Osteoarthritis and Cartilage 21 (2013) 1738–1745

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Estrogen reduces mechanical injury-related cell death and proteoglycan degradation in mature articular cartilage independent of the presence of the superficial zone tissue

J. Imgenberg †, B. Rolauffs ‡, A.J. Grodzinsky §, M. Schünke †, B. Kurz ||*

† *Anatomisches Institut, Christian-Albrechts-Universität Kiel, Germany*
‡ *Klinik für Unfall- und Wiederherstellungschirurgie, Eberhard-Karls-Universität, Tübingen, Germany*
§ *MIT, Cambridge, MA, USA*
|| *School of Medicine, Bond University, Gold Coast, 4229 Queensland, Australia*

ARTICLE INFO

Article history:
Received 14 February 2013
Accepted 6 July 2013

Keywords:
Articular cartilage
Estrogen
Injury
Compression
Nuclear blebbing
TUNEL
Apoptosis
Glycosaminoglycan

SUMMARY

Objective: To study the effect of 17β-estradiol (E2) and the superficial zone (SFZ) on cell death and proteoglycan degradation in articular cartilage after a single injurious compression *in vitro*.
Method: Cartilage explants from the femoropatellar groove of 2 year old cows with or without the SFZ were cultured serum-free with physiological concentrations of E2 and injured by an unconfined single load compression (strain 50%, velocity 2 mm/s). After 96 h cell death was measured histomorphometrically (nuclear blebbing (NB) and TUNEL staining) and release of glycosaminoglycans (GAG) by DMMB assay.
Results: Injurious compression increased significantly the number of cells with NB and TUNEL staining and release of GAG. Physiological concentrations of E2 prevented the injury-related cell death and reduced the GAG release significantly in a receptor-mediated manner (shown by co-stimulation with the antiestrogen fulvestrant/faslodex/ICI-182,780). The presence of the SFZ did not alter the NB response to either the mechanical injury or E2, but reduced the overall release of GAG significantly.
Conclusion: E2 prevents injury-related cell death and GAG release, and might be useful for the development of treatment options for either cartilage-related sports injuries or osteoarthritis (OA). The SFZ does not seem to play an important role in (1) the E2-related tissue response and (2) the mechanically-induced cell death in deeper regions of the explants and GAG release. The latter might be related to the unconfined nature of the injury model.

Introduction

Many studies suggest that estrogen-deficiency is involved in the onset or progression of osteoarthritis (OA) and that both, endogenous and exogenous estrogens affect the joint health[1,2]. Postmenopausal women demonstrate a higher prevalence of OA compared to male patients[3,4], and those who receive an estrogen-replacement-therapy show reduced cartilage loss[5,6]; some clinical studies, however, show different results[7,8]. The clinical data are supported by studies showing that 17β-estradiol (E2) reduces cartilage damage in experimental arthritis models[9–11] and inhibits spontaneous or substance-induced apoptosis in chondrocytes[12–15]. However, the impact of E2 on mechanically-induced cell death has not been studied so far. E2 also affects the chondrocyte-mediated extracellular matrix (ECM) turnover or degradation after stimulation with oxidative stress, iodoacetate (IA) or interleukin-1 (IL-1) by altering the expression of matrix-degrading enzymes, TIMPs, growth factors or VEGF or by increasing the GAG synthesis[16–20]. However, whether E2 might prevent injury-related GAG loss has also not been investigated to date. Mechanical overload is a known risk factor for the development of OA[21], and has been studied extensively *in vitro*[22]. Single-compression loading[23–25] or prolonged cyclic compression of articular cartilage[26,27] can result in cell death, such as necrosis and apoptosis[23–25,28–31], collagen network damage, and release of GAG[28,32].

During maturation articular cartilage develops a zonal structure, which consists of a superficial (SFZ), middle, and deep zone. The estrogen-sensitive osteoprotegerin (OPG)/receptor activator of

* Address correspondence and reprint requests to: B. Kurz, School of Medicine, Bond University, Gold Coast, Queensland 4229, Australia. Tel: 61-410000503.
E-mail addresses: jan_imgenberg@web.de (J. Imgenberg), berndrolauffs@googlemail.com (B. Rolauffs), alg@mit.edu (A.J. Grodzinsky), mschuenk@anat.uni-kiel.de (M. Schünke), bkurz@bond.edu.au, bkurz@anat.uni-kiel.de (B. Kurz).

1063-4584/$ – see front matter © 2013 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.joca.2013.07.007

nuclear factor kappaB (RANK) system and the RANK ligand are well known as important mediators of estrogen-deficiency-related osteoporosis and specifically expressed in the SFZ of cartilage[33,34]. OPG expression increases in response to IL-1, and the expression of IL-1 can be up-regulated by mechanical stimulation of cartilage[35], which suggests that the SFZ might be a specific target for changes in E2 levels. Additionally, the SFZ plays an important role in the biomechanical behavior of cartilage by distributing loads away from directly-loaded regions[36]. Because of a relatively low equilibrium confined compression modulus, compression induced stiffening properties, as well as the ability to buffer shear-related forces superficial tissue has been suggested to greatly affect the biomechanical behavior of cartilage[37–40]. Superficial tissue of immature cartilage is particularly soft and more vulnerable to compressive injury, causing extensive compaction and surface ruptures with immediate failure of the biomechanical functioning in that zone compared to tissue from deeper layers[41]. Taken together the data suggest that the SFZ affects the compression- and E2-related responses in tissue from deeper zones.

The aims of this study therefore were to (1) test the hypothesis that E2 alters the mechanical injury-induced loss of tissue viability and GAG in a single load injury model using mature bovine articular cartilage and (2) investigate whether the presence of the SFZ in said model significantly alters the outcome.

