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Osteoarthritis and Cartilage

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The rate of hypo-osmotic challenge influences regulatory volume decrease (RVD) and mechanical properties of articular chondrocytes

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S U M M A R Y

Objectives: Osteoarthritis (OA) is associated with a gradual reduction in the interstitial osmotic pressure within articular cartilage. The aim of this study was to compare the effects of sudden and gradual hypo-osmotic challenge on chondrocyte morphology and biomechanics.
Methods: Bovine articular chondrocytes were exposed to a reduction in extracellular osmolality from 327 to 153 mOsmol/kg applied either suddenly (<5 s) or gradually (over 180 min). Temporal changes in cell diameter and the existence of regulatory volume decrease (RVD) were quantified along with changes in cortical actin and chromatin condensation. The cellular viscoelastic mechanical properties were determined by micropipette aspiration.
Results: In response to a sudden hypo-osmotic stress, 66% of chondrocytes exhibited an increase in diameter followed by RVD, whilst 25% showed no RVD. By contrast, cells exposed to gradual hypo-osmotic stress exhibited reduced cell swelling without subsequent RVD. There was an increase in the equilibrium modulus for cells exposed to sudden hypo-osmotic stress. However, gradual hypo-osmotic challenge had no effect on cell mechanical properties. This cell stiffening response to sudden hypo-osmotic challenge was abolished when actin organization was disrupted with cytochalasin D or RVD inhibited with REV5901. Both sudden and gradual hypo-osmotic challenge reduced cortical F-actin distribution and caused chromatin decondensation.
Conclusions: Sudden hypo-osmotic challenge increases chondrocyte mechanics by activation of RVD and interaction with the actin cytoskeleton. Moreover, the rate of hypo-osmotic challenge is shown to have a profound effect on chondrocyte morphology and biomechanics. This important phenomenon needs to be considered when studying the response of chondrocytes to pathological hypo-osmotic stress.

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Introduction

The biomechanical properties of living cells provide valuable information on cellular structure and the dynamic response to mechanical stimuli. Numerous studies have quantified chondrocyte biomechanical properties using a variety of experimental and computational techniques including cytocompression[1], indentation[2], atomic force microscopy (AFM)[3], compression in hydrogels[4,5] and micropipette aspiration[4,6–8]. These studies provide insight into

changes in the mechanical properties of cells associated with their position in the tissue, mechanical loading and disease.

In articular cartilage, chondrocytes live in a complex and dynamic physicochemical environment with associated changes in osmolality. Negatively charged proteoglycans attract cations and thereby water into the extracellular matrix[9]. Consequently changes in the concentration of the proteoglycans causes the osmolality to vary from approximately 350 mOsm/kg in the surface zone to 450 mOsm/kg in the deep zone[9]. In addition, during compressive loading, water is exuded from the tissue, increasing the local proteoglycan concentration and therefore exposing chondrocytes to hyper-osmotic stress. Furthermore, an increase in hydration of cartilage and a reduction in osmolality is an indicator of early stage osteoarthritis (OA) due to degradation of the collagen network and tissue swelling[10]. Previous studies have shown that chondrocytes are

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sensitive to both hyper and hypo-osmotic challenge which influence cell morphology[11–15], metabolism[16–19] and biomechanics[20,21].

Chondrocytes have the capacity to regulate cell volume in response to osmotic challenge which is pivotal for a variety of cell functions[12–14]. When chondrocytes are exposed to a hypo-osmotic challenge, they swell passively due to the uptake of water such that cell volume increases in proportion to the magnitude of osmolality reduction. Within minutes, cell volume then reduces towards the initial value by extrusion of water and solutes until a new osmotic equilibrium with the extracellular environment is achieved. This adaptive mechanism is termed regulatory volume decrease (RVD). Similarly hyper-osmotic challenge causes chondrocyte shrinkage followed by regulatory volume increase (RVI). However this occurs only in cultured chondrocytes[14]. This study focuses on chondrocyte response to hypo-osmotic challenge and RVD which is currently poorly understood. Loss of chondrocyte RVD is associated with chondrocyte death and the progression of OA[22,23] Many transmembrane channels have been linked to volume regulation including Cl^- and K^+ channels[24,25], hemichannels[26] and especially the transient receptor potential vanilloid type 4 channel (TRPV4) which is essential for chondrocyte RVD[27–29]. The actin cytoskeleton regulates these channels[30,31] and is itself influenced by osmotic challenge[19–21]. Hypo-osmotic challenge influences chondrocyte stiffness, probably through changes in actin organisation[20]. However osmotic challenge effects chromatin condensation[32] which also influences cell stiffness[33].

In the majority of studies, hypo-osmotic challenge has been applied rapidly in contrast to the gradual pathological changes. Therefore this study examines the relative influence of sudden and gradual hypo-osmotic challenge. We investigate alterations in chondrocyte structure and morphology and the activation of RVD and the consequences for cell biomechanics. We show that the rate of hypo-osmotic challenge regulates chondrocyte morphology and the activation of RVD. Hypo-osmotic challenge also affects the cellular mechanical properties, but only when RVD is activated in response to a sudden hypo-osmotic challenge. These findings demonstrate that the rate of hypo-osmotic challenge affects cell morphology and biomechanics which have important implications for studies examining chondrocyte response to pathological osmotic challenge.

Methods

Chondrocyte isolation and osmotic challenge

Articular chondrocytes were isolated from full depth cartilage taken from bovine metacarpal-phalangeal joints by a process of sequential enzyme digestion with pronase and collagenase as previously described[4]. Due to pooling of cells from multiple individuals, no estimate of biological variability can be provided. The cartilage digest was filtered through a 70 μm pore size sieve and the resulting cell suspension was washed twice in fresh DMEM + 16% FCS. Cells were re-suspended in culture medium at a density of 0.5×10^6 cells/ml and used within 8 h post isolation. A freezing-point depression osmometer (3250, Advanced Instruments, Norwood, USA) was used to measure media osmolality. In all experiments, Dulbecco's minimal essential medium supplemented with 16% (v/v) fetal calf serum (DMEM + 16% FCS, Gibco, Parsley, UK) was used as an iso-osmotic medium (327 mOsm/kg). Sterile distilled water was used to achieve a hypo-osmotic media (153 mOsm/kg) as in previous studies[20]. To apply sudden hypo-osmotic challenge, a rapid decrease in osmolality was induced by adding distilled water to cell suspension and mixing for 5 s prior to pipetting onto the microscope stage. Although this approach adding distilled water is widely used, it also influences ion concentration. An alternative is

to replace the media with a hypotonic media but this is not suitable for cells in suspension without centrifuging the cells. For gradual hypo-osmotic challenge, two syringe pumps were used to slowly perfuse the cell suspension with distilled water within a custom built rig mounted on an inverted microscope. In this case, the osmolality of the cell suspension was slowly reduced during 173 min. In some experiments, cells were pretreated with 4 μM cytochalasin D (Santa Cruz Biotechnology, USA) for 3 h to inhibit actin polymerization or 75 μM REV5901 (Sigma–Aldrich, Poole, UK) for 30 min to inhibit RVD. These concentrations were selected based on previous studies in chondrocytes[12,34]. All experiments were performed at room temperature unless otherwise stated.

