This article was published in Medical Engineering and Physics, 37(8), 719-728, 2015
http://dx.doi.org/10.1016/j.medengphy.2015.05.015

From mechanical stimulus to bone formation: A review

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Abstract

Bone is a remarkable tissue that can respond to external stimuli. The importance of mechanical forces on the mass and structural development of bone has long been accepted. This adaptation behaviour is very complex and involves multidisciplinary concepts, and significant progress has recently been made in understanding this process. In this review, we describe the state of the art studies in this area and highlight current insights while simultaneously clarifying some basic yet essential topics related to the origin of mechanical stimulus in bone, the biomechanisms associated with mechanotransduction, the nature of physiological bone stimuli and the test systems most commonly used to study the mechanical stimulation of bone.

1. Introduction

Understanding the influence of mechanical stimuli on the structure of bone has long been a topic of scientific interest. To the best of our knowledge, Galilei [1], noticed a relationship between body weight and bone size and shape. However, mechanical forces were not identified as responsible for shaping the architecture of the skeleton until the 19th century, in studies developed by Meyer [2], Culmann [3] and Roux [4].

The German anatomist von Meyer identified arched trabecular patterns in a sagittally sectioned human first metatarsal and calcaneus, and Culmann, a pioneer of graphical methods in engineering, suggested that the patterns appeared to be aligned along principal stress directions produced by functional loading [5]. In 1881, Roux proposed that the apposition and absorption of bone is a biological stress-controlled process [6,7].

However, Julius Wolff [8] – influenced by von Meyer–Culmann interactions in 1867 – became associated to the concept of bone adaptation. He claimed that the shape of bone is related to mechanical stress by *Wolff's law of bone transformation*. Although

this law is an overly simplified mathematical approach, the concept has been accepted by the scientific community. Recent interpretations of "Wolff's Law" have proposed that bone mass and architecture are to some extent governed by adaptive mechanisms that are sensitive to their mechanical environment [9–11].

Over the years, remarkable work has been done to elucidate bone mechanotransduction and its response to mechanical stimulation. The first contact with this subject can be overwhelming due to the complexity and multidisciplinary mechanisms involved. This review paper aims to establish the state of the art of this area while simultaneously clarifying some basic yet important questions on which light has been shed during recent years, such as

- What is the origin of the mechanical stimulus? How is it triggered?
- How does bones mechanotransduction work?
- What are the normal physiological bone stimuli?
- What test systems are commonly used to study bone's mechanical stimulation?

2. Mechanical stimulus

Bone mass is maintained by and adapted to mechanical strain, primarily as the result of muscular contraction [12,13]. Some key aspects are currently accepted by the scientific community at large and should be mentioned.

First, long bones deformation is obtained by an orchestrated muscle activity as demonstrated by Duda et al. [14]. Using a finite element strain distributions model, these authors concluded that simplified load regimes produced differences in strain as high as 26% compared with regiments that included all thigh muscles. Although this study focused only on the proximal femur situation, this concept can be generalized to other bones in the human body.

Second, the forces experienced by bone arise from muscle action rather than from mere gravitational forces [15]. Hence, muscle mass/strength correlates with bone strength [12]. This concept was demonstrated in a study by Sievänen et al. [16]. The patella bone mineral apparent density and average strain magnitude were measured in a chained event experiment that included one-year unilateral strength training interventions, an accidental knee ligament rupture and a two-year rehabilitation period. The patella was selected as the target bone because it is a non-weight-bearing bone that receives mechanical stimuli from only the quadriceps activity. Sievänen et al. showed that a decline in muscle mass precedes a decline in bone strength under conditions of disuse and that the recovery of muscle mass increases before bone mass. In another study, Schönau et al.[17] compared the muscle development with age as well as muscle development and bone strength.

Disuse can be asserted to cause muscle wasting and bone loss, whereas physical activity increases muscle strength and bone mass. However, according to Rittweger et al. [15], this relationship only holds to a certain extent. The authors claim that muscular exercise can only enhance bone strength up to 1–2% because tendon stiffness may limit the musculoskeletal peak forces.

In several studies [9,12,16,18], a time lag of up to 5 days was registered between a

single period of mechanical loading *in vivo* and the onset of collagen and mineral apposition increases on the bone sur- face. This phenomenon is justified by the delay between the initial formation of new bone and the establishment of fully mineralized and mature bone.

The third key aspect was stated in one of the earliest far-reaching interpretations of bone loading made by Pauwels [19], who suggested that bending moments are transmitted along limbs by a combination of tensile forces in the muscle and compressive forces on bone. Hence, gravitational forces tend to lower and collapse our body segments in any upright posture. However, bending moments are accentuated rather than reduced due to the physiological curvature of long bones. In response to these external loads, muscles not only provide the necessary moment equilibrium in joints, but they counteract the passive bending moments along bones in an energetically efficient manner, as stated by Munih et al. [20]. While reducing the bending stress, muscles increase the axial compressive load irrespective of the posture to ensure minimal bone stress and minimal bone weight [21]. From all registered loading modes in long bone, bending is the most significant for bone adaptation [22–24].

