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**Review**

# In vitro models for the study of osteoarthritis

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**Highlights**

- No in vitro model of osteoarthritis has been validated against the native disease
- Cytokine and compression models are most commonly used
- Cytokine based models often use concentrations far greater than values measured in vivo
- Supraphysiological loads are also used often to exaggerate the response
- The development of an in vitro model might require a combinatorial, multi-modal approach


## Abstract

Osteoarthritis (OA) is a prevalent disease of most mammalian species and is a significant cause of welfare and economic morbidity in affected individuals and populations. In vitro models of osteoarthritis are vital to advance research into the causes of the disease, and the subsequent design and testing of potential therapeutics. However, a plethora of in vitro models have been used by researchers but with no consensus on the most appropriate model. Models attempt to mimic factors and conditions which initiate OA, or dissect the pathways active in the disease. Underlying uncertainty as to the cause of OA and the different attributes of isolated cells and tissues used mean that similar models may produce differing results and can differ from the naturally occurring disease.

This review article assesses a selection of the in vitro models currently used in OA research, and considers the merits of each. Particular focus is placed on the more prevalent cytokine stimulation and load-based models. A brief review of the mechanism of these models is given, with their relevance to the naturally occurring disease. Most in vitro models have used supraphysiological loads or cytokine concentrations (compared with the natural disease) in order to impart a timely response from the cells or tissue assessed. Whilst models inducing OA-like pathology with a single stimulus can answer important biological questions about the behaviour of cells and tissues, the development of combinatorial models encompassing different physiological and molecular aspects of the disease should more accurately reflect the pathogenesis of the naturally occurring disease.

*Keywords:* Cytokine; In vitro; Loading; Model; Osteoarthritis

## Introduction

Osteoarthritis (OA) is the most common form of arthritis and is one of the leading causes of disability worldwide. Most mammalian populations are affected, including humans and domesticated animal species including sheep (Vandeweerd et al., 2013), horses (Ireland et al., 2013), cats (Clarke et al., 2005) and dogs (Clements et al., 2009). Estimates in human populations suggest that 9.6% of men and 18% of women over the age of 60 years have symptomatic OA (Woolf and Pfleger, 2010). In 2006-2007 in the United Kingdom 94% of hip and 97% of knee replacements were carried out for relief of OA, costing an estimated £809 million[1]. Consequently OA is a major concern, particularly in ageing populations (Nguyen et al., 2011).

Symptoms of OA most commonly include pain, swelling and stiffness in the affected joint, resulting from the degradation of articular cartilage (Madry et al., 2012), changes in the composition of the subchondral bone (Sniekers et al., 2008; Saito et al., 2012) and synovitis (Goldhammer et al., 2010). Historically, OA was primarily observed in elderly individuals which led to the idea that OA was a 'wear-and-tear' type disease (Berenbaum, 2013). However, this idea is now less favoured because younger patients often display symptoms of OA secondary to injuries or because of a genetic predisposition to the disease (Da Silva et al., 2009).

OA is a multifactorial disorder and no single aetiological mechanism has been found common to all forms of the disease (Iliopoulos et al., 2007). Large genetic studies have identified numerous genetic risks for OA (Reynard and Loughlin, 2013), although the odds ratios for most single nucleotide polymorphism (SNP) associations are low, and rarely do

[1]See: http://www.arthritisresearchuk.org/arthritis-information/data-and-statistics/osteoarthritis.aspx (accessed 24 November, 2014)
£1 = approx. US$1.50, €1.36 at 06 April 2015.

they apply to more than one form of the disease. The mechanisms which underpin genetic risks are often unidentified, and in vitro models are critical if we are to expand our understanding of their role in disease progression.

Both in vivo and in vitro models of OA have been used in the past (Goldring et al., 2008; Grenier et al., 2014; Fang and Beier, 2014). Whilst models can be broadly grouped according to the primary mechanism by which the catabolic process is stimulated, each with their own strengths and weaknesses (Table 1), subtle variations mean that an almost infinite number of variations exist for a single model (Benam et al., 2015). Similarly, whilst in vivo models may give the most accurate reflection of the naturally-occurring whole-joint disease, the ease of manipulating an in vitro system, as well as a shift towards the 3R philosophy of refining, reducing and replacing the use of animals in animal science (Madden et al., 2012) makes in vitro modelling of the disease desirable. The observation that spontaneous OA in domestic animals has a similar pathogenesis to that observed in humans (Clements et al., 2006), and the availability of naturally-occurring, early-stage diseased tissue, for example at slaughter in food animal species or following a veterinary surgical intervention in companion animals (Clements et al., 2009) makes domestic animals an important source of clinical material for such models.