Measurement of cell diameter change

Bright field images of cells were obtained by a confocal microscope system (Ultra View, Perkin Elmer, UK) which connected to an inverted microscope (TE eclipse, Nikon, UK) with a ×60/1.4 NA oil immersion objective lens. This produced images with a pixel size of 0.11×0.11 μm. The diameters of single cells were calculated from the mean of the two orthogonal diameters measured using image J.

To monitor cell swelling and subsequent RVD, sequential time-lapse images of the same individual cells were recorded at 1 frame per 30 s for 60 min after sudden hypo-osmotic challenge or for the entire period (173 min) of gradual hypo-osmotic challenge. Cells were considered to show RVD following hypo-osmotic challenge if the diameter reduced by at least 1 μm during the imaging period in line with previous studies[11].

In addition cell diameters were measured from bright field images captured for separate samples of live cells under iso-osmotic condition and at 5, 60, 120 and 180 min intervals after sudden hypo-osmotic challenge or 40, 80, 120 and 173 min after the onset of a gradual hypo-osmotic challenge. Approximately 200 cells were imaged at each time interval (±2 min). The percentage recovery in cell diameter was calculated based on measurements obtained from the timelapse images. However as it was not possible to capture the initial iso-osmotic volume, this was estimated based on the final volume and the percentage difference between the final and initial volumes measured from the separate sample populations.

Micropipette aspiration

Micropipette aspiration was used to determine the viscoelastic properties of individual chondrocytes at various time points follow the onset of sudden and gradual hypo-osmotic challenge. The overall technique was similar to that previously described[8]. Although this is a well-established technique, it should be noted that cells are responsive to mechanical stimuli and have been shown to respond directly to the aspiration with changes in calcium signalling and actin organisation which may influence cellular mechanical properties[35]. Micropipettes were constructed from glass capillaries (G-1, Narshinghe Intentional, UK) and pulled by a commercial system (P97, Sutter Instrument, CA, USA) and fractured on a microforge to an inner diameter of approximately 4.5–5.5 μm. The micropipettes were coated with silicone solution (Sigmacote, Sigma, MO, USA) to prevent cell adhesion. For all the micropipette aspiration experiments, the ratio of cell diameter to micropipette diameter was maintained between 2.5 and 3.0 as required by the analytical model used to calculate viscoelastic modulus[36].

Cells were tested at three different time intervals (5–15 min, 15–45 min, 60–90 min) after exposure to sudden hypo-osmotic challenge. In gradual hypo-osmotic experiments, cells were exposed to a gradual hypo-osmotic challenge for 173 min and tested within the following 30 min. A tare pressure of 0.5 cm H_2O

was applied followed by a step pressure of 7 cm H_2O. Precise temporal control of this aspiration pressure was achieved using a PC-controlled peristaltic pump and LabView control. For all experiments, the aspiration pressure ramp rate was 5.48 cm H_2O/sec. Following the step aspiration pressure, sequential bright field images of the cell were recorded at 1 frame every 2.15 s for 180 s using a ×60/1.4 NA objective.

Additional experiments were conducted in which cells were pretreated with either cytochalasin D or REV5901 and then exposed to sudden hypo-osmotic challenge for 15–45 min before micropipette aspiration. For cells treated with cytochalasin D, it was necessary to reduce the aspiration pressure in order to prevent complete aspiration into the micropipette. In this case a tare pressure of 0.1 cm H_2O was used followed by a step pressure of 2 cm H_2O.

The cell volume during micropipette aspiration was calculated based on the following equation where L is the aspiration length into the micropipette.

$$V = \frac{1}{6} \Pi D_h^2 D_v + \frac{1}{4} \Pi D_p^2 \left(L - \frac{D_p}{2} \right) + \frac{2}{3} \Pi \left(\frac{D_p}{2} \right)^3 \quad (1)$$

D_h and D_v represent the horizontal and vertical diameters of the cell outside of the micropipette which is assumed to be an oblate ellipsoid. Previous studies assumed a spherical morphology for this portion of the cell[8], however our measurements indicate that the it has an elliptical cross section such that $D_h < D_v$, (data not shown). D_p is the internal diameter of the micropipette and L is the aspiration length. The leading edge of the cell within the micropipette was assumed to be hemispherical with a diameter equal to that of the micropipette.

The viscoelastic parameters were calculated using the standard linear solid (SLS) model[37]. Three viscoelastic parameters, k_1, k_2 and μ were determined by fitting the following equation using nonlinear regression analysis.

$$L(t) = \frac{\varnothing a \Delta P}{\Pi k_1} \left[1 - \left(\frac{k_1}{k_1 + k_2} - 1 \right) e^{-t/\tau} \right] \quad (2)$$

$$E_{in} = \frac{3}{2}(k_1 + k_2) \quad (3)$$

$$E_{eq} = \frac{3}{2} k_1 \quad (4)$$

$$\mu = \tau \left(\frac{k_1 k_2}{k_1 + k_2} \right) \quad (5)$$

Where $L(t)$ is the aspiration length of the cell at time t, E_{in} and E_{eq} represent the instantaneous moduli and equilibrium moduli respectively, and μ is the apparent viscosity and τ is the time constant. $\varnothing$ is defined as the wall function with a value of 2.1 for the micropipettes used in this study[38].

