Why does intermittent hydrostatic pressure enhance the mineralization process in fetal cartilage?

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Abstract

The purpose of this study was to determine which factor is the most likely one to have stimulated the mineralization process in the in vitro experiments of Klein-Nulend et al. (Arth. Rheum., 29, 1002–1009, 1986), in which fetal cartilaginous metatarsals were externally loaded with an intermittent hydrostatic pressure, by compressing the gas phase above the culture medium. Analytical calculations excluded the possibility that the tissue was stimulated by changes in dissolved gas concentration, pH or temperature of the culture medium through compression of the gas phase. The organ culture experiments were also mechanically analyzed using a poroelastic finite element (FE) model of a partly mineralized metatarsal with compressible solid and fluid constituents. The results showed that distortional strains occurred in the region where mineralization proceeded. The value of this strain was, however, very sensitive to the value of the intrinsic compressibility modulus of the solid matrix ($K_s$). For realistic values of $K_s$ the distortional strain was probably too small (about 2\textmu strain) to have stimulated the mineralization. If the distortional strain was not the factor to have enhanced the mineralization process, then the only candidate variable left is the hydrostatic pressure itself. We hypothesize that the pressure may have created the physical environment enhancing the mineralization process. When hydrostatic pressure is applied, the balance of the chemical potential of water across cell membranes may be disturbed, and restored again by diffusion of ions until equilibrium is reached again. The diffusion of ions may have contributed to the mineralization process. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The process of endochondral ossification in the fetal skeleton commences in the diaphysis where the primary ossification center is formed by hypertrophied chondrocytes. In a short period of time, a calcium phosphate is deposited in the matrix around these hypertrophied cells. Hence, the chondrocytes hypertrophe before the matrix mineralizes. The processes of hypertrophy and mineralization progress towards the distal ends of the fetal bone, where the epiphyses are formed. Finally, the mineral is resorbed by osteoclasts and replaced by bone tissue. In vivo, the mineralization process in fetal mouse metatarsals starts at 16 days of gestational age. It was observed that this process coincides with the first muscle contractions of the feet (Burger et al., 1991). The deposition of calcium phosphate might, therefore, be stimulated by mechanical loads.

To study the influence of mechanical loads on the mineralization process, Klein-Nulend et al. (1986) performed in vitro culture experiments on 16-day-old fetal mouse metatarsals. The organs were loaded for a period of five days with a cyclic hydrostatic pressure (13 kPa, 0.3 Hz). The pressure was applied through compression of the gas phase (5\% CO\textsubscript{2} in air) above the culture medium (Klein-Nulend et al., 1986). The results showed that loaded metatarsals had a mineralized diaphyseal part which was two to three times longer than that of the unloaded controls (Klein-Nulend et al., 1986). Hence, it was concluded that the mineralization process in fetal cartilage is stimulated by mechanical load. However, it is
It is known that the physical conditions of the culture medium may be important for the mineralization process of the fetal metatarsal. For instance, whether the metatarsal mineralizes or not depends, amongst others, on the pH and the temperature of the medium. It has already been shown that chondrocytes in vitro can alter their activity by changes in their biophysical environment, such as osmotic pressure (Urban, 1994; Urban and Hall, 1994), fluid flow (Kim et al., 1994, 1995), hydrostatic pressure (Urban, 1994; Urban and Hall, 1994; Lammi et al., 1994; Parkkinen et al., 1994, 1995), electrical potential gradients (Kim et al., 1995; Frank and Grodzinsky, 1987), and pH (Gray et al., 1988). As the hydrostatic pressure in the in vitro culture experiments was applied through a gas phase, it was suggested that it is possible that the mineralization process was stimulated by changes in dissolved gas concentration or pH, rather than by the pressure itself (Urban, 1994).

Wong and Carter (1990) performed a finite element (FE) stress analysis of the organ culture experiments of Klein-Nulend et al. (1986). They suggested, based on their results, that the mineralization process might have been stimulated by shear stresses at the cartilage/mineralized cartilage interface, as local effects of the external hydrostatic pressure. In this FE analysis, however, they considered the tissue as a linear elastic material. In fact, cartilage consists of a solid phase, mainly collagen and proteoglycans, and a fluid phase of interstitial water. Biphasic tissues like this are known to display strong non-linear, time dependent deformational behavior when loaded (Mow et al., 1980; Spilker et al., 1988). Hence, it is questionable whether the stress patterns determined in a linear-elastic FE analysis are realistic. In addition, not only deformation, but also pressure gradients and interstitial fluid flow play roles that might affect the mineralization process.

The following questions were addressed in this study: (1) Is it likely that the mineralization process in the in vitro organ culture experiments of Klein-Nulend et al. (1986) was stimulated by one of the physical changes in the culture medium, e.g. dissolved gas concentration, pH, or temperature? (2) Is it likely that the mineralization process was stimulated by one of the mechanical factors in the tissue, e.g. internal strain, stress, fluid pressure and fluid flow, as local effects of the external hydrostatic pressure? To answer these questions, we analyzed the physical changes of the culture medium under the experimental loading conditions. In addition, a poroelastic FE analysis was performed to determine the distribution of mechanical variables at the mineralization front. Information about the mineralization process may be important for the prevention and treatment of musculoskeletal developmental deformities.

2. Methods

2.1. Physical analysis of the culture medium

As the hydrostatic pressure in the organ culture experiments was applied through the gas phase (Fig. 1), the volume in the culture system decreased during compression, leading to an increase in partial CO$_2$ and O$_2$ pressures, which resulted in their increased absorptions in the

![Fig. 1. Schematic drawing of the intermittent hydrostatic compression (IHC) apparatus. IHC was generated by intermittently compressing the gas phase (one second of loading, followed by two seconds of unloading) of a closed culture chamber with 98% humidity, which contained the culture dishes and was placed in a 37°C incubator. The maximum pressure was 13 kPa. (A) 37°C incubator; (B) culture chamber; (C) culture dishes containing organ cultures and fluid culture medium; (D) H$_2$O; (E) tap inlet; (F) tap outlet; (G) gas phase of 5% CO$_2$ in air; (H) piston-rod; (I) membrane; (J) compressed air cylinder; (K) inlet; (L) pressure supply; (M) electronic valve; (N) pulse generator; (O) pressure transducer.](image-url)
culture medium. The compression period is 1 second, which is followed by two seconds of relaxation in which the volume in the culture system returns to normal again. When the gas is compressed from pressure $p_1$ to $p_2$, the partial CO$_2$ and O$_2$ pressures will increase with a factor $p_2/p_1$, as will the quantities of CO$_2$ and O$_2$ absorbed in the liquid. In the experiment, the total pressure changed between 100 and 113 kPa. Since the medium was buffered by bicarbonates, a change in the CO$_2$ concentration led to a change in the pH of the medium due to a shift in the chemical equilibrium

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+. \quad (1)$$

To calculate the change in pH during compression, the following equation was solved:

$$\text{pH} = -\log [\text{H}^+] = -\log \left( K_a \frac{[\text{CO}_2]}{[\text{HCO}_3^-]} \right), \quad (2)$$

where $K_a$ is the equilibrium constant which can be calculated under control conditions with the equation

$$K_a = \frac{[\text{HCO}_3^-][\text{H}^+]}{[\text{CO}_2]}, \quad (3)$$

where the concentration of HCO$_3^-$ is known from the added amount of NaHCO$_3$ in the medium as 2.2 g/l (i.e. [HCO$_3^-$] = 0.0262 mol/l), and the concentration H$^+$ is known from the pH value of 7.4 (i.e. [H$^+$] = 10$^{-7.4}$). The concentration of CO$_2$ is unknown and can be calculated from Henry’s law (Perry, 1950) with the assumption that the quantity of CO$_2$ absorbed in the medium is similar to that in water, such that