Fourth and last, in addition to mechanical stimuli, bone remodelling may also be regulated by hormones, such as estrogen and parathyroid hormone [25,26], and induced by the nervous system [18,27] and inflammatory reactions [28].

3.Mechanotransduction system

Over the last several years, osteocytes have become generally accepted as the mechanosensory cells within the bone. Osteocytes coordinate the remodelling process by converting external mechanical forces into biochemical responses – a process known as mechanotransduction. However, the mechanism by which these cells sense the mechanical loads and facilitate adaptive alterations in bone mass and architecture is not yet completely understood [10,18,29,30].

3.1. *Stimuli perceived by osteocytes*

Osteocytes are generally assumed to react to bone deformation or to one of the consequences of bone deformation, such as shear stress due to load-induced fluid flow, electric fields caused by stress- generated streaming potentials, and hydrostatic pressure [22,31,32].

3.1.1. *Cell deformation*

The immediate consequence of mechanical loading is strain, which is a small deformation throughout the calcified matrix. These stimuli will stretch the osteocytes to the same extent as the sur- rounding bone tissue. When stretched in one direction, bone tends to slightly contract in the perpendicular direction. Hence, direct biaxial osteocyte strain is common [9,33]. Several authors [33–35] suggest that the strains experienced by an osteocyte are much higher than those measured on the bone surface,

with registered amplification factors that are up to 9 times larger than the applied global strain. This difference may be due to a magnification effect caused by the cell's complex surrounding pericellular and extracellular matrix. In a recent study, Wang et al. [36] proposed that the strain amplification factor positively correlates with the loading frequency and loading strain.

3.1.2. *Shear induced by fluid-flow*

Loading the bone first pressurizes the interstitial fluid around the osteocytes before flow is initiated [32]. A study developed by Gardinier et al. [37] predicted that *in vivo* osteocytes could experience hydrostatic pressures of up to 5 MPa. The interstitial fluid within the lacuno-canalicular (LC) is then driven to flow through the thin layer of non-mineralized pericellular matrix surrounding the osteocytes and towards the Harversian or Volkmann's channels [22,32]. In this sense, bone can be compared to a water-soaked sponge. A compressive force on the sponge will squeeze water out of it. Similarly, mechanical loading will result in a flow of interstitial fluid through the LC network of bone (see Fig. 1) [38]. The flow of interstitial fluid through the LC net- work places shear stress on the cell membranes. This stress is thought to initiate a biochemical response from the cells [39].

Piekarski [40] was the first researcher to propose that mechanical loading induces fluid-flow in bone. He stated that this flow enabled nutrition and waste removal.

The effect of the three-dimensional LC network complex geometry of bone on the fluid flow shear stress stimuli mechanism and its role in osteocyte mechanobiology are not yet fully understood. However, a recent study developed by Verbruggen et al. [41] showed that individual osteocytes may be subjected to a maximum shear stress stimulus of approximately 11 Pa and an average fluid velocity of 60.5 μm/s in response to vigorous activity. Mechanosensing bone cells also seem to be able to sense low fluid-flow stress values, as demonstrated by Morris et al. [42], Delaine-Smith et al. [43], Young et al. [44].

Several studies have also evaluated the responsiveness of bone cells to different flow profiles. Of these studies, we would like to highlight the important work developed by Jacobs' group [45–47], in which they studied oscillating, pulsatile and steady fluid flow. These studies showed that pre-osteoblast cell lines recognized both steady unidirectional and oscillating fluid flow as an osteogenic stimulus, but the latter was considered to be more realistic in physiologic terms than steady or pulsatile flow. This condition occurs because induced flow through the LC network is reversed when the bone is unloaded [46,47]. Case et al. [46] also claimed that the flow duration and inclusion of rest periods may influence flow effects.

3.1.3. *Streaming potentials*

In the mid-1960s, researchers observed that mechanical strains generated electrical potential differences along the lateral and longitudinal axes of compact bone. These differences may exert direct effects on bone cells because the *in vivo* application of an electromagnetic field to bone is known to inhibit bone resorption and stimulate bone formation. Of all of the mechanisms proposed to explain the strain-generated

potentials, two were selected for analysis: streaming potentials and piezoelectricity [9,10,48].

Streaming potentials are electric fields caused by stress-generated fluid-flow. Initially, streaming potentials were thought to be generated by electrokinetic effects that are associated with the collagen- apatite porosity system of connected micropores. Currently, pores are considered to be the canaliculi in mineralized bone. The bone surface is negatively charged; thus, the interstitial fluid cations that are being forced through channels are attracted to the surface, producing a surplus of ions in the fluid. The voltage that results from this imbalance of ions is positive in the direction of flow. The streaming potential produced by interstitial fluid-flow in bone is believed to be able to produce a number of responses in osteocytes, including the activation of voltage-operated channels in the cellular membrane. Thus, this streaming potential serves to trigger the mechanotransduction process [32,49–51].