F-actin staining

Freshly isolated chondrocytes were centrifuged after exposure to sudden hypo-osmotic challenge for 5, 15 and 60 min or gradual hypo-osmotic challenge for 173 min. Cells were fixed for 30 min at room temperature with 3% glutaraldehyde (Agar Scientific, Stansted, UK) buffered with 8 mM sodium cacodylate (Sigma–Aldrich, Poole, UK) creating a solution of 300 mOsm/kg. Fixed cells were subsequently washed twice with phosphate buffered saline (PBS) and stained with 5 μL Alexa Phalloidin 555 (Life Technologies, Paisley, UK) in 200 μL of PBS for 30 min at 37°C. The intensity of

cortical F-actin was measured from confocal microscopy images (SP2, Leica, UK) obtained with a ×63/1.4 NA oil immersion objective (pixel size 0.09 × 0.09 μm, 543 nm excitation, emission collected above 565 nm). Photobleaching was prevented by minimising laser power and exposure time. All imaging settings, including photodetector gain and offset, were kept constant. Several different methods have been reported for quantification of F-actin organisation[19,39,40] however this study opted for a relatively simple approach in which the mean intensity was measured within a circular beigel region of interest, 2 μm in thickness, which was positioned over the cell cortex[41].

Chromatin condensation in suspension and monolayer culture

Osmotic induced changes in nuclear chromatin condensation were assessed in order to investigate their contribution to changes in cell stiffness. Chondrocytes in suspension were subjected to sudden and gradual hypo-osmotic challenge as described above. In addition cells in monolayer were also examined since they have flatten nuclei which more clearly demonstrate changes in chromatin condensation[32]. After osmotic challenge, the cells were fixed with 1% glutaraldehyde (30 min, room temperature). The nuclei were stained with 8 μM Hoechst 33,342 (10 min, 37°C) and imaged by confocal microscopy. The chromatin condensation parameter (CCP) was measured using a validated image processing algorithm which quantifies the granularity of staining within the nucleus. Full details of this methodology are extensively described in previous studies[32].

Data analyses

All statistical analyses were conducted using SPSS® Version 13.0 (SPSS-IBM Inc., IL, USA). Cells were isolated from a pool of cartilage taken from two donors for each experiment. Micropipette aspiration experiments under each condition were performed at least twice. The numbers of cells analysed for each experimental condition is summarized in Table I. Normality testing (Kolmogorov–Smirnov test) was performed for all experimental groups. For parametric statistics, data was presented as mean with 95% confidence intervals (CI) and assessed by unpaired Student's t test. For non-parametric statistics, data was presented as the median and interquartile range and assessed by Mann–Whitney U test or Wilcoxon signed-rank test. Chi-square test was used to examine the differences between two proportions. In all cases, 2-tailed tests were employed. Differences were considered statistically significant at $P < 0.05$.

Results

Sudden hypo-osmotic challenge induced chondrocytes swelling followed by RVD

Due to slight cell movement following addition and mixing of the distilled water, it was not possible to capture images immediately after the application of a sudden hypo-osmotic challenge. Therefore the first measurement of cell diameter was taken 2 min after the start of a sudden hypo-osmotic challenge. A total of 61 cells were monitored to record the real-time change in cell diameter. The majority of cells (65.6%) exhibited RVD [Fig. 1(A) and (B)]. In addition, 24.6% cells exhibited no RVD [Fig. 1(C)], whilst 6.6% burst during swelling. For cells that exhibited RVD, the mean time to reach maximum cell diameter was 10.9 ± 1.5 min ($n = 40$ cells).

From measurement of the whole population, the mean cell diameter of 10.8 ± 0.1 μm ($n = 389$ cells) at 0 min was increased

Table I
The numbers of cells analysed for each experimental condition including the numbers of cells rejected from the analysis, the ratio between cell diameter and micropipette diameter and the osmolality. Data is presented as median and interquartile

Condition	Cell number				Cell diameter/pipette diameter	Osmolality (mOsm/kg)
	Total cells tested	Successfully aspirated	Convergence	K > 0.95		
Sudden hypo 0—15 min	42	41	39	31	2.56 (0.24)	321
	28	25	23	14	2.72 (0.21)	153
Sudden hypo 15—45 min	53	48	48	39	2.66 (0.3)	321
	51	48	48	28	2.64 (0.23)	153
Sudden hypo 60—90 min	33	30	30	25	2.67 (0.21)	321
	26	26	26	20	2.56 (0.25)	153
Gradual hypo	27	25	25	14	2.67 (0.26)	153
Sudden hypo 15—45 min (CytoD)	44	36	36	35	2.73 (0.2)	317
	33	24	24	22	2.75 (0.19)	153
Sudden hypo 15—45 min (REV5901)	41	36	36	35	2.53 (0.24)	324
	47	40	40	35	2.8 (0.26)	153

significantly ($P < 0.001$) by 20% to 13.0 ± 0.2 μm ($n = 232$ cells) at 5 min after the sudden hypo-osmotic challenge [Fig. 1(D)]. Cell diameters were then decreased over time, with value at 60 min of 11.3 ± 0.2 μm ($n = 227$ cells), 11.1 ± 0.2 μm at 120 min ($n = 230$ cells) and 11.3 ± 0.2 μm at 180 min ($n = 207$ cells). At all time points cell diameter was significantly greater than that measurement at 0 min ($P < 0.001$ in all cases) [Fig. 1(E)]. The % volume recovery of the whole cell population 180 min after the onset of a sudden hypo-osmotic challenge was 84%.
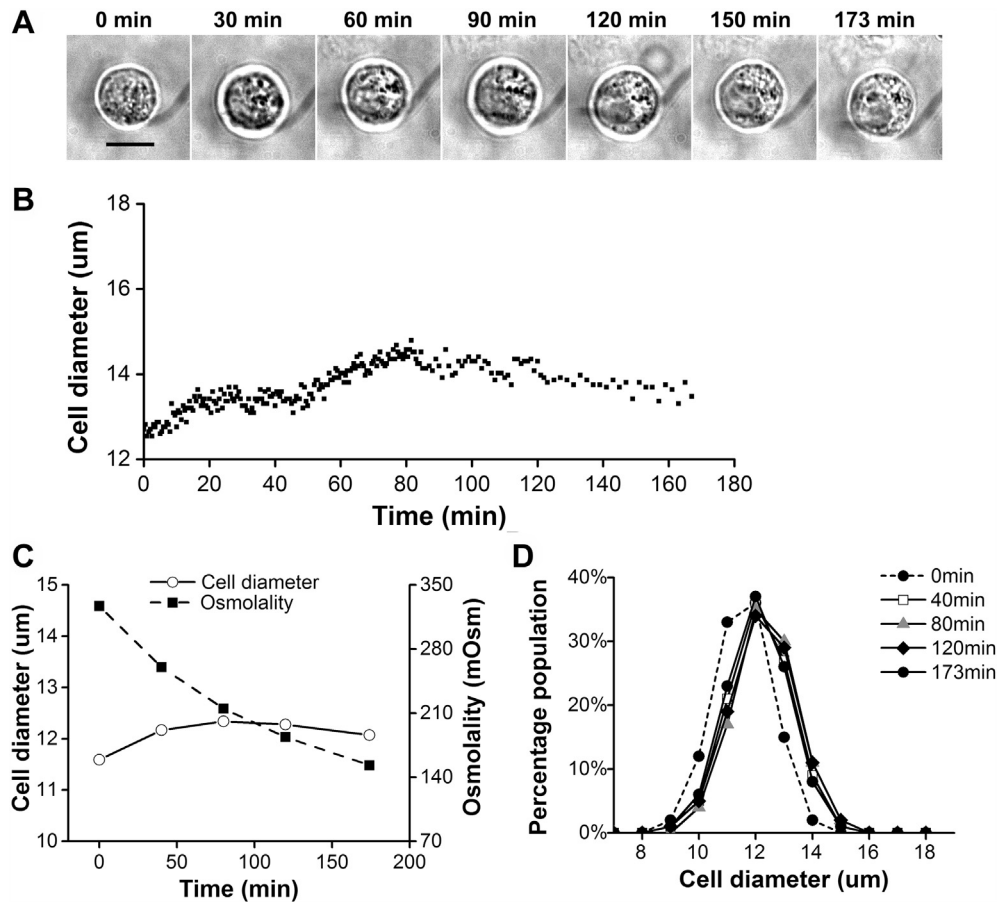
Gradual hypo-osmotic challenge induced limited cell swelling without RVD

