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ORIGINAL ARTICLE

Influence of cyclic bending loading on in vivo skeletal tissue regeneration from periosteal origin

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Summary
Introduction: Periosteum osteogenic and chondrogenic properties stimulate the proliferation then differentiation of mesenchymal precursor cells originating from its deeper layers and from neighboring host tissues. The local mechanical environment plays a role in regulating this differentiation of cells into lineages involved in the skeletal regeneration process.
Hypothesis: The aim of this experimental animal study is to explore the influence of cyclic high amplitude bending-loading on skeletal tissue regeneration. The hypothesis is that this mechanical loading modality can orient the skeletogenesis process towards the development of anatomical and histological articular structures.
Material and methods: A vascularised periosteal flap was transferred in close proximity to each knee joint line in 17 rabbits. On one side, the tibiofemoral joint space was bridged and loading occurred when the animal bent its knee during spontaneous locomotion. On the other side, the flap was placed 12 mm distal to the joint line producing no loading during bending. Tissue regeneration was chronologically analyzed on histologic samples taken from the 4th day to the 6th month.
Results: The structure and mechanical behavior of regenerating tissue evolved over time. As a result of the cyclic bending-loading regimen, cartilage tissue was maintained in specific areas of the regenerating tissue. When loading was discontinued, final osteogenic and fibrogenic differentiation occurred in the neoformed cartilage. Fissures developed in the cartilage aggregates resulting in pseudo-gaps suggesting similar processes to embryonic articular development. Ongoing mesenchymal stem cells stimulation was identified in the host tissues contiguous to the periosteal transfer.

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Discussion: These results suggest that the pseudarthrosis concept should be reconsidered within the context of motion induced articular histogenesis.

Introduction

Mature skeletal tissue: bone, cartilage, tendons and ligaments all develop from common cell precursors: mesenchymal progenitor cells [1]. This is the result of a cascade of genetically controlled cellular and molecular events, which control cell proliferation and differentiation and lead to the architectural organization and functional specialization of tissue. Mechanical environmental stimuli also influence the steps of embryogenesis. In joints, these stimuli influence all levels of tissue organization, from the molecular structure of the extracellular matrix [2] to the macroscopic morphology of the organ [3,4]. Indeed, mechanotransduction modulates the metabolism and synthesis of immature cells as well as their differentiation into different cell lineages.

There are numerous similarities between the process of skeletal tissue regeneration, observed in clinical and experimental studies, and embryonic skeletogenesis [5—7]. The molecular signals of intercellular communication as well as the cascade of events during proliferation and differentiation are similar during these two processes, except that in a post-traumatic situation, an inflammatory mechanism initiates the process [8]. Mechanoregulation also influences consecutive molecular and cellular events.

Models of skeletal tissue regeneration have been developed based on studies on the effect of local mechanical stimulation on precursor cell differentiation [9]. High local strain directs precursor cell differentiation into fibrous tissue. On the other hand, mild stress directs precursor cell differentiation into osteochondrogenic cells with direct ossification associated with weak hydrostatic stresses while cartilage growth is favored by higher compressive stresses [10—15].

In vivo experimental models have confirmed some of these theoretical hypotheses. Numerous implants or external devices have made it possible to exert controlled mechanical pressure on regenerating skeletal tissue. Thus the effects of shear stress, cyclic loading and low amplitude bending have been studied [15—18].

However, there are no experimental studies on this histogenic process during cyclic high amplitude bending-loading, similar to that which occurs in diathrodial joints. The goal of our study was to create this type of mechanical loading to determine its influence on skeletal regeneration. Thus, we initiated a process of skeletal regeneration by transferring a vascularised periosteal flap to the medial knee of New-Zealand rabbits. The mesenchymal precursor cells brought to the surgical bed by the periosteum and the host tissues proliferate before differentiating [19,20].

Our hypothesis is that the process of skeletal tissue regeneration integrates the numerous elements of the mechanical environment that it is exposed to. As a result this process, which is determined by the control of cell differentiation also organizes the structure of the regenerating tissue. The aim of this study was to analyze the architectural and structural changes in regenerating tissue as it evolved during in vivo high amplitude mechanical articular bending-loading. In this experiment, mechanical loading was obtained from the spontaneous knee movements of the animal during locomotion. We compared the neotissue that developed during bending-loading to that without bending.