Method

Isolation and culturing of articular cartilage explants

The experimental design of the study is summarized in Fig. 1. Articular cartilage explants were isolated from the femoropatellar groove of knee joints from 16 to 24 months old cows procured from a local abattoir authorized by the relevant meat inspectors. Cartilage/bone cylinders (9 mm diameter) were drilled perpendicular to the cartilage surface as described previously[23,24]. For explants without the superficial zone (SFZ) 50–100 μm of superficial tissue were removed with a microtome, and then a 1 mm thick cartilage disk was sliced and 3 mm diameter explants were punched out of each disk using biopsy punches (HEBUmedical, Tuttlingen, Germany). Explants with intact SFZ were punched directly from the femoropatellar groove and the top 1 mm tissue was sliced with a scalpel parallel to the surface (thickness was measured using a calliper rule); for an experiment two (or one) knee joint(s) were used and up to six explants with appropriate thickness were isolated and randomly distributed per experimental group. These experiments were repeated independently up to five times (for details see results). Explants were cultured individually in 200 μl medium in 96-well plates, equilibrated overnight at 37°C in an atmosphere of 5% CO_2 in serum-free culture medium (low-glucose Dulbecco's modified Eagle's medium (Biochrom) supplemented with 10 mM HEPES buffer (Biochrom), 1 m*M* sodium pyruvate (PAA), 0.1 mM nonessential amino acids (Sigma–Aldrich, St. Louis, MO, USA), 0.4 mM proline (Sigma), 1× ITS Liquid media supplement (Sigma), L-glutamine (PAA), 100 units/ml of penicillin G, 100 μg/ml of streptomycin, and 0.25 μg/ml of amphotericin B (PAA)).

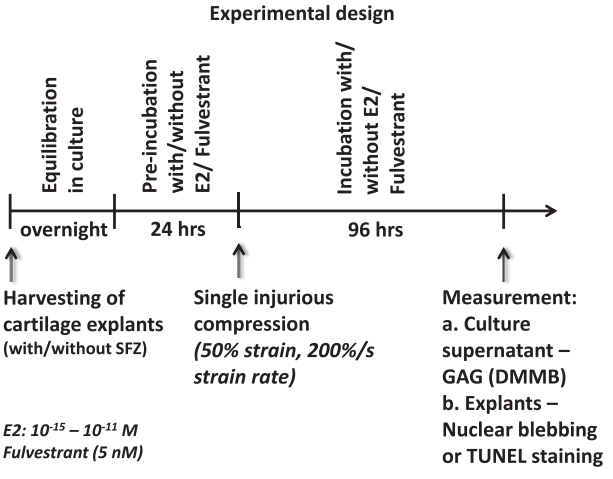

Fig. 1. Flow chart of the experimental design. Articular cartilage explants (1 mm thick, 3 mm diameter) have been isolated with or without superficial zone tissue (SFZ), cultured overnight, pre-incubated for 24 h with or without E2 or fulvestrant, injured with a single load compression, and incubated with or without E2 or fulvestrant for another 96 h. Paraffin sections of the explants have been used for detection of cell death, culture supernatants for detection of released GAG by DMMB assay. For detailed description see methods section.

Injurious compression and incubation with E2

After equilibration the explants received new medium containing E2 (Sigma) in physiological concentrations (10^{-15}–10^{-11} M) and/or the antiestrogen fulvestrant (Faslodex, ICI 182.780, or 7α-[9-(4,4,5,5,5-pentafluoro-pentylsulphinyl)nonyl]oestra-1,3,5(10)-triene-3,17βdiol; from Sigma; final concentration 5 n*M*[42]). A 10^{-1} M stock solution of both chemicals was produced and further diluted in ethanol (>99.8%; Roth); the final ethanol concentration in medium (also in control cultures) was 1 μl/ml. Explants were incubated for 24 h, then some explants were compressed (see below), and all explants were further cultivated for 96 h receiving new medium with/without E2 or fulvestrant. Then supernatants were frozen and explants fixed in 4% paraformaldeyde, embedded and sectioned for further biochemical or histomorphometric measurements.

Mechanical injury was applied by a single load compression (radially unconfined) using an incubator-housed loading device[23]. Controlled displacement ramps to 50% final strain were applied to individual explants, using the original explant cutting thickness as starting point; ramp velocity was 2 mm/s (strain rate 200%/second). The non-porous platen was held for 10 s and then leveled back to the starting position. Platen displacement and the force produced during compression were recorded, which allowed identification of the peak stress (MPa). Strain, velocity, and length of compression were developed as an evolution of a previous protocol successfully used in our study group to yield a higher percentage of apoptotic cells[24] without causing too severe an injury to the tissue as to render any hypothetical protective effect of E2 undetectable.

Histologic detection of cell death

Explants were fixed overnight using 4% paraformaldehyde (in PBS), embedded in Paraplast, serial sections (7 μm) were cut sagittally through the entire thickness of the explants, immobilized on glass slides, and stained with Mayer's hematoxylin or TUNEL (according to the manufacturer's protocol ApopTag peroxidase *in situ* apoptosis detection kit; Oncor, Gaithersburg, MD) in order to visualize cells with nuclear blebbing (NB) (an indicator for apoptosis[23,43]) or DNA fragmentation (an indicator of cell death[24]), respectively. 3–5 sections from each explant disk were evaluated for NB- or TUNEL-positive cells, respectively. The margins of the sections (about 150 μm thickness) were excluded, since cutting of the explants during the initial isolation process induces apoptosis at the tissue edges (therefore the SFZ has also been excluded in those cases with SFZ). Using a Zeiss Axiophot microscope (Zeiss, Wetzlar, Germany) with a 40× objective and 10× eye piece, positive and negative cells were counted in three optical fields in each section (one field in the center and two near the long ends of the

sections, excluding the margins). The mean number of cells per visual field was 74 (71 in explants with, 77 in explants without the SFZ). The mean value of NB- or TUNEL-positive cells from each explant was calculated (% of total cells). Encoded labels were used on all samples during histomorphological analysis to ensure blind scoring.

Measurement of glycosaminoglycans (GAG)

GAG content in the media was determined spectrophotometrically by DMMB assay at a wavelength of 520 nm (Photometer Ultraspec II, Biochrom, Cambridge, UK) using shark chondroitin-sulfate as standard[24]. Values are presented as μg GAG/mm^3 tissue of the explants; tissue volume was calculated based on the original thickness and diameter of the explant disks.

Statistics

For the data presentation and analysis n represents measurements of individual explants or corresponding culture supernatants; all explants were separately isolated, injured, cultured and measured. Per experiment up to six explants were distributed randomly per experimental group sourced from two knee joints. These experiments were repeated independently two times (for dose–response experiments, TUNEL staining, fulvestrant testing) or five times (for main NB and GAG experiments). Therefore, as examples, $n = 10$ means: 10 individual explants sourced from four knee joints in two experiments, or $n = 29$ means: 29 individual explants sourced from 10 knee joints in five experiments. The Kolmogorov–Smirnov-test was used to test for normality. For GAG and TUNEL data a Box–Cox-transformation was made prior to analysis with a General Linear Model (GAG or TUNEL data as dependent variables, experimental group as fixed and animal/experiment as random factors); the TUKEY posthoc test was used for subsequent pairwise comparisons with $P < 0.05$ indicating significant differences. NB data were analyzed with the Kruskal–Wallis test with a subsequent Mann–Whitney U test for pairwise comparisons with $P < 0.05$. All data are presented as means with 95% confidence intervals in text and tables; figure charts show means with standard error of the mean.