3.1.4. *Piezoelectricity*

Yasuda [52] was the first researcher to observe piezoelectric behaviour in bone tissue. Subsequently, Fukada et al. [53] systematically investigated and measured the direct and converse piezoelectric effect in dry specimens cut from human and ox femurs. The centro- symmetric crystal structure of hydroxyapatite excludes the possibility of observing these piezoelectric properties, as demonstrated in de-collagenated bone [54]. Minary-Jolandan et al. [55] found that isolated collagen fibrils have unipolar axial polarizations and behave mainly as shear piezoelectric materials with a shear piezoelectric constant of $d15 \approx 1$ pm V^{-1} (or pC N^{-1}).

In a recent study, Ahn et al. [10] explored the possible influence of bone piezoelectricity on streaming potentials. The piezoelectricity of collagen may influence the magnitude of the zeta potential and thus the streaming potential. Therefore, it may indirectly modify the stiffness and fluid dynamics of bone. The load may also create a local fixed charge density that may modify the steady state fluid content of the bone and consequently the amount available for transfer from the collagen-hydroxyapatite microporosity to the LC system. This influence in turn affects the fluid-flow around the osteocytes. In a complementary multiscale approach study developed by Lemaire et al. utilized a coupled poro-elastic model of cortical tissue to deter- mine that in vivo electric measurements at the organ scale are due to streaming currents.

Although the precise stimuli bone cells experience in vivo are not yet fully understood, a number of theoretical and experimental studies over the past decade have uncovered strong evidence favouring direct cell strain and interstitial fluid-flow as the most likely stimuli for mechanosensation, instead of streaming potentials or hydrostatic pressure. These studies further support that shear stress induced by fluid-flow is the predominant stimuli recognized by osteocytes, as opposed to direct mechanical strain by substrate stretching [32,33,37,39,57,58].

3.2. *Mechanism of stimuli perception*

Osteocytes may sense loads via several mechanisms. Both cell body and dendritic processes have been proven to perceive mechanical forces. Several *in vitro* studies [33,59–61] have attempted to de- cipher the part of the cell – body or dendritic process – that is more sensitive to mechanical forces. Although it remains a controversial subject, the prevalent, widely accepted, hypothesis proposes that the osteocyte cell process is responsible for mechanosensation [62].

Several excellent papers on the mechanisms for the initial detection and conversion of a mechanical force into a biochemical signal, such as [21,32,63] and references within these papers, have been written, and the reader is referred to these studies for many of the details. Integrins, cation non-selective channels and the bone cell primary cilium are proposed to be involved in the osteocyte perception process of the mechanical signal.

One critical transduction pathway consists of strengthening ligand-integrin-cytoskeleton linkages in response to a force. Integrins are a superfamily of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands, and soluble ligands. The transfer of forces across cell adhesions allows focused stresses applied on the surface membrane to affect distant sites, such as the mitochondria and nucleus [22,56,64,65].

Ion channels, which are located in bone cell membranes, form strain-sensitive systems that respond to several stimuli, such as ligand binding, voltage changes, stretching and fluid shearing, via cellular ion fluxes. Several of these channels have been detected in osteocyte cultures: a Gd^{3+} sensitive non-selective cation channel; the volume sensitive epithelial-like Na^{+} channel ENaC; secondary driven Ca^{2+} channels, such as the voltage-dependent L-type channels or Na^{+}/Ca^{2+} exchange channels; and Cl^{-} and K^{+} channels. Integrins and stretch-activated channels also seem to be linked. Hence, cell stretching may lead to an increase in lateral membrane tension, which activates mechanically gated ion channels, *i.e.,* stretch-activated channels. Stretch-activated cation channels are also thought to be responsible for mechanotransduction in osteoblasts. [64,66–68].

Primary cilia are structures that project from the cell surface and deflect under fluid-flow. Currently, research points to the follow- ing flow-induced cilia response: increase in *cox-2* gene expression, prostaglandin E2 release (which is an important chemical mediator in the mechanotransduction process, as outlined in the following section) and an increase in the OPG/RANKL ratio. The cilia-based osteocyte response is independent of intracellular calcium. Many interesting aspects of the role of primary cilia in bone mechanotransduction remain to be studied [30,69,70].

Although osteocyte sensing mechanisms have been individually presented, researchers strongly believe that these mechanisms are highly associated; therefore, there is no single transduction pathway [9].

3.3. *Osteocytes biochemical stimuli-induced responses*

The *in vivo* and *in vitro* osteocyte responses to load include the production of several biochemically relevant messengers [57,71,72], such as Ca^{2+}, nitric oxide and prostaglandin E2.

One of earliest osteocyte responses to mechanical stimulation is calcium (Ca^{2+}) exchange between the extracellular and intracellular medium. This response propagates to the neighbouring cells, suggesting that the cellular network communicates and synchronizes via this mechanism. This response initiates a number of essential down- stream signalling pathways, *e.g.* ATP, nitric oxide and prostaglandin E2 (PGE2) release [9,60,73,74].

Nitric oxide (NO) and PGE2 are considered potent anabolic regulators of bone growth. Studies have shown [75,76] that the inhibition of only one of the two rapidly released small molecules at the time of mechanical stimulation suppresses the osteogenic response to mechanical stimulation.