Additionally, models of OA offer the opportunity to study early features of the development of the disease, prior to the development of a fulminant catabolic process, which have been difficult to dissect because of the lack of available tissue from early disease and the limited molecular changes associated with it (Aigner et al., 2006). No consensus on the most appropriate model for the representation of particular features of OA has been made, as each model has its own mechanisms for the induction of a general catabolic process. Furthermore,

the molecular phenotypes of different forms of OA also show distinct differences (Xu et al., 2012), and such subtleties can be considered when designing models of OA rather than ignoring them when using more general in vitro models.

The relevance of in vitro models to clinical disease always needs to be interpreted with caution. For example, numerous publications report the chondroprotective effects of glucosamine and chondroitin sulphate on in vitro models (Dechant et al., 2005; Chan et al., 2007), but clinical trials have failed to show such effects in vivo (Wandel et al., 2010; Sawitzke et al., 2010). Whilst some effects of the differences can be ascribed to delivery, complexity, duration and variation of the phenotype, ultimately in vitro models should be designed to better reflect the natural in vivo disease. This particularly applies to the disease state, where almost all models are designed to replicate the symptoms of end-stage OA with little or no regard to the early disease where chondroprotection is likely to yield greater benefits.

This review summarises the two most commonly used in vitro models of OA, namely, cytokine-based models and load-based models, and then discusses their various merits and how they reflect the naturally-occurring processes.



**Cytokine induction of OA-like processes**

Classic research on OA has focused on cartilage, but other tissues such as bone, synovium, ligament, infrapatellar fat (Maccoux et al., 2007) and periarticular muscles (Geyer et al., 2009) are also involved (Fig. 1). The changes seen in these tissues are attributed to diffusible factors, including proteolytic enzymes, such as matrix metalloproteinases (MMPs) and members of the ‘a disintegrin and metalloproteinase with thrombospondin-motif’

(ADAMTS) family that are present in the joint environment during the disease (Little et al., 2005; Clements et al., 2006).

*Cytokines in OA*

During OA, synoviocytes, mononuclear cells or chondrocytes may increase their expression of catabolic proteins (Fernandes et al., 2002) following stimuli such as cytokine or chemokine exposure, including interleukin (IL)-1β and tumour necrosis factor (TNF)-α, which are present in the joint following synovial inflammation (Sohn et al., 2012). Pro-inflammatory cytokines make ideal candidates for the induction of OA-like biological changes in articular cells or tissues in culture, in which temporal and concentration effects can be explored.

Models of OA where cytokines are the primary method of induction are very common and are generally well understood. The model is usually inexpensive and is very easily manipulated. The ability to expand cells in vitro also means that many replicates are possible, allowing multiple hypotheses to be tested from single sources of tissue. Nevertheless, cells in culture (particularly chondrocytes) are prone to dedifferentiate to fibroblasts after only a small number of passages (Caron et al., 2012; Minegishi et al., 2013), and isolating cells from their matrix removes possible matrix-effects. Additionally, inter-tissue crosstalk is difficult to characterise in vitro and both time- and concentration-dependent effects are not well understood (Table 1).

Evidence for a role of IL-1β in OA is well established, and it has been used as a potential therapeutic target, for example through the design of vectors activated by IL-1 that protect against its catabolic effect (Campbell et al., 2005) or through the antagonism of the

IL-1 receptor (IL-1R) (Chevalier et al., 2009). Exposure to IL-1β stimulates chondrocytes and synovial cells to produce catabolic proteases (Maccoux et al., 2007) with apocrine signalling further enhancing MMP release and the resulting degradative cascade. The catabolic response can be blocked by the inhibition of IL-1β through antagonism with the IL1-R antagonist (IL-1Ra) (Bujak and Frangogiannis, 2009).

Inflammatory molecules produced by chondrocytes in response to IL-1β, include prostaglandin (PG)$E_2$, cyclooxygenase (COX)-2, IL-6, IL-8 and leukaemia inhibitory factor (LIF). IL-1β also leads to the accumulation of reactive oxygen species, through expression of inducible nitric oxide synthase (iNOS) by the transcription factor nuclear factor kappa B (NF-κB), ultimately leading to apoptosis (Fig. 2a). This mechanism can also be accelerated by IL-1β-mediated damage to mitochondrial DNA, leading to a further release of reactive oxygen species and enhancing apoptosis (Loeser, 2011).