In contrast, the response of isolated chondrocytes exposed to a gradual hypo-osmotic challenge was completely different with a gradual but reduced increase in cell diameter with no RVD [Fig. 2(A) and (B)].

The mean cell diameter increased gradually from 11.6 ± 0.1 μm ($n = 406$ cells) at 0 min to 12.2 ± 0.1 μm ($n = 264$ cells) at 40 min,
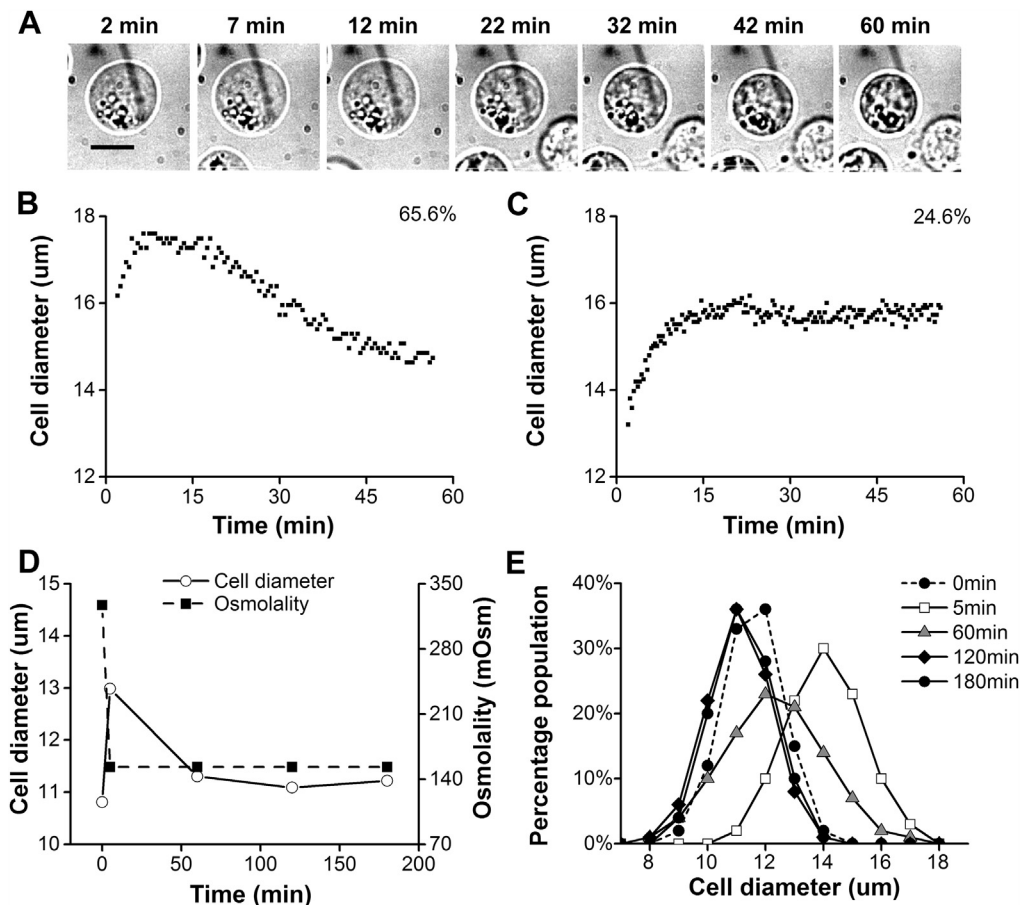
Fig. 1. Sudden hypo-osmotic challenge induces chondrocyte swelling followed by RVD. (A) Bright field images of an isolated chondrocyte showing RVD in response to sudden hypo-osmotic challenge. (B) Corresponding temporal changes in cell diameter for the same cell exhibiting RVD and (C) for a separate cell not showing RVD. The % of each response is indicated. (D) Mean cell diameter for chondrocyte populations measured at 0, 5, 60, 120 and 180 min after the onset of a sudden hypo-osmotic challenge, $n > 200$ cells at each time point. (E) Corresponding frequency distributions of cell diameter at each time point.

Fig. 2. Gradual hypo-osmotic challenge induces limited cell swelling without RVD. (A) Bright field images of an isolated chondrocyte exhibiting no RVD in response to gradual hypo-osmotic challenge. (B) Corresponding temporal change in cell diameter. (C) Mean cell diameter of chondrocyte populations measured at 0, 40, 80, 120 and 173 min after the onset of a gradual hypo-osmotic challenge, $n > 200$ cells at each time point. (D) Corresponding frequency distributions of cell diameter at each time points.