$$p = Hx, \quad (4)$$

in which $p$ is the partial CO$_2$ pressure, $H$ is the constant of Henry’s law, and $x$ is the mole fraction of the gas solved in the total liquid. The concentration of O$_2$ is calculated in the same way. The partial pressures $p$ under control conditions are 5.07 \times 10^3 \text{ Pa (}= 5\%) \text{ for CO}_2 \text{ and } 2.13 \times 10^4 \text{ Pa (}= 21\%) \text{ for O}_2, \text{ whereas the values of H at 37}^\circ \text{C are calculated from the values at 35 and 40}^\circ \text{C, assuming a linear scale between 35 and 40}^\circ \text{C, giving } 2.22 \times 10^8 \text{ Pa for CO}_2 \text{ and } 5.25 \times 10^9 \text{ Pa for O}_2 \text{ (Perry, 1950). The mole fraction (x) is calculated from Eq. (4). The quantity of CO}_2 \text{ (m}_{\text{CO}_2}/44) \text{ or O}_2 \text{ (m}_{\text{O}_2}/32) \text{ in moles, dissolved in a defined mass of water (m}_{\text{H}_2\text{O}} \text{ of 37}^\circ \text{C can now be calculated under control conditions and during compression from}

$$x = \frac{m_{\text{CO}_2}}{44} + \frac{m_{\text{H}_2\text{O}}}{18} \quad \text{or} \quad x = \frac{m_{\text{O}_2}}{32} + \frac{m_{\text{H}_2\text{O}}}{18}. \quad (5)$$

However, the calculated quantities of CO$_2$ and O$_2$ will certainly not be reached within 1 s of compression, as the diffusion process of these molecules in the liquid proceeds very slowly. Hence, the changes calculated in dissolved gas concentrations and the pH are only valid for that part of the culture medium in which the gas is diffused. The mean distance traveled by the gas particles (⟨x⟩) during compression should, therefore, be calculated by (Atkins, 1990)

$$\langle x \rangle = \frac{\sqrt{2kT}}{3\pi \eta a} \sqrt{\tau} \quad (6)$$

in which $k$ is the Boltzmann constant (1.3807 \times 10^{-23} \text{ J/K}), $T$ the temperature of the gas (K), $\eta$ the viscosity of the solvent (for culture medium: $\eta = 1.027 \times 10^{-3} \text{ Pa s}$, experimentally determined by C.M. Semeins, ACTA, Amsterdam), $a$ the radius of the gas particles (for CO$_2$: 1.615 \times 10^{-10} \text{ m}; for O$_2$: 1.460 \times 10^{-10} \text{ m}) \text{ (Weast, 1970), and } \tau \text{ the time (s). The gas temperature } T \text{ varies during the dynamic experiment. Since the compression of the gas phase is an adiabatic process, e.g. there is no heat flow in or out of the system, the laws for adiabatic processes must be applied in order to calculate the increase in gas temperature during compression, hence (Griffiths and Thomas, 1962; Marion, 1979)}$

$$pV^{1/\gamma} = \text{constant}. \quad (7)$$

Using the universal gas law (Marion, 1979) this results in

$$T^1/p^{1-\gamma} = \text{constant} \quad (8)$$

in which $p$ is the pressure (Pa), $V$ is the volume (m$^3$), $\gamma$ is the specific heat ratio, and $T$ is the temperature (K). For air the value of $\gamma$ is about 1.4 (Washburn, 1928). During the adiabatic compression of gas from pressure $p_1$ to $p_2$, the temperature raises from $T_1$ to $T_2$ ($\Delta T = T_2 - T_1$). With the use of Eq. (8) temperature $T_2$ can be calculated as

$$T_2 = T_1 \left( \frac{p_1}{p_2} \right)^{1-\gamma}. \quad (9)$$

Adiabatic expansion of the gas from $p_2$ to $p_1$, i.e. during the relaxation period, causes an equal decrease in temperature. As the mean temperature of the system is 37°C (i.e. 310 K) the temperature will fluctuate between 37°C - $\frac{1}{2} \Delta T$ and 37°C + $\frac{1}{2} \Delta T$. Finally, the mean diffusion distance of the gasses for the most unfavorable situation, which is at a gas temperature of 37°C + $\frac{1}{2} \Delta T$, can be calculated. The consequences of this variation must be analyzed, in the perspective that the height of the culture medium in the culture well is 1700 μm and that the thickness of a fetal metatarsal is about 300–400 μm.

