Inter-species investigation of the mechano-regulation of bone healing: Comparison of secondary bone healing in sheep and rat

Sara Checa, Patrick J. Prendergast, Georg N. Duda

Abstract

Inter-species differences in regeneration exist in various levels. One aspect is the dynamics of bone regeneration and healing, e.g. small animals show a faster healing response when compared to large animals. Mechanical as well as biological factors are known to play a key role in the process. However, it remains so far unknown whether different animals follow at all comparable mechano-biological rules during tissue regeneration, and in particular during bone healing.

In this study, we investigated whether differences observed in vivo in the dynamics of bone healing between rat and sheep are only due to differences in the animal size or whether these animals have a different mechano-biological response during the healing process. Histological sections from in vivo experiments were compared to in silico predictions of a mechano-biological computer model for the simulation of bone healing.

Investigations showed that the healing processes in both animal models occur under significantly different levels of mechanical stimuli within the callus region, which could explain histological observations of early intramembranous ossification at the endosteal side. A species-specific adaptation of a mechano-biological model allowed a qualitative match of model predictions with histological data. Specifically, when keeping cell activity processes at the same rate, the amount of tissue straining defining favorable mechanical conditions for the formation of bone had to be increased in the large animal model, with respect to the small animal, to achieve a qualitative agreement of model predictions with histological data.

These findings illustrate that geometrical (size) differences alone cannot explain the distinctions seen in the histological appearance of secondary bone healing in sheep and rat. It can be stated that significant differences in the mechano-biological regulation of the healing process exist between these species. Future investigations should aim towards understanding whether these differences are due to differences in cell behavior, material properties of the newly formed tissues within the callus and/or differences in response to the mechanical environment.

1. Introduction

Distinct dynamics of the bone healing process are observed between small and large animal models. Several rat osteotomy models stabilized with external fixator have shown a strong initial healing response characterized by periosteal and endosteal bone formation (Fig. 1) (Kaspar et al., 2008, Mark et al., 2004). On the other hand, externally stabilized sheep osteotomy models showed no signs of endosteal intramembranous ossification during the early stages of healing (Fig. 1) (Schell et al., 2006). In addition, healing is a significantly faster process in rats when compared to sheep (Fig. 1). These differences on tissue patterning could be a hint towards differences in the bone healing cascade.

It is well known that mechanical conditions influence the course of bone healing (e.g. Claes et al., 1997; Klein et al., 2003; Larsson et al., 2001); however despite the significant number of experimental studies, so far, little is known about how the mechano-regulation of bone healing is translated across species. The question arises whether tissue patterning is driven by similar mechano-biological rules in different animals or if substantial different rules are followed across species.

Mechano-biological computer models have been previously used to investigate the influence of the mechanical conditions during bone healing (Loboa et al., 2001; Andreykiev et al., 2008; Geris et al., 2008; Hayward and Morgan, 2009; Epari, 2006; Isaksson et al., 2006; Garcia-Aznar et al., 2006; Gomez-Benito et al., 2005; Lacroix et al., 2002c). These models have used different mechano-regulation
The rat animal model is based on a well established hypophysial cycle with an internal marrow cavity. Intra-species differences were not accounted for in model geometry. Last, the callus area was divided into a very fine grid in which each of the cell types and maximal differentiation of stem cells into the specific cell phenotype. The mechanical environment within the callus immediately post-surgery was calculated assuming that the callus region was occupied by granulation tissue (Table 1). Compression and bending loads were applied as axial ramps (1 Hz) and strain levels were determined at peak load. Bending loads were applied in the direction perpendicular to the fixator plane. Loads were applied to the proximal bone, while the distal part was fully constrained. Loading magnitudes were set as follows:

- Sheep tibia:
  - Compression: 1200 N; equivalent to 2 times animal body weight (BW) during normal walking (Duda et al., 1998).
  - Bending: 75 N. Applying the Euler–Bernoulli beam theory, this load would result in a maximum bending moment of 25 BWmm at the fixed side in an identical intact bone (without osteotomy), as reported by Duda et al. (1998).

- Rat femur:
  - Compression: 14.4 N; equivalent to 6 times animal body weight during normal walking (Wehner et al., 2010).
  - Bending: 1.7 N. This load would result in intact bone in a maximum bending moment of 10.7 BWmm at the femoral mid-shaft (calculated using beam theory), as reported by Wehner et al. (2010).