12.3 ± 0.1 μm ($n = 283$ cells) at 80 min, 12.3 ± 0.1 μm ($n = 238$ cells) at 120 min and 12.1 ± 0.1 μm ($n = 345$ cells) at 174 min. At all time points the diameter was significantly greater than under iso-osmotic conditions at 0 min ($P < 0.001$ in all cases) [Fig. 2(C) and (D)]. The % volume recovery of the whole cell population was reduced to 37% under gradual hypo-osmotic challenge confirming a lack of RVD based on previous criteria for a 50% recovery[11].

Sudden hypo-osmotic challenge temporarily increased the equilibrium modulus of isolated chondrocytes

Using the micropipette aspiration technique, the effect of hypo-osmotic rate on chondrocyte mechanics was examined. Representative bright field images of two individual cells under iso-osmotic and sudden hypo-osmotic conditions are shown along with associated temporal changes in aspirated length and cell volume [Fig. 3(A)–(D)]. Under both conditions, chondrocytes exhibited viscoelastic creep behaviour in response to a step increase in pressure, such that cells were initially aspirated rapidly into micropipette followed by a decreased aspiration speed until reaching the equilibrium length. This response was accurately fitting using the SLS model (Table I).

Chondrocytes from all groups showed a significant decrease in volume of approximate 20%, 180 s after aspiration [$P < 0.001$ in all cases; data was assessed by Wilcoxon signed-rank test; Fig. 3(E)]. In addition, compared to the corresponding iso-osmotic control group a significant greater decrease in volume was found at 15–45 and

60–90 min after sudden hypo-osmotic challenge, and after the onset of gradual hypo-osmotic challenge ($P = 0.003$, 0.005, and 0.019 respectively). In terms of cell mechanics, the equilibrium modulus was significantly increased at 5–15 and 15–45 min after sudden hypo-osmotic challenge [$P = 0.008$ and 0.014, compared with the corresponding iso-osmotic group; Fig. 3 (F) and (G)]. Interestingly, this effect disappeared at 60–90 min ($P = 0.268$). By contrast, gradual hypo-osmotic challenge had no significant effect on chondrocyte mechanical properties ($P = 0.392$).

Sudden and gradual hypo-osmotic challenge reduced cortical F-actin distribution in isolated chondrocytes

After sudden hypo-osmotic challenge, the intensity of cortical F-actin staining was significantly decreased to 52% at 15 min and 57% at 60 min ($P < 0.001$ in both groups, Fig. 4). After gradual hypo-osmotic challenge, the intensity was also significantly decreased in this case to 46% ($P < 0.001$). The changes in cell diameter were in accordance with those measured for the population of cells described previously (Figs. 1 and 2).

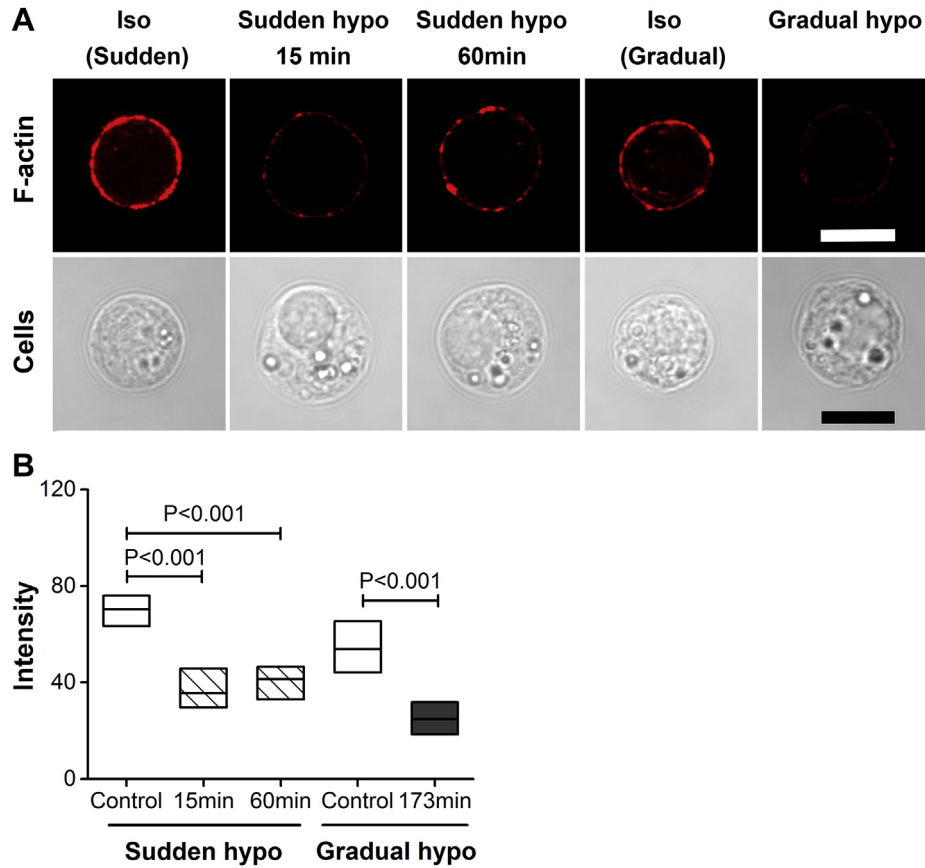*Sudden and gradual hypo-osmotic challenge induced chromatin decondensation*

