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CARTILAGE SELF-HEATING CONTRIBUTES TO CHONDROGENIC EXPRESSION

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Abstract

Articular cartilage is a soft tissue showing inelastic properties. Under cyclic loading, inelastic materials may dissipate mechanical energy into heat. In knee cartilage, due to an intrinsic reduced heat convection related to its avascularity, a local temperature increase could be observed. This phenomenon is referred to as self-heating. As cells are sensitive to temperature variation, the energy dissipation could influence their metabolism. The goal of this study was to evaluate the effect of cartilage self-heating on chondrogenic expression. In the first part of this study, using a custom-made deformation calorimeter, we quantified the heat generated in cartilage submitted to cyclic loading at different frequencies. We calculated the corresponding local increase of cartilage temperature. At the cellular level, we then assessed the effect of these temperature variations on chondro-progenitor cell metabolism by measuring the gene expression of transcription factors involved in chondrogenesis. An up-regulation in mRNA expression levels of Sox9 and its co-activator PGC-1α was observed with an increase of temperature. Taken together, the results of this study suggest a dissipation contribution to chondrogenic gene expression. Dissipation phenomena might then be considered as a new variable in mechanobiology.

Keywords: Cartilage; dissipation; deformation calorimetry; Sox9; mechanobiology.

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Introduction

Articular cartilage is a soft tissue showing inelastic properties (Hayes and Mockros, 1971). Under cyclic loading, inelastic materials dissipate mechanical energy through heat production (Ratner and Korobov, 1966). Given that articular knee cartilage is avascular and has a low coefficient of thermal conductivity (Bowman *et al.*, 1975), heat could accumulate in this tissue. A local temperature increase in the cartilage could then be obtained. The aspect of local temperature increase subsequent to a mechanical loading, also called self-heating, has not been studied for knee cartilage.

It has long been stated that mechanical stimulation on cartilage generates a number of signals within the tissue that act on cells present within (Mouw *et al.*, 2007; Mow *et al.*, 1999). Those latter integrate various signals arising from the extracellular matrix and modulate their biosynthetic activities, a mechanism called mechanobiology. As protein biosynthesis is thermosensitive (Bernstam, 1978), a temperature increase through a dissipation process could influence the gene expression in cartilage cells. Hence, the temperature of a healthy human knee cartilage, which is normally at 33 °C (Harris and McCroskery, 1974), would be raised during daily activities such as walking or running. In this case, the dissipation that occurs locally in cartilage and acts directly on the cells could be appreciated as a mechanobiology variable.

The transcription factor Sox9 (SRY-related high mobility group-box gene 9) is an early marker of chondrogenic expression (Zhao *et al.*, 1997). Sox9 expression is also expressed in non-chondrogenic tissues, suggesting the existence of molecular partners required for Sox9 to control chondrogenic gene expression (Asahara, 2011). Kawakami *et al*. (2005) reported the identification of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) as a co-activator for Sox9 during chondrogenesis. In parallel, PGC-1α expression has been shown to be temperature-dependent and to play a role in mitochondrial biogenesis and thermoregulation (Liu and Brooks, 2012; Puigserver *et al.*, 1998; Yamaguchi *et al.*, 2010; Yu and Auwerx, 2009). Sirt1 (a NAD-dependent histone deacetylase), associated with Sox9 and PGC-1α, was reported to enhance the transcription of Col2a1 (Dvir-Ginzberg *et al.*, 2008). SirT1 also plays a role in cell differentiation and thermogenesis (Lee *et al.*, 2011; Yu and Auwerx, 2009), as well as in osteoarthritis and regulation of heat shock proteins (Fujita *et al.*, 2011; Westerheide *et al.*, 2009). On the other hand, Twist1 (Basic helix-loop-helix transcription factor) inhibits the transactivator function of Sox9 (Gu *et al.*, 2012). The interplay between these transcription factors, co-activators

