 ml d'Imalgène 1000 ND), Azapérone (2,2 mg/kg – 1,5 à 2,5ml of Stresnil ND) and Atropine (1ml of Atropine 0,5mg/ml). Using a punch biopsy, 6 mm mucosal pellets were removed on the right and left side of the palate, 5 mm lateral from the mid-palatal suture of each pig (Fig. 1). The pellets were separated and removed under the periosteum. The use of the punch biopsy allows the realization of an identical and reproducible wound for each pig.

The right side was treated with LIPUS and the left side was not treated (control group).

2.3. Insonification

Ultrasounds were generated using a signal generator (Hewlett-Packard 8116A 50 MHz pulse/function Generator, Germany) and a power amplifier (Kalmus model 150CF power amplifier, Engineering International, Woodinville, WA, USA). A latex membrane on the sensor was mounted and filled with degassed water to provide acoustic coupling. A cooling system has been attached to the insonation device to minimize temperature increase during the sonication. The coolant velocity is 170 ml / min and the cooling bath represented by a water tank was at room temperature (22 ° C). The LIPUS parameters used were: 1 MHz frequency, pulsed 1:4, 2 ms signal duration. This material allowed the creation of an acoustic intensity of 300 mW/cm2 measured in water. Parameters was chosen according the habits of the research department and previous studies [15–17].

To facilitate the implementation of the sensor on the palate of the animal, the sensor was mounted on a rigid support.

LIPUS was applied 5 minutes per day, on 5 consecutive days according to previous study [15–18] and until complete healing of the defect.

The animals were anesthetized (isoflurane 1.5%) during the insonification.

Although LIPUS has minimal thermal effects due to its low intensity and pulsed output mode [19,20], the temperature of mucosa during insonification was controlled using a K thermocouple. The local temperature rise was 0.8°C to 300 mW.

2.4. Measure of the size and depth of the defect

The wound morphology was evaluated using a silicone polymer molding (Silflo®, Flexico Developements, UK). The imprint was performed with a mixture of 4 drops of catalyst to 3 ml of silicone polymer and spreading the mixture over the target region.

Mucosa molding was analysed by using fringe projection techniques, which allowed to reconstruct in three-dimensions (3D) every micro and macro-reliefs of the surface. It also allowed to quantify the roughness, volume and depth of the wound. The system consisted in a charge coupled device camera (CDD camera) and a fringe projection. These were projected onto the surface to be analysed and images of fringes were acquired by the camera at an angle of 45 ° to the fringe projector (Fig. 2).

The resolution of the system depends on the width of the projected fringe and the angle between the optical axis of the camera and the projector. Deformation fringes over the reference plane were proportional to the height between the object of this plan.

Thus, series of fringe images, containing information on the height of the analysed object, were reconstructed into a single 3D image (Fig. 3, Fig. 4).

The applied fringe projection system was Dermatop ® software with

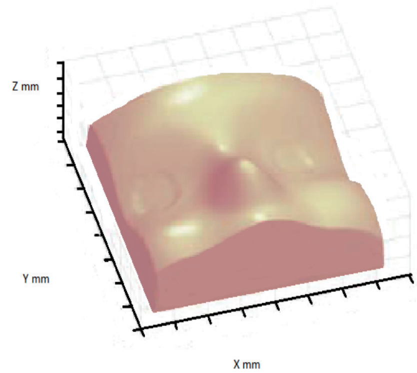

Fig. 3. Three-dimensional image of the analyzed palate.

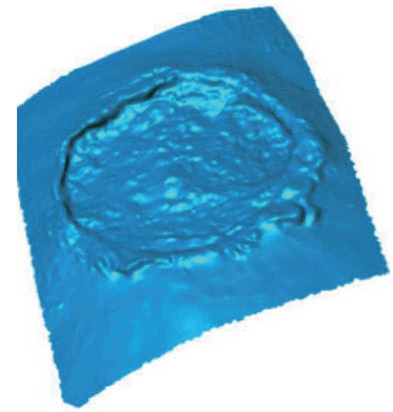

Fig. 4. Three-dimensional image of the soft tissue defect.

Table 1. Evolution of the wound volume (mm^3) at different time points on the treated side.