Under both hypo-osmotic conditions, DNA staining appeared more homogeneous in the nuclei, reflected by the lower level of the CCP (Fig. 5). For the cells in monolayer, the values of CCP were
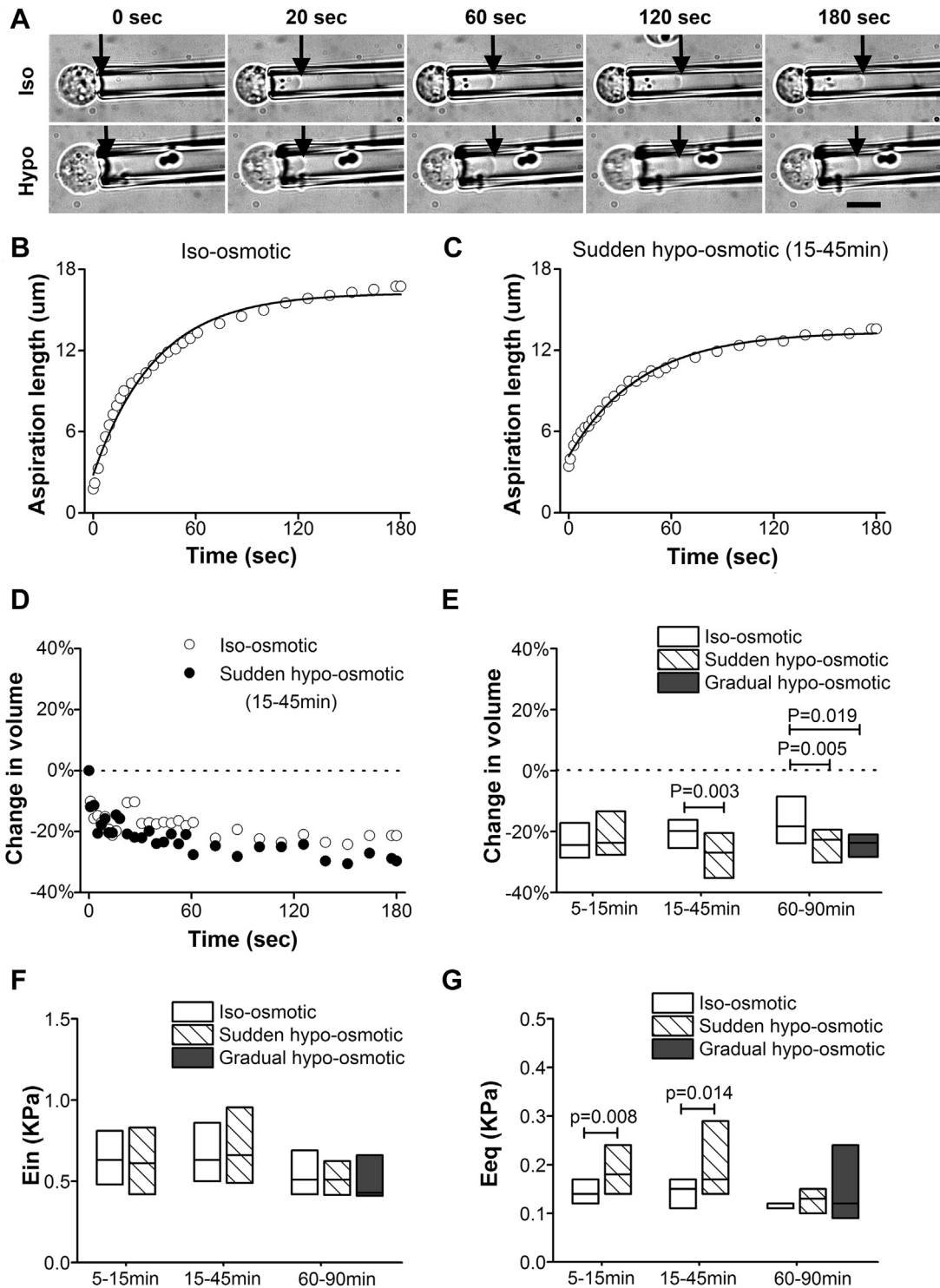
Fig. 3. Sudden but not gradual hypo-osmotic challenge induces an increase in cell equilibrium modulus. (A) Representative bright field images showing micropipette aspiration of two chondrocytes subjected to a 7 cm H_2O aspiration pressure under iso-osmotic conditions and following sudden hypo-osmotic challenge. The arrows indicate the extent of the aspiration length into the micropipette. Scale bar represents 10 μm. (B, C) Corresponding temporal changes in aspiration length fitted using the SLS model, and (D) percentage changes in cell volume during aspiration. For separate population of cells subjected to either sudden or gradual hypo-osmotic challenge we measured (E) the change in volume after aspiration ($t = 180$ s), (F) instantaneous modulus and (G) equilibrium modulus. Data presents median and quartiles ($n = 14$–39 cells). Data was analysed by Mann–Whitney U test.

significantly decreased to 19% following sudden hypo-osmotic challenge and 21% following gradual hypo-osmotic challenge ($P < 0.001$ in both cases). For the cells in suspension, the CCP was significantly decreased to 17% and 7% following sudden and gradual hypo-osmotic challenge respectively. No significant differences were found between sudden and gradual hypo-osmotic groups in either suspension or monolayer indicating that the observed differences in cell stiffness are not likely to be due to changes in chromatin condensation. Furthermore previous studies suggest that a decrease in chromatin condensation is likely to decrease cell stiffness rather than the increase observed here in response to hypo-osmotic challenge.
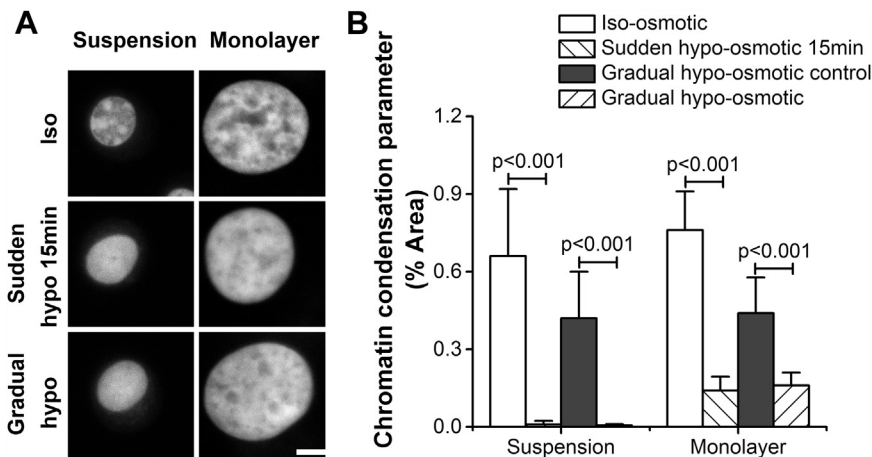
Fig. 4. Sudden and gradual hypo-osmotic challenge reduces cortical F-actin distribution in isolated chondrocytes. (A) Representative confocal images of cortical F-actin within an iso-osmotic environment, or following either sudden or gradual hypo-osmotic challenge. F-actin cytoskeleton was labelled with Alexa Phalloidin 555. Scale bar represents 10 µm. (B) Quantification of cortical F-actin for cells exposed to hypo-osmotic challenge. Data is presented as median and quartiles ($n = 33$–43 cells). Data was analysed by Mann—Whitney U test.