Materials and methods

Seventeen three-month old skeletally immature New Zealand rabbits weighing 2.5 kg were used as animal models (INRA-ENSA Montpellier France) in the surgical protocol. Rabbits underwent surgery on both hind legs at once. The surgery was performed in an accredited experimental surgery laboratory of Montpellier Medical School, in accordance with French regulations on animal care and use of laboratory animals.

A vascularised periostal flap was harvested from the medial tibia along the axis of the saphenous bundle and was transferred to the knee joint. In one of the two legs, which was part of the ''loaded'' group, the flap was sutured to the medial side of the knee to undergo mechanical loading during knee movements, mainly cyclic bending on the saggital plane during spontaneous locomotion of the animal. In the controlateral leg, which was part of the ''control'' group, the flap was sutured 12 mm distally so that it would not undergo bending-loading during regeneration (Figs. 1 and 2). The choice of limbs for the experimental and control groups was randomized. Tissue regeneration was analyzed chronologically under two experimental conditions: mechanical loading from spontaneous knee movements, or no mechanical bending-loading.

Surgical procedure

Under general anesthesia (xylazine, intravenous ketamine) and strict aseptic conditions, the medial side of the tibias and knees were exposed by medial approach. The first step included the sectioning and raising a 30 mm long and 10 cm wide vascularised periosteal flap from the medial tibia. The saphenous bundle which provides anterograde vascularisation was interrupted distally for transplantation of this flap near the knee joint. Both hind legs were operated on during the same operation. In the ''loaded'' group, the flap was sutured to fibrous elements attached proximally to the femur and distally to the tibia with its deep layer turned towards the knee. Particular attention was paid not to perforate the joint capsule. The middle third of the flap was centered on the tibiofemoral joint space to undergo a maximum of cyclic bending during locomotion of the animal. The controlateral limb served as a control.

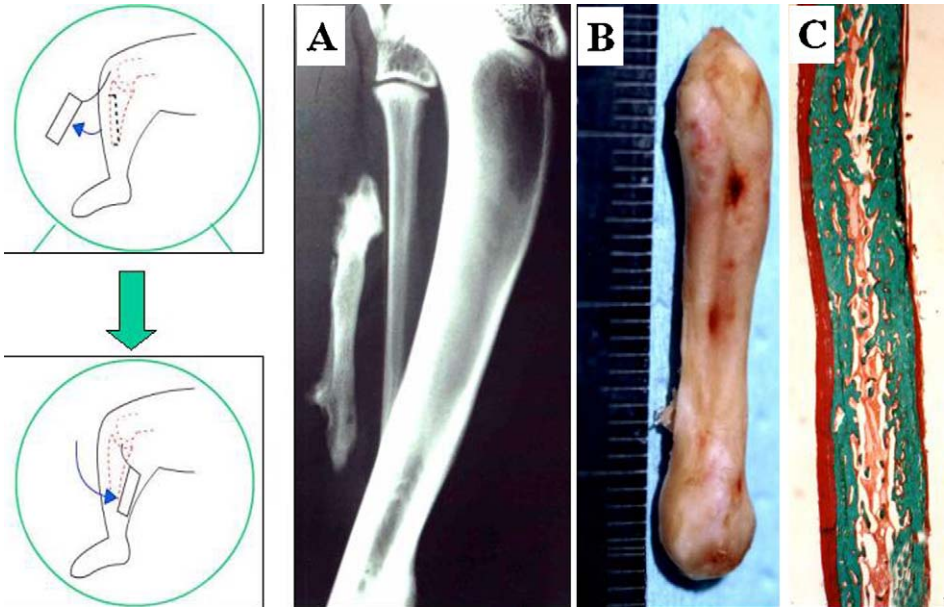

Figure 1 ''Control'' group. The periosteal flap raised from the medial side of the midtibial diaphysis is transferred just distal to the knee joint space to prevent bending loading. Analysis of heterotopic bone neoformation at 2 months. (A) Radiography. (B) Macroscopic view of bone neoformation after dissection. (C) Histologic view of a longitudinal section of the newly formed bone. The medullary cavity is formed (undecalcified, methylmethacrylate inclusion, Goldner's trichrome coloration, low magnification).

The periosteum was transferred to a similar position but was distal to the joint space so that there would be no loading during bending. It was sutured to the medial gastrocnemius muscle which provided support. After suturing the skin and when the animal had awakened, they were allowed to move around spontaneously outside their cages for one hour per day, otherwise movement was limited to the cage.