IL-1 plays a role in bone pathophysiology relevant to OA, particularly IL-1α which is also known as osteoclast activating factor (Lee et al., 2010). In bone, there is an increase in the activity of $PGE_2$ in osteoblasts and stromal cells, as well as an increase in the expression of receptor activator of NF-κB Ligand (RANKL). RANKL is critically involved in the activation, maturation and survival of osteoclasts (Tanaka et al., 2005). IL-1 has also been shown to induce multinucleation of osteoclasts, thus potentiating the function of the cells. In vivo, when adult rats were injected with a moderate amount of IL-1β (1 μg/kg bodyweight), an increase in serum and urinary $Ca^{2+}$ concentration was noted, as well as an increase in osteoclast number, implying an increase in bone resorption (Nguyen et al., 1991)

TNF-α has also been used to induce OA-like changes in in vitro experiments, because it is found in diseased synovial fluid (Horiuchi et al., 1999; Fujita et al., 2005), and is able to induce catabolism and inhibit anabolic pathways in joint tissues and cartilage cells (Liacini et al., 2003). While IL-1β and TNF-α are the most commonly used cytokines in modelling OA, other cytokines may also play important roles. Concentrations of IL-6, IL-8, vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) are all increased in the synovial fluid of OA joints (Sohn et al., 2012). Osteoclasts are recruited by IL-6, and thus it may be an important modular of the bone remodelling observed in OA (Silfverswärd et al., 2004). However, in model designs, these cytokines are rarely considered, possibly because they are characterised as chondrocyte-derived and thus they can be induced by other cytokines such as IL-1β or TNF-α (Bunning et al., 1990). Using cytokines in combination may allow for the induction of OA-like cell and tissue responses that more closely replicate the natural disease, particularly in lieu of synovial effects in the model design.

*In vivo determination of cytokine concentrations*

Cytokine-based models use a wide variety of concentrations and durations of cytokine stimulation, namely those which produce a measurable downstream effect, rather than a concentration that reflects that in naturally occurring disease. Besides, OA is a slowly-progressing disease, and relatively small increases in cytokine concentrations have been identified in naturally-affected joints.

When OA synovial fluid is assayed, the quantities of IL-1 (< 2 ng/mL) and TNF (almost 3 ng/mL) are highly variable between experiments, but are low in comparison with those used to exert an effect in vitro (Table 2). The variation in physiological concentrations

is evident and may be the result of several factors, including the method used to quantify the cytokines, or the phenotype of the disease. In contrast, the concentrations used in models are typically much higher at up to 100 ng/mL of IL-1β (Macrory et al., 2009), and up to 50 ng/mL of TNF-α (Gabriel et al., 2010).

*Explant-based models of cytokine stimulation*

Explant-based models are simple and easy to produce, and have the major advantage that they can be used to examine the response of cells in their natural extracellular matrix and, once removed from their extracellular matrix, the cell phenotype is altered (Zien et al., 2001). Using explanted tissue also allows features such as matrix degradation to be observed. However, the use of tissue explants creates new problems; for example, cells at the explant edge die (Hunziker and Quinn, 2003; Gilbert et al., 2009), there are limitations to the number of samples which can be obtained from the same source and more than one tissue might be required to maintain viability (Amin et al., 2009).

Cartilage is highly sensitive to TNF-α and physiologically relevant concentrations as low as 0.25 ng/mL (Westacott et al., 1990) are sufficient to increase the release of glycosaminoglycans (GAGs) from OA cartilage (human) when compared with healthy cartilage in a 14 day period (Westacott et al., 2000). Species-specific differences may exist in the stimulation required to elicit a particular response (such as GAG release); thus, GAG release from feline cartilage explants requires stimulation with both recombinant human IL-1β and oncostatin-M (OSM) in combination (Gabriel et al., 2010), although a feline-specific stimulus may have elicited a different response.

A possible autocrine network has been suggested because both IL-β and TNF-α show strong positive protein staining in the superficial zone of cartilage as well as in the synovial fluid in late-stage OA. Meanwhile, deep zone cells only demonstrate marginal staining in the most severe cases (Tetlow et al., 2001), illustrating the differential responses of chondrocytes in disease. Notably, the early stages of disease rarely demonstrate any chondrocyte expression of cytokines, implying that any inflammatory cytokines present in the joint at the early stage of the disease are most likely to be synovial in origin (Tetlow et al., 2001).

*Chondrocyte culture-based cytokine models*

The choice of whether to use a monolayer, a cell scaffold or intact tissue will influence the cells' response to the cytokine stimulus applied. The sensitivity of chondrocytes to their molecular and loading environment dictates that ideally they should not be isolated from their matrix, or if they are, the matrix in which they are embedded should closely match the behaviour of normal, healthy tissue. However, the low cellularity of cartilage tissue necessitates the demand for large explants, thereby reducing the number of replicates which can be obtained from a single tissue source. Monolayer cultures allow the expansion of the cellular resource, although this is finite for tissues such as cartilage (Nicholson et al., 2007), as the cell phenotype changes in monolayer culture (Zien et al., 2001).