2.2. Finite element analysis

To simulate the in vitro culture experiments of Klein-Nulend et al. (1986), a FE model of a fetal mouse metatarsal was developed using a poroelastic description of
the tissue. An axisymmetric model was used which consisted of 175 eight-noded elements with quadratically interpolated displacements and pressures (Fig. 2). Because the project was aimed at the process of enhanced mineralization through the loading experiment, the model contained mineralized cartilage in the central part and unmineralized cartilage in the diaphysial part of the metatarsal (Fig. 2). The surface of the metatarsal was assumed to be permeable to fluid. The model was loaded with a cyclic hydrostatic pressure of 13 kPa at 0.3 Hz. This load was applied to the boundary elements of the FE mesh.

Table 1 presents the material properties of the fetal cartilage which were implemented in the FE model. The tissue properties of fetal cartilage are unknown but are probably close to the properties of growth plate cartilage, as the process of development (i.e. cell proliferation, cell hypertrophy, and mineralization around hypertrophied chondrocytes) is similar. The Young’s modulus (E) and permeability (k) for the unmineralized cartilage were obtained from average values of literature data on hypertrophied cartilage (Bachrach, 1995; Cohen et al., 1992), and for the mineralized cartilage from the results of this study.

<table>
<thead>
<tr>
<th>Material property</th>
<th>Unmineralized cartilage</th>
<th>Mineralized cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>E (MPa)</td>
<td>0.44</td>
<td>117</td>
</tr>
<tr>
<td>k (m4/N.s)</td>
<td>$6.7 \times 10^{-15}$</td>
<td>$6.7 \times 10^{-16}$</td>
</tr>
<tr>
<td>v</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>n</td>
<td>0.31</td>
<td>0.25</td>
</tr>
<tr>
<td>$K_s$ (MPa)</td>
<td>2200</td>
<td>2200</td>
</tr>
<tr>
<td>$K_e$ (MPa)</td>
<td>1-10000</td>
<td>10000</td>
</tr>
</tbody>
</table>
whereas the average Poisson’s ratio (ν) of articular cartilage was used (Mow et al., 1991). For the porosity value (n), or fluid volume fraction, only the free movable water of the tissue was considered. We assumed that the water in the chondrocytes is fixed and moves with the solid matrix. Hence the porosity was calculated by quantifying the fraction of matrix in the fetal tissue (0.54, using image analysis) (data not shown), times the fraction of water in the matrix (0.75) (Mow et al., 1991, 1984), times the fraction of free water in the matrix (0.77) (Torzilli, 1988). This resulted in a porosity estimate of 0.31. In mineralized cartilage, the matrix contains calcium phosphate which increases its Young’s modulus (117 MPa) (Huiskes et al., 1993), while reducing permeability and porosity. The permeability and porosity values were estimated, as no proper literature data was available. The Poisson’s ratio of bone was used (Table 1). The value of Kₜ is known to be 2200 MPa (Tamura et al., 1993) (Table 1). However, little is known about the values of Kₛ, the unmineralized cartilage was varied between 1 MPa (highly compressible) and 10000 MPa (virtually incompressible), and that of the mineralized cartilage was held constant at an average value of cortical bone of 10000 MPa (S. Tepic, personal communication, 1996).

As in the organ culture experiments (Klein-Nulend et al., 1986) the dynamic hydrostatic pressure was applied to the FE model. The distributions of shear stresses, shear strains, fluid velocity and fluid pressure were determined for the whole range of Kₛ values, in order to analyze which of the factors is the most likely one to have stimulated the mineralization process in the in vitro experiments.