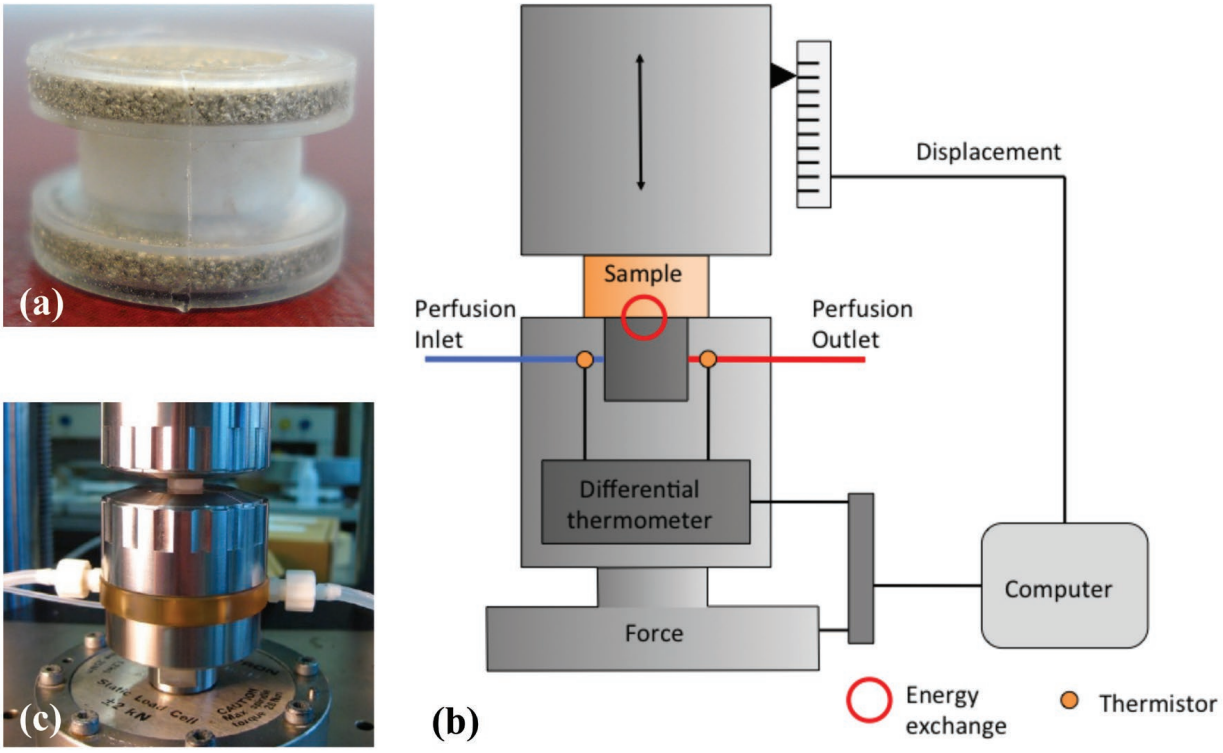

Fig. 1. (a) The encapsulation chamber made of a silicon membrane enclosing the cartilage sample between a steel filter and a non-porous rigid disc. **(b)** The principle of the calorimetric measurement is based on the differential temperature measurement of the inlet and outlet perfusion. The cartilage sample, placed in the encapsulation chamber, is clamped between two mechanical actuators mounted on a standard tension machine. **(c)** Picture of the deformation calorimeter.

and co-repressors regulates the expression of Col2a1, one of the main components of cartilage extracellular matrix. Therefore, the hypothesised temperature effect on chondrogenic expression would, by some means, affect either the expression or the activation of the above-mentioned factors.

This study aims firstly to measure the heat generated in knee cartilage due to dissipation and secondly to assess the corresponding effect of temperature increase on chondrogenic gene expression.

Materials and methods

Deformation calorimetry

Experiments on cartilage

Heat dissipation was measured with a custom-made deformation calorimeter developed in our laboratory (Vogel, 2011; Vogel and Pioletti, 2012). Briefly, the cartilage sample is placed in an encapsulation chamber (Fig. 1A). This chamber is composed of a medical grade 40 µm pore size sintered-steel filter, a non-porous rigid disc, and a 100 µm thin polydimethylsiloxane (Sylgard 184, Midland, MI, USA) elastomer deformable membrane. The chamber containing the cartilage sample is positioned in the centre of the calorimeter (Fig. 1B). A perfusion system goes through the chamber allowing heat transfer and providing isothermal conditions to the sample. The difference in temperature between the inlet and the outlet perfusion is measured by thermistors forming a Wheatstone bridge. The induced potential difference is correlated to the heat generated in the sample. Isothermal boundary conditions are imposed by the perfusion system, so that all the generated heat in the samples could be transferred to the thermistors. The calibration of the system was performed using a resistive heater placed instead of the sample and releasing heat in the range of 0.1 to 16 mW by Joule effect. For more details, the reader may refer to Vogel (2011). The calorimeter is placed in a universal testing machine allowing simultaneous cyclic loading and calorimetry measurements (Fig. 1C).

Cartilage samples were taken from young calf knees (less than 6 months old, known to have thicker cartilage than older animals and thus providing sufficient material) supplied by a local slaughterhouse. The samples were dissected immediately prior to testing. They were taken from the patellofemoral groove, where cartilage thickness was maximal. Samples were full-thickness cartilage excluding subchondral bone. The geometric parameters were as follows: cylinders of 4 mm height and 8 mm diameter. The superficial zone of the cartilage faced the porous platen and the deep zone faced the non-porous disc.

Mechanical testing was performed with an Electropuls Dynamic Test System E3000 (Instron, Norwood, MA, USA). A 5 % pre-strain was applied to ensure full contact with the sample. Sinusoidal loadings at frequencies of

0.1, 1, 2 and 5 Hz were applied on each sample with 15 % strain amplitude. The applied strain corresponds to the mean value of cartilage deformation during the stance phase of gait (Liu *et al.*, 2010). A *t*-test was used to examine the differences in heat dissipation at the four different frequencies for all the samples ($n = 5$).

Calculation of temperature increase in cartilage during walking

By experimentally quantifying the dissipation in cartilage during cyclic loading and assuming adiabatic conditions partially due to the tissue avascularity, the evolution of cartilage temperature (dT/dt) can be obtained as follows (Sonntag *et al.*, 2003):

$$mc\frac{dT}{dt} = P_v \qquad (1)$$

where P_v is the dissipation power normalised per unit volume, obtained from the deformation calorimetry experiment. T and t are the temperature and time respectively. m is the cartilage mass and c is its heat capacity.

Temperature effect on cells

Human epiphyseal chondro-progenitors cells (ECPs) culture

Human ECPs were isolated from the proximal ulnar epiphysis of a 14-week gestation donor (Centre Hospitalier Universitaire Vaudois Ethics Committee Protocol # 62/07), kindly supplied by Prof. Lee Applegate (UNIL, Lausanne, Switzerland). The isolation procedure was carried out according to our previous work (Darwiche *et al.*, 2012). Monolayer expansions were performed in standard tissue culture polystyrene flasks containing 10 mL Dulbecco's Modified Eagle Medium (DMEM) with 25 mM dextrose and 1 mM sodium pyruvate, 5.97 mM L-glutamine (Life Technologies Ltd, Paisley, UK), 10 % foetal bovine serum (Sigma, St. Louis, MO, USA). Human ECPs were seeded at 3300 cells/cm^2, then placed in standard humidified tissue culture incubators at 37 °C with 5 % CO_2 until reaching 90 % confluence.

Temperature stimulation of cells

After expansion, cells were seeded in 6-well plates at 20,000 cells/well with F12 medium complemented with 5.97 mM L-glutamine, 1 % ITS solution IV (PAN-Biotech GmbH, Aidenbach, Germany), 100 U/mL penicillin and 100 µg/mL streptomycin. After 24 h, half of the plates were placed in the incubator at 33 °C and the other half at 37 °C, both under a 5 % CO_2 environment. There were several phases to this *in vitro* investigation. The first phase was the expansion of the cells in order to obtain sufficient numbers. This is why cells were initially cultured at 37 °C, following their thawing, to obtain a consistent cell population (Darwiche *et al.*, 2012) as starting material. Once sufficient cells were obtained, the quantification of the temperature effect on cells commenced by acclimating them for 24 h at 33 °C. This represented the baseline for cell behaviour, as it has been shown that this is the temperature of knee cartilage at rest (Becher *et al.*, 2008). The return to a temperature of 37 °C corresponded to the situation where the cells are stimulated, as this temperature is similar to the temperature increase due to a mechanical loading.