The ease of using chondrocytes in monolayer combined with their rapid response to cytokine stimulation has resulted in this being the most widely used model. Numerous models that use cytokines added to cell or tissue culture medium have been shown to produce OA-like responses in chondrocytes in monolayer, such as a decrease in the expression of type 2 collagen and aggrecan, and an increase in the expression of MMP-13, across multiple species (Miyaki et al., 2009; Novakofski et al., 2012; Yang et al., 2014).

Alternatively, stimulating chondrocytes with the synovial fluid from OA patients (Hoff et al., 2013), a more physiologically-relevant stimulus, produces similar results, including the expression of the pro-inflammatory cytokines IL-6, IL-8, IFN-γ, MCP-1, granulocyte-colony stimulating factor (G-CSF) and VEGF. However, this method of stimulation is also limited by the imprecise understanding of the relative contribution of different mediators which are driving catabolism in this model, and the lack of repeatability because of the limited synovial fluid volume that can be obtained from a single source.

*Co-culture-based cytokine models*

Recognising that OA is a disease that affects and involves the interaction between multiple tissues are co-culture experiments that permit the study of these interactions in vitro. Cytokine or osmotic pressure stimuli can be easily applied to co-culture models, though the tissues might require different culture conditions, necessitating some compromise on the culture conditions used when cultured together. The co-culture of synovial membrane with chondrocytes is one method by which the complexity of the pro-inflammatory cascade can be reproduced in vitro, because synovium is the primary source of these mediators (Ushiyama et al., 2003).

Co-culturing synovium from OA patients with healthy cartilage explants produces an increase in the expression of IL-1, IL-4, IL-7, IL-8, IL-10, IL-13 and osteoprotegerin (OPG), similar to synovial fluid from OA joints, as well as reducing GAG production in the cartilage (Beekhuizen et al., 2011). Whilst it may be desirable to use synovium to model OA in vitro, it is composed of two different, but interacting, cell types and shows highly variable lesion patterns both across different OA joints, and within a single joint with clinical OA (Rhodes et

al., 2005; Goldhammer et al., 2010; Smith, 2011). Consequently, deconstructing the effects within the synovial co-culture model is complex, although characterisation of the factors responsible for the response will help to standardise across experiments. The use of bone in co-culture experiments is also important, because it appears to have a role in maintaining the long-term viability of chondrocytes in the superficial zone of articular cartilage (Amin et al., 2009).

*Bone cytokine models*

Evaluation of the response of bone to cytokine stimulation in models of OA is scarce, which is unsurprising because it is difficult to ascertain the precise cytokine environment to which bone cells are exposed to in OA. Most data on cytokine roles in bone focus on the specific roles of TNF-α, IL-6 and IL-1 release during osteocyte injury (Komori, 2013). Further, generation of RANKL is induced by IL-6 and IL-1, and mice lacking RANKL completely lack osteoclasts (Kong et al., 1999). Osteoclasts driven to apoptosis release soluble RANKL, and conditioned media from these cells further induce osteoclastogenesis (Al-Dujaili et al., 2011). Notably, bone plays a larger role than previously considered in cartilage health, and removal of cartilage explants from the underlying bone tissue leads to a higher percentage of cell death in chondrocytes than if chondrocytes were left attached to the bone (Amin et al., 2009).

Mouse calvarial cultures incubated with IL-1 showed bone resorption, demonstrating that cytokines act on cultured bone (Gowen et al., 1983), and cultured osteoblasts actively synthesised NO in response to IL-1α in a dose-dependent fashion, although IL-1β, TNF-α and IFN-γ failed to elicit a response (Ake et al., 1994). Evidently, cytokines play a role in bone turnover, and bone is responsive to inflammatory stimuli. IL-1β has been shown to induce the