3. Results

3.1. Physical analysis of the culture medium

During the dynamic hydrostatic pressure experiments, the physical changes in dissolved gas concentration, pH, and temperature in the culture medium were small and only present in the top layer of the culture medium; hence not at the bottom where the metatarsals were lying (Table 2). One second compression of the gas phase from p₁ = 100 kPa to p₂ = 113 kPa increased the concentration of O₂ and CO₂ in the culture medium from 0.225 to 0.254 mmol/l, and from 1.271 to 1.436 mmol/l, respectively (Table 2). In addition, the pH value was decreased from 7.400 to 7.347 (Table 2). The temperature of the gas phase was increased with 5.5 K, which led to mean diffusion distances in the culture medium of 44.3 and 42.1 μm for O₂ and CO₂, respectively (Table 2). The temperature variation in the culture medium was negligible compared to the temperature variation in the gas phase because of the poor heat transmission from gas to liquid (data not shown). During the 2 s of relaxation, i.e. from p₂ = 113 kPa to p₁ = 100 kPa, the dissolved gas concentration and pH return to their original values.

3.2. Finite element analysis

For each intrinsic compressibility modulus of the solid phase (Kₛ) of the unmineralized cartilage, and for each point in time during the loading cycle, the gradient of fluid pressure in the tissue was small, resulting in relatively small fluid velocities. The fluid pressure in the unmineralized cartilage during loading was between 0.001 and 0.980 kPa higher than the applied hydrostatic pressure of 13 kPa, if Kₛ of the unmineralized cartilage was lower than Kₜ, i.e. lower than 2200 MPa. This was caused by the solid phase, which volume was decreased more under the applied hydrostatic pressure than the volume of water. As a consequence, the pressure of the fluid was increased. As fluid always flows from a high pressure towards a lower one, the fluid flowed predominantly outwards (Fig. 3a), with maximum velocities between 4.8 × 10⁻⁸ and 9.0 × 10⁻⁵ mm/s, for Kₛ values between 1000 and 1 MPa, respectively. When the load was released, the fluid flow reversed its direction (Fig. 3b). In the case that Kₛ of the unmineralized cartilage was higher than Kₜ, the pressure in the tissue was about 0.0005 kPa lower than the applied pressure during loading. As a consequence, the fluid flowed inwards with a maximum velocity of about −4.0 × 10⁻⁸ mm/s (Fig. 3a). When the load was released the direction of the fluid flow was again reversed (Fig. 3b).

The pattern of deformation in the unmineralized cartilage was about the same for every Kₛ value, but its quantitative significance depended on the Kₛ value (Figs. 4, 5). Due to the different material properties of mineralized and unmineralized regions, distortional

<table>
<thead>
<tr>
<th>Gas pressure (kPa)</th>
<th>T (K)</th>
<th>[O₂] (mol/l)</th>
<th>[CO₂] (mol/l)</th>
<th>pH</th>
<th>⟨x⟩₁₄ O₂</th>
<th>⟨x⟩₁₄ CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>310.2</td>
<td>0.225 × 10⁻³</td>
<td>1.27 × 10⁻³</td>
<td>7.400</td>
<td>43.92</td>
<td>41.76</td>
</tr>
<tr>
<td>113</td>
<td>315.7</td>
<td>0.254 × 10⁻³</td>
<td>1.44 × 10⁻³</td>
<td>7.347</td>
<td>44.31</td>
<td>42.13</td>
</tr>
</tbody>
</table>
Fig. 3. Schematic presentation of the fluid flow direction during loading (a) and unloading (b). (a) When the $K_s$ value of unmineralized cartilage was lower than $K_f$, then the fluid mainly flowed outwards. Fluid was also transported through the mineralization region. In case that $K_s$ was higher than $K_f$, the pressure in the unmineralized tissue was lower than the applied pressure. As a consequence, the fluid flowed inwards, (b) When the load was released the direction of the fluid flow was reversed.

strains (or octahedral shear strains) occurred in the region of hypertrophied cells (Fig. 4), whereas peak shear stress values were prominent in the already mineralized area (data not shown). At the end of the loading phase, the maximum distortional strain in the hypertrophied area was between 0.4 and 4707.0 $\mu$strain for $K_s$ values between 10000 and 1 MPa, respectively (Fig. 5). When the load is released, this value was reduced, but not entirely to zero, because of the time inertia effect (Fig. 4). Thus, the quantitative value of the distortional strain changed with the modulus $K_s$ (Fig. 5). When the $K_s$ value increased, the total change in volume in the unmineralized cartilage decreased, resulting in decreased shear strain values.