Pig	Day 0	Day 7	Day 14	Day 21
1	82	40.3	11.2	0.9
2	85.2	36.6	13.8	0.7
3	87.1	46.2	12.5	1.2
4	89.9	43.7	5.5	0.8
5	79.9	42.2	12.4	1.1
6	85.1	41.6	10.2	1.2
7	86.4	43.5	9.7	0.7
8	84.1	40.3	12.1	0.5
9	84.9	40.8	7.8	1.2
10	85.2	43.4	15.3	0.7

Table 2. Evolution of the wound volume (mm^3)at different time points on the untreated side.

Pig	Day 0	Day 7	Day 14	Day 21
1	85.2	47.1	27.7	20.3
2	86.1	48.5	22.4	20.6
3	84.8	49.6	25	17.4
4	83.6	50.2	33.1	19.3
5	84.2	48.5	24.9	21.5
6	86.7	49.2	28.1	20.1
7	86.1	49.9	29.5	19.5
8	84.1	51.1	29.4	18.6
9	84.9	50.5	28.4	20.1
10	84.2	46.5	30.1	8.7

Table 3. Mean of the wound volume (mm^3) at different time points on the both sides.

	Mean in the treated side (SD)	Mean in the no treated side (SD)	Mean of the difference	p Value *
D0	84,9 (2,7)	85,0 (1,0)	0,1 (2,9)	0,96
D7	41,9 (2,6)	49,1 (1,5)	7,3 (2,9)	< 0,0001
D14	11,1 (2,9)	27,9 (3,1)	16,8 (5,3)	< 0,0001
D21	0,9 (0,3)	19,6 (1,2)	18,7 (1,2)	< 0,0001

* Paired Student t--test

OPTOCAT ® (Breuckmann-Germany) image acquisition. This system was used indirectly on impressions or silicone polymer replicas on the defect surface. Toposurf® (Digital Imaging, Besançon, France) software image processing analyses the reconstructed 3D topography with standard roughness parameters and tools for studying depth and volume. With these tools, the depth profiles of the lesions were generated to calculate their average depth.

The process was performed on day 0, day 7, day 14, and day 21.

The primary endpoint of the study was the comparison of wound volume between LIPUS-treated and untreated sides at day 21.

Secondary endpoints were the difference of wound volume between the two sides at day 7 and day 14 in order to determine if and how soon this difference seems to appear.

2.5. Statistical analysis

The first species risk was set to 5%.

The difference of treated and non-treated sides was also assessed at day 0, to ensure comparability at the beginning of the study.

Since the aim of the experience was to compare wound healing of both sides in the same pig, we used a paired test for each analysis (principal and secondary endpoints, comparability at day 0)

To determine which test to use (parametric or non-parametric), the normality of the distribution of the difference between the two sides (Validity conditions of a paired t-test) was assessed at each of the time points (day 0, 7, 14 and 21.). Graphical distributions were studied using histograms and density curves, and Shapiro-Wilk tests were performed. Since all the distributions of the differences followed a normal law at each of the times points (normal graphical distribution and non-significant Shapiro-Wilk test), a paired t-test was used for each of the analyses.

The study was used statistical software R 3.5.0.

3. Results

Data is presented in Table 1 and Table 2. The results of statistical analyses are presented in Table 3.

At day 0, immediately after surgery, no difference between wound volumes was measurable between both sides (mean difference: 0.1, p value =0.96, Table 3)

At day 21, the volume of the wound on the treated side was significantly smaller than the one on the untreated side (mean difference: 18.7, p value <0.0001). The wound was completely healed on all pigs treated with LIPUS 300 mW / cm^2 and only 80% of the soft tissue defect was healed without any treatment (Fig. 5).

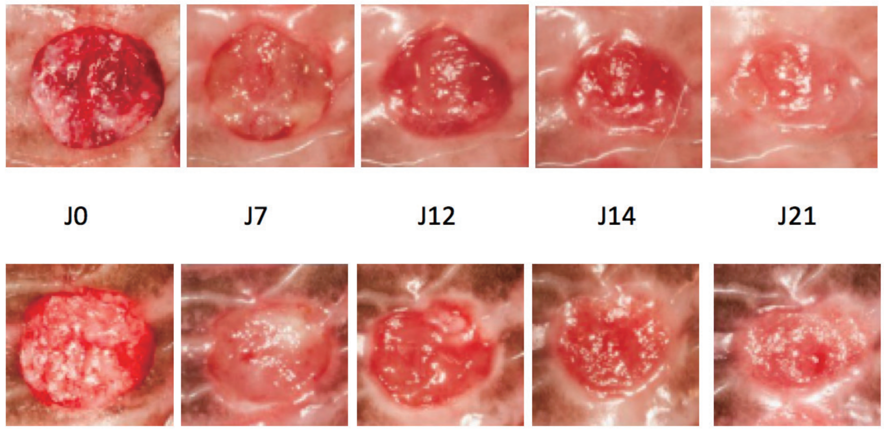

Fig. 5. Photographs of the soft tissue defect at each time. Top: with LIPUS application. Buttom : without LIPUS application.