2. Methods

2.1. Animal models

The sheep model has been already described in detail elsewhere (Schell et al., 2005). Merino-mix sheep (N=24) underwent a 3 mm tibia osteotomy, which was stabilized with an external fixator (Fig. 1). The animals were sacrificed at 2, 3 and 6 weeks post-surgery. The rat animal model is based on a well established externally stabilized femoral osteotomy model (Strube et al., 2008). Sprague-Dawley rats (N=24) underwent a 1 mm osteotomy and fixed by external fixator (Fig. 1). Animals were sacrificed after 2, 3 and 4 weeks post-surgery. In both animal models, the final time points correspond to phases of the healing process where bone bridging occurred. Since bone remodeling was not included in this study, we excluded later remodeling phases. Histological sections are only used here as a qualitative comparison with model predictions; a detailed analysis of the sections has already been performed elsewhere (Lienau et al., 2005; Schwarz et al., 2011).

2.2. Mechanical stimuli within the callus post-surgery

Finite element models (FEM) of the sheep and rat osteotomies were developed using Cubit 11.1 and then imported in Abaqus 6.6 for further analyses. The models included the external fixator, the cortical bone, the marrow cavity and the callus (Fig. 2A). Intraventricular species differences were not accounted for in model geometry. Last time point histological sections, shown in Fig. 1, were used to define the dimensions of a simplified callus (Fig. 2B). Bones were idealized as cylindrical entities with an internal marrow cavity.
same parameter values (Table 2). Loading was applied as axial ramps (1 Hz) simulating the loading for one day. Time dependent loading (Fig. 2C) was included to simulate the unloading of the operated limb due to the surgical procedure (Seebeck et al., 2005). Since both animal models present a very similar unloading of the limb, an averaged data fitting of the unloading in both animals at each time point was performed.

3. Results

3.1. Mechanical stimuli within the callus post-surgery

The bending load applied in the rat would result in intact bone (without osteotomy) in strain levels at the surface of the mid-shaft of about 900 με (calculated using beam theory: \(\varepsilon = My/IE\)), while the applied compression load would result in intact bone in strain levels of 300 microstrain (calculated using beam theory: \(\varepsilon = F/AE\)). In the externally stabilized osteotomy model, strain levels in the rat under bending were similar to those predicted under compression (Fig. 3). Fluid flow velocities were significantly lower in bending compared to compression (Fig. 3).

In the case of the sheep osteotomy, the strain levels under bending were of the order of 10 times lower than those predicted under compression, the latter being around twice the value predicted in the rat (Fig. 3). In the same non-osteotomized bone, surface strains under the applied bending load would be of the

Fig. 2. (A) Finite element models of the externally stabilize osteotomy models in the rat and sheep. The models include the external fixator, the bone, the marrow cavity and the callus. (B) Dimensions of the sheep and rat osteotomy models. Callus shapes were based on histological sections at the end time points. (C) Percentage of limb loading during healing (based on Seebeck et al. measurements in the sheep and unpublished data in the rat). Scaled to the amount of limb loading for the simulation of bone healing. After 49 days, full loading was applied.

Table 1

<table>
<thead>
<tr>
<th>Tissue material properties (Lacroix and Prendergast, 2002a).</th>
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<td>Granulation tissue</td>
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<td>Bulk modulus grain (MPa)</td>
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* Martin et al. (1998).

Table 2

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<th>Cell type</th>
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* Isaksson et al. (2008).
* Appeddu and Shur (1994).
order of 2200\(\mu\varepsilon\), while strains under the applied compression load would be about 600 microstrain (calculated using beam theory). The externally stabilized osteotomy shifted the main straining of the bone from occurring under bending to occur under compression. Based on this finding, bending will be omitted in the rest of the paper.

Fig. 3. Minimum principal strains (A) and mechanical stimuli used in the bone healing simulations (fluid flow: B and shear strain: C) in the rat and sheep callus under different loading conditions, immediately after surgery. The sheep callus exhibited higher strains when compared to the rat. In addition bending loads resulted in lower strain levels than compression, especially in the sheep.