4. Discussion

In the present study the organ culture experiments of Klein-Nulend et al. (1986) were analyzed to determine which factor is the most likely one to have stimulated the mineralization process. It has to be appreciated that the hydrostatic pressure of 13 kPa applied to the fetal metatarsals is much less than what is exerted on adult articular cartilage (Afoke, 1987). The value of 13 kPa is estimated to be the stress exerted on the cartilage in vivo as a result of the first muscle contractions (Klein-Nulend et al., 1986). Although muscle contractions cause other deformation patterns than pure hydrostatic load does, the experimentally applied pressure may be of the same order of magnitude as the resulting pressures in the tissue due to muscle forces.

We showed that the changes in dissolved gas concentration, pH and temperature in the culture medium were relatively small. Although Lo et al. (1994) showed that small changes in CO$_2$ concentration may affect cell behavior, we calculated that these changes were only
present in the superficial layer (42 µm) of the culture medium. The 1 s compression period from 100 to 113 kPa was far too short for CO₂ to reach (and affect) the tissue. Hence, when the height of the culture medium column (1700 µm) and the thickness of the fetal metatarsal (300–400 µm) lying on the bottom of that column are considered, it seems unlikely that the mineralization process was stimulated by fluctuations in dissolved gas concentration, pH or temperature during the experiment. Therefore, we assume that the tissue was stimulated by the intermittent hydrostatic pressure itself. An additional point in favour of this argument is that the calculated change in pH during the experiments would work against mineralization. It should be noted that the effect of serum protein, amino acids and other medium components are ignored in the calculation of changes in the pH of the culture medium; the medium was treated as a simple mineralization. It should be noted that the effect of serum proteins, amino acids and other medium components are ignored in the calculation of changes in the pH of the culture medium; the medium was treated as a simple bicarbonate buffer. However, the effect of these components is probably negligible as they are likely to minimize pH changes resulting from fluctuation in partial CO₂ pressure.

The first mechanical variable considered in the FE analysis was the fluid flow. It was shown that the influence of the fluid flow on the mineralization process was probably negligible as the fluid pressure gradients in the tissue were very small.

Wong and Carter (1990) simulated the same dynamic culture experiments with a linear elastic FE model. They suggested that a combination of shear stress and hydrostatic pressure, the so-called osteogenic index, stimulated the mineralization process. We could not confirm this hypothesis in the present study. This is not surprising, since the poroelastic tissue properties differ considerably from linear elastic ones. In our poroelastic analysis, the intrinsic compressibility of the solid matrix showed to have a major impact on the resulting distributions of the mechanical variables. The distortional strain was prominent in the hypertrophied region, which is precisely the region where the mineralization process proceeds. For this reason, the distortional strain seemed to be a factor likely to have stimulated the mineralization process. The value of the strain, however, is very sensitive to the compressibility K₄. This implies that the value of K₄ must be known, before the probability of distortional strain as a mechanical stimulus can be assessed. If the solid phase is assumed to be intrinsically incompressible, i.e. K₄ is infinite, then the pressure inside the tissue is exactly the same as the hydrostatic pressure externally applied. Hence, the pressure varies at every point in the tissue between 0 and 13 kPa, depending on the time in the loading cycle. As a consequence, the fluid velocity is zero and no deformation occurs.