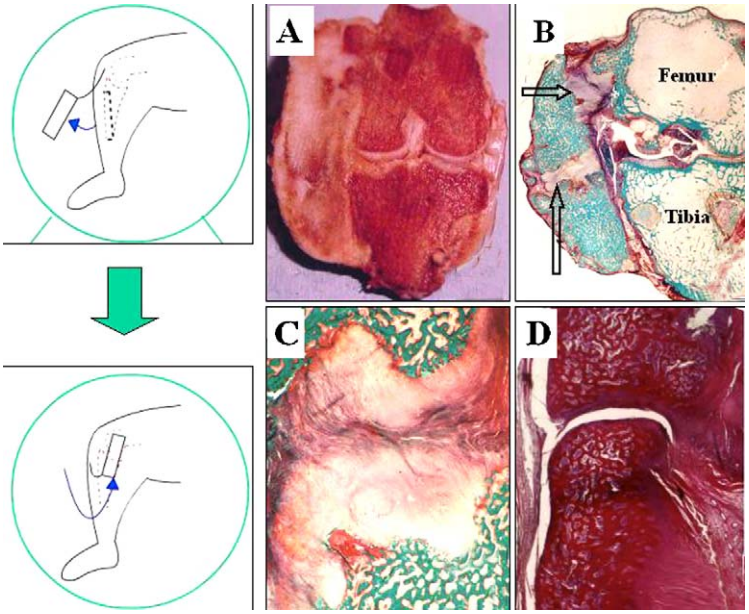

Figure 2 In the ''loaded'' group, the flap bridges the femoral-tibial joint line to be submitted to cyclic bending. (A) Macroscopic section of the knee joint in a coronal plane with at its medial side the massive newly formed skeletal tissue. (B) Very low magnification of the previous macroscopic section. The newly formed skeletal tissue is mainly composed of bone (green) with areas of cartilage maintained in its midportion and at the junction of the end of the femur (arrows) (Goldner's trichrome coloration). (C) Same view, intermediate magnification. The two masses of cartilage facing each other are separated by fissuring fibrocartilage which constitute the symmetrical axis of the midsegment of this newly formed tissue. (D) The coalescence of microcleft leads to macroscopic fissures that may persist until the end of the differentiation process, simulating a joint space within the new skeletal tissue.

Histological analysis

After a predetermined time, the rabbits were killed with an overdose of pentobarbital. Animals were killed on the 4th, 7th, 15th, 30th, 45th, 60th, 90th, and 180th day for the chronological study of cellular events occurring during the process of tissue regeneration. Once the animals had been sacrificed, tissue samples were taken for histological study. Skeletal regeneration samples were cut longitudinally on the frontal plane to obtain a histological field in relation to the joint space. The histological study was performed on samples fixed in formol, then decalcified and imbedded in paraffin, as well as on non-decalcified samples fixed in 80% alcohol then imbedded in methyl methacrylate.

Hematoxylin-Eosin-Saffron (HES) and Giemsa stains were used on paraffin samples. Goldner and May-Grünwald-Giemsa (MGG) stains were used for methyl methylacrylate samples. During microscopic study, the predominant phenotype of each histological field was determined. Thus, the following predominant fields were identified: blastema, cartilage, fibrocartilage, fibrous or bone.

Results

None of the animals died during surgery or postoperatively. At sacrifice, none of the surgical fields was infected. The ''loaded'' group knees presented with at least 90° of flexion, and a swelling of various sizes along the medial side. The members of the ''control'' group did not seem to be affected by the presence of a long palpable mass in the posterior compartment of the leg.

Evolution of cell differentiation in the regenerating tissue

Significant proliferation of precursor cells constituting an undifferentiated blastema in the area of flap, and the first step in cell differentiation was found in both groups on the 4th day. In the ''control'' group, the development of neotissue was observed along the medial gastrocnemius (Fig. 1A). In the ''loaded'' group, it developed on the medial side of the knee, and remained separate from the intact joint capsule (Fig. 2A). Condensation of these blastema cells provided the morphological contours of the final regenerated tissue.

After the 4th day, chondrogenic differentiation of mesenchymal precursor cells, which is a key step in enchondral ossification, was similar in both experimental groups. In the ''control'' group, a process of ossification of the neotissue matrix gradually replaced all of the cartilage with bone. Between the 15th and 30th day, all the cartilage had disappeared and was replaced either with bone or fibrous tissue. After the 30th day, a segment of long bone, whose mean length was identical to that of the flap (27—32 mm), had formed in the posterior compartment of the muscle (Fig. 1B). A medullary cavity had developed and usually included bone marrow (Fig. 1C). Osteoclasts were identified on the surfaces of newly formed bone. At 6 months, the regenerated tissue was composed of 90% bone and 10% fibrous tissue.

In the ''loaded'' group cartilage and fibrocartilage, differentiation continued until the 3rd month. The presence of cartilage was gradually limited to the ends and to the middle of the newly formed tissue (Fig. 2B). These areas extended to the initial junction with the support bone and to the tibiofemoral joint space, respectively. After the 3rd month, the newly formed skeletal tissue was detached from at least one of its points of attachment to the support bone. Knee bending no longer caused the regenerated tissue to bend. The cartilage had completely disappeared from the newly formed tissues. At 6 months, a bone segment with a medullary cavity had finally developed on the medial side of the knee. It barely interfered with articular range of motion because it was structurally separate from its initial support bone.

Development of microfissures

Fissures developed as the regenerating tissue in the ''loaded'' group went through the different stages of maturation. They were mainly located around the tibiofermoral joint space and at the junction between the regenerating tissue and the support bone, in the area where the cartilage phenotype was maintained. In the early stages, they were microfissures organized in a strip of fibrous or cartilaginous tissue. They created a symmetrical area between two masses of cartilage facing each other, whose architectures were mirror images of each other (Fig. 2C). In a few cases, coalescence of these cavities resulted in the formation of a macroscopically mobile area. In later stages of the protocol, these zones were mobile areas within the new skeletal tissue or on the interface with the support bone (Fig. 2D). This newly formed joint space was thus made up of fibrous tissue.

Changes in the host tissue

After transfer of the periosteal flap, histological changes were observed in the host tissues. In the earliest stage (4th day), lytic activity was observed in the tissues in contact with the transfer. This corresponded to necrosis of the superficial layers of muscle in immediate contact with the periosteum. This first stage was followed by a process of muscular regeneration which systematically resulted in complete repair without scar tissue in less than 14 days. This sequence was mainly observed in the ''control'' group, in particular on the surface of the medial gastrocnemius, which supported the periosteal transfer. At the same time, significant osteoclatic activity was observed in the cortical and cancellous bone located under the periosteum in the ''loaded'' group. Intense osteoblastic activity followed the initial osteolytic process, contributing to the formation of a callus between the regenerated bone and its support bone.

Moreover, in the ''loaded'' group, we observed stimulation of mesenchymal precursor cells in all of the spaces around the periosteal transfer. It was systematically found in neighboring muscles, in the medullary cavity of the bone, in the bone marrow (Fig. 3A), in the joint space and in the subserous bursa. It appeared to be multidirectional differentiation of precursor cells into various mesenchymal cell lines. It resulted in the spontaneous and ectopic production of bone, elements of cartilage, pseudo-ligamental structures and even muscle tissue (Fig. 3B—D).

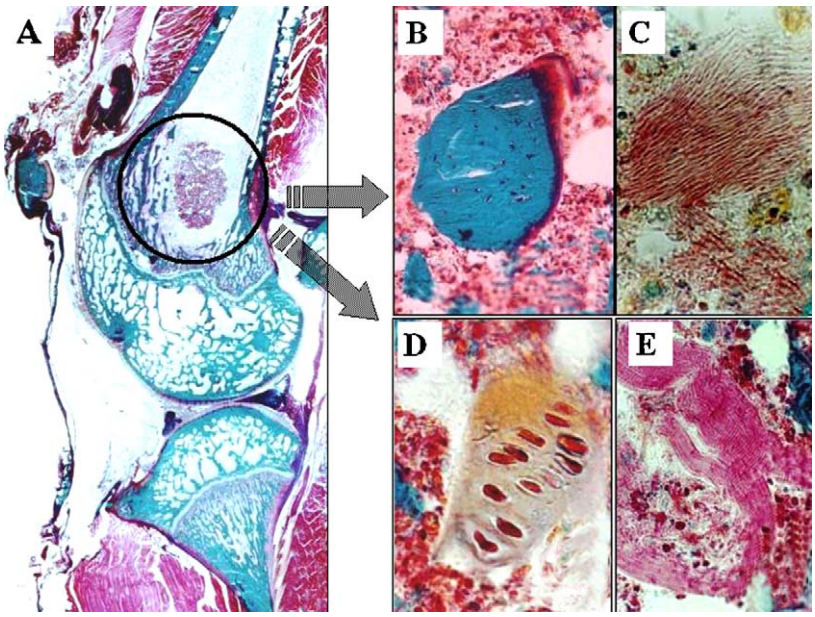

Figure 3 Host tissue changes neighboring the periosteal flap transfer observed in the ''flexion'' group. (A) Stimulation of marrow mesenchymal precursors with in situ skeletal tissue production in the distal femur (Goldner's trichrome coloration, undecalcified methylmethacrylate inclusion intermediate magnification). (B) A bone ossicle (green) is accompanied by smaller sparse bone elements at the periphery of this slide (high magnification). (C) Arc-shaped fibrotendinous tissue neoformation (high magnification). (D) Characteristic appearance of newly formed cartilage (beige) and small elements of newly formed bone (green) (high magnification). (E) Striated muscle neoformation and elements of bone (green) (high magnification).

Discussion

Structural modification of regenerating tissue in response to cyclic loading

When exposed to functional loading, the structural properties of bone tissue, and more generally, skeletal tissue adapt to the local mechanical environment. Although the general morphological characteristics are genetically determined, certain anatomical details as well as the microstructure of these tissues are influenced by the mechanical stresses and strains they undergo during the process of modeling and remodeling [18]. The results of our experimental study suggest that this adaptive capacity is not limited to mature skeletal tissue. The precursor cells involved in the postnatal development of these skeletal tissues also respond to stimuli from mechanical loading. The latter plays a role in orienting cell differentiation and the structural organization of both the regenerating tissue and the host tissue.

In this experimental protocol, bending-loading was begun in the immediate postoperative period thus influencing the mesenchymal stem cells as soon as they appeared on the site of tissue regeneration. Continuous bending-loading maintained and preserved the cartilage phenotype in specific areas of the regenerating tissue such as in the middle and at the ends. The middle area of the regenerating tissue extended towards the tibiofemoral joint space while the ends were at a junction between the regenerating tissue and the support bone. These areas were submitted to specific mechanical conditions, resulting in delayed ossification and the maintenance of cartilage tissue until the 3rd month, while cartilage disappeared completely between day 15th and 30th day in ''control'' group neotissue.

Variations in hydrostatic pressure influence the mechanisms that regulate the proliferation and differentiation of mesenchymal precursor cells. They stimulate proliferation in vitro, while [21] in vivo, they redirect differentiation of precursors of bone tissue towards a cartilage phenotype [17].

Differentiation into chondrogenic cell lines is favored by a local mechanical environment associating high hydrostatic pressures and mild strains [10,11]. High amplitude strain inhibits angiogenesis thus influencing enchondral ossification [22].

In our experimental protocol, loading of neotissue by cyclic bending generated a complex mechanical environment which could be described by numerous physical variables such as strain, variations in pressure or fluid as well as shear stress or movements at the cell/matrix and cell/cell interfaces. All of these interdependent variables can play a role in the transduction of mechanical signals into a biological response. They resulted in a change in the architecture of the regenerated tissue which, besides maintaining a cartilage phenotype, affected its anatomical organization. Indeed, the presence of identical volumes of tissue with symmetrically arranged fissures located in masses of cartilage (Fig. 2C), resembles the histomorphology of early articular development in the embryo. This includes the development of an interzone with the formation of cavities, then microfissures which coalesce to form the joint space. These various genetically programmed stages are highly dependent upon mechanical strains and stresses on

the embryo. The fissures observed in our study could be associated with a biological process which resembles the embryonic stages of joint development generating a pseudo ''joint space'' by a phenomenon of cavitation. Nevertheless, they could merely be the result of a purely mechanical process, causing a breakdown in cartilaginous material due to unduly high levels of strain on the tissue. Nevertheless, according to existing phenomenological models, high strain on mesenchymal tissue orients differentiation towards the development of fibrous tissue [10—14], making a fatigue fracture improbable in regenerating tissue.