NO is responsible for stimulating bone formation by inhibiting osteoclast formation and inducing osteoblasts differentiation. This anabolic regulator is also responsible for maintaining the viability of osteocytes and enhancing PGE2 [77]. Klein-Nulend et al. [78] examined the effect of pulsating fluid flow (0.5 ± 0.02 Pa, 5 Hz and 0.4 Pa/s) stimulation on chicken calvarial osteocytes. NO showed a maximum effect after 5 min, decreasing afterwards. PGE2 effect was significant after 10 min, which was maintained throughout 60 min.

PGE2 is an important signalling molecule because it not only stimulates osteogenic function in existing osteoblasts but also increases the production of osteoblasts by recruiting and promoting the differentiation of precursor cells. Another molecule that has similar effects to PGE2 on the signalling of the osteogenic process is the insulin-like growth factor I [9,32,78–82].

During bone remodelling, a cutting cone of osteoclasts, followed by a reversal or transition zone of osteoblast precursors and a closing cone of osteoblasts, constitute the basic multicellular units (BMU), which move in tandem. In modelling, bone resorption driven by osteoclasts and osteoblast-mediated bone formation actuate independently on different surfaces of the skeleton as bone is reshaping to adapt to different loading conditions [29,71,83–85].

*4.*Bone strain *in vivo*

The quantification of human normal bone strain is an important step in understanding the response of bone to mechanical stimuli [86]. One of the first contributions to this subject was made by Hert et al. [87], Hert et al. [88]. By applying loads to rabbit tibiae diaphysis using transcutaneous pins and Bowden cables, they showed that dynamic, but not static strains, increase bone formation. Today, the response of bone cells to mechanical stimuli is well accepted to be modulated by the parameters of the applied strain, namely, the magnitude, rate and duration of the applied load [9,55,89].

Although Hert et al. [88] could determine the magnitude of the applied load in their experiment, they could not determine either the physiological strains in that region or the strains that the loading engendered [86]. The *in vivo* measurement of strain in bone tissue surface was not possible until the development of strain gauges, the gold-standard for measuring bone strain [90–92]. The use of strain gauges on the bone surface was reportedly introduced as early as the mid-twentieth century [86,93].

Following Roux's footprints, Frost [94] developed an important concept: the "mechanostat" for bone adaptation to strain. This theory proposes a mechanical usage window and introduces the concept of minimal effective strain (MES). Frost compiled his various works that concern mechanical stimuli for bone regeneration in a 2003 paper [95], which describes the "mechanostat" as follows (see Fig. 2): he claimed a threshold for disuse-mode remodelling (MESr), 50–100 με, below which bone is removed and weakened. He defined the modelling region (MESm) between 1000 and 1500 με, where mechanically controlled modelling begins and could increase if strains exceed this upper limit. Frost believed that strain stimuli between MESr and MESm could define the region of naturally acceptable whole-bone strength relative to the typical peak voluntary mechanical load on a loadbearing bone during typical physical activities and the span of a normal "bone-strength/bone-load" ratio. According to this theory, the microdamage threshold (MESp) is approximately 3000 με and loads that can fracture a healthy young adult bone cause strains centred near 25,000 με (Fx).

Even though several researchers [23,96–98] support Frost's MESm concept of an increase in bone mass and remodelling when a mechanical load surpasses a threshold, criticism has also been raised. Some researchers [7,86] claim that Frost's theory is a qualitative theoretical construction of several hypotheses and that the precise threshold limits that control bone remodelling remain unknown. A dataset based on several studies was created to evaluate the ability of various types of vigorous physical activities to "stimulate bone formation" according to the MES concept (see Table 1). In all selected studies, the test subjects were healthy, young adult or middle- aged humans of both genders, and the peak strains were obtained by directly attaching strain gauges to the tibia bone surface.

An analysis of Table 1 indicates that of the vigorous activities considered, bicycle riding at 60 cycles/s produced the lower peak strain values both in compression and tension sites, with magnitudes on the order of 291 and 271 με, respectively. Normal walking activity, depending on the type of floor or grade (levelled, uphill or downhill gait), can lead to compression bone strain values ranging from 308 to 950 με. Table 1 also shows that, when considering the same type of physical activity and muscular contraction, different sites on the tibia present different local deformations.

Running can lead to compressive bone strains from 879 με during jogging to 2104 με during 17 km/h sprints. The highest strain was registered during a forward jump and vertical drop, which led to compressive deformations of up to 3450 με. In addition to the maxi- mum strain values mentioned, *in vivo* measurements of human tibiae strains do not seem to exceed 2000 με, even during vigorous running activity.

The data presented in Table 1 suggest that the peak strain com- pressive values tend to be higher than the tension values, with the exception of walking and running downhill.