293 release of $PGE_2$, MMP-3 and MMP-13 from osteoblasts (Pecchi et al., 2012), although this
294 can be inhibited with chondroitin sulphate.
295
296 **Load-based models of inducing OA**
297 Chondrocytes are sensitive to load, and must always be under sufficient force to
298 maintain extracellular matrix homeostasis, yet below that which induces apoptosis or
299 stimulates an inflammatory cascade within the tissue (Henrotin et al., 2012). Subchondral
300 bone is also mechanosensing, and responds in vivo by changing its thickness and reducing its
301 resorption when loading is increased (Murray et al., 2001). Identifying the load thresholds
302 that alter the balance from maintenance of homeostasis to injury is important to our
303 understanding of the magnitude of a beneficial or deleterious load.
304
305 Load models are easily manipulated and, as a result, high throughput experiments can
306 be performed. Signalling pathways associated with mechanotransduction are becoming well
307 understood (Millward-Sadler and Salter, 2004; Mobasheri et al., 2005), allowing better
308 appreciation of the processes associated with this model. Removing cells from their native
309 matrix and embedding them into an artificial scaffold alters, at least theoretically, the native
310 signalling network, and the force used in the experiment is innately dependent on the ability
311 of the scaffold to withstand that force.
312
313 Cell loading models require high cell numbers that might not be available from some
314 sources. Using entire tissues overcomes this problem, and allows cells to use natural cell-
315 matrix interactions and cross-tissue communication as well as much greater forces.
316 Conversely, native tissue experiments are limited to larger species and lower numbers of
317 replicates. Additionally, cells have been shown to undergo substantial cell death at the cut

edge (Huntley et al., 2005; Huntley et al., 2005a) so distorting observations at these sites (Table 1).

*In vivo determination of cartilage load*

Several in vivo studies have attempted to determine the physiological pressures experienced by articular cartilage during loading. The pressure passing through the load-bearing region of a human acetabular prosthesis has been determined to be approximately 3.5 MPa during locomotion (Hodge et al. 1989), and the articular contact pressure of the human knee does not exceed 8 MPa, even when the menisci have been removed (Fukubayashi and Kurosawa, 1980). Loads of a similar magnitude have been reported in both the medial and lateral compartments of canine elbow joints, with mean contact pressures between 3.0 and 4.0 MPa and peak pressures between 6.6 and 9.1 MPa (Cuddy et al., 2012). These data suggest that the articular loading experienced by different joints in different species are physiologically comparable, and concurs with estimates of the articular cartilage compressive stress in different mammalian species, which only vary within one order of magnitude from mice to cows (Simon, 1970).

Chondrocytes sense the loading of their environment through integrin receptors (Bader et al., 2011). When activated, the integrins stimulate stress pathways leading to cytoskeletal disruption and release of inflammatory cytokines, such as IL-1β and TNF-α (Valhmu et al., 1998; Durrant et al., 1999; Bader et al., 2011). Cytokine-induced proteolytic enzyme release is mediated by nitric oxide, $PGE_2$ and reactive oxygen species. The extracellular proteins cleaved by the activated proteases are then capable of further induction of both proinflammatory cytokines and matrix proteases, though the receptors activated by collagen fragments remain elusive (Klatt et al., 2009).

The signalling pathways induced by static loading and cytokine induction are similar and the mechanism that governs both is similar in both models (Fig. 2b). The compression pathway, however, appears reliant on the magnitude and duration of the stress (Fanning et al., 2003).

*Explant based models of cartilage loading*

The use of tissue explants, assessing the response of cells embedded in the natural matrix, is the simplest method for assessing the effects of load. Load is applied to tissue explants through various methods, based on the variable in question. Most typically, 'drop towers' in which a free weight is released from a predetermined height onto the tissue are used to impart a single impact load. This is believed to replicate the development of 'post traumatic' OA, which occurs following an injurious articular load. Load can also be applied in a cyclical manner with devices such as pneumatic or hydraulic loading chambers.

Static loading can induce similar deleterious changes in cartilage explants when applied at an appropriate magnitude. For example a compressive strain of 50% applied to a cartilage explant results in a decrease in the synthesis of collagen type II and proteoglycans (Chen et al., 2001). Static compression of calf patellofemoral cartilage to 25% or 50% strain for 24 h produces deleterious changes in cartilage metabolism, resulting in an increase in expression of *MMP3, 9* and *13* mRNA and decrease in *COL2A1* and aggrecan (*ACAN)* within 1-2 h post loading (Fitzgerald et al., 2004). IL-1 receptor activation and activation of the extracellular-signal related kinase 1/2 (ERK1/2), p38, mitogen-associated protein kinase (MAPK) family member pathways in a time-dependent manner meditate these changes (Fanning et al. 2003).

A load equivalent to 1.5 × bodyweight placed on a human knee joint caused only 10% strain in the patellofemoral cartilage following 10 min of static loading (Wong and Sah, 2010). Similarly, intact human femoral head cartilage loaded to the equivalent of a single leg stance (less than 2.3 × bodyweight) is subject to a strain of 33% (Greaves et al., 2010). Consequently the use of higher strains in in vitro models exaggerates the maximal normal physiological load experienced by a joint in vivo, although this reflects the requirement to induce an effect within a shorter timeframe. Furthermore, the elastic (Young's) modulus varies across cartilage within a joint (Shepherd and Seedhom, 1999), and thus the load required to induce a specific strain, or strain produced from a specific load will also differ across samples from the same joint.