Results

Cell death

First the most effective concentration of E2 in a physiological range (10^{-15}–10^{-11} M) was identified by using 58 explants with SFZ (thickness 1.33 mm ± 0.14 SD, Min. 1.00, Max. 1.5, 95% CI 1.3–1.4) sourced from six knee joints in three experiments. Compression (peak stresses: 11.9 ± 4.4 SD MPa, Min. 3.7, Max. 21.7, 95% CI 10.6–13.2) introduced NB in 7.7% of the chondrocytes [±3.3 SD, Min. 4.0, Max. 14.6; 95% CI 5.6–9.8; $n = 12$; see examples of normal cells and cells with NB in Fig. 1(A) and (B)], which was a significant increase ($P < 0.001$) compared to control tissue (1.6% ± 1.6 SD; Min. 0.0, Max. 5.0; 95% CI 0.5–2.6; $n = 12$). E2 10^{-15} M did not alter the injurious response, whereas E2 10^{-13} M diminished NB non-significantly ($P = 0.135$) by about 50% (4.2% ± 5.0 SD; Min. 0.0, Max. 12.0; 95% CI 0.0–8.3; $n = 8$). E2 10^{-11} M, however, reduced NB significantly ($P < 0.001$) almost to control levels (1.9% ± 2.4 SD; Min. 0.0, Max. 6.7; 95% CI 0.3–3.5; $n = 11$; $P = 0.948$ vs control). This concentration was also reported as being effective in a study about E2 effects on mechanical cartilage integration[44]. Therefore the following experiments were conducted with a concentration of E2 10^{-11} M.

The next part of the study was performed to (1) confirm the effect of E2 on NB by using a larger number of explants and (2) see whether the presence of the SFZ might have an impact on the tissue response or on the protective effect of E2. 87 explants with intact SFZ (mean thickness: 1.27 mm) and 114 explants without SFZ (mean thickness 1.02 mm; see Table I) were used; peak stresses during compression were not significantly different in both types of explants (with SFZ: 16.7 MPa, without SFZ: 17.73 MPa; $P = 0.133$; see Table I). For both types of explants injury increased the percentage of cells with NB to levels significantly higher than in all other experimental groups [all $P < 000.1$; see Fig. 1(E) and Table II]: explants with SFZ demonstrated 12.41% NB compared to 0.87% in uninjured controls, and in explants without SFZ injury-related NB was found in 13.21% compared to 1.76% in controls. E2 alone lowered slightly the NB level in both types of explants compared to controls (with SFZ: 0.44 %, $P = 0.665$; without SFZ: 0.48 %, $P = 0.08$). However, injury-dependent NB was diminished by E2 significantly in both types of explants by 77.1% with SFZ and 69.8% without SFZ, respectively, but the levels were still significantly higher than the controls (with SFZ the combined compression/E2 showed 2.84% NB, $P < 0.001$ vs compression, $P = 0.009$ vs control, and without SFZ this combination resulted in 3.99 % NB, $P < 0.001$ vs compression, $P = 0.034$ vs control). The data from explants with and without SFZ did not differ significantly, indicating that the presence of the SFZ does not alter the injurious NB response or the effect of E2 on NB in mature articular cartilage.

TUNEL staining in explants without SFZ [see examples in Fig. 1(C) and (D) and data in Fig. 1(G)] confirmed the injurious cell death-related response and the effect of E2; however, the overall rate of TUNEL-positive cells was higher than that of cells with NB. The explants had a thickness of 1.04 mm (±0.13 SD; Min. 0.91, Max. 1.1, $n = 33$) and compression induced peak stresses about 16.75 MPa (±8.11 SD, Min. 5.3, Max. 31.4). In controls 5.05 % of the cells were TUNEL-positive (±3.26 SD; Min. 0.0, Max. 13.2; 95% CI −0.24 to 10.4, $n = 10$), and compression increased the number significantly ($P < 0.001$) to 54.14 % (±33.76 SD; Min. 6.3, Max. 94.3; 95% CI 28.2–80.1, $n = 9$). E2 did not alter the basic amount of TUNEL-positive cells (8.73% ± 4.4 SD; Min. 0.0, Max. 13.3; 95% CI 5.1–12.4, $n = 8$; $P = 0.748$ vs control), but reduced the injury-dependent response significantly by 78% ($P < 0.001$), so that just 11.86% (±6.55 SD; Min. 1.6, Max. 21.9; 95% CI 7.2–16.6, $n = 10$) of cells were TUNEL-positive in the co-treated compression/E2 group.

The antiestrogen fulvestrant had no significant impact on the basic amount of cells with NB (with: 0.65% ± 1.04 SD, Min. 0.0, Max. 2.4; 95% CI −0.4 to 1.7, $n = 6$; control: 0.67% ± 1.63 SD, Min. 0.0, Max. 4.0; 95% CI −1.0 to 2.4, $n = 6$; $P = 0.674$). In the subsequent injury study [see Fig. 1(F) and Table III] compression increased NB significantly compared to the control (compression: 18.05%, Con: 0.00 %; $P < 0.001$). E2 did not alter the basic level of NB (0.54%; $P = 0.068$ vs control), but reduced the injury-dependent response significantly by 84% (2.83%; $P = 0.001$). Addition of fulvestrant reduced the E2 mediated effect significantly, so that the reduction of the injury-dependent NB by E2 was only about 35% and not

Table I
Thickness of articular cartilage explants and peak stresses during injurious compression

Parameter	*N*	Mean	SD	Minimum	Maximum	95% CI mean (upper/lower limit)
Thickness A	114	1.02	0.06	0.86	1.10	1.01–1.03
Thickness B	87	1.27	0.15	1.00	1.50	1.24–1.3
Peak stress A	51	17.73	7.32	5.30	31.38	15.67–19.79
Peak stress B	54	16.74	6.94	3.72	29.09	14.85–18.64

Thickness (mm) of articular cartilage explants without (A) or with intact SFZ (B) and peak stresses (MPa) during compression (50% strain, velocity 2 mm/s) of the explants. *N* represents the number of explants per group which have been isolated, treated and measured separately from 10 knee joints in five experiments.