Both the strain magnitude and strain rate are considered essential parameters in the stimuli process. In Table 1, the strain rates were again lower during bicycle riding at 60 Hz, with both compressive and tensile values of approximately 5 Hz. Higher strain rates were registered during sprint running, with values of up to 26 and 31 Hz in compression and tension, respectively. These data are in the physiological strain rate range claimed in other studies [9,55,89,99,100], between 1 and 60 Hz.

The data in Table 1 should be considered an approximation of real values because the effect of muscle fatigue on the bone strains and strain rates, as has often been identified *in vivo*, cannot be excluded in all studies. However, physical activities, such as sprinting or running in a zigzag, forward jumping and vertical dropping, may increase bone formation if we consider these data to be physiological values and 1000 με at 1 Hz cyclic mechanical loading is assumed as the MESm [101–103]. Importantly, only tibiae shaft measurements were considered, and conclusions based on these data should not be extrapolated to the spine and may or may not be valid for other bones in the body [98,104–106].

Normally a large portion of human daily routine includes events that are associated with far smaller strain magnitudes than vigorous physical activities, such as standing and sitting. According to Huang et al. [107], very low strains, those significantly smaller than 5 με, at high frequency strains are constantly bombarding the human skeleton. This finding was confirmed by Fritton et al. [90], who counted the daily (12–24 h) strain events and showed that large strains (exceeding 1000 με) seldom occur throughout the day and that very small strains (less than 10 με) occur thousands of times per day. Thus, the importance of small strains in bone adaptation and/or bone maintenance needs to be assessed.

Rubin et al. [108] asserted that the strain magnitude and strain rate of bone are related. In this study, they noticed that cortical bone mass could be maintained via the application of a 800 με peak- induced load at a frequency of 3 Hz for 600 s per day. Furthermore, only 200 με was necessary to maintain cortical bone mass for the same loading regimen if the strain was applied at 30 Hz. In a study by Weinbaum et al. [99], 250 με at 15 Hz produced a fluid shear stress that was 3.75 times that of a 1000 με at 1 Hz stimulus and exceeded the threshold for excitation. Moreover, Rubin et al. [108] showed that the combination of an even lower strain amplitude than the ones tested by [99], i.e., less than 10 με, with a high-frequency physiological strain rate between 10 and 100 Hz could stimulate bone growth by doubling its formation rate. These findings led to the development of several studies based on the potential use of such stimuli [109–111].

These findings indicate that low-amplitude high-frequency postural strains due to muscular contractions could be as or even more effective in maintaining bone mass than high-amplitude low-frequency strains due to locomotion. This behaviour may explain why astronauts lose bone mass in a microgravity environment, where the need to maintain posture is absent, despite rigorous exercise, or why 3 h/day of quiet standing has been shown to prevent bone loss in bed rest patients [90,112,113].

5. Test system for mechanical stimulation study

Selecting the most suitable test model for a stimulation study of bone mechanical behaviour is very important. Several test systems are available and are grouped in four categories: *in vivo*, *in vitro*, *ex vivo* and *in silico*.

5.1. *In vivo*

For *in vivo* testing, although no species fulfils the requirements of an ideal animal model, the dog is perhaps described as having the most similar bone structure to humans. However, using companion animals for medical research is associated with ethical implications. Other species, such as sheep and pigs, have also been suggested. Some researchers have also shown a preference for using rats and mice as experimental animal models [114–118]. According to the literature, rabbits may be the least similar to humans in terms of bone structure and properties. When possible, other bone study test models should be used prior to *in vivo* experimentation because the latter is expensive, leads to animal sacrifice, presents a large degree of systemic complexity and often is not considered satisfactory for the investigation of the mechanism that underlies cellular processes in bone [119–122].

5.2. *In vitro and ex vivo*

In vitro bone research includes three main branches: cell culture, for which cells are mechanically or enzymatically harvested from tis- sue and proliferate in a suspension or attached to a surface or monolayer; tissue culture, for which tissue fragments are maintained with- out not necessarily preserving architecture; and organ culture, for which organs (in whole or in part) or tissues are maintained or grown *in vitro* [123]. Several methodologies to provide mechanical stimuli for experimental study are reviewed by Ehrlich et al. [86], Brown [124], Brown [125].

Since Glucksmann [126] first used *in vitro* bone cell cultures to study the effects of mechanical stimuli in bone formation, this system has been considered to be an acceptable and common approach. This test system was also adapted to meet the purposes of more specific research, such as the study of cell responsiveness to fluid flow, first via the development of the parallel plate flow chamber by Frangos et al. [127], as well as in a number of other studies [47,128] that developed similar experimental devices. The advantage of using cell culture systems is that the local environment can be tightly controlled, such as ensuring the absence of growth hormones. A great number of molecular and biochemical tools are also available to ensure that experimental replicates are almost identical, which simplifies statistical analysis and quantification [119,123,129].

The most common bone cell cultures used for *in vitro* mechanical stimulation experiments are osteoblastic (e.g., MC3T3-E1), osteocytic (e.g., MLO-Y4) and less often,

osteoclastic-like cell cultures. Due to their availability and ease of use, osteoblast cell lines are used in many studies to infer osteocytes behaviour. While osteocytes are the descendants of osteoblasts and similarities would be expected for cells of the same lineage, these cells have distinct differences, particularly in their responses to mechanical loading [9,32,33,130].