Disappearance of the cartilage phenotype

The structure and mechanical response of regenerating tissue evolves over time. As it matures, the regenerating tissue ossifies and mineralisation occurs so that it gradually becomes rigid. This process, which is incompatible with high amplitude knee movements, caused the regenerating tissue to break off from the anchor points of its support bone so that bending-loading no longer occurred. We then observed the disappearance of neocartilage, although it had been maintained until this event at the 3rd month. Thus, the process of enchondral ossification was interrupted, and the cells did not finish their differentiation into cartilage [23]. Nevertheless the deep layer of the periosteum contains cell precursors which are engaged in chondrogenic differentiation, and which form cartilage during monoclonal cell cultures [24].

In our study, the segments of new cartilage sandwiched between two ossifying structures were not in a physiochemical environment that favored the stability of the cartilage phenotype. The molecular constituents of the extracellular matrix send signals of differentiation to its mesenchymal precursor cells [25,26]. Thus, although the environment of the articular cavity and the new cartilaginous tissue are chondrogenic, contact with the extracellular bone matrix directs precursors towards osteogenic differentiation [27]. Moreover, the elasticity of the extracellular matrix is a physical factor which influences the differentiation of the mesenchymal stem cells that it contains. Its increasing rigidity directs cells towards the development of osteogenic cell lines [28]. As a result, under our experimental conditions, the maintenance of the cartilage phenotype became dependent upon continued cyclic mechanical loading.

Modification of host tissues

The general notion of tissue regeneration is based upon deterministic models of differentiation in which regenerating tissue adapts to functional and anatomical locoregional prerequisites. However, to obtain the complete integration rather than the simple adhesion of newly formed tissue to preexisting tissue, the surrounding host tissue must also adapt to the regenerating tissue [29]. Muscular necrosis/regeneration as well as osteoclastic/osteoblastic bone activity can probably be explained by this essential reciprocal, ''preadaptation''.

Host tissues do not passively submit to tissue development from the periosteum they are in contact with. Host tissue makes a quantitative contribution to the histogenic process by providing precursor cells [20]. This mechanism could be stimulated by the diffusion of growth factors from the periosteal transfer or proliferating blastemic cells [30]. The bone and articular capsule which was left intact during periosteal transfer were probably an anatomical barrier against cell migration. Thus the recruiting and multidirectional differentiation of mesenchymal precursor cells observed inside the articular cavity and the medullary cavity suggests that these factors are influencing resident [31] or circulating [32] stem cells.

Critical analysis of the experimental model

To identify the influence of mechanical loading on tissue regeneration, the periosteal flap was placed in different anatomical positions. In the ''loaded'' group, the deep layer of the periosteum was placed upon the articular capsule, while in the ''control'' group, it faced the medial gastrocnemius fascia. The aim of this choice was to maintain normal range of motion in both knees with identical muscular activity, and probably similar vascular flows. This seemed important because partial oxygen pressure seems to influence chondrogenic differentiation. However, this choice may have influenced the regeneration process, because the tissue environments of the transfers were different.

Conclusion

This is a purely descriptive experimental study. Indeed, the limited number of samples at each phase of the study made it impossible to perform a statistical analysis. Nevertheless, these results support mechanobiological theories and suggest that the mechanical factors of the cell environment play a significant role in regulating cell differentiation during in vivo skeletal regeneration. In this study, mechanical loading from cyclic physiological knee movements, temporarily favored chondrogenic differentiation of mesenchymal precursors in selective areas of neotissue. Moreover, besides cell differentiation, the complex local mechanical environment also contributed to the architectural organization of the regenerating tissue.

Numerous models of pseudarthrosis have been described in the literature based on reproducing the mechanical environment observed under therapeutic conditions of unsuccessful internal fixation or primary fixation of implants (revision arthroplasty). They include bending-loading or shear stress at relatively limited amplitudes. The tissues observed under these conditions are mainly fibrous. Our model is original because it produces a mechanical environment with high amplitude bending-loading. Knee range of motion in rabbits resulted in at least 90° flexion throughout the experiment, applied to regenerating tissue immediately and until the third month. Our model can also be used for hypertrophic pseudarthrosis but under specific mechanical conditions of high amplitude cycle bending-loading. Our results raise the question of the conceptual definition of hypertrophic pseudarthrosis. Normally seen as unsuccessful union of two bone segments, the results of this experimental study suggest that it may be the result of incomplete articular genesis.

This is a classic histological study. To better understand the routes of differentiation and the determinism of these steps of tissue maturation, a phenotypical study of regenerating tissue could be performed as the tissue matures. Moreover, technical modifications could be made when attaching the regenerating tissue to prolong bending-loading throughout the entire experiment.

Conflict of interest statement

None.

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