Table II
Cells with NB in articular cartilage explants after injurious compression or E2 treatment

Experimental group	*N*	Mean (%)	SD	Minimum	Maximum	95% CI mean (upper/lower limit)
Con A[c]	28	1.76	3.43	0.00	15.38	0.43–3.07
Con B[c]	22	0.87	1.45	0.00	5.00	0.23–1.51
Comp A[a]	28	13.21	15.03	0.00	62.50	7.39–19.04
Comp B[a]	22	12.41	7.59	4.00	31.11	9.04–15.77
Comp + E2 A[b]	29	3.99	4.04	0.00	11.21	2.45–5.53
Comp + E2 B[b]	21	2.84	3.26	0.00	13.64	1.36–4.33
E2 A[c]	29	0.48	0.81	0.00	2.27	0.18–0.76
E2 B[c]	20	0.44	0.9	0.00	3.28	0.02–0.86

Cells with NB (% of total cells); A without and B with intact SFZ; Comp: 50% strain, velocity 2 mm/s; E2 treatment (10^{-11} M); Con = control culture; *N* represents the number of explants per group which have been isolated, treated and measured separately from 10 knee joints in five experiments. a,b,c indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed *P*-values are given in the text).

significantly different from the compression group any more (11.71% cells with NB; $P = 0.049$ vs compression + E2, $P = 0.082$ vs compression), which indicates that fulvestrant had a significant impact on the E2-related effect, and that the latter must be receptor-mediated.

Release of GAG

Throughout all experimental groups the accumulated amount of GAG in cultures with intact SFZ was significantly lower (three- to five-fold) than that in the explants which had their SFZ removed [$P < 0.001$, Fig. 3(A) and Table IV]. The release of GAG in controls was 4.48 μg/mm^3 tissue with SFZ and 20.71 μg/mm^3 without the SFZ. Compression induced a significant increase in GAG release compared to controls (11.41 μg/mm^3 with SFZ and 33.0 μg/mm^3 without the SFZ; both $P < 0.001$). E2 did not affect the basic GAG release (4.03 μg/mm^3 with SFZ and 20.37 μg/mm^3 without the SFZ; $P = 0.898$ and $P = 1.0$ vs controls, respectively), but decreased the injury-related GAG release significantly, so that GAG levels in the media were about 40% lower in cultures with SFZ (6.85 μg/mm^3; $P = 0.044$) and 24.4% lower in cultures without the SFZ (24.94 μg/mm^3; $P = 0.011$) compared to the corresponding compression group, which indicates that E2 reduces the injury-related release of GAG significantly. The Comp + E2 groups were not significantly different from the controls (with SFZ: $P = 1.0$; without SFZ: $P = 0.849$) or E2 groups (with SFZ: $P = 0.897$; without SFZ: $P = 0.954$).

Fulvestrant had no impact on the basic GAG release in cultures with SFZ (5.46 μg/mm^3 ± 4.59 SD, Min. 1.93, Max. 14.24, 95% CI 0.6–10.3, $n = 6$; controls: 5.61 μg/mm^3 tissue ± 1.96 SD, Min. 3.83, Max. 9.2, 95% CI 3.6–7.7; $n = 6$; $P = 0.94$). In the subsequent injury experiment [Fig. 2(B) and Table V] compression (9.51 μg/mm^3) increased significantly 2.64-fold the GAG release compared to the control (3.6 μg/mm^3; $P = 0.011$). E2 did not alter the basic GAG release (3.65 μg/mm^3; $P = 0.999$ vs control), but reduced the injury-dependent release significantly down to control levels (3.78 μg/mm^3; $P = 0.003$ vs compression). Fulvestrant reduced the E2 effect, leading to (non-significantly) higher GAG levels than the compression/E2 group (6.29 μg/mm^3; $P = 0.321$). However, this group was also not significantly different from the compression group ($P = 0.265$), suggesting that the impact of E2 on the injury-dependent release of GAG might be partially receptor-mediated.

Table III
Impact of fulvestrant on NB in articular cartilage explants without SFZ after injurious compression and E2 treatment

Experimental group	*N*	Mean (%)	SD	Minimum	Maximum	95% CI mean (upper/lower limit)
Con[c]	10	0.00	0.00	0.00	0.00	0.00–0.00
E2[c]	10	0.54	1.06	0.00	3.28	−0.22 to 1.29
Comp[a]	10	18.05	7.49	6.06	31.11	12.69–23.41
Comp + E2[b]	9	2.83	1.83	0.00	5.88	1.42–4.24
Comp + E2 + Ful[a]	10	11.71	8.85	1.33	31.43	5.34–18.10

Cells with NB (% of total cells); Comp: 50% strain, velocity 2 mm/s; E2 treatment (10^{-11} M); Con = control culture; Ful = fulvestrant (5 nM); *N* represents the number of explants per group which have been isolated, treated and measured separately from four knee joints in two experiments. a,b,c indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed *P*-values are given in the text).

Discussion

In the present study we found a significant increase in NB and TUNEL-positive cells in response to injurious compression which supports previous studies[24,25,28,29,31]. Physiological concentrations of E2 (10^{-11} M corresponds to the range of E2 concentrations found in the peripheral blood of cows[45]) did not alter the basic rate of cell death, but reduced the injury-dependent cell death-response of the tissue. While a protective function of E2 related to the induction of apoptosis by molecular agents has been shown previously[12–15] the impact of E2 on mechanically-induced cell death in articular cartilage is shown here for the first time. Due to its artificial nature our *in vitro* model does not attempt to simulate the precise three-dimensional forces and deformation patterns that cartilage would experience in a clinical joint injury, which is a quite complex matter, but it shows that mechanical injury can trigger cell death and that E2 is able to prevent this significantly. Apoptotic cell death has been found in intraarticularly fractured joints[46] and based on our data we suggest, that application of a drug with E2-properties should reduce apoptotic cell death in such injured tissue. It could even be speculated that people with a very high risk of joint injury (for example athletes) could reduce the risk of cell death by preventive treatment based on a drug with E2-like properties.

Cells which have been identified with NB using light microscopy have been confirmed to be apoptotic by transmission electron microscopy previously[24,43], which suggests that the NB data represent apoptosis. Still the method might underestimate the level of apoptosis, since cells which have not yet reached the state of NB in the execution of their apoptotic program will be missed. Likewise, NB outside of the sectional plane of a particular slide cannot be detected. The TUNEL assay on the other hand has been repeatedly reported to yield positive staining in apoptotic as well as necrotic cells in different types of tissue[43,47,48], and indeed TUNEL staining yielded higher percentages of positive cells than via detection of NB in our study. But most importantly both methods revealed the same general patterns of response to injury and the protective effect of E2 and thus served as independent confirmation of the validity of our E2/cell death-related results.

Mechanical injury induced a significant increase in cartilage GAG release which supports previous findings showing damage to the ECM after mechanical overload[28,32] and E2 reduced this GAG release significantly. Previous studies showed that E2 prevents GAG loss from articular cartilage triggered by oxidative stress, restores IL-1-related decrease in proteoglycan levels in chondrocyte cultures, or up-regulates GAG synthesis in isolated chondrocytes[18–20]; however, the prevention of injury-related GAG release from mature articular cartilage has not been shown previously. Due to the fact that mechanical injury is able to increase the transcription of matrix-degrading enzymes, and E2 has been described to reduce the transcription rate of such enzymes in chondrocytes in general or triggered by other stimuli than mechanical injury[16–20], we

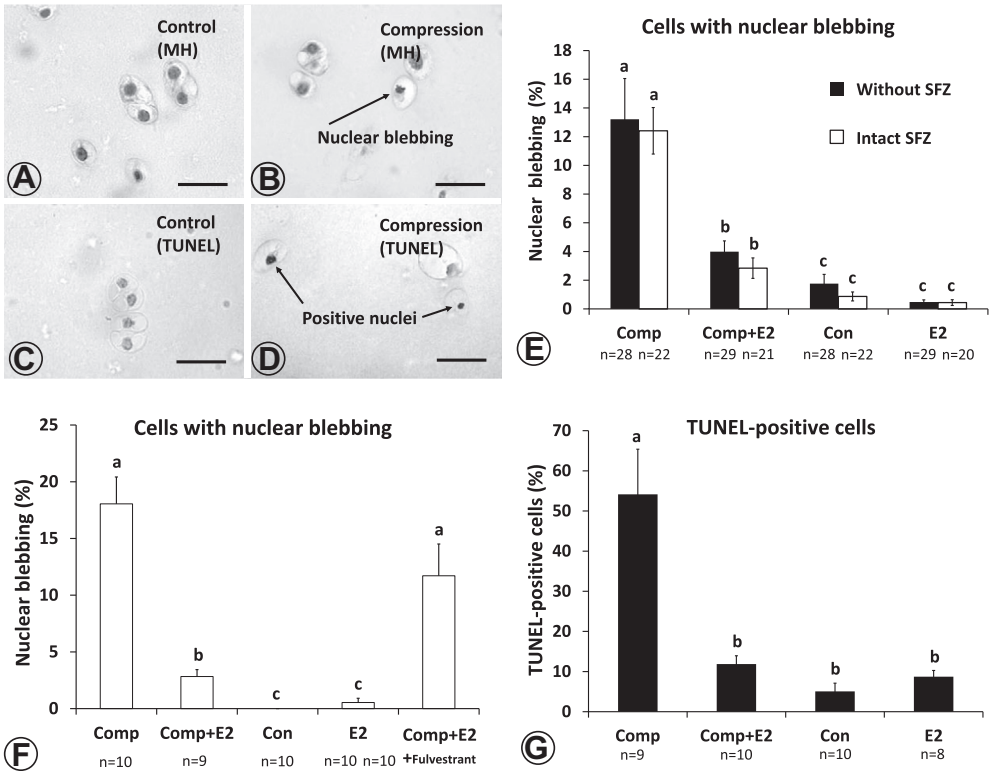

Fig. 2. NB and TUNEL-positive cells in mature bovine articular cartilage explants under normal culture conditions (Con), the influence of E2, (10^{-11} M), or after a single load compression (Comp; strain 50%, velocity 2 mm/s). A and B: Examples of chondrocytes with normal nuclei in control tissue (A) or cells with NB (arrow in B), sections stained with Mayer's Hemalaun (MH). C and D: Examples of chondrocytes with normal nuclei without staining in control tissue (C) or cells with dark nuclear TUNEL staining (arrow in B). A–D bar = 15 μm. E and F: Relative amount of cells with NB (% of total cell number). Fulvestrant = antiestrogen (5 nM). G: Relative amount of TUNEL-positive cells (% of total cells). a,b,c,d indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed P-values are given in the text). All mean ± S.E.M. of mean (95% CI are given in text or tables); n represents the number of explants per group which have been isolated, treated and measured separately from 10 knee joints in five experiments for E, and four knee joints in two experiments for F and G.

speculate that the reduction of the transcription and activation of matrix-degrading enzymes by E2 might be involved in the present findings. This has, however, to be investigated in further studies.

The steroidal estradiol receptor (ER) antagonist fulvestrant binds with an affinity of 0.89 compared to E2[49]. It induces increased degradation of the receptor, inhibits dimerization and thus further translocation of the receptor into the nucleus, and reduces binding of the receptor complex to the estrogen-responsive elements (ERE) and therefore reduces the receptor-dependent gene transcription[50]. Fulvestrant did not show any toxic effect in the present study, but reduced the protective effect of E2 on the injury-dependent cell death significantly, which suggests that this effect is mediated via ERs. Even though fulvestrant diminished some of the protective effect of E2 on the GAG release, the results were less striking as the NB data. Therefore, the question if the effect on injury-related GAG release is also receptor-mediated cannot be resolved based on the available data. Since membrane-bound receptors for E2 have recently been identified (mER), namely GPR30, and fulvestrant has been shown to be able to bind and activate GPR30 in human breast cancer cells[51], it remains also unclear what the importance of mER in articular cartilage might be with respect to injury-induced cell death. It has been shown that GPR30

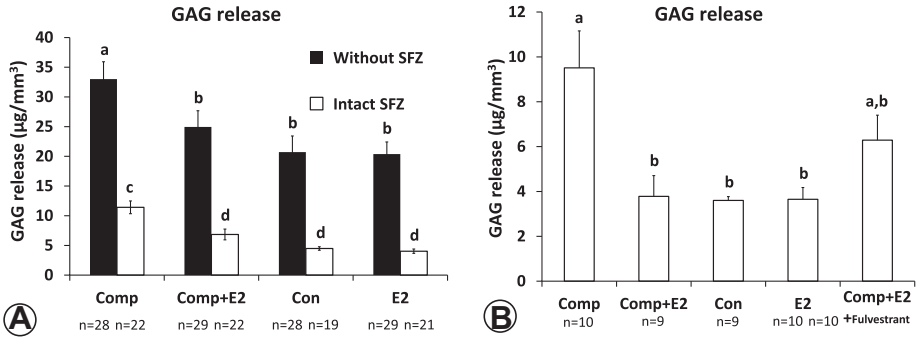

Fig. 3. Glycosaminoglycan (GAG) release in mature bovine articular cartilage explant cultures under normal culture conditions (Con), the influence of E2, (10^{-11} M), or after a single load compression (Comp; strain 50%, velocity 2 mm/s). Fulvestrant = antiestrogen (5 nM). a,b,c,d indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed P-values are given in the text). All mean ± S.E.M. of mean (95% CI are given in the text or tables); n represents the number of explants per group which have been isolated, treated and measured separately from 10 knee joints in five experiments for A and four knee joints in two experiments for B.