For studies of the osteocyte physiological response to mechanical loading, two-dimensional cell culture conditions are arguably far too simple and hardly representative of the complex bone environment. This approach overlooks many parameters known to be essential for accurately reproducing the mechanotransduction process. Three- dimensional cell-growth environments have been developed in an at- tempt to overcome some cell culture disadvantages, mimic the physiological complexity of real tissue, and avoid the use of bone organ cultures [121]. Several materials have been tested as scaffolds, such as collagen glycosaminoglycan [131,132] and porous chitosan [133]. Un- der static culture conditions, cell proliferation in scaffolds is limited by diffusion, due to increases in the cell mass and decreases in the effective porosity resulting from matrix deposition. Fluid transport and cell distribution, as well as cell stimulation and differentiation may be improved via the use of bioreactors for three-dimensional scaffold systems [134,135]. Flow perfusion bioreactors are more commonly used than any other bioreactor for three-dimensional bone stimulation studies. These bioreactor systems pump culture medium through the scaffold's interconnected pores, which are held in place across the continuously circulating flow. These devices enable the close monitoring and precise control of several environmental conditions, such as the temperature, pH, oxygen and nutrient supply inside the scaffold and removal of waste products and metabolites. They also provide a high degree of reproducibility and automation, which favours the development and maturation of bone cells in scaffolds [134,136,137].

An understanding of the behaviour and responses of cells cultured on scaffolds should guide the scaffold optimization process. The pore size, pore interconnectivity and total porosity are essential parameters for scaffold development. The pore size affects cell migration into the scaffold and influences the amount of fluid that reaches the cells. It also determines the mechanical load to which the cells are exposed, i.e., the wall shear stress and the hydrostatic wall pressure that acts on the cells [138–143].

In vivo, osteocytes are attached to their mineralized matrix either via tethering filaments or perhaps via integrin-based focal adhesions. When these cells are seeded on a stiff two-dimensional surface, they are not surrounded by a pericellular matrix. Thus, they will spread out and form only integrin-based attachments with the substrate. In three-dimensional cultures, the pores dimensions of the scaffold will influence the initial cell attachment levels and the morphology of attachment, such as a flat morphology (akin to a two-dimensional monolayer culture), which occurs for large pores, and a bridging morphology. The pore size affects the cell's ability to span the void space [32,144,145].

Cell attachment can influence the dynamics of surface-cell-flow relationships and thus impact the magnitude of cytoskeletal deformations. According to Klein-Nulend et al. [32], round non-adherent osteocytes are more sensitive to mechanical stimuli than adherent ones. In a study by Jungreuthmayer et al. [143], three-dimensional

culture conditions required lower fluid flow rates to obtain bone formation than two-dimensional culture conditions, i.e., the increased cell deformability leads to increased cellular shear stress sensitivity. One advantage of two-dimensional systems over three-dimensional cell-scaffolds systems is that cellular loss due to fluid shear stress investigations is not a major concern in two-dimensional systems. In these systems, the levels of shear stress required to induce cellular detachment are orders of magnitude greater than those expected to cause osteogenesis *in vivo*. In three-dimensional cell-scaffolds systems, if a cell adapts a bridging morphology type, it will experience greater levels of cytoskeletal deformation than a flat cell when subjected to the same flow conditions [146]. Furthermore, according to Klein-Nulend et al. [32], the flow-induced stimulus for two- dimensional cell cultures is the same on the cell process and cell body in nearly all experiments.

Although tremendous advances have been made in the development of three-dimensional scaffold-based systems for bone cells studies that could reproduce both the formation of an organized bone-like matrix and cell-mediated substrate degradation, this methodology is also associated with limitations depending on the aim of a study [121,136].

To study bone's physiological strain profile *in vitro*, the bone's natural microenvironment must be mimicked. The osteocyte mechanosensation response depends on the type of material to which they are attached. Hence, scaffolds that mimic the properties of native bone should be used. However, the osteocyte mechanical conditions *in vivo* are not clearly understood, which precludes the re- liable recreation of these conditions in an *in vitro* experiment. Variables such as the complex lacuna-canalicular geometry and the distribution of the cell's focal adhesions play an important role in the osteocyte mecanotransduction process [60,147,148].

The critical requirement for *in vitro* models that represent the physiological diversity and complexity of the bone formation process have led to the development of different test systems, such as bone organ cultures or *ex vivo* bone culture systems.

Organ culture has been used for more than 50 years in an at- tempt to bridge the gap between cell culture and *in vivo* models [123,149,150]. The pioneering work of Fell et al. [151] in this field is known worldwide.

Organ culture provides a model that is, in certain aspects, more similar to the *in vivo* situation. This testing system respects the bone's natural three-dimensional structure and retains the extracellular matrix, which allows normal cell-matrix attachment sites to be maintained. The conservation of the tissue architectural organization will most likely simulate the physiological distinct mechanical con- sequence of loading, such as strain, fluid shear stress, and streaming potentials. Another great advantage of using organ culture technology is that they are not as complex as the whole animal and local effects, such as mediators and mechanical stress, can be isolated from systemic ones, such as steroids, other hormones and toxins [89,120,149,152].

The main disadvantages of using organ cultures are (1) the loss of a vascular system, which limits the organ sample size that can be harvested; (2) explants cannot be used for experiments longer than 24 h; and (3) the artificial environment created requires that the investigator exercises caution in the interpretation of the results and their

extrapolation to an *in vivo* situation [86,89,120,123,149,150].

In recent years, the evolution of bioreactor devices for the application of specific mechanical stimuli and controlled medium recirculation has favoured cell viability and prolonged culture time of bone explants [121]. A system that shows a successful applicability of these strategy was developed by Davies et al. [153]. In this *ex vivo* system, a mechanical loading and measurement system are combined with a cancellous bone diffusion culture-loading chamber named ZetOS^{TM}. This bioreactor maintains bone biopsy cultures for extended periods of time whilst preserving the natural three-dimensional architecture and inter-cellular interactions of several cell types.

53. *In silico*

The use of conceptual and mathematical models has already proven valuable in bone research [154]. Advances in scientific knowledge, mathematical modelling, and computer technology has facilitated the integration of numerical equations into finite element models [155]. A variety of computational approaches [156–161] have been developed in an attempt to understand the mechanotransduction process and response to mechanical stimulus in bone, such as interstitial fluid flow [41,162] and direct cell strain [36].

A good example of the integration of *in silico* analysis with *in vivo* and *in vitro* studies is the work developed by Yang et al. [163]. The authors studied the strain field in mice tibiae using micro CT-based finite element analysis together with diaphyseal strain gauge measurements during *in vivo* dynamic compression loading. Furthermore, as suggested by Webster et al. [164], future computational approaches should focus on merging models from different scales into a fully integrated multiscale modelling approach, which would enhance both the predictive and descriptive ability of computationally models and consequently facilitate the generation of new hypotheses and new experimental studies.

6.Conclusion

The scientific community has long recognized the importance of mechanical loading conditions in defining the mass and structure of bone. Bending moment stimuli are experienced by and transmitted along long bones via a combination of its physiological curvature, gravitational load (body weight) and the load applied by balanced muscle activities. Hence, muscle and bone are coupled as a functional unit. During mechanotransduction, osteocytes play the role of sensory cells within the bone, and their response is most likely mediated by strain-derived fluid flow shear stress through the lacuno-canalicular network. Osteocytes will respond to this mechanical stimuli by opening stretch-activated ion channels and increasing the levels of intracellular Ca^{2+} and protein Kinase C, which consequently stimulate the release of potent anabolic regulators of bone growth, such as NO and PGE2.

The mechanical stimulus strain magnitude and rate are important parameters. Low frequency, high magnitude strains occur during vigorous activities, such as running and jumping, and exert a recognized positive effect on bone formation/remodelling. Conversely, the relevance of high frequency, low magnitude strains associated with events such as standing or sitting as bone mechanical stimuli is now starting to be taken into consideration.

Isolated cell lines in mechanical stimulation studies are important to investigate the behaviour of individual bone cell populations during mechanotransduction. For studies of the bone mechanical and biological environments *in vivo*, two-dimensional osteoblast- or osteocyte-like cell cultures and the use of cells scaffolds do not fully mimic the real bone environment, which is an organ system with complex and distinct cell interactions. Thus, defining the type of study and the variables that need be included in the system is important to select the proper test model.

With this paper, we attempt to clarify important notions related to the adaptation behaviour of bone to external load stimuli. Although mechanical forces clearly affect the bone's behaviour, this relationship remains poorly understood. Continued studies of both the mechanical (e.g., bone muscle interactions) and biological fields (e.g., mechanisms and pathways underlying bone remodelling and mechanotransduction) are necessary. Moreover, biomechanical cross-talk should not be neglected. New research tools, such as advanced algorithms and techniques to assess the mechanical environment of bone *in vivo*, should allow integrated approaches to capture the complex dynamics of bone biomechanical behaviour. A better understanding of bone's response process to mechanical stimuli should provide new insights into diseases, such as osteoporosis, hyperparathyroidism, hyperthyroidism, Paget's disease and osteopetrosis.

Conflicts of interest

None declared.

Ethical approval

Not required for this study.

Acknowledgements

We acknowledge support from the Foundation for Science and Technology (FCT), Lisbon, Portugal, through Project PEst- C/CTM/LA0025/2013 and research grant SFRH/BD/87089/2012 (NR).