In vitro studies have been used to determine the critical stress thresholds of cartilage explant, in which apoptosis, collagen degradation and nitrite accumulation are observed. Values range between 4.5 MPa for cyclic loading (six compressions to a final strain of 30-50%, held for 5 min rested for 25 min, Loening et al., 2000) and 15 MPa for a single impact load (Torzilli et al., 1999) for bovine cartilage, although the results between experiments are highly variable. Notably, bovine cartilage explants subjected to a 0.5 MPa cyclic loading increase proteoglycan synthesis across various cycle lengths (Parkkinen et al., 1992) supporting the hypothesis that moderate loading is beneficial to cartilage health.

*Chondrocyte culture-based loading models*

The response of cells to load can be dissected further by isolating cells in culture, embedding them in an artificial matrix, and compressing them using a bioreactor. This has the advantage of permitting very precise changes in loading parameters, as it is highly

reproducible, as well as looking at the effects of different matrices on the cellular response. However, a large caveat to such experiments is that the cell response observed in vitro may not represent that observed in vivo where the interaction of the matrix is critical to the effect produced.

In vitro loading of isolated chondrocytes seeded in a 3-dimensional (3-D) culture (typically agarose) results in both an increase in cell proliferation and proteoglycan synthesis when cyclically loaded at a physiological strain of 15% (Lee and Bader, 1997). 3-D culture also allows for the application of bi-axial cyclic loading (direct compression or tension, and shear) (Pingguan-Murphy and Nawi, 2012), and addresses the observation that in vivo several loads may simultaneously impact on a joint during normal activity. When subjected to biaxial loading (10% compressive strain with 1% sheer strain) for two 12 h periods, separated by a 12 h resting period, both cell proliferation and an increase in GAG production were observed, demonstrating the importance of closely representing the joint environment in in vitro experiments.

Cyclic loading of bovine chondrocyte-embedded calcium polyphosphate scaffolds at 1 kPa and 1 Hz for 30 min, activates Erk1/2 and c-Jun N-terminal kinase (JNK) as seen with static loading, and causes an increase in activator protein-1 (AP-1) binding. This stimulus induces *MMP-3* and *13* expression and results in MMP-13 mediated extracellular matrix (ECM) degradation. However, following the loss of functional tissue, collagen type II and aggrecan gene expression occurs after 12 h, and synthesis occurs by 24 h post loading (Fig. 2c) (De Croos et al., 2006).

*Co-culture based loading models*

Co-culture methods have been used to explore the effect of loading on articular tissues in parallel, although they are challenged by the necessity to use different culture conditions for each tissue, or to compromise the culture conditions. For example, alginate-embedded chondrocytes cultured on a porous filter above mechanically stretched osteoblasts become more hypertrophic during stimulation of the osteoblasts. This change was most pronounced when the osteoblasts were subjected to tensile loads, suggesting molecular 'cross-talk' occurs between the two cell types in response to mechanical stress in bone (Lin et al., 2010).

Co-culture of OA osteoblasts with healthy alginate bead-embedded chondrocytes result in a phenotypic shift to chondrocyte hypertrophy and matrix mineralisation, which does not occur with healthy osteoblasts stimulated with IL-1, IL-6 or OSM (Sanchez et al 2005). This demonstrates the limits of artificially stimulating cells and the phenotypic differences of naturally-diseased cells and highlights the need for better characterisation of the soluble factors released by these cells as well as better definition of the molecular stimulation required to induce the OA phenotype in healthy cells.

*Bone loading models*

Osteocytes are the major mechanosensors of bone, although they are rarely included in models of OA because they are notoriously difficult to culture in vitro. Analysis of the osteocyte (in contrast to osteoblast) response to compression has been hindered due to this challenge (Kato et al., 1997). Osteocyte cell lines seeded into type I collagen gels layered with osteoblasts on their surface respond to mechanical loading, with co-cultured constructs increasing type I collagen expression with loading, and osteocyte embedded gels expressing $PGE_2$ after mechanical stimulation (Vazquez et al., 2014).

*Relevance of load: From in vivo to in vitro models*

The validity of a loading model depends, at least to some extent, on its relationship with the natural environment of the joint and its loading in vivo. The loading parameters of a selection of cell and tissue loading models are presented in Table 3. The use of scaffolds can impart some structure to the cells for culture-based models, and permit cyclic loading of isolated cells, albeit within ranges that are governed by the strength of the matrix in which they are embedded.

The heterogeneity of the cartilage structure means that precise reconstruction of the tissue in vitro may not be possible, and so compromises must be made when constructing a load based model (Gannon et al., 2012). The individual phenotype of each chondrocyte is related to their location in the tissue (Fujioka et al., 2013; Schuurman et al., 2015) and therefore in homogeneous tissue models, chondrocytes may not behave in the same manner.

**Future directions**

The multifactorial nature of OA should be considered when designing a model to reproduce it, even if it is only testing a single parameter, such as the response to a load or catabolic stimulus. A deeper understanding of the pathways evoked in in vitro models, and their relevance to the changes seen in naturally-occurring OA phenotypes is important in order to improve the translational relevance of the conclusions drawn.

To date the vast majority of explant- and culture-based models have assessed cartilage in isolation. While this might reflect the ease of manipulating cartilage and the resilience of chondrocytes in cell culture, progress in tissue engineering and cell culture techniques will

allow for the development of more advanced models including other cell types. The responsiveness of bone to stimuli, and the cross talk that occurs between the different tissues in OA joints dictate that models should consider the role of multiple tissues when assessing the response to a given stimulus so as to enable more meaningful translation to the anticipated response in vivo.

There is no all-encompassing model that is suitable for all studies of OA, and no single model can be used to perfectly simulate naturally-occurring events. Whilst models seek to answer specific biological questions, more standardised end-points for the molecular and physiological parameters assessed are necessary, as at present it is impossible to directly compare the outcomes of the many in vitro OA models published in the scientific literature.

Other areas of articular health research, such as those looking at histological assessments of cartilage repair and damage[2] (Glasson et al., 2010), have developed guidelines upon which assessments should be made through the consensus of experts and the publication of their recommendations. In molecular biology, similar guidelines have been developed for performing microarray and quantitative polymerase chain reaction studies (Brazma et al., 2001; Bustin et al., 2009). Whilst the in vitro models of OA have many different functions and outcomes, guidelines could still be developed to determine the endpoints which are matrix- and chondro-protective, and the minimum number of features of a model (such as measures of matrix release and turnover, transcription changes and/or cellular morphometric changes) which are agreed to represent an 'OA-like' scenario. Similarly, models seeking to investigate the pathogenesis of OA must justify the nature of stimulatory conditions, relative to the in vivo disease, beyond simply that required to produce a response. At the very least in

[2] See: http://www.cartilage.org/_files/contentmanagement/ICRS_evaluation.pdf (accessed 7 April, 2015)

vitro models should be standardised to a particular disease phenotype, with reasoning for the source of the cells and/or tissues used and the outcomes to be measured, as there is no single OA phenotype which can be encompassed by all models.

## Conclusions

The molecular pathways underpinning cytokine-stimulation and load-based in vitro models of disease are similar. The combination of different models types may permit the use of stimuli which are physiologically relevant, and which allow us to understand the development and progression of the disease, particularly the early phase, rather than simply the catastrophic downstream events after it has begun. Standardisation of the approaches, both within and between different species will allow the wider applicability of results between studies, which in turn will enhance our understanding of the disease.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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885

886 **Tables**

887 **Table 1**

888 Advantages and disadvantages of some of the most commonly used in vitro models

| Model Type | Variables that can be applied | Advantages | Disadvantages | Examples |
|---|---|---|---|---|
| **Monolayer culture** | Cytokine stimulation, osmotic pressure | Allows expansion of cellular resource from a single sample, investigation of distinct pathways in isolation | Altered phenotype of isolated cells due to isolation from tissue and absence of normal extracellular matrix | (Sylvester et al., 2012; Novakofski et al., 2012) |
| **Co-culture** | Cytokine stimulation, osmotic pressure | Considers cross-talk between cell types | Altered phenotype of isolated cells<br><br>Different cell types require different culture conditions, or compromise if culture together | (Lin et al., 2010; Beekhuizen et al., 2011; Vazquez et al., 2014) |
| **3D-culture** | Cytokine stimulation, osmotic pressure, physical injury and loading regimens. | Affords structure and force to sensitive cells. | Magnitude of force is scaffold dependent, and may not reflect that of the normal tissue<br><br>Isolation and expansion of cell types first | (Mizuno and Ogawa, 2011; Bougault et al., 2012; Pingguan-Murphy and Nawi, 2012) |
| **Explant** | Cytokine stimulation, osmotic pressure, physical injury and loading regimens. | Inexpensive, easily produced, cells maintained in normal extracellular matrix. | Cell death at cut edge of tissue, few replicates available from same source, more than one tissue type may be required to maintain viability, physical attributes may change in culture | (Fitzgerald et al., 2004; Bush et al., 2005; Jeffrey and Aspden, 2006; Gabriel et al., 2010) |