Table IV
GAG release from articular cartilage explants after injurious compression or E2 treatment

Experimental group	*N*	Mean	SD	Minimum	Maximum	95% CI of mean (upper/lower limit)
Con A[b]	28	20.71	14.35	2.58	59.37	15.15–26.28
Con B[d]	19	4.48	1.28	3.86	5.10	2.45–6.55
Comp A[a]	28	33.00	15.49	7.05	76.87	26.98–39.00
Comp B[c]	22	11.41	5.03	3.96	20.22	9.18–13.63
Comp + E2 A[b]	29	24.94	14.72	8.28	71.18	19.34–30.54
Comp + E2 B[d]	22	6.85	4.26	0.16	16.85	4.95–8.75
E2 A[b]	29	20.37	11.01	9.05	56.00	16.18–24.56
E2 B[d]	21	4.03	1.67	1.59	8.03	0.36–3.27

GAG release (μg/mm^3 tissue); A without and B with intact SFZ; Comp: 50% strain, velocity 2 mm/s; E2 treatment (10^{-11} M); Con = control culture; *N* represents the number of explants per group which have been isolated, treated and measured separately from 10 knee joints in five experiments. a,b,c,d indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed *P*-values are given in the text).

mediates E2-related inhibition of chondrogenesis, if E2 is applied in higher concentrations (10^{-8} M or higher[52]). Therefore, further studies are needed in order to clarify the E2 signaling pathways involved in the inhibition of injury-related cell death and GAG release.

Explants with the original tissue surface (SFZ) were significantly thicker than those without. Using the microtome instead of a scalpel was more precise in order to produce explants of a defined thickness, which is the reason why the deviation of thickness was much smaller in the group without SFZ. However, the peak stresses during compression were not significantly different. Even though the SFZ cells have been described to be unique by expressing estrogen-sensitive OPG/RANK[33], the presence of the SFZ had no impact on the outcome of the E2 experiments, which suggests that the SFZ does not mediate the E2-related protection of injury-induced cell death or GAG release. The SFZ is important in transferring loads from the directly-loaded area to neighbor areas[36]; however this concept might not be transferable to the presented injury model, since the platen of the loading device had a larger diameter than the explants, so that there were no areas without loading. This could be one reason why the peak stresses during compression were not significantly different in the two groups of explants. We previously found zone-specific types of damage by performing a texture analysis of images taken from injured superficial and deeper zones explants with texture regularity, homogeneity, and entropy being significantly lower in injured superficial tissue; we therefore assumed that an unconfined compression of explants with intact SFZ might lead to a different form of matrix-disruption[41]. Superficially the fibrils are oriented parallel to the platen and might stabilize the integrity of that particular side of the explants. Due to the impermeable nature of both platen and bottom of the compression chamber, fluid flow during compression has to go laterally forcing especially the non-intact bottom side of the explants to spread out (or top and bottom side equally in explants without SFZ). Indeed explants with intact SFZ showed a different macroscopic appearance, with the bottom ends showing larger swelling laterally than the upper end of the explants (not shown). However, neither the peak stresses during compression were significantly different in the two groups, nor the impact of compression on the rate of cell death. This suggests that the SFZ has no cell viability-related protective function with respect to deeper zone tissue in the unconfined injury model.

Table V
Impact of fulvestrant on GAG release from articular cartilage explants without SFZ after injurious compression and E2 treatment

Experimental group	*N*	Mean (%)	SD	Minimum	Maximum	95% CI mean (upper/lower limit)
Con[b]	9	3.6	0.51	2.95	4.62	3.21–4.99
E2[b]	10	3.65	1.66	1.59	5.85	2.47–4.84
Comp[a]	10	9.51	5.21	3.96	17.57	5.78–13.23
Comp + E2[b]	10	3.78	2.93	0.16	10.46	1.68–5.88
Comp + E2 + Ful[a,b]	10	6.29	3.51	0.2	11.97	3.78–8.8

GAG release (μg/mm^3 tissue); Comp: 50% strain, velocity 2 mm/s; E2 treatment (10–11 M); Con = control culture; Ful = fulvestrant (5 nM); *N* represents the number of explants per group which have been isolated, treated and measured separately from five knee joints in two experiments. a,b,c indicate clusters of experimental groups which are significantly different from each other with $P < 0.05$ (detailed *P*-values are given in the text).

However, the overall release of GAG was up to five-fold lower in cultures with SFZ compared to the corresponding cultures without SFZ. This might seem contradictory to our previous findings showing that a similar injury induces a two-fold higher relative (to explant GAG content) GAG release in superficial explants compared to deep tissue explants without SFZ[41]; but the overall GAG content in the superficial explants in that study was 2.5- to three-fold lower than that of deep tissue explants, which means that the actual levels of GAG in the superficial explants cultures were lower. Additionally, the SFZ is relatively thin (35–100 μm in femoral or tibial canine cartilage, which is less than 4.5–12% of the total thickness of the non-calcified part of cartilage tissue[53]) and softer than deeper zones[40]. It might therefore be speculated that it is too thin and soft, and that its relative contribution to the effects measured for the total tissue in a full area-loaded and unconfined 50% compression model are negligible.

Conclusion

Taken together we conclude that E2 prevents injury-related cell death in a receptor-mediated pathway and reduces GAG release significantly, which might have implications for the development of treatment options for cartilage-related sports injuries or OA. The SFZ, however, does not seem to play an important role in the E2-related tissue response. The missing influence of the SFZ on mechanically-induced cell death and GAG release might be related to the unconfined nature of the injury model.