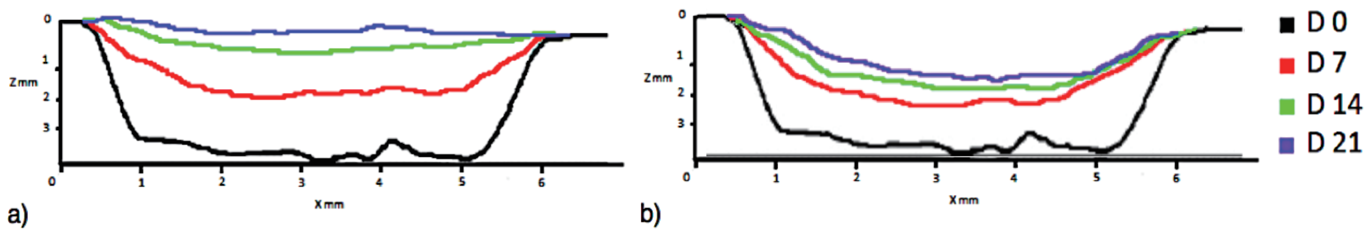

Fig. 6. a) Depth profile after LIPUS treatment at each time, average of 10 pigs; b) Depth profile of the control group at each time, average of 10 pigs.

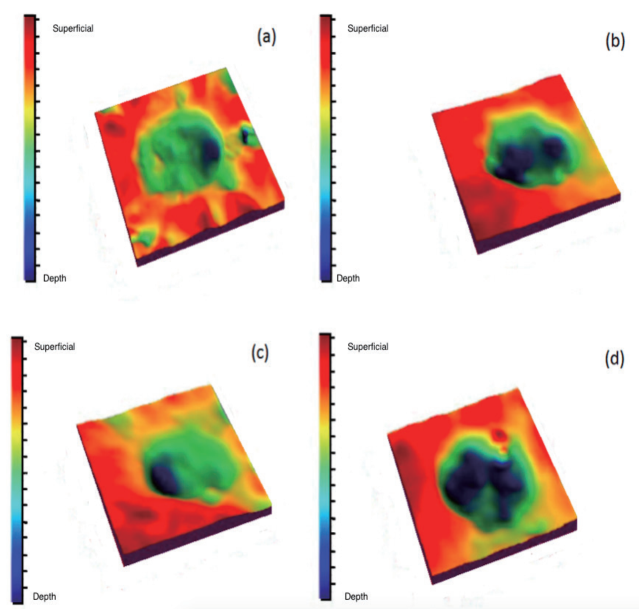

Fig. 7. Example of three-dimensional reconstructed images of palatal mucosal defects in two mini-pigs on day 14: a) and c) with LIPUS application, b) and d) without LIPUS application.6 a) Depth profile after LIPUS treatment at each time, average of 10 pigs; b) Depth profile of the control group at each time, average of 10 pigs.

Furthermore, the difference was significant from day 7 (mean difference: 7.3, p value <0.0001) with an increase of the difference at each further time point (day 14 mean difference: 16.8, p value <0.0001) .

For each group, we extracted the mean profile of the depth of the wound at each time for the 10 pigs. Figure 6 represents the average profile of the depth and the surface deficiency of the wound at each time, for the LIPUS group and the control group (Fig. 6).

The figure 7 also shows an example of three-dimensional reconstructed images of palatal mucosal defects in two mini-pigs on day 14: a) and c) with LIPUS application, b) and d) without LIPUS application). The color scale represents the depth of the defect.

4. Discussion

To our knowledge, this is the first study evaluating the ability of LIPUS to stimulate the wound healing of the palatal mucosa in a porcine model. Results show a significant and positive influence of the LIPUS treatment on the mucosal wound healing. A significant difference was seen from day

7 of treatment, and continued to grow until the end of the study after 21 days. Moreover, LIPUS is effective for wound width and depth healing.