Gene expression

Total RNA was extracted using NucleoSpin® RNA XS kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The RNA was quantified using a Nanodrop ND-1000 Spectrophotometer (Witec AG, Pfäffikon, Switzerland), and 500 ng of RNA was reverse transcribed into cDNA using Taqman® Reverse Transcription Reagents (Applied Biosystems, Life Technologies Ltd, Paisley, UK). Quantitative PCR was performed using the Fast SYBR® Green PCR Master Mix (Applied Biosystems) as well as primers synthesised by Microsynth (Balgach, Switzerland) for Sox9, Twist1, Sirt1, PGC-1α and the housekeeping gene GAPDH (Table 1). Efficiency of the genes was determined using cDNA dilutions over 4 orders of magnitude, and ranged from 90-113 %. 10 ng cDNA were added to each well of the reaction plate (MicroAmp Fast Optical 96-well, Applied Biosystems). The StepOnePlus Real-Time PCR system (Applied Biosystems) was used to perform the quantitative PCR. Gene expression data were processed following the comparative Ct method (Pfaffl, 2001), normalising

Table1: qRT-PCR primer sequences**.**

Gene name	Symbol	GenBank Accession No	Efficiency	Sequence
Sry-related HMG box 9	SOX9	NM_000346.3	100 %	F: 5'-TGGAAACTTCAGTGGCGCGGA-3' R: 5'-AGAGCAAAAGTGGGGGCGCTT-3'
Twist-related protein 1	TWIST1	NM_000474.3	91 %	F: 5'- AGCAGGGCCGGAGACCTAGATGTCA-3' R: 5'- ACGGGCCTGTCTCGCTTTCTCT-3'
Sirtuin 1	SIRT1	NM_001142498.1	90 %	F: 5'- CGGTTCCTACTGCGCGAGCTG-3' R: 5'-GAAGGTTATCTGGCTGCTGCGGAA-3'
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	PGC-1α	NM_013261.3	113 %	F: 5'-AGACACCGCACGCACCGAAAT-3' R: 5'- AGCTGTCATACCTGGGCCGACG-3'
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	NM_002046.3	105 %	F: 5'-TCTTTTGCGTCGCCAGCCGAG-3' R: 5'-TGACCAGGCGCCCAATACGAC-3'

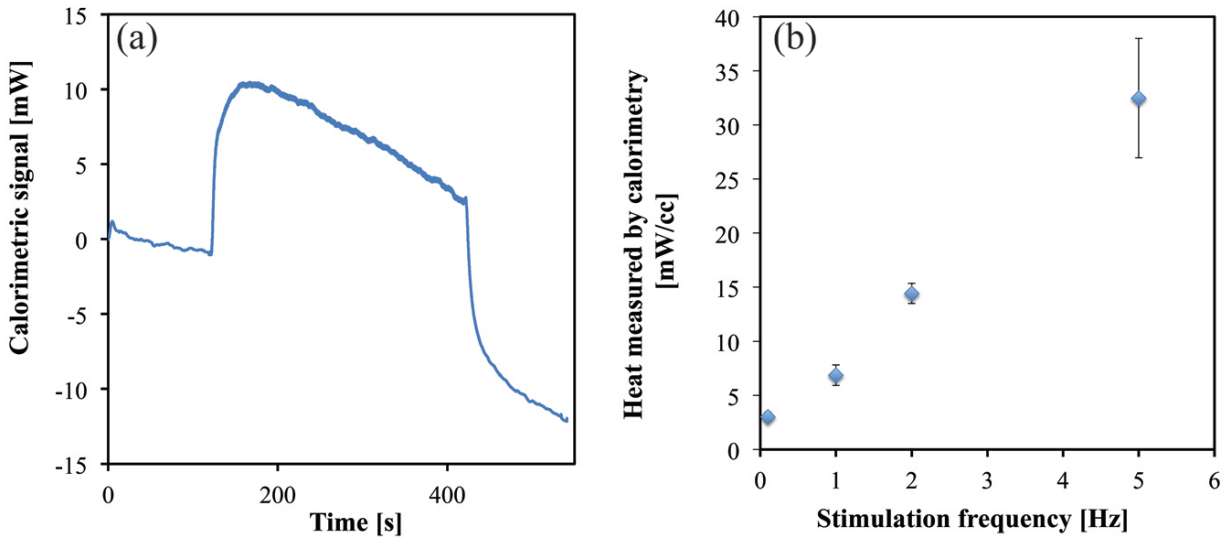

Fig. 2. (a) Calorimetric measurement for a cartilage specimen subjected to a cyclic loading at 1 Hz. **(b)** Heat dissipation of cartilage as function of stimulation frequency (cyclic loading at 0.1, 1, 2 and 5 Hz). The result is presented for each frequency, as the mean value of heat power measured on cartilage samples extracted from five different animals.

gene expression levels to that of the housekeeping gene and using, as a biological reference, the normalised gene expression levels of cells cultured at 33 °C. A *t*-test was used to examine the differences in RT-PCR results for all the experimental conditions ($n = 6$).

ATP quantification

In order to assess the effect of a single short-duration of temperature increase on cell metabolism, we quantified the ATP generated after a temperature increase for 1 h and 2 h, respectively. After expansion, cells were seeded in four 96-well plates at 20,000 cells/well and kept at 33 °C. F12 medium was used with 5.97 mM L-glutamine, 1 % ITS solution IV, 100 U/mL penicillin and 100 µg/mL streptomycin. After 24 h, 2 plates were placed in the incubator at 37 °C with 5 % CO_2 and the two other plates remained at 33 °C. After 1 and 2 h at an increased temperature, ATP was quantified for each group ($n = 12$) using the CellTiter –Glo luminescent assay according to manufacturer's instructions (Promega, Madison, WI, USA).

Results

Deformation calorimetry

The calorimetric signal during a mechanical stimulation sequence is shown in Fig. 2A. Initially, we did not apply any mechanical stimulation in order to determine the baseline of the calorimetric signal. Then, when subject to a cyclic loading, the sample generated heat that was translated in the signal as an increase relative to the baseline. This returned to its initial value at the end of the sequence, when the loading stopped. We quantified the heat power generated in the sample for all the stimulation frequencies, namely 0.1, 1, 2 and 5 Hz. As shown in Fig. 2B, the heat power generated by the specimens under loading was frequency-dependent ($p < 0.01$) and increased with frequency. However, the energy dissipated per cycle remained similar for all the tested frequencies. If we consider a walking gait frequency of 1 Hz, the corresponding measured heat power due to energy dissipation had a value of 7.4 mW. Using this value, equation 1 indicated that cartilage temperature would rise from 33 °C to 37 °C after 40 min of walking.

Temperature effect on cells

In order to assess the cumulative heat-effect due to daily activities on cell behaviour, we cultured human ECPs at 33 °C and 37 °C for both 48 h and 96 h. Indeed, as the self-heating phenomenon may occur several times per day, its effect on gene expression is most probably cumulative. The profiles for gene expressions (Sirt1, PGC-1α, Sox9, Twist1) after 48 h and 96 h are illustrated in Fig. 3. In general, an increase of temperature to 37 °C induced an up-regulation for Sox9 expression by 1.9- and 4.1-fold after 48 h and 96 h, respectively. The same trend is observed for PGC-1α by 2.3-fold up-regulation after 96 h. In parallel, to assess the effect of a short duration heat-shock on cell metabolism we quantified the ATP generated after a 1 h and 2 h heat-shock, respectively. The results showed that significant metabolic changes already occur after a 1 h heat-shock, and is enhanced after the 2 h heat-shock (Fig. 4).

Discussion

The dissipative properties of cartilage are usually characterised by a hysteresis stress-strain curve (Szarko *et al.*, 2010). Hysteresis may encompass a multiple of inelastic phenomena including intrinsic viscosity, poroelasticity, plasticity and latent energy of deformation (Adams and Farris, 1989). These phenomena may or may