Author contributions

JI made the acquisition of data and most of the analysis of the data, and was also involved in the design of the study and drafting of the manuscript. BR, AJG and MS helped with the interpretation of the data and the drafting of the manuscript. BK was involved in the conception and design of the study, analysis and interpretation of the data, and did most of the drafting of the manuscript. All authors read and approved the final manuscript.

Role of funding source
The study sponsor had no involvement in the study design, data collection, analysis or interpretation of data, manuscript writing or decision making related to the publication of the data.

Competing interest statement
The authors declare that they have no financial or non-financial competing interests related to this study.

Acknowledgments

The authors would like to thank Rita Kirsch and Frank Lichte for their excellent technical support. The study was funded by the

Medical Faculty of the Christian-Albrechts-University, Kiel, Germany.

References

1. Roman-Blas JA, Castañeda S, Largo R, Herrero-Beaumont G. Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther 2009;11:241.
2. Tanamas SK, Wijethilake P, Wluka AE, Davies-Tuck ML, Urquhart DM, Wang Y, *et al.* Sex hormones and structural changes in osteoarthritis: a systematic review. Maturitas 2011;69:141–56.
3. Hanna FS, Wluka AE, Bell RJ, Davis SR, Cicuttini FM. Osteoarthritis and the postmenopausal woman: epidemiological, magnetic resonance imaging, and radiological findings. Semin Arthritis Rheum 2004;34:631–6.
4. Richette P, Corvol M, Bardin T. Estrogens, cartilage, and osteoarthritis. Joint Bone Spine 2003;70:257–62.
5. Wluka AE, Davis SR, Bailey M, Stuckey SL, Cicuttini FM. Users of oestrogen replacement therapy have more knee cartilage than non-users. Ann Rheum Dis 2001;60:332–6.
6. Parker D, Hwa SY, Sambrook P, Ghosh P. Estrogen replacement therapy mitigates the loss of joint cartilage proteoglycans and bone mineral density induced by ovariectomy and osteoarthritis. APLAR J Rheumatol 2003;6:116–27.
7. Nevitt MC, Felson DT, Williams EN, Grady D. The effect of estrogen plus progestin on knee symptoms and related disability in postmenopausal women: The Heart and Estrogen/Progestin Replacement Study, a randomized, double-blind, placebo-controlled trial. Arthritis Rheum 2001;44:811–8.
8. Sahoyoun NR, Brett KM, Hochberg MC, Pamul ER. Estrogen replacement therapy and incidence of self-reported physician diagnosed arthritis. Prev Med 1999;28:458–64.
9. Jochems C, Islander U, Erlandsson M, Engdahl C, Lagerquist M, Ohlsson C, *et al.* Effects of oestradiol and raloxifene on the induction and effector phases of experimental postmenopausal arthritis and secondary osteoporosis. Clin Exp Immunol 2011;165:121–9.
10. Engdahl C, Jochems C, Windahl SH, Börjesson AE, Ohlsson C, Carlsten H, *et al.* Amelioration of collagen-induced arthritis and immune-associated bone loss through signaling via estrogen receptor alpha, and not estrogen receptor beta or G protein-coupled receptor 30. Arthritis Rheum 2010;62:524–33.
11. Dulos J, Vijn P, van Doorn C, Hofstra CL, Veening-Griffioen D, de Graaf J, *et al.* Suppression of the inflammatory response in experimental arthritis is mediated via estrogen receptor alpha but not estrogen receptor beta. Arthritis Res Ther 2010;12:R101.
12. Kumagai K, Imai S, Toyoda F, Okumura N, Isoya E, Matsuura H, *et al.* 17β-Oestradiol inhibits doxorubicin-induced apoptosis via block of the volume-sensitive Cl(-) current in rabbit articular chondrocytes. Br J Pharmacol 2012;166:702–20.
13. Li S, Luo Q, Huang L, Hu Y, Xia Q, He C. Effects of pulsed electromagnetic fields on cartilage apoptosis signalling pathways in ovariectomised rats. Int Orthop 2011;35:1875–82.
14. Chagin AS, Chrysis D, Takigawa M, Ritzen EM, Sävendahl L. Locally produced estrogen promotes fetal rat metatarsal bone growth; an effect mediated through increased chondrocyte proliferation and decreased apoptosis. J Endocrinol 2006;188:193–203.
15. Hattori Y, Kojima T, Kato D, Matsubara H, Takigawa M, Ishiguro N. A selective estrogen receptor modulator inhibits tumor necrosis factor-α-induced apoptosis through the ERK1/2 signaling pathway in human chondrocytes. Biochem Biophys Res Commun 2012;421:418–24.
16. Claassen H, Steffen R, Hassenpflug J, Varoga D, Wruck CJ, Brandenburg LO, *et al.* 17β-estradiol reduces expression of MMP-1, -3, and -13 in human primary articular chondrocytes from female patients cultured in a three dimensional alginate system. Cell Tissue Res 2010;342:283–93.
17. Sniekers YH, van Osch GJ, Jahr H, Weinans H, van Leeuwen JP. Estrogen modulates iodoacetate-induced gene expression in bovine cartilage explants. J Orthop Res 2010;28:607–15.
18. Ganesan K, Tiwari M, Balachandran C, Manohar BM, Puvanakrishnan R. Estrogen and testosterone attenuate extracellular matrix loss in collagen-induced arthritis in rats. Calcif Tissue Int 2008;83:354–64.
19. Maneix L, Beauchef G, Servent A, Wegrowski Y, Maquart FX, Boujrad N, *et al.* 17Beta-oestradiol up-regulates the expression of a functional UDP-glucose dehydrogenase in articular chondrocytes: comparison with effects of cytokines and growth factors. Rheumatology (Oxford) 2008;47:281–8.
20. Tinti L, Niccolini S, Lamboglia A, Pascarelli NA, Cervone R, Fioravanti A. Raloxifene protects cultured human chondrocytes from IL-1β induced damage: a biochemical and morphological study. Eur J Pharmacol 2011;670:67–73.
21. Arden N, Nevitt MC. Osteoarthritis: epidemiology. Best Pract Res Clin Rheumatol 2006;20:3–25.
22. Natoli RM, Athanasiou KA. Traumatic loading of articular cartilage: mechanical and biological responses and post-injury treatment. Biorheology 2009;46:451–85.
23. Kurz B, Jin M, Patwari P, Cheng DM, Lark MW, Grodzinsky AJ. Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orthop Res 2001;19:1140–6.
24. Kurz B, Lemke A, Kehn M, Domm C, Patwari P, Frank EH, *et al.* Influence of tissue maturation and antioxidants on the apoptotic response of articular cartilage after injurious compression. Arthritis Rheum 2004;50:123–30.
25. Torzilli PA, Grigiene R, Borrelli Jr J, Helfet DL. Effect of impact load on articular cartilage: cell metabolism and viability, and matrix water content. J Biomech Eng 1999;121:433–41.
26. Blain EJ, Gilbert SJ, Wardale RJ, Capper SJ, Mason DJ, Duance VC. Up-regulation of matrix metalloproteinase expression and activation following cyclical compressive loading of articular cartilage in vitro. Arch Biochem Biophys 2001;396:49–55.
27. Steinmeyer J, Knue S. The proteoglycan metabolism of mature bovine articular cartilage explants superimposed to continuously applied cyclic mechanical loading. Biochem Biophys Res Commun 1997;240:216–21.
28. Ewers BJ, Dvoracek-Driksna D, Orth MW, Haut RC. The extent of matrix damage and chondrocyte death in mechanically traumatized articular cartilage explants depends on rate of loading. J Orthop Res 2001;19:779–84.
29. Chen CT, Burton-Wurster N, Borden C, Hueffer K, Bloom SE, Lust G. Chondrocyte necrosis and apoptosis in impact damaged articular cartilage. J Orthop Res 2001;19:703–11.
30. Loening AM, James IE, Levenston ME, Badger AM, Frank EH, Kurz B, *et al.* Injurious mechanical compression of bovine articular cartilage induces chondrocyte apoptosis. Arch Biochem Biophys 2000;381:205–12.
31. Quinn TM, Allen RG, Schalet BJ, Perumbuli P, Hunziker EB. Matrix and cell injury due to sub-impact loading of adult bovine articular cartilage explants: effects of strain rate and peak stress. J Orthop Res 2001;19:242–9.
32. Thibault M, Poole AR, Buschmann MD. Cyclic compression of cartilage/bone explants in vitro leads to physical weakening, mechanical breakdown of collagen and release of matrix fragments. J Orthop Res 2002;20:1265–73.

