In vivo assessment of the effect of controlled high- and low-frequency mechanical loading on peri-implant bone healing

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The aim of this study was to investigate the effect of controlled high- (HF) and low-frequency (LF) mechanical loading on peri-implant bone healing. Custom-made titanium implants were inserted in both tibiae of 69 adult Wistar rats. For every animal, one implant was loaded by compression through the axis of tibia (test), whereas the other one was unloaded (control). The test implants were randomly distributed among four groups receiving different loading regimes, which were determined by ex vivo calibration. Within the HF (40 Hz) or LF (2 Hz) loading category, the magnitudes were chosen as low- (LM) and high-magnitude (HM), respectively, leading to constant strain rate amplitudes for the two frequency groups. This resulted in the four loading regimes: (i) HF-LM (40 Hz–0.5 N); (ii) HF-HM (40 Hz–1 N); (iii) LF-LM (2 Hz–10 N); and (iv) LF-HM (2 Hz–20 N) loading. Loading was performed five times per week and lasted for one or four weeks. Tissue samples were processed for histology and histomorphometry (bone-to-implant contact, BIC; and peri-implant bone fraction, BF) at the cortical and medullar level. Data were analysed statistically with ANOVA and paired t-tests with the significance level set at 0.05. For the one-week experiments, an increased BF adjacent to the implant surface at the cortical level was exclusively induced by the LF-HM loading regime (2 Hz–20 N). Four weeks of loading resulted in a significant effect on BIC (and not on BF) in case of HF-LM loading (40 Hz–0.5 N) and LF-HM loading (2 Hz–20 N): BIC at the cortical level significantly increased under both loading regimes, whereas BIC at the medullar level was positively influenced only in case of HF-LM loading. Mechanical loading at both HF and LF affects osseointegration and peri-implant BF. Higher loading magnitudes (and accompanying elevated tissue strains) are required under LF loading to provoke a positive peri-implant bone response, compared with HF loading. A sustained period of loading at HF is needed to result in an overall enhanced osseointegration.

Keywords: implant; mechanobiology; animal; osseointegration; loading frequency

1. INTRODUCTION

In orthopaedics and implant dentistry, a variety of strategies have been explored to achieve a solid and rapid osseointegration, which is required for early implant loading. Accordingly, the biological basis of such an objective is a controlled and fast peri-implant bone healing, which implies bone regeneration and remodelling. The mechanical environment plays an important role in bone (re)modelling by which the existing bone’s mass, shape and structure is adapted to its mechanical challenge [1,2]. In line with this general concept, the effect of mechanical loading on bone regeneration and remodelling also applies to bone surrounding titanium implants [3–5]. Findings from in vivo studies have shown that well-controlled mechanical loading at low frequency (LF) can improve bone formation at the implant surface and in the peri-implant region [4–7].

The interest in high-frequency (HF) mechanical loading, i.e. a frequency beyond the physiological
frequency (approx. 3 Hz of human mastication), grows because an increasing number of studies indicate its stimulating effect on bone formation and fracture healing [8–11]. Together with the findings of clinical studies [1,12,13], the advantages of mechanical loading at HF are believed to be safe and efficient. The latter refers to the evidence that HF mechanical loading improves the bone’s mechanical properties, thereby promoting its properties to withstand the physiological demands [2]. This makes HF mechanical loading a potent non-pharmacological intervention for osteoporosis, fracture healing and beyond [2,12–14].

Several in vivo systems for applying HF loading have been successfully used, such as whole-body vibration (WBV) [8,11] and individual limb compressive loading [15,16]. WBV experiments have evidenced the beneficial effect of HF loading on bone [8,10,11] and on titanium implant osseointegration [17,18]. On the other hand, the accessibility of local strain quantification and the feasibility of controlling the individual loading parameters render the compressive loading mode appropriate for performing a parametric study. However, the compressive loading model has not been tested for the peri-implant setting thus far. Moreover, the specific contribution of the individual loading parameters (magnitude, frequency, rate and duration of loading) on the resulting peri-implant bone tissue response to HF loading remains partly unknown.