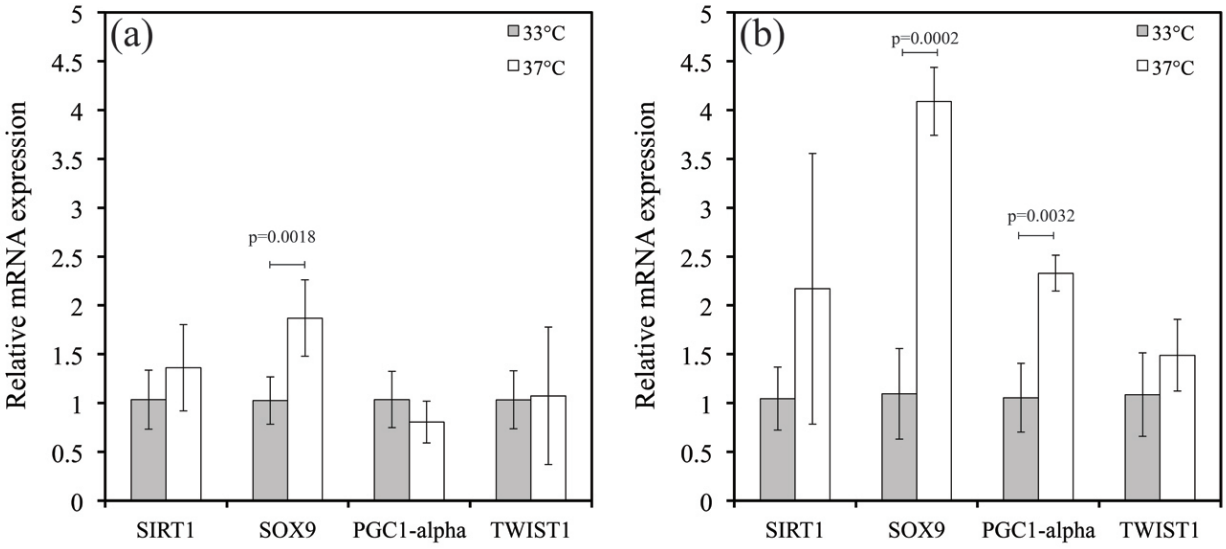

Fig. 3. Gene expression levels for Sirt1, Sox9, PGC1-alpha, and Twist1 **(a)** after 48 h and **(b)** after 96 h. The data are normalised to the housekeeping gene GAPDH (n = 6). Fold changes in mRNA levels are expressed to cells cultured at 33 °C, as a biological reference.

not generate heat. Hysteresis alone is thus not sufficient to assess if mechanical energy would be transformed into heat and assumptions on the nature of inelasticity must be drawn. Calorimetry overcomes this issue, as it can assess heat production of the cartilage directly and thereby furnishes a means to assert dissipation, in particular self-heating behaviour, as a relevant mechanobiological signal.

Evaluation of the self-heating behaviour of knee cartilage has not been reported in the literature. Our results demonstrated that this phenomenon is important and can induce a consequent temperature increase in the cartilage. We calculated that cartilage self-heating may be sufficient to raise the temperature in knee cartilage from 33 °C to 37 °C already after a 40 min walk. The predicted 4 °C temperature increase in 40 min is close to what can be found in the literature. Temperature has been measured in 6 healthy males, having a probe for intra-articular measurement inserted into the notch of the right knee. Median intra-articular temperatures increased by 4 °C and 5.8 °C after 30 and 45 min of jogging (Becher *et al.*, 2008). However, intra-articular temperature variations may be due to muscle thermogenesis and not to the cartilage self-heating. The intra-articular temperature may also not directly correspond to the cartilage temperature. The contribution of the dissipative properties of the cartilage to the temperature rise could then not be established. In another study, it has been shown that 50 min flexion-extension applied on cadaveric human hip joints, having probes in the acetabulum subchondral bone, increased the temperature up to 2.5 °C (Tepic, 1982). Here again, no distinction was possible to determine if the temperature increase was due to intrinsic cartilage dissipation or friction between the joint surfaces. Indeed, the author mentioned that the latter was the cause of the observed temperature increase. In our study, temperature increase was obtained from direct measurement of cartilage dissipation subject only to deformation. The dissipation is an intrinsic property

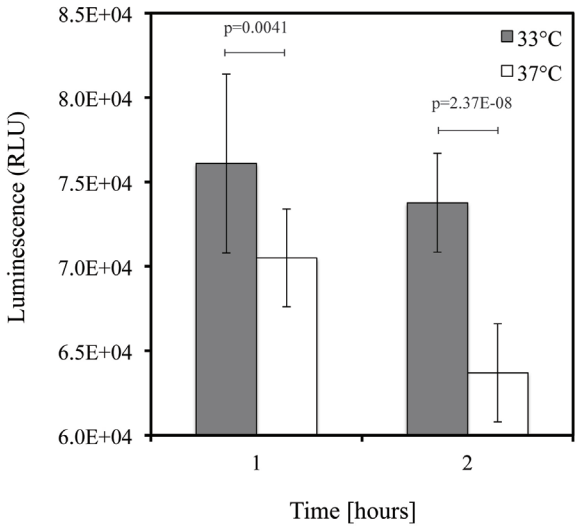

Fig. 4. Luminescence of cells after 1 h and 2 h at 37 °C compared to a baseline at 33 °C. Luminescence level is linearly correlated with the amount of ATP produced by cells.

of the cartilage making this quantity a relevant variable to evaluate its effect directly on cells.