33. Komuro H, Olee T, Kühn K, Quach J, Brinson DC, Shikhman A, *et al*. The osteoprotegerin/receptor activator of nuclear factor kappaB/receptor activator of nuclear factor kappaB ligand system in cartilage. Arthritis Rheum 2001;44:2768–76.
34. Jules J, Ashley JW, Feng X. Selective targeting of RANK signaling pathways as new therapeutic strategies for osteoporosis. Expert Opin Ther Targets 2010;14:923–34.
35. Murata M, Bonassar LJ, Wright M, Mankin HJ, Towle CA. A role for the interleukin-1 receptor in the pathway linking static mechanical compression to decreased proteoglycan synthesis in surface articular cartilage. Arch Biochem Biophys 2003;413:229–35.
36. Bevill SL, Thambyah A, Broom ND. New insights into the role of the superficial tangential zone in influencing the microstructural response of articular cartilage to compression. Osteoarthritis Cartilage 2010;18:1310–8.
37. Buckley MR, Gleghorn JP, Bonassar LJ, Cohen I. Mapping the depth dependence of shear properties in articular cartilage. J Biomech 2008;41:2430–7.
38. Chen AC, Bae WC, Schinagl RM, Sah RL. Depth- and strain-dependent mechanical and electromechanical properties of full-thickness bovine articular cartilage in confined compression. J Biomech 2001;34:1–12.
39. Schinagl RM, Gurskis D, Chen AC, Sah RL. Depth-dependent confined compression modulus of full-thickness bovine articular cartilage. J Orthop Res 1997;15:499–506.
40. O'Connor P, Orford CR, Gardner DL. Differential response to compressive loads of zones of canine hyaline articular cartilage: micromechanical, light and electron microscopic studies. Ann Rheum Dis 1988;47:414–20.
41. Rolauffs B, Muehleman C, Li J, Kurz B, Kuettner KE, Frank E, *et al*. Vulnerability of the superficial zone of immature articular cartilage to compressive injury. Arthritis Rheum 2010;62:3016–27.
42. Hoffmann J, Bohlmann R, Heinrich N, Hofmeister H, Kroll J, Künzer H, *et al*. Characterization of new estrogen receptor destabilizing compounds: effects on estrogen-sensitive and tamoxifen-resistant breast cancer. J Natl Cancer Inst 2004;96:210–8.
43. Patwari P, Gaschen V, James IE, Berger E, Blake SM, Lark MW, *et al*. Ultrastructural quantification of cell death after injurious compression of bovine calf articular cartilage. Osteoarthritis Cartilage 2004;12:245–52.
44. Englert C, Blunk T, Fierlbeck J, Kaiser J, Stosiek W, Angele P, *et al*. Steroid hormones strongly support bovine articular cartilage integration in the absence of interleukin-1β. Arthritis Rheum 2006;54:3890–7.
45. Schallenberger E, Schondorfer AM, Walters DL. Gonadotrophins and ovarian steroids in cattle. I. Pulsatile changes of concentrations in the jugular vein throughout the oestrous cycle. Acta Endocrinol (Copenh) 1985;108:312–21.
46. Kim HT, Lo MY, Pillarisetty R. Chondrocyte apoptosis following intraarticular fracture in humans. Osteoarthritis Cartilage 2002;10:747–9.
47. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995;21:1465–8.
48. Charriaut-Marlangue C, Ben-Ari Y. A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 1995;7:61–4.
49. Wakeling AE, Furr BJ, Glen AT, Hughes LR. Receptor binding and biological activity of steroidal and nonsteroidal antiandrogens. J Steroid Biochem 1981;15:355–9.
50. Howell A, Osborne CK, Morris C, Wakeling AE. ICI 182,780 (Faslodex): development of a novel, "pure" antiestrogen. Cancer 2000;89:817–25.
51. Thomas P, Pang Y, Filardo EJ, Dong J. Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. Endocrinology 2005;146:624–32.
52. Jenei-Lanzl Z, Straub RH, Dienstknecht T, Huber M, Hager M, Grässel S, *et al*. Estradiol inhibits chondrogenic differentiation of mesenchymal stem cells via nonclassic signaling. Arthritis Rheum 2010;62:1088–96.
53. Arokoski JP, Hyttinen MM, Helminen HJ, Jurvelin JS. Biomechanical and structural characteristics of canine femoral and tibial cartilage. J Biomed Mater Res 1999;48:99–107.