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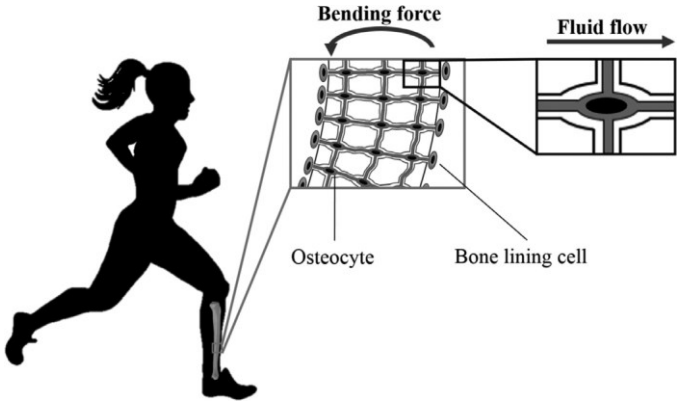

Fig. 1. Schematic representation of mechanical loading that causes interstitial fluid flow through bone's lacuna-canalicular network (adapted from Duncan et al. [9]). The tension/compression stresses associated with bending cause a pressure gradient that promotes fluid flow along the osteocytes.

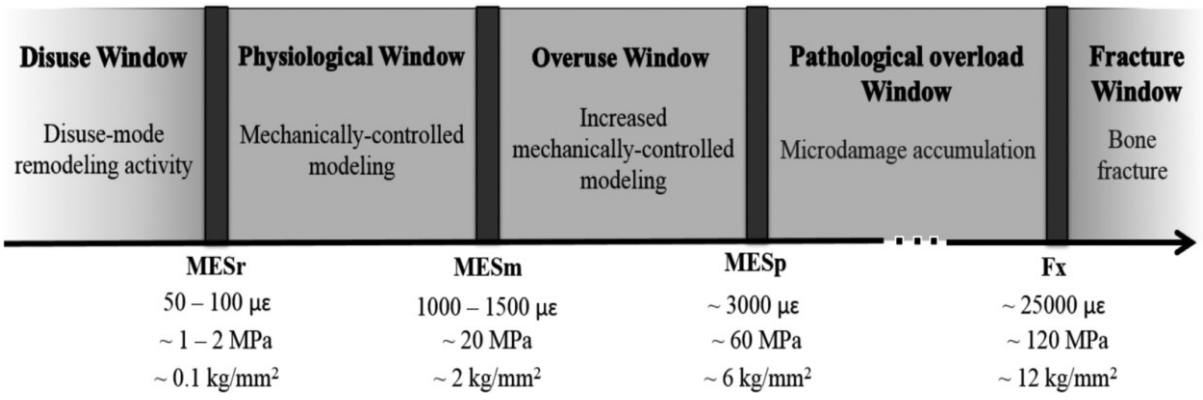

Fig. 2. Mechanical usage window defined by Frost's "mechanostat" theory of bone adaptation to strain (adapted from Duncan et al. [9] and Frost [95]). The

horizontal arrow at the bottom shows the typical minimum effective strain (MES) levels and the set point values for bone's thresholds and ultimate strength - microstrain (με), stress (MPa) and unit-load (kg/mm^2).

Table 1

In vivo humans bone strain values measured during different types of physical activities, reported in the literature

Activity	Type	Strain (με)		Strain rate (με/sec)		Region[a]	Test[b]	References
		Compressive	Tensile	Compressive	Tensile			
Walking	Level	308	–	2300	–	(A)	(a)	[92]
		544	437	7183	11006	–		[96]
		334	–	–	–	(B)	(b)	[97]
		950	–	–	–	(C)		
		454	840	3306	3955	(D)	(c)	[106]
	Uphill	630	440	8176	7113	–	(a)	[96]
	Downhill	414	531	10846	9566	–		
Walking (≥ 20 kg)	Level	557	381	6437	11434	–		
	Uphill	680	509	10645	8989	–		
	Downhill	751	601	17892	9839	–		
Walking (21–45 kg)	Level	393	–	2500–2700	–	(A)		[92]
Walking (≤ 46 kg)	Level	412	–	–	4200			
Running	Jogging	879	625	27376	13913	–		[96]
	Sprint (13.88 km/h)	1321	646	34457	20237	–		
	Sprint (17 km/h)	2104	1415	14543	7780	(D)	(c)	[98]
	Uphill	954	633	23834	17475	–	(a)	[96]
	Downhill	517	707	26337	16710	–		
Zigzag running	Uphill	1226	743	20930	13638	–		
	Downhill	1147	707	30039	15653	–		
Bicycle	60 cycles/s	291	271	1510	1286	(D)	–	[106]
Forward jump	30 cm	1600	–	–	–	(B)	(b)	[97]
		3450	–	–	–	(C)		
Vertical drop	Height of 26 cm	1905	896	13178	7621	(D)	(c)	[98]
	Height of 39 cm	1990	921	11342	5021			
	Height of 45 cm	2128	–	–	–	(B)	(b)	[97]
		436	–	–	–	(C)		
	Height of 52 cm	2098	1007	8663	4796	(D)	(c)	[98]

[a] The anatomical regions considered in these articles were: (A) Anteromedial aspect of the tibial midshaft, (B) Anterior middiaphysis of the distal tibia, (C) Posteromedial part of the distal tibia and (D) Medial aspect of the mid-diaphysis of the tibia.

[b] The subjects used in the studies were all active healthy adult: (a) male. (b) female and (c) males and female group tests.