It should be mentioned that the temperature calculation was obtained following the hypothesis that the cartilage was considered as an adiabatic system. While, in reality no system can be purely adiabatic, one can assume adiabaticity when the power of heat generation is higher than the power of heat transfer; in such a case heat accumulation occurs in the system, and temperature increase is observed. This effect of heat accumulation may be spectacular in biomechanics, as it has been shown that an increase in temperature of 6.5 °C can be generated during intense running in horse tendon (Wilson and Goodship, 1994). The avascular region of the tendon was also the site of the most marked temperature increase, suggesting that the key point to obtain an increase in temperature in a

tissue is an insufficient blood supply to transport the generated heat. The other phenomenon that may influence heat accumulation is the convection by synovial fluid flow. Nevertheless, only little fluid exudation occurs in the middle and deep layers of articular cartilage. Indeed, the ability of cartilage to support load through interstitial fluid pressurisation is dependent on its low hydraulic permeability, which is nothing more than the ease of fluid flow through a material. For articular cartilage, it is governed by the extracellular matrix and increases in the middle and deep zone with osteoarthritis as described by Hwang and colleagues (Hwang *et al*., 2008). Therefore, low heat transfer is expected *via* fluid movement for normal healthy cartilage, especially in the middle and deep zone, which sustains our hypothesis of adiabatic conditions (at least for middle and deep zone of cartilage). Equally, gradients of temperature may be present in the tissue. However as a first approach we were interested in global/bulk effects, and calorimetry is particularly useful for that purpose.

It is interesting to note that heat dissipated per cycle was similar for the different frequencies used in this study. With the limitation of the adiabatic hypothesis, it means that the number of loading cycles determines the temperature increase. For instance, while jogging, the frequency of cartilage stimulation would be higher resulting in a faster temperature increase compared to walking.

To determine the effect of temperature augmentation on chondrogenesis, human epiphyseal chondro-progenitors were cultured at 33 °C and 37 °C. RT-PCR analysis revealed an increase of Sox9 mRNA levels in cells at 37 °C after 48 h. The same trend was observed after 96 h confirming the effect of temperature on chondrogenic expression, as Sox9 is a key regulator of the Col2a1 gene, whose product is an early and abundant marker of chondrocyte differentiation (Zhao *et al.*, 1997). Equivalently, we assessed the effect of a short duration temperature increase on cell metabolism. Results have shown significant ATP level variation between 33 °C and 37 °C, presenting thus significant metabolic changes already within 1 h. The fact that ATP content at 37 °C is lower than at 33 °C might be due to lower rate of ATP supply, as it has been shown that cell differentiation may be associated with a reduction in ATP demand (Birket *et al.*, 2011). Alternatively, it might be explained by a higher ATP turnover, as ATP is pivotal in signalling pathways *via* protein phosphorylation, inter alia TGF-β signalling, which is required for downstream chondrogenic expression (Kwon, 2012).

In this study, we used chondro-progenitor cells to evaluate the effect of temperature on chondrocyte metabolism. We have chosen to use these cells mainly because they spontaneously keep their chondrocyte phenotype in culture over many passages. We have demonstrated that this cell source is very stable and gives reproducible behaviour related to chondrogenic phenotype viewpoint (Darwiche *et al.*, 2012), making then a cell source of choice for studying the proposed temperature effect in this study.

Unless cultured under hypoxic conditions, GAPDH is known to be a stable housekeeping gene for experiments on chondrocytes (Toegel *et al.*, 2007). However, to rule out possible temperature sensitivity of this housekeeping gene, we also normalised the data with a second housekeeping gene (B2M) and obtained the same results as with GAPDH (data not shown).