In an effort to further optimize mechanical loading protocols that enhance implant osseointegration, we tested the hypothesis that a HF signal may be more effective in stimulating implant osseointegration than a low-frequency (LF) signal, and that the differential sensitivity is dependent on the induced loading magnitude. To do so, the peri-implant tissue response to mechanical loading at HF and LF was assessed by means of the rat tibia compression model.

2. MATERIAL AND METHODS
2.1. Animals and surgical procedure
Seventy-seven male Wistar rats (three months old) with an average weight of 353.7 g (s.d. ± 12.9) were used in the present study. Eight of these rats were used for the determination of the loading protocol by means of ex vivo strain gauge measurements. The remaining 69 rats were used for the in vivo evaluation of peri-implant tissue response to loading.

Custom-made cylindrical screw-type implants (ø: 2 mm × length: 8 mm) were obtained from titanium rods (99.6% Ti, Goodfellow Cambridge Ltd, Huntingdon, UK; figure 1a). The implants were ultrasonically cleaned with distilled H2O and etched with a solution of HF (4%) and HNO3 (20%), resulting in a roughness value Ra of 0.45 μm. Implants were sterilized prior to surgery.

Implantation was performed under full anaesthesia induced by 2.5 per cent isoflurane inhalation (Isoflurane USPR, Halocarbon, NJ, USA). Implants were installed bi-laterally in the medio-proximal site of tibia. Both cortices were perforated with low rotational speed under constant saline cooling. The final surgical drill was 0.3 mm undersized compared with the implant diameter. After manual implant insertion by means of a custom-fit wrench, the wound was closed with resorbable sutures (Vicryl 3-0, Ethicon, USA). At the end of the experiment, the animals were sacrificed by cervical displacement under isoflurane-induced anaesthesia.

2.2. Quantification of peri-implant bone strains
Bone strains measured on eight excised rat hindlimbs before and after implant placement [19,20] were used to establish the relationship between the applied load and the resulting bone strain. In short, a single element strain gauge (type FLG-02-11, TML, Tokyo Sokki Kenkyujo Co., Ltd) was glued onto the exposed lateral bone surfaces of the intact tibiae at 25 per cent of the tibia length. The limbs were placed in between two loading cups of a testing device (Bose TestBench LM1, EnduraTEC Systems Group, Bose Corp., Minnetonka, MN, USA).

Figure 1. Implant design, ex vivo calibration and in vivo loading are illustrated. (a) Commercially pure titanium custom-made screw-shaped implant (ISO M2 screw-thread protocol). (b) Ex vivo strain gauge measurements were performed on excised hind limbs in order to determine the relation between the applied loading force and the resulting strain in proximity of the implant. (c) An in vivo axial compressive loading was applied by two custom-made loading cups of a testing system (Bose TestBench LM1, EnduraTEC Systems Group, Bose Corp., Minnetonka, MN, USA).

J. R. Soc. Interface (2012)
Minnetonka, MN, USA), and strain was measured at compressive forces of 2.5, 5.0, 7.5 and 10 N, respectively, at a rate of 0.5 mm s\(^{-2}\). Measurements were performed five times after complete removal and repositioning of the limbs into the loading cups. The experiments were repeated on each specimen after the insertion of a titanium screw-shaped implant of 2 mm diameter 1.5 mm distal to the strain gauge site. Mean strain and standard deviation over the five repetitions were calculated for each limb before and after implant insertion.

### 2.3. In vivo mechanical loading

Rats were randomly allocated to eight groups, corresponding to four different loading regimes and two experimental periods (table 1). The one- and the four-week loading groups were loaded consecutively. The loading regimes consisted of HF (40 Hz) and LF (2 Hz) protocols. Within each frequency category, the strain amplitude varied twofold (i.e. LM and HM, respectively). The combined loading frequency and magnitude resulted in identical strain rate amplitudes between the frequency categories (identical strain rate amplitudes for HF-LM and LF-LM, and for HF-HM and LF-HM). The test tibiae (=loaded) were held in loading cups and subjected to dynamic axial loads (figure 1c). Loading was initiated one day post implant installation. The load application took 10 min per session and was performed five times a week. The implant in the contralateral tibia served as unloaded control (=unloaded). Anaesthesia induced by isoflurane inhalation (Isoflurane USPR, Halocarbon, NJ, USA) was applied during the loading.