The squamous cell type of the oral mucosa is like to the skin, but differs in the absence of appendages (hair follicles, sweat glands, sebaceous glands) and the small number of melanocytes. The particularity of this cell type is the permanent moistening with saliva and a rapid cellular turnover in the epithelium (25 days instead of 50 to 75 days for the skin). In this present study, masticatory mucosa was stimulated [21]. The masticatory mucosa lines on the gingiva and the hard palate, and is used for mechanical compression of the food. It consists of a keratinised surface, firmly anchored to the underlying bony structures (palatal and alveolar bone) and presenting long epithelial ridges. It is rich in collagen fibers.

LIPUS parameters were those used in previous works [15,16,22] and according to the habits of the laboratory : 1 MHz frequency, pulsed 1:4, 2 ms signal duration, acoustic intensity of 300 mW/cm2. The 5 min/day sonication is the one used in our previous in vitro studies [15,17] and in an other study which reported significant increase in cell proliferation with 6 min/day of LIPUS treatment [18].

The effects of LIPUS are well known today. Regarding osseous healing, it was shown to enhance osteoconduction in porous scaffolds, directly or through cartilage differentiation, by accelerating the migration and maturation of osteogenic and chondrogenic cells. Tsai et al. demonstrated that a LIPUS exposure at 1,5 MHz was more effective than 3 MHz for the bone consolidation of a rabbit fibula fracture: the bone mineral content was higher [23]. Gleizal et al. demonstrated that LIPUS at 1 MHz stimulated the gene associated with the Runx2 pathway and stimulated the bone formation on calvarial osteoblasts [15]. Thus, the frequency of 1 or 1.5 MHz may be used for fracture healing in orthopedic fields and shortening the overall bone healing time.

Regarding osseous growth, Kaur et al. demonstrated that a daily application of LIPUS for 20 minutes in growing rats enhances mandibular condylar growth in growing rats, either alone or in combination with a functional appliance [24]. Raza et al. demonstrated that LIPUS at 1,5 MHz accelerated bone formation in mandibular bone distraction [25].

Regarding dental fields, LIPUS proved its beneficial effect on osseointegration around dental implants [10]. It accelerates bone regeneration and bone integration with titanium implants under osteoporosis conditions [6]. Moreover, LIPUS exposure would reduce the atrophic changes of alveolar bone by inducing the upregulation of periostin and CTGF expression to promote periondotal ligament healing. LIPUS would reinforce the periodontal ligament and regenerate alveolar bone when they are atrophic after occlusal hypofunction malocclusion [26].

Although there is evidence for insonation causing a rise in tissue temperature, low-intensity pulsed ultrasounds reduces heating compared to continuous ultrasound [19,20]. LIPUS has minimal thermal effects due to its low intensity and pulsed output mode while maintaining the transmission of acoustic energy to the target tissue. In this study, the local temperature rise was only 0.8°C. However, vibration effect of LIPUS is proven [19,20] and can increase angiogenesis, vascular permeability, the secretion of growth factors, nutrient delivery and differentiation of the fibroblasts, chondroblasts, and osteoblasts with the opening of membrane channels [18,27–30]. This might explain the positive effect on soft tissue healing such as tendon, cartilage, ligament and mucosae [20,31–33].

Few thousand genes would be upregulated by LIPUS exposure by producing micromechanical strains in tissues that trigger several cellular responses. LIPUS up-regulates the expression level of integrin α6β4 that plays a role in stabilizing epidermal to dermal layers in skin [27]. In soft tissue healing, integrin α6β4 induces tissue remodeling, controls various cell function and promotes contraction of the wound site. It also increases CCN2/CTGF, a connective tissue growth factor which plays an important role in wound healing and regulation of the extracellular matrix periodontal tissue [14]. Finally, Maeshige and al. showed that ultrasound promotes the TGF beta1 which induced expression of alpha-smooth muscle actin (αSMA), a specific marker for myofibroblasts, in human dermal fibroblasts [34]. Thus, ultrasound therapy tends to accelerate wound closure due to enhanced collagen synthesis by fibroblasts [35].

5. Conclusion

Our study showed that the use of LIPUS on the oral mucosa defect accelerated the healing of masticatory mucosa, which had the property to be securely attached to the palatal bone. To our knowledge, this present study is the first that evaluated the effects of LIPUS on porcin models. LIPUS appears to be a very useful method to improve mucosal regeneration.

Conflict of interest

The authors declare to have no conflict of interest.

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