Interaction of F-actin and RVD during sudden hypo-osmotic challenge

In order to investigate the effect of the actin cytoskeleton on the RVD during sudden hypo-osmotic challenge, cytochalasin D was used to disrupt F-actin. Cytochalasin D had no effect on cell diameter under iso-osmotic conditions [Fig. 6(A)]. Following cytochalasin D

treatment, 40.9% cells exhibited RVD after sudden hypo-osmotic challenge, which was significantly less than the result observed in untreated chondrocytes (Chi-square test, $P = 0.008$). However there was no significant difference in % volume recovery [Fig. 6(B)]. The time to reach maximum cell diameter was significantly decreased from 10.9 ± 1.5 min in untreated chondrocytes to 8.3 ± 2.0 min following cytochalasin D treatment ($P = 0.047$). In addition, both

Fig. 5. Sudden and gradual hypo-osmotic challenge induces chromatin decondensation. (A) Representative confocal images of chondrocytes nuclei in suspension and on monolayer culture under different osmotic conditions. Scale bar represents 5 µm. (B) CCP quantified from the nuclei images of each osmotic group. Data is presented as mean $\pm$ CI. ($n = 40$ cells). Data was analysed by unpaired Student's t test.
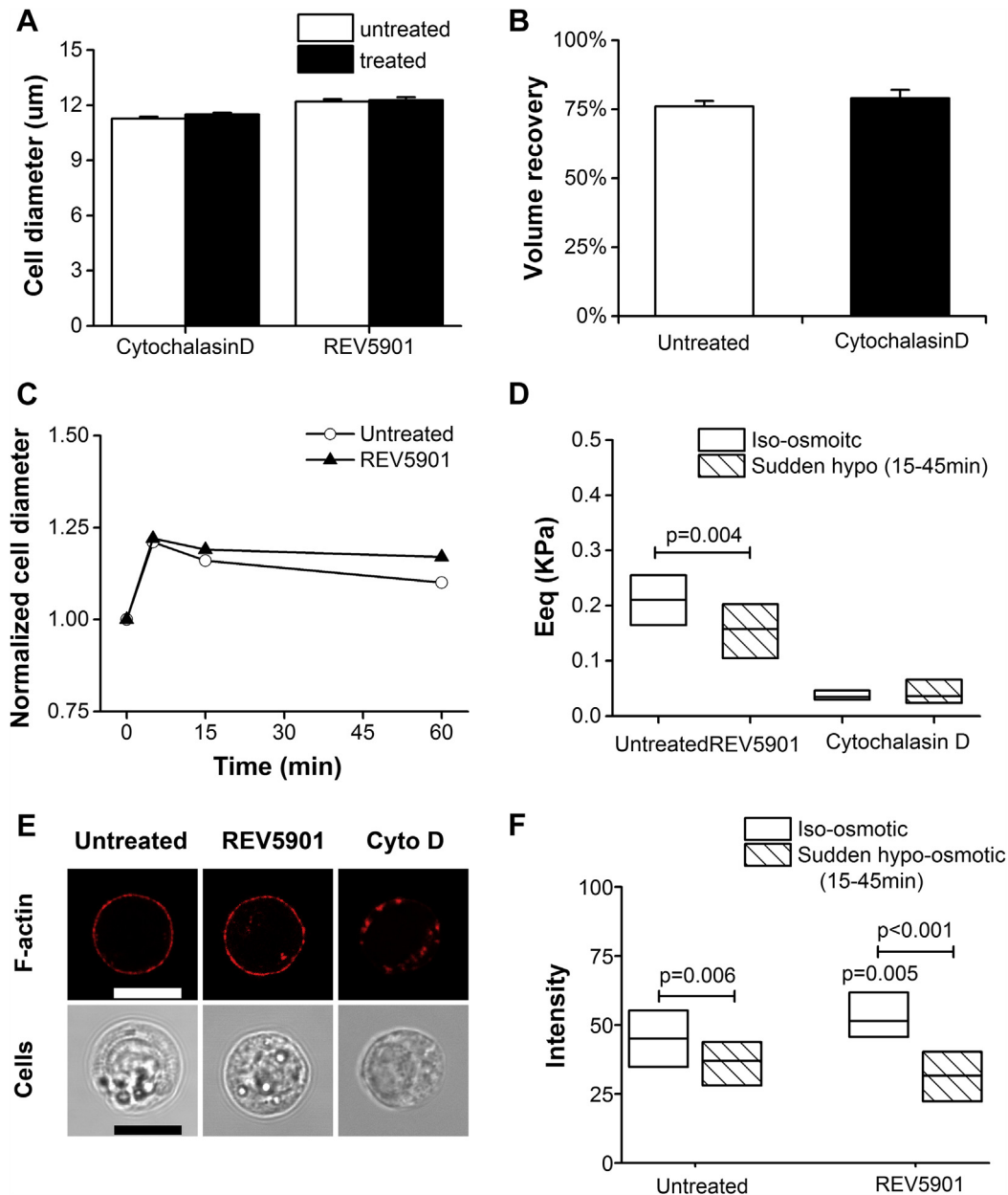
Fig. 6. RVD and actin are required for hypo-osmotic induced increase in cell equilibrium modulus. (A) Cytochalasin D and REV5901 treatments have no effect on cell diameter of isolated chondrocytes under iso-osmotic conditions. Data is presented as mean ± CI (n = 122−486 cells). Statistical significance was assessed by unpaired Student's *t* test. (B) Cytochalasin D treatment has no effect on the % volume recovery after sudden hypo-osmotic challenge. Data is presented as mean ± CI (n = 20−24 cells). (C) Changes in cell diameter for isolated chondrocytes with REV5901 treatment after sudden hypo-osmotic challenge. Data is normalized to the mean value of the corresponding iso-osmotic group. (D) Micropipette aspiration experiments were performed within 15−45 min after initiation of a sudden hypo-osmotic challenge for cells treated with either REV5901 or cyto-chalasin D. Data is presented as median and quartiles (n = 22−35 cells). (E) Representative confocal images of cortical F-actin stained with Alexa phalloidin in chondrocyte under iso-osmotic condition following treatment with either REV5901 or cytochalasin D. Scale bar represents 10 μm. (F) Quantification of cortical F-actin for the REV5901 treated cells exposed to sudden hypo-osmotic challenge. Data represents median and quartiles (n = 36−47 cells). Data was analysed by Mann−Whitney *U* test. *P*-value shown for the comparison between REV5901 treated and corresponding untreated iso-osmotic groups.

equilibrium and instantaneous modulus of chondrocytes under iso-osmotic condition were dramatically reduced to 0.12 and 0.03 KPa respectively. Furthermore cytochalasin D abolished the increase in equilibrium modulus observed after the application of sudden hypo-osmotic challenge (P = 0.909; Fig. 6(C) and (D)).