### 2.4. Specimen preparation

After sacrifice, the implants and surrounding bone tissues were isolated and immediately fixed in a CaCO\(_3\)-buffered formalin solution, dehydrated in an ascending series of ethanol concentration and embedded in polymerized methylmethacrylate resin. The tissue blocks containing the implants were sectioned along the longitudinal direction of the tibia and the implant’s axis by means of a diamond saw (Leica SP1600, Wetzlar, Germany). After polishing to a final thickness of 20–30 \(\mu\)m (Exakt 400 CS, Exakt Technologies Inc., Germany), the sections were stained with a combination of Stevanel’s blue and Von Gieson’s picrofuchsin red, visualizing mineralized (red) and non-mineralized (blue) tissues.

![Figure 2. Illustration of the defined regions of interest (ROI) for histomorphometrical analysis. Bone is highlighted in green. In the region of either cortex or medulla, three different ROIs were defined according to their distance relative to the implant surface: (ROI 1) 0–100, (ROI 2) 100–500 and (ROI 3) 500–1000 \(\mu\)m.](http://rsif.royalsocietypublishing.org/}

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**Table 1.** The applied loading regimes, the resulting strain and strain rate amplitudes in the peri-implant environment and the animal distribution.

<table>
<thead>
<tr>
<th>loading regime</th>
<th>estimated strain and strain rate amplitude</th>
<th>group size ((n))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>frequency (Hz)</td>
<td>magnitude (N)</td>
</tr>
<tr>
<td>HF-LM</td>
<td>40</td>
<td>0.5</td>
</tr>
<tr>
<td>HF-HM</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>LF-LM</td>
<td>2</td>
<td>10</td>
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<tr>
<td>LF-HM</td>
<td>2</td>
<td>20</td>
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**2.5. Histological and histomorphometrical analysis**

The sections were histologically evaluated using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Histomorphometrical analyses were performed under 10\(\times\) objective lens of a light microscope (Leica Laborlux, Wetzlar, Germany) equipped with a high sensitivity video camera (AxioCam MRc5, Zeiss, Göttingen, Germany). The analyses were carried out on both distal and proximal sides of the implant, at both cortical and medullar level. The following parameters were measured using an image-analysing software package (Axiovision 4.0, Zeiss, Göttingen, Germany):

- bone-to-implant contact (BIC, %) = summation of the lengths of contact between bone and implant/ the implant length from the first till the last BIC; and
- bone fraction (BF, %) = area occupied by bone/area of the region of interest. Three different regions of
interest (ROI) were defined: 0–100 (ROI 1), 100–500 (ROI 2) and 500–1000 μm (ROI 3) away from the implant surface (figure 2).

2.6. Statistical analysis

Two-way ANOVA followed by Tukey’s Honestly Significant Difference (HSD) test for multiple comparisons were performed to assess the effect of (i) loading, (ii) time, and (iii) region of interest on the peri-implant tissue response of each loading regime (SPSS v. 13.0, Chicago, IL, USA). When significant interactions of loading and time/ROI in the assessed loading regimes were detected by two-way ANOVA, the pairwise comparison (paired t-test) between the unloaded and loaded was applied to spot their differences at each time/ROI level. Data were reported as mean ± standard error of the mean (s.e.m.). The significance level of $P < 0.05$ was acknowledged.

3. RESULTS

3.1. Animal and implant outcome

Implant surgery and in vivo mechanical loading were uneventful for both experimental periods. From a total of 138 implant samples, five samples were lost during histological processing (two from the one-week group and three from the four-week group) and another two samples (from the four-week group) were excluded owing to peri-implant infection.

3.2. Quantification of peri-implant bone strains

Compressive loading of the rat limbs resulted in tensile strains on the medio-proximal surface of the tibiae. Over the range of load magnitudes applied, we found for each limb that the mean strains exhibited a linear relation to the applied loads both before and after implant insertion ($R^2 > 0.99$). Repeated measurements on each specimen showed high precision (intra-individual differences were as low to 6.45 ± 2.27% and 9.22 ± 2.67% for the intact and implanted tibiae, respectively). When compared with the measured strains in intact bone, the strains after implant placement were on average 35.3 per cent lower.

As the experimental data were strongly affected by strain gauge placement, they could not be used directly for defining the desired strain rates. Instead they were used to validate established micro-finite element models, providing a three-dimensional quantification of the strain throughout the whole tibiae [19,20]. We estimated maximum tensile strains at 25 per cent of the tibia length (hence corresponding to the strain gauge position and alignment) of approximately 260 μstrain for the implanted tibiae subjected to 10 N loading. These data were adopted to define the in vivo loading protocols by taking into account the linearity of the relationship applied loading-induced strain (i) a maximum loading force of 20 N (ii) and assuming strain rate to be given by the product between strain magnitude and loading frequency (iii); hence, we estimated peak strains of 13, 26, 260 and 520 μstrain to correspond to the HF-LM, HF-HM, LF-LM and LF-HM loading, respectively, and strain rates of 520 and 1040 μstrain s$^{-1}$ for the LM and HM loading regime, respectively (table 1).

3.3. Histology

The histological images revealed bicortical bone apposition to the implant for both loaded and unloaded implants and for both healing periods (figure 3). After one week, newly formed bone was observed along the implant surface in the medullar cavity, whereas active remodeling occurred at the cortex. After four weeks, the newly formed bone in the medulla was rearranged into denser and more homogeneous bone close to the implant surface. Furthermore, the healing of the peri-implant cortex was complete. No obvious differences between loaded and unloaded implants could be noticed on the histological sections.

3.4. Histomorphometry

3.4.1. Bone-to-implant contact

In the peri-implant region of the cortex, four weeks of loading significantly increased BIC in case of HF-LM loading (40 Hz–0.5 N) and LF-HM loading (2 Hz–20 N; 79.8 ± 1.9% versus 83.4 ± 1.3% and 78.6 ± 2.2% versus 87.3 ± 1.1%, respectively; unloaded versus loaded; $P < 0.05$; two-way ANOVA followed by pairwise comparison; figure 4a). In the medulla, a significant increase of BIC in response to loading was also found after four weeks of loading, but only in case of HF-LM loading (73.4 ± 4% versus 79.1 ± 3.7%; unloaded versus loaded; $P < 0.05$; two-way ANOVA followed by pairwise comparison; figure 4b). No significant effect of the four loadings on BIC was found for the one-week experiment.

Concerning BIC change over time, BIC at both cortex and medulla level significantly increased from one to four weeks for all loading regimes ($P < 0.01$; ANOVA).

3.4.2. Bone fraction

In the cortical peri-implant region, one week of loading significantly increased BF in ROI 1 in case of LF-HM loading (2 Hz–20 N; 36.2 ± 2.7% versus 45.8 ± 3%; unloaded versus loaded; $P < 0.05$; two-way ANOVA followed by pairwise comparison; figure 5). No effect of loading on BF, however, was identified either in the other ROIs or after four weeks of loading. In the medullar peri-implant region, no significantly different BF was detected between the unloaded and loaded implants of the four loading regimes. In all assessed samples, peri-implant BF evolution over time (from one to four weeks of healing) was found to increase at the cortical level but to decrease at the medullar level ($P < 0.01$; ANOVA).

Similarly, with regard to BF relative to the distance to the implant surface, opposing results were found for the cortex and the medulla of all samples. At the cortex, the amount of bone was significantly lower in the region closest to the implant surface (ROI 1), compared with the other ROIs ($P < 0.01$; ANOVA followed by Tukey’s HSD). At the medulla, BF decreased with increasing distance from the implant surface (BF in
ROI 1 > 2 > 3; P < 0.01; ANOVA followed by Tukey’s HSD).

4. DISCUSSION

In this study, different loading regimes were applied in order to evaluate the stimulatory effect of HF (i.e. 40 Hz) and LF (i.e. 2 Hz) mechanical loading on implant osseointegration and peri-implant bone formation. In order to assess additionally the influence of the loading magnitude, two loading forces varying by twofold within each frequency category were assigned.

According to Frost [21], loads on bones cause bone strains that generate signals detected by osteocytes and hence lead to cellular response and eventually to bone remodelling. The anabolic effect of loading on bone is evident when the bone strain induced by LF loading goes beyond the 1000 \( \mu \text{e} \) threshold [21]. However, this does not apply to a HF loading protocol, where LM events have been shown to be stimulatory to the bone [2]. We intended to test whether the latter also applies to for peri-implant bone healing. With respect to the configuration limitations of the loading setup, 40 and 2 Hz were selected as HF and LF, respectively. Specifically, the resonance of the loading system was observed during ex vivo tests when the loading frequency exceeded 50 Hz. To eliminate the systemic resonance, which is harmful to the loading device and causes extra stimuli to the implant, the HF regime was limited to 40 Hz. For both frequency categories, the magnitudes of loading were determined in such a way that identical strain rate amplitudes were obtained. In this way, the role of the strain rate amplitude—a determinant parameter of bone response to loading [22]—could be controlled. In a series of studies from our group [23–25], the amplitude of peri-implant strain favouring bone formation was found to be 250–1000 \( \mu \text{e} \) at LF (3 Hz) with strain rate amplitudes ranging from 267 to 1600 \( \mu \text{e} \cdot \text{s}^{-1} \). In the present study, a strain of 260 and 520 \( \mu \text{e} \) was induced when loading at LF (2 Hz), resulting in strain rate amplitudes of 520 and 1040 \( \mu \text{e} \cdot \text{s}^{-1} \), respectively. Accordingly, for the HF loading (40 Hz), the estimated strain magnitudes of 13 and 26 \( \mu \text{e} \) by loading would result in identical strain rate amplitudes. All loading categories could thus be considered lying within the range of positively influencing the peri-implant bone response.

Owing to the advantages including the comparable bone-remodelling rate with human [26], the accessibility for implant surgery and mechanical loading, the rat tibia was applied in the present research to study peri-implant bone healing. Histological observations revealed a normal healing response after implantation, irrespective of the loading regime. These observations were corroborated by the histomorphometrical data: (i) bone remodelling in the cortical peri-implant region led to an increased BIC and BF over time. Compared with the distant host bone, however, BF in the direct implant vicinity remained inferior, even after four weeks of healing and (ii) in the peri-implant medullar area—an initial bone tissue-free region—massive woven bone was formed soon after implantation. The formed bone originated from the endosteum of the peri-implant cortex and grew along the implant surface. Subsequent remodelling of this newly formed bone led to less but denser bone, in close contact with the implant. With regard to the changes over time in this medullar region, implant osseointegration (as quantified by BIC) was found to increase from one to four weeks, whereas the bone volume in the vicinity of implant decreased for the same time period. This is in line with previous findings with the same animal model [18].

The anabolic effects of HF loading have been reported in several animal [8–11] and clinical trials [12–14]. Hence, it has been confirmed that bone can sense and respond to extremely small mechanical signals if they are applied at HFs. Only a few studies investigated the effect of HF loading on bone surrounding implants. Rubin & McLeod [27] investigated the effect of mechanical loading on bone growth into implants. Strains of 150 \( \mu \text{e} \) were generated in the cortex immediately adjacent to the implant by means of host bone bending in the turkey ulna disuse model. The results showed that a 20 Hz loading regime induced the most favourable bone response, whereas the 1 Hz loading only prevented the bone resorption caused by disuse. Other studies on implants were performed using a rat tibia model in which HF loading (12–100 Hz or 50 Hz) was applied via WBV. This loading was found to increase peri-implant bone formation in normal [18] and in oestrogen-deficient animals [17]. In the current experimental setup, BIC, the criterion of osseointegration, was significantly increased in the peri-implant region in case of HF-LM loading (40 Hz–0.5 N). HF-HM loading (40 Hz–1 N), however, showed only a trend towards an increased BIC. These findings agree with the notion that HF mechanical loading can be osteogenic [8]. However, this stimulatory effect may not require a high-loading magnitude.

The effect of bio-physical stimulation at LF loading (less than 3 Hz) on bone adaptation and regeneration has been well documented in rodent models using compressive loading [15,28–30], and implant healing setups [23–25,31]. Evidence is provided that, under LF loading, bone is sensitive to the applied loading magnitude, with higher magnitude being more osteogenic [32–34]. The findings of the LF regimes applied in the current study demonstrated increased BIC and BF in the cortical bone solely when the load was applied at HM (i.e. 20 N). Applying 10 N at 2 Hz did not influence the peri-implant healing response. Owing to the animal and technical restraints of the present study, the effect of higher loading forces (greater than 20 N) on the bone response could not be investigated. Despite these limitations, the findings suggest that the anabolic effect of LF loading, which increases with increasing magnitude, also applies to peri-implant bone healing.

Although the osteogenic potential of HF loading is evident, the peri-implant tissue response to the loading via different modes of application varies. Specifically, WBV seems to be superior to the localized loading. WBV has been found to extensively influence peri-implant bone remodelling and hence led to significant increases of BIC and BF [17,18,35]. Compared with
Figure 3. Representative histological sections. Active osteogenesis was found adjacent to the implant in medulla after one week of healing for both (a) loaded and (b) unloaded implants. After four weeks of healing, remodelling of peri-implant bone led to denser medullar bone formation around both (c) loaded and (d) unloaded implants.

Figure 4. Bone-to-implant contact (BIC) at (a) cortex and (b) medulla level for the four-week experiment. (Data from the one-week experiment are not shown as no significant differences were observed; *P < 0.05; two-way ANOVA followed by pairwise comparison.)

Figure 5. Bone fraction (BF) for the region adjacent to the implant surface (ROI 1) at (a) cortical and (b) medullar level for the one-week experiment. (Data from the four-week experiment are not shown as no significant differences were observed; *P < 0.05; two-way ANOVA followed by pairwise comparison.)
the control, more than 10 per cent extra BIC was found in case of WBV after four weeks. This increase in BIC is almost twofold times the bone-stimulating effect found with HF-LM loading. Moreover, the improved osseointegration by WBV can already be observed after one week of loading [35]. On the other hand, when the HF loading was directly applied onto the implant, the loading failed to induce a pronounced peri-implant bone response [36]. The possible explanations for the superior stimulatory effect of WBV are (i) WBV may lead to stochastic resonance of the applied vibrations, which could serve as an extra bone stimulus [28,37]; (ii) the frequency span tested in WBV is higher than the one used in the present study (up to 150 versus 40 Hz). As more efficient mechanotransduction occurs with increasing loading frequency [38], more osseous response could be associated with loading at a higher frequency; (iii) as anaesthesia is not required during the application of WBV, the side-effects and/or complications of daily usage of full anaesthesia, which is performed on the animals in this compressive loading study, can be precluded; and (iv) the peri-implant strain distribution induced by different loading applications varies and inherently causes a different tissue response. However, measuring the exact peri-implant bone strains during WBV is difficult. Meanwhile, the strain measured in the present study corresponds to the bone surface level, thereby not exactly representing the strain at the BIC interface. Hence, for a better understanding of the biomechanical conditions with different experimental setups, further in vitro testing and numerical modelling is required.

In conclusion, mechanical loading at both HF and LF can contribute to peri-implant bone healing. Higher loading magnitudes (and accompanying elevated tissue strains) are required under LF loading to provoke a positive peri-implant bone response, compared with HF loading. A sustained period of loading at HF is needed to induce overall enhanced osseointegration.

The research protocol was approved by the local ethical committee for laboratory animal research of the Katholieke Universiteit Leuven (P029/2008) and was performed according to the Belgian animal welfare regulations and guidelines.

The authors declare no conflicts of interest. This study was supported by the Research Council of the Katholieke Universiteit Leuven (OT/07/059).

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