Little is known about the intrinsic compressibility modulus of fetal cartilage. Ultrasound measurements showed that adult articular cartilage has a compressibility modulus of 3400 MPa (Tepic et al., 1983). As this value mostly depends on the collagen content, it is likely that the value for fetal tissue is between the value of adult articular cartilage (3400 MPa) and that of water (2200 MPa). In that case, the maximum distortional strain in the loading cycle is reduced to about 2 µstrain (Fig. 5). To the knowledge of the authors it has never been shown that 2 µstrain stimulates cell expressions significantly. However, Wright et al. (1996) showed that chondrocytes might react with cell hyperpolarization to strain values of about 10–15 µstrain. These values are, however, much larger than those found in the FE analysis, for realistic K₄ values as mentioned above. It is therefore not likely that the distortional strain enhanced the mineralization process in the culture experiments.

As the material property values might be critical for the validity of the computational model, additional analyses were performed (data not shown) in which extreme values of the other material properties were implemented to determine their significance for the results and conclusion. For the unmineralized cartilage, the permeability was varied between 2 × 10⁻¹⁵ and 2 × 10⁻¹⁴ m²/Ns, a porosity value of 0.80 was used (which would include all the water in the tissue), the Young’s modulus was varied between 0.34 and 1.0 MPa, and the Poisson’s ratio was varied between 0.10 and 0.4. For the mineralized cartilage, the permeability was varied between 2 × 10⁻¹⁶ and 2 × 10⁻¹⁵ m²/Ns, a porosity value of 0.65 was used, a Young’s modulus of 250 MPa was used, and a K₄ value of 5000 MPa was used. It was found that these variations had only minor effects on the results, compared to variation of K₄ for the unmineralized cartilage (data not shown). Hence, the central conclusion remains unchanged if material properties are varied in the above-mentioned range.

If the distortional strain was not the factor to have stimulated the mineralization process in the experiment, then the only candidate variable that is left is the hydrostatic pressure itself. Other studies have already shown that hydrostatic pressure may change cell activity (Urban, 1994; Urban and Hall, 1994; Lammi et al., 1994; Parkkinen et al., 1994, 1995; Hall, 1997; Klein-Nulend et al., 1995; Takano-Yamamoto et al., 1991). The pressure might create the physical environment which promotes the mineralization process. A possible explanation for such a mechanism could be the effect of the chemical potential of water (Gu et al., 1993, 1997). This notion is based on the triphasic theory (Lai et al., 1991), according to which the chemical potential of extracellular water has to be equilibrated by the chemical potential of the intracellular water. If the equilibrium across the cell membranes is disturbed, the chemical potential is the driving force to restore the balance. Chemical potential is a function of, among others, the fluid pressure and the concentration of ions (Lai et al., 1991). This means that if the pressure in the extracellular matrix changes, then the chemical potential of the water in the extracellular matrix
changes as well. In this way it is possible that the chemical potential of the water in the extracellular matrix is not in equilibrium with the chemical potential of water in the cells, as there is a membrane in between. This disturbance can be restored by changing the ion concentration across the cell membrane. Hence, when the cyclic hydrostatic pressure is applied to the tissue in the organ culture experiments, the balance across the cell membranes may possibly be disturbed, and restored again by diffusion of ions until equilibrium is reached again. As a consequence, the intracellular and extracellular concentrations of various ions may change, which may have an effect on intracellular signal transduction, eventually leading to changes in the speed of mineralization during the dynamic loading experiment. Without the externally applied pressure, the mineralization process in vitro still takes place, although the production of mineral is less than in the loaded case. It can be hypothesized that chondrocytes, during the process of cellular hypertrophy, start to exert pressure on the extracellular matrix (Pauwels, 1980). This cell-derived pressure may, in an as yet unknown manner, help to create the natural physical environment to initiate the mineralization process. In that case the externally applied hydrostatic pressure of the culture experiments may have enhanced the mineralization process.

In conclusion, it is not likely that the distortional strain enhanced the mineralization process in the cyclic hydrostatic pressure experiments. The only other candidate is the hydrostatic pressure itself which may have created the right physical environment, enhancing the mineralization process through diffusion of ions. However, further studies are required to test this possibility in more detail.

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