889

890 **Table 2**

891 Osteoarthritic synovial fluid interleukin (IL)-1β and tumour necrosis factor (TNF)-α

892 concentrations in vivo

| Condition | Cytokine | |
|---|---|---|
| | IL-1β | TNF-α |
| Control (human)<br>(Kahle et al., 1992) | <20 pg/mL | 2890 pg/mL |
| Control (canine)<br>(Fujita et al., 2005) | 490 pg/mL | 105.3 pg/mL |
| OA (human)<br>(Kahle et al., 1992) | 21 pg/mL | 80 pg/mL |
| OA - Hip dysplasia (canine)<br>(Fujita et al., 2005) | 2010 pg/mL | 600 pg/mL |
| OA - Mild (porcine)<br>(McNulty et al., 2013) | 109 pg/mL | - |
| OA - Moderate (porcine)<br>(McNulty et al., 2013) | 122 pg/mL | - |

893

894 **Table 3**

895 A summary of load-induced changes in OA models

| Species studied | Regimen tested | Duration of experiment | Findings of experiment | Citation |
|---|---|---|---|---|
| Dog (in vivo) | 20-40 km/day | 15 weeks | Increased water content. Decreased collagen content. Decreased proteoglycan content. | (Kiviranta et al., 1992) |
| Bovine explant | Six on-off cycles of 30-50% strain | 5 min compression: 25 min release | Apoptosis of cells. Degradation of collagen network. Glycosaminoglycan release. | (Loening et al., 2000) |
| Bovine explant | 100 g, 500 g and 1 kg dropped from 2, 5, 10 and 20 cm | N/A | Linear decrease in cell viability. Increased hydration of tissue. Partial depth fissures. | (Jeffrey et al., 1995) |
| Canine shoulder explant | 5 MPa at 0.3 Hz | 20 or 120 min | Cell death increased with increased loading time. Glycosaminoglycan and NO were not significantly altered. | (Levin et al., 2001) |
| Canine shoulder explant | 5 MPa at 0.3 Hz | 0, 2, 20, 120 min | Necrosis and apoptosis of cells increased with loading time. Proteoglycan increased in 2 and 20 minute groups but decreased at 120 minutes. | (Chen et al., 2001) |
| Agarose embedded equine chondrocytes | 15% strain either statically or cyclically (0.3-3 Hz) | 48 h | Dynamic strain increased cell proliferation. Static strain decreased glycosaminoglycan content, while cyclic strain increased glycosaminoglycan. | (Lee and Bader, 1997) |
| Bovine explant | 25-50% strain over 3 min period | Maintained for 1, 2, 4, 8 and 24 h | Relative expression of matrix genes decreased. Relative expression of proteases increased. | (Fitzgerald et al., 2004) |
| Human chondrocyte monolayer | 1, 5 or 10 MPa hydrostatic pressure at 1 Hz | 4 h per day for either 1 or 4 days | mRNA and protein expression of aggrecan and collagen type 2 upregulated after 4 days. No difference at 1 day. | (Ikenoue et al., 2003) |
| Full thickness human cartilage explant | Single mechanical load of 14 MPa for 500 ms | Measurements taken 96 h after injury | DNA fragmentation in 34% of loaded chondrocytes (vs. 4% of control) GAG release increased in loaded explants (1.9% vs. 0.8% total GAG content) | (D'Lima et al., 2001) |

896

## Figure legends

Fig. 1. Healthy and diseased synovial joint showing the changes in the entire joint organ. Bone weakness and wearing has been reported, as well as synovial thickening and swelling, subchondral bone thickening, osteophyte formation and cartilage degradation. In addition, tendons can become weak and inflamed and ligaments can become lax.

Fig. 2. Mechanistic pathways of in vitro models of OA. Both cytokines (a) and injurious loading (b) combine to cause the typical OA-like phenotype, showing apoptosis of cells, tissue degradation and inflammatory gene expression. In these cases, feedback loops occur either through inflammation, causing further cytokine stimulation, or abnormal loading, caused by tissue degradation. However, normal homeostatic loading is vital to tissue health, and works through similar pathways, as shown by the green line in (c), leading to tissue growth.

MAPK, mitogen-activated protein-kinase; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; NFκB, nuclear factor kappa B; COX2, cyclooxygenase-2; PGE2, prostaglandin-$E_2$; MMP, matrix metalloproteinase; ADAMTS, a distintegrin and metalloproteinase with a thrombospondin motif; ROS, reactive oxygen species.