Fig. 4. (A) Bone healing as predicted in the rat (top) and sheep (bottom) models under axial compression load. The rat model shows initial endosteal and periosteal bone formation, which agrees with histology. However, no bone bridging was observed after 4 weeks. The sheep model shows no bridging after 6 weeks and an early formation of bone in the central gap region, which does not agree with histology. (black: bone; purple: fibro-cartilage). (B) and (C) Amount of differentiated tissues during bone healing in the rat and sheep models, under axial compression load. Both models resulted in a steady-state combination of fibrous and cartilaginous tissues in the gap region, representing a non-union situation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.2. Bone healing simulations

In the rat, initial endosteal and periosteal bone formation were predicted (Fig. 4A). This is in accordance with histological observations at the 2 week time point (Fig. 1). Cartilage formation was predicted at the osteotomy gap after 3 weeks and remained after 4 weeks (Fig. 4A). Complete bridging was not predicted after 4 weeks. The gap area resulted in a steady-state combination of fibrous and cartilaginous tissue, representing a non-union situation (Fig. 4B).

In the sheep model, after 2 weeks, periosteal bone formation was predicted (Fig. 4A). This was in agreement with experimental observations (Fig. 1). After 3 weeks, bone formation was predicted in the central region of the osteotomy gap, which was not observed in the experimental study (Fig. 1). Bone bridging was not predicted after 6 weeks (Fig. 4A), contrary to that observed in the histology (Fig. 1). As for the rat, a steady-state combination of cartilage and fibrous tissue resulted in the gap region, representing a non-union situation (Fig. 4C).

Bone bridging was predicted in the rat and sheep when the limits of the mechanical stimuli driving the different cellular processes (Table 3) resulted in endochondral ossification after 8 weeks (Fig. 5B). After 10 weeks, complete bone bridging was predicted when the level was set as 5 times the baseline value (Fig. 6), being the time to complete healing longer than that observed experimentally (Fig. 1).

The same limits on the mechano-regulation of cellular processes resulted in significantly higher amounts of bone formation in the intracortical region in the rat compared to the sheep (Fig. 7A). Only when ossification in the sheep occurred under high mechanical stimuli, when compared to the rat, the sheep model showed a significant increase in the formation of bone over time.

Based on these results, we investigated the influence of load magnitude on bone healing predictions. By increasing 25% the compression load applied in the rat and decreasing 25% the load applied in the sheep, we still observed marked differences in the limits of the mechanical stimuli driving bone healing, so that model predictions would qualitatively agree with experimental observations (Fig. 7B). These simulations suggest that the ossification process in sheep occurs under higher levels of mechanical stimulation compared to rat (Fig. 7C).

4. Discussion

Inter-species comparisons of bone healing experiments clearly show a slower healing in large animals when compared to small animals (Schell et al., 2005; Strube et al., 2008). In addition, it is known that mechanical stability influences the bone healing outcome (Klein et al., 2003); however it remains unknown

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**Table 3**

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<thead>
<tr>
<th></th>
<th>Baseline (Prendergast et al., 1997)</th>
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<th>Level 3</th>
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<td>$S &gt; 5$</td>
<td>$S &gt; 6$</td>
<td>$S &gt; 7$</td>
</tr>
</tbody>
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$s = \frac{\gamma}{b} \frac{v}{u}$

where $s$ represents the mechanical stimulus, $\gamma$ the shear strain, $v$ the fluid solid velocity, $a=0.0375$ and $b=3 \mu$m/s (Huskes et al., 1997).

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![Fig. 5. Percentages of bone (top) and fibro-cartilage (bottom) tissue for different limits of the mechanical stimuli driving the different cellular processes (Table 3).](image-url)
whether different species respond in the same way to mechanical stimulation. In this study, we investigated whether differences in bone healing patternning in two different animal models could be due to differences in the mechanical conditions under which bone healing takes place. Finite element models of sheep and rat osteotomy, which experimentally show significantly different healing patterns (Fig. 1), were developed to investigate the mechanical conditions within the callus. In addition, a lattice-based mecanobiological model that explicitly includes migration, proliferation and differentiation of the cells (Byrne et al., 2007; Checa and Prendergast, 2009a, 2009b; Prendergast et al., 2010; Sandino et al., 2010; Khayyeri et al., 2009, 2010) was implemented to simulate the bone healing process. Model predictions were compared with histological sections at several time points post-surgery.

Finite element analysis was used to determine the mechanical environment within the callus. The geometry of the FEM was to some extent simplified; bones were idealized as cylinders and the callus had an initially pre-defined shape. Being the most external regions of the callus occupied by differentiated tissue only at the late time points, callus size is not expected to significantly influence the results at the early time points. Since callus size is based on histological data at the end time points, this approach gives us a good approximation of the mechanical conditions within the callus.