Increase in temperature of the knee cartilage, above its physiological temperature of 33 °C, can then influence cells chondrogenic expression. The evaluation of temperature effect on chondrocyte behaviour has already been studied. For instance, Yalcin *et al.* (2007) demonstrated the effect on chondrocyte differentiation of intermittent thermal alterations during long time culture. It has also been shown that chondrocyte proliferation and extracellular matrix volume strongly correlate with cartilage temperature in mice (Serrat *et al.*, 2008). The novelty of the present study is to link the temperature increase to a self-heating process, allowing then to indirectly relate a mechanical stimulation to a cell behaviour through the tissue dissipation properties. In particular for knee cartilage, its dissipation properties can cause this temperature increase. We may then conclude that self-heating following a mechanical deformation of the cartilage can be a mechanobiological variable influencing chondrogenesis.

Conclusion

The goal of this study was to evaluate the effect of dissipation on chondrogenic expression. First, we measured the self-heating generated by dissipation in cartilage subject to deformations. We calculated that this heat may be sufficient to raise the temperature in knee cartilage from 33 °C to 37 °C after 40 min of walking. On a cellular level, we showed that the elevation of the temperature by 4 °C increased the chondrogenic expression of human chondro-progenitors cells. Taken together, the results of this study suggest a dissipation contribution to chondrogenic gene expression, and show that dynamic loading of the cartilage can contribute to chondrogenic expression *via* self-heating.

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Discussion with Reviewers

Reviewer I: If the *in vivo* temperature of articular cartilage is indeed 33 °C, why do not all laboratories culture chondrocytes at this «physiological» temperature? I guess at this low temperature chondrocytes do not proliferate *in vitro*? Would one not expect that the optimal growing temperature would be at 33 °C for chondrocytes in general?
Authors: To our knowledge, there is no study comparing the *in vitro* proliferation of chondrocytes at 33 °C and 37 °C. Generally, cells are cultured at 37 °C because it is the human core body temperature, which is assumed to be the optimal temperature. The aim of our study was not to look at the proliferation of the cells, but on their gene expression. However, from what we could observe on our 4 d experiment, the cells cultured at 33 °C showed viability that was as good as the cells cultured at 37 °C. This can be easily verified experimentally with an *in vitro* proliferation study.

Reviewer II: The analysis was performed using young or immature cartilage or chondrocytes. Do the authors think that their findings extend to mature cartilage and chondrocytes?
Authors: We expect that the findings can be extended to mature cartilage, because this tissue still has viscoelastic properties implying dissipation/self-heating under dynamic loading. Likewise for mature chondrocytes, we expect the same results, as the human chondro-progenitor cells used keep their chondrocyte phenotype in culture over many passages, are very stable, and give reproducible behaviour related to the chondrogenic phenotype.

Reviewer II: Given that the cells are sensitive to temperature fluctuations, would there be any benefit, or would the authors expect different results, if chondro-progenitor cells underwent subculture or longer durations of maintenance at 33 °C prior to «heat shock» of 37 °C? Is the 24 h at 33 °C sufficient time for cells to equilibrate/acclimate at this temperature?
Authors: We expect that longer duration of maintenance at 37 °C or increasing the frequency or number of temperature-increase events would alter the results more than keeping the cells at 33 °C prior to the temperature increase. Usually, the expression of the genes we looked at are quite fast (Kawakami *et al*., 2005), and we assume that 24 h at 33 °C is sufficient to let cells acclimate at this temperature.

Reviewer II: The rigid, non-porous boundary condition imposed by the experimental system does not truly represent the physiologic cartilage-subchondral bone interface. What effects would this boundary condition be expected to have, from both mechanical and biological perspectives?
Authors: From a mechanical perspective, since the superficial zone of the cartilage faced the porous platen and the deep zone faced the non-porous disc, it may be expected that more fluid-flow would occur at the superficial zone. From a biological perspective, it has been shown that the shear stress generated by the fluid-flow may affect the molecular response in chondrocytes. Therefore, with this imposed boundary condition we might expect a zone-dependent response to the fluid-flow.

Additional Reference

Kawakami Y, Tsuda M, Takahashi S, Taniguchi N, Esteban CR, Zemmyo M, Furumatsu T, Lotz M, Belmonte JC, Asahara H (2005) Transcriptional coactivator PGC-1alpha regulates chondrogenesis *via* association with Sox9. Proc Natl Acad Sci USA **102**: 2414-2419.