By using REV5901, RVD was totally abolished after sudden hypo-osmotic challenge such that there was no significant reduction in cell diameter once cells reached maximum swelling [Fig. 6(C)]. Interestingly, the equilibrium modulus at 15−45 min was significantly decreased (P = 0.004) compared to iso control [Fig. 6(D)] in contrast to the increased modulus in untreated cells

exposed to the sudden hypo-osmotic challenge (Fig. 3). In addition, treatment with REV5901 induced a significant increase in the intensity of cortical F-actin staining under iso-osmotic conditions (P = 0.005). Sudden hypo-osmotic challenge reduced cortical actin but the effect was greater in REV5901 treated cells than untreated cells [Fig. 6(E) and (F)].

Discussion

In this study, we compared the effects of sudden and gradual hypo-osmotic challenge on the morphology and biomechanics of

freshly isolated articular chondrocytes. Following sudden hypo-osmotic challenge chondrocytes swelled rapidly, reaching a peak within 5–15 min, followed by a reduction in volume. In previous studies the speed of RVD in isolated chondrocytes reduces at temperatures below 37°C[11,12,15,42]. However, a recent study by Huttu et al. showed temperature doesn't affect chondrocyte volume regulation in situ[43]. In our study, conducted at room temperature, 65.6% of chondrocytes exhibited RVD in response to sudden hypo-osmotic challenge. This is in close agreement with Kerrigan MJ et al., who reported ~50% of isolated chondrocytes exhibited RVD following hypotonic shock at 37°C[11]. It is unclear why RVD is not observed in all cells, but this may represent depth-dependent heterogeneity in the chondrocyte population and ion channel expression or variations in cell cycle position.

In contrast to the response to sudden hypo-osmotic challenge, gradual hypo-osmotic challenge at a rate of approximately 0.98 mOsm/kg per min, induced much less cell swelling and no detectable RVD. It could be argued that a form of RVD may still occur, but at a rate masked by the gradual swelling therefore producing no visible reduction in volume. Previous studies in other cell types have also observed reduced cell swelling and a lack of visible RVD in response to gradual hypo-osmotic challenge[44–46]. However, this is the first time this behaviour has been reported in chondrocytes.

The present study used micropipette aspiration to estimate chondrocyte viscoelastic mechanical properties. For all conditions micropipette aspiration induced a decrease in cell volume in agreement with studies on normal and OA chondrocytes[6]. Sudden hypo-osmotic challenge induced an initial increase in chondrocyte equilibrium modulus. This effect was lost once RVD was completed 60 min after the onset of osmotic challenge. By contrast, gradual hypo-osmotic challenge had no effect on cell stiffness. The increased cell stiffness following sudden hypo-osmotic challenge is different to previous studies from Guilak's group who found a reduction in chondrocytes modulus following sudden hypo-osmotic challenge[20]. To investigate the mechanism through which sudden hypo-osmotic challenge influences cellular mechanical properties and the difference between these two studies, we examined actin cytoskeletal organization and chromatin condensation, both of which have been shown to influence cell stiffness[34,47–50]. Sudden hypo-osmotic challenge reduced cortical F-actin in agreement with previous studies in various cell types including annulus fibrosus cells[51], astrocytes[52] and chondrocytes[20,21]. Gradual hypo-osmotic challenge also reduced chondrocyte actin organization. Both sudden and gradual hypo-osmotic challenge reduced the level of chromatin condensation in agreement with recent work from our group[32]. Interestingly, this actin de-polymerization and chromatin de-condensation were not associated with any reduction in cell stiffness. Instead we hypothesized that the increase in stiffness with sudden hypo-osmotic challenge is due to activation of RVD not observed during gradual hypo-osmotic challenge. Therefore chondrocytes were treated with REV5901 to block RVD following hypo-osmotic challenge. REV5901 is an antagonist of cysteinyl-leukotriene receptors, a family of G-protein coupled receptors[53], and has been shown to inhibit RVD in a variety of cell types including chondrocytes[12,54]. Recently REV5901 was shown to supresses the activity of large-conductance Ca^{2+}-activated K^+ channel, and thereby inhibit RVD by preventing K^+ efflux[55]. In our study, inhibition of RVD prevented the increase in stiffness after sudden hypo-osmotic challenge and instead resulted in a significant reduction in chondrocytes equilibrium modulus, possibly due to changes in cortical actin organization. This confirmed the importance of RVD in regulating cell mechanical properties following osmotic challenge. Furthermore, this finding may account for the reduction in cell stiffness reported by Guilak

et al. since they observed no RVD following a rapid reduction in osmolality[20]. In addition, disruption of cortical actin with cytochalasin D caused a substantial softening of the cells in agreement with previous studies[34]. Cytochalasin D reduced the percentage of cells exhibiting RVD and the time taken to swell to maximum volume. This differs from a previous study in which disruption of actin with latrunculin B had no effect on the percentage of chondrocytes showing RVD[56]. In our studies actin disruption also prevented the increase in cell equilibrium modulus associated with sudden hypo-osmotic challenge. These findings suggest the temporal increase in chondrocyte modulus following sudden hypo-osmotic challenge is dependent on activation of RVD and the presence of an intact actin cytoskeleton. These results agree with recent studies that showed a transient increase in cortical tension following sudden hypo-osmotic challenge, which disappeared when RVD or the actin cortex was disrupted[57].

In summary, we show for the first time that whilst sudden hypo-osmotic challenge activates cell swelling and characteristic RVD, this behaviour is completely absent in response to a gradual decrease in osmolality. Moreover we show that sudden, but not gradual, hypo-osmotic challenge increases chondrocyte stiffness, and that this is due to the activation of RVD and the interaction with actin cytoskeleton. Thus chondrocyte RVD is unlikely to be activated by the gradual reduction in osmolality which occurs with the development of OA. We therefore suggest that studies seeking to replicate these pathological changes in osmolality should adopt a gradual hypo-osmotic challenge in order to prevent non physiological changes in cell morphology and biomechanics which may impact on downstream signalling pathways.

Author contributions

Study conception and design: Zhao Wang, Jerome Irianto, Martin M. Knight.
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Analysis and interpretation of data: Zhao Wang, Jerome Irianto, Wen Wang, Martin M. Knight.

Competing interests
The authors declare no conflict of interest.

Acknowledgements

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