After osteotomy, the sheep model resulted in a more mechanically stimulated callus than the rat (Fig. 3). Axial compression loads as high as 15 BW would be needed in the rat to reach strain levels similar to those found in the sheep (Fig. 8A). This difference in the initial mechanical conditions within the callus may be in part responsible for the differences in the healing patterns shown at later time points. In the sheep, histological sections show no bone formation in the gap region during the first weeks post-surgery, while a significant amount of bone is shown in the rat (Fig. 1). The higher strain levels shown in the sheep when compared to the rat may explain these observations (Fig. 8B).

To further investigate the differences in the bone healing process between these two animals, a mecanobiological model for the simulation of the healing process was implemented. Tissue material properties were based on previous mecanobiological models where we incorporated known inter-species differences in mature bone elasticity (Manjubala et al., 2009; Leong and Morgan, 2008). Other tissue material properties might as well show inter-species differences; however to our knowledge, this remains unknown. Similarly, although, some evidence exists about inter-species differences in cell response (Torricelli et al., 2003), the number of studies is very limited and quantitative data are not available. Our approach was to isolate the relative effect of the level of mechanical stimuli defining the tissue boundaries in the mecanobiological theory implemented here. What can be inferred is that the same mecanobiological model is not able to capture the bone healing process in these two animal models. If the material properties of the newly formed tissues within the callus and biological parameters would be similar, then our results suggest that they have a significantly different sensitivity to mechanical stimulation (Fig. 7C). On the other hand, if rat and sheep cells would respond to the same levels of mechanical stimulation, then simulations suggest that a significant difference in the activity of the cells and/or the evolution of the tissue within the callus, in terms of material properties, exists between these two species. What we can conclude is that differences in the dynamics of bone healing between sheep and rat cannot be completely explained by the differences in animal size. In addition, differences in the mechanism-sensitivity and/or biological response of the cells are expected. Future investigations on the characterization of species-specific cell behavior and a profound characterization of the mechanical properties of the tissues within the callus will allow a further corroboration of these observations.

Previous mecanobiological models of bone healing in the sheep have shown complete healing after 8 weeks (Lacroix and Prendergast, 2002b; Isaksson et al., 2008). Results cannot be easily translated across studies due to differences in the implementation of the models (simulation of the cellular processes) and model parameters (e.g. different geometry). In those studies the external fixator was not explicitly included. Isaksson et al. showed that an increase in compression load from 300 to 400 N doubled the time to bony bridging, and a further load increase to 500 N resulted in a steady-state non-union. Initial inter-fragmentary movements in our sheep model under a 1200 N compression load were 0.4 mm, which agree with previous experimental measurements (Duda et al., 1998).

Histomorphometry measurements in the sheep after 6 weeks showed that 67% of the callus area was occupied by mineralized bone and 8% by cartilaginous tissue (Lienau et al., 2005). In our model predictions, bone bridging was observed when approximately 75% of the callus was occupied by bone and around 5% by cartilage. In the rat osteotomy model, Schwarz et al. (2011) found that after 4 weeks, 35% of the callus was occupied by bone while 22% by cartilage. In our model, after 4 weeks, approximately 45% of the callus was occupied by bone and 10% by cartilage.

The availability of experimental data has led to the use of two different bones (tibia vs. femur). The biological environment of the tibia is different compared to the femur (lower amounts of soft tissues surround the tibia), which could have an effect on the bone healing process. However, the specific role of soft tissue coverage in bone healing remains unknown.

It is to be noted that model predictions in this study are almost identical on the lateral and medial side, while histologically there is often a marked difference, especially at the late stages of healing (Fig. 1). We could expect that bone shape could be responsible for these differences; however, we do not exclude
biological mechanisms, e.g. related to the surgical procedure. The fact that our model predictions were rather symmetric when compared to the histology is a limitation of the model. However, it is not expected that this limitation influenced the general conclusions drawn.

In conclusion, two different animal models of bone healing in two distinctly different species have been investigated in relation to their differences in speed of healing and distinct healing patterns. A significant difference in the mechanical stimuli within the callus region and healing patterns was found. Compression loading could explain histological observations of early intramembranous ossification at the endosteal side. Under this loading condition, a species-specific mechano-biological regulation of the bone healing process was predicted, which could be due to a differential cell response to mechanical loading and/or to differences in biological and material properties. These findings need to
be carefully considered, if conclusions from one animal model are transferred to another animal or even to humans.

Conflict of interest statement

The authors have no conflict of interests.

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