Estrogen effects on cartilage and bone changes in models for osteoarthritis

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Estrogen Effects on Cartilage and Bone Changes in Models for Osteoarthritis

Oestrogeeneffecten op kraakbeen- en botveranderingen in modellen voor artrose

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Contents

List of abbreviations

ACTG1	actin gamma
ADAMTS	a disintegrin and metalloproteinase with thrombospondin-like motifs
AGC1	aggrecan
ANOVA	analysis of variance
BP	bisphosphonate
BV/TV	bone volume fraction
COL2	type II collagen
ConnD	connectivity density
CT	computed tomography
Ctrl	control
CTX-II	C-telopeptide of type II collagen
DMEM	Dulbecco's modified Eagle medium
E	estradiol
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
ERT	estrogen replacement therapy
ESR	estrogen receptor
GAG	glycosaminoglycan
GPR	G-protein coupled receptor
IA	(mono)iodoacetate
IGF	insulin-like growth factor
IL-1	interleukin-1
MMP	matrix metalloproteinase
OA	osteoarthritis
ORX	orchiectomy
OVX	ovariectomy
PGK	3-phosphoglycerate kinase
ROI	region of interest
RPL15	ribosomal protein L15
Sal	saline
SERM	selective estrogen receptor modulator
SMI	structure model index
TbTh	trabecular thickness
TGFB	transforming growth factor β
TGFβ	transforming growth factor β
TIMP	tissue inhibitor of matrix metalloproteinase
TNFα	tumor necrosis factor α
VEGF	vascular endothelial growth factor
wt	wild type

Chapter 1

General introduction

Osteoarthritis

Osteoarthritis (OA) is one of the most frequently occurring disorders of the musculoskeletal system. OA is characterized by progressive damage to the cartilage, osteophyte formation, subchondral bone changes, mild synovial inflammation, and weakness of muscles and tendons. This leads to joint pain, limitation of movement, deformity and disability, and therefore may cause many restrictions in daily life. Apart from the consequences for the patient, OA has serious economic impact due to its chronic course and high costs of interventions [1-3]. OA most frequently occurs in the knee, hip, hand, spine, and foot, but it can affect any synovial joint [4]. Although all tissues in the joint are affected by OA, articular cartilage and subchondral bone are the tissues of interest in this thesis.

Articular cartilage consists of a hydrated extracellular matrix in which a small number of chondrocytes (1-5% of the volume) is embedded. The major components of the extracellular matrix are collagens and proteoglycans. Type II collagen is the most abundant collagen type in articular cartilage. It forms a cross-linked network, also including type IX and XI collagen. The collagen network defines the form of the articular cartilage and provides strength and stiffness. The proteoglycans, which are highly negatively charged, are immobilized in the collagen network. To balance the negative charge, positively charged ions are attracted, leading to an osmotic pressure. This attracts water into the matrix, resulting in swelling of the matrix and a water content of 60-80% of wet weight. The swelling is constrained by the tensile stiffness of the collagen fibres. When the joint is loaded, water is squeezed out of the cartilage. After loading, water is absorbed again, driven by the osmotic pressure provided by the proteoglycans. In this way, the proteoglycans provide elasticity of the articular cartilage.

In osteoarthritis, the extracellular matrix is degraded, leading to progressive loss of articular cartilage. Degradation of either collagen or proteoglycans results in reduced resistance to mechanical loading (reduced strength/stiffness or reduced elasticity) and further damage of the extracellular matrix. This can lead to loss of cartilage at the surface and in the deeper zones, eventually exposing the subchondral bone. Degradation of the extracellular matrix is catalysed by a wide range of enzymes which are produced by the chondrocytes themselves, or diffuse into the cartilage from adjacent tissues. These enzymes include matrix metalloproteinases (MMPs) and aggrecanases termed "A Disintegrin And Metalloproteinase with ThromboSpondin-like motifs" or ADAMTS. MMP-1 and MMP-13 are mainly involved in the degradation of type II collagen, while MMP-3 and ADAMTS-1, -4, and -5 are mainly involved in the degradation of proteoglycans.

Apart from the cartilage damage, bone changes are also part of the disease process. These bone changes occur close to the joint, in the epiphysis. Since the osteoarthritic alterations are not uniform is this area, it is useful to distinguish separate anatomic entities that include the subchondral plate, the subchondral trabecular bone, and the bone at the joint margins (figure 1.1). Bone changes associated with osteoarthritis include progressive increase in subchondral plate thickness, alterations in the architecture and

mineralization of subchondral trabecular bone, formation of new bone at the joint margins (osteophytes), and development of subchondral bone cysts [5].

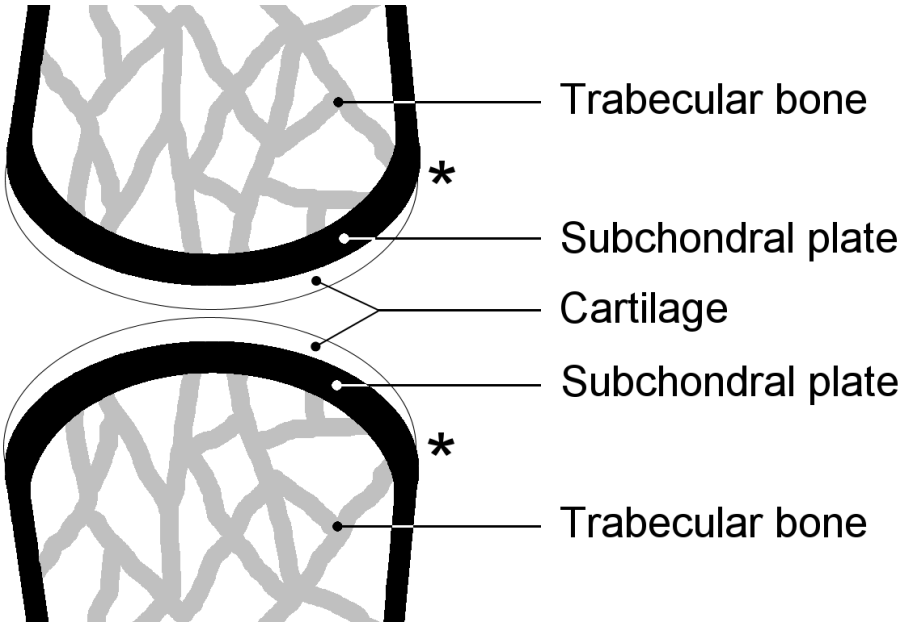

Figure 1.1: Schematic representation of cartilage and bone structures in a joint. The asterisks indicate the joint margins.

Whether these bone changes may initiate or contribute to the progression of cartilage damage is still a matter of debate. Radin and Rose [6] were among the first to suggest a possible role for subchondral bone in the initiation and progression of cartilage degeneration. They proposed that the increased thickness and volume in the subchondral bone in OA was associated with increased stiffness in the bone tissue and that these changes adversely affected the biomechanical environment of the overlying cartilage. However, their hypothesis has never been proven. Bone volume is not the only factor that determines the mechanical properties of bone. Additional factors include the architecture and material properties of the tissue. Due to increased remodelling rates in osteoarthritic bone, the tissue is hypomineralized and the effective tissue modulus (stiffness) is decreased [7] rather than increased. Still, the bone alterations may affect the biomechanical environment of the overlying cartilage.

Apart from this biomechanical role of bone in OA, it may also have a biochemical/nutritional role. Since cartilage is avascular, it depends on synovial fluid and subchondral bone for its nutrients supply [8]. In addition, cytokines and growth factors may also be transported from bone to cartilage. In OA, different cytokines and growth factors may be released from the bone [9]. Besides, changes in the subchondral bone may also affect its vasculature, and thus influence the nutrient supply towards the cartilage [10].

Estrogens and OA

From epidemiological studies, several risk factors for OA have been identified. Aging, obesity, gender and hormonal status, ethnicity, joint deformities, and abnormal loading are all known risk factors for OA [11]. However, not all risk factors offer a clear

mechanistic explanation. For instance, obesity could increase the risk for OA due to increased loading of the joint, or due to adipokines released from the fat tissue [12, 13].

One of the risk factors for OA is gender and hormonal status. Already in 1925, Cecil and Archer [14] described "arthritis of the menopause" as rapid development of hand and knee osteoarthritis coinciding with cessation of menses. More recent epidemiological studies have shown that men younger than 50 have a higher prevalence and incidence of OA than women younger than 50, whereas after the age of 50 women have a higher prevalence and incidence of the disease than men [15, 16]. The prevalence increases with age in both women and men, but in women, the prevalence of OA increases dramatically around the age of 50 [15, 17, 18]. This coincides with the start of menopause.

Estrogen replacement therapy (ERT) has been prescribed to postmenopausal women to relieve unpleasant menopause symptoms (e.g. hot flushes) and to prevent or treat osteoporosis. The effect of ERT on OA has also been studied. A recent systematic review showed that there is some evidence for a protective effect of ERT for hip OA, but for knee OA the results are conflicting [19].

Also in animal models the link between estrogens and OA has been investigated. In these studies, ovariectomy is used to simulate the postmenopausal situation in women. For instance, in rats and sheep ovariectomy has been shown to have an adverse effect on articular cartilage [20-22]. In addition, estrogen replacement therapy reduced cartilage degradation [20, 23]. However, a few other studies showed different results, with no effect of ovariectomy [24, 25], and an adverse effect of estrogen treatment [26].

To better understand the OA-protective effect of estrogens, more information about estrogen is needed: Estrogens are a group of steroid compounds. The three major naturally occurring types of estrogen are estrone, estradiol, estriol (figure 1.2). Estradiol is the most potent estrogen, with a potency 12 times that of estrone, and 80 times that of estriol. For this reason, estradiol is considered to be the major estrogen.

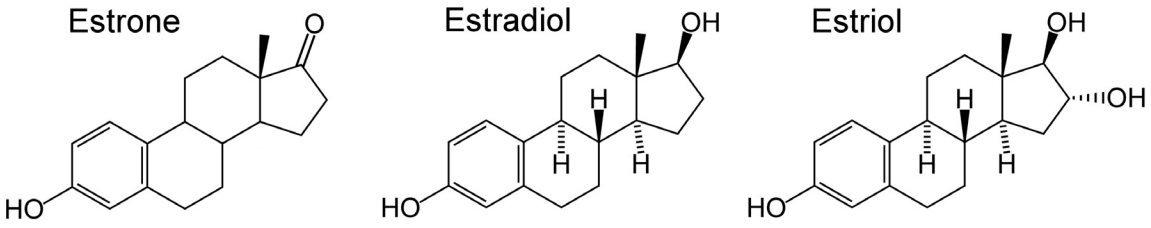

Figure 1.2: Chemical structure of the three estrogens: estrone, estradiol, and estriol.

In pre-menopausal women, estradiol is synthesized in the ovaries from cholesterol derived from the blood. Cholesterol is converted in several steps to androstenedione in the theca folliculi cells. In the granulosa cells of developing follicles androstenedione is aromatized to estrone, followed by conversion of estrone to estradiol. Alternatively, androstenedione is converted to testosterone, which in turn can undergo aromatization to estradiol. Smaller amounts of estradiol are also produced by the adrenal cortex, and (in men) by the testes. In addition, precursor hormones, specifically testosterone, are converted by aromatization to estradiol in other tissues than the gonads, such as muscle, fat, bone and nervous tissue [27-30]. These secondary sources of estrogen are especially important in postmenopausal women.

Estrogen signals by binding to estrogen receptors. There are two main subtypes of estrogen receptors: estrogen receptor α (ERα) and estrogen receptor β (ERβ). They vary

in structure and are encoded by different genes (ESR1 and ESR2, respectively). Both estrogen receptors are expressed in cells of many tissues. The tissue distributions of ERα and ERβ differ, although there is some overlap. These receptors are intracellular receptors, located in the cytoplasm. As free estrogen diffuses into the cell, it binds to the ligand-binding domain of the receptor, which dissociates from its cytoplasmic chaperones; the complex of estrogen and estrogen receptor then diffuses into the cell nucleus. These estrogen–estrogen receptor complexes bind to specific sequences of DNA called estrogen-response elements and initiate transcription [31]. Apart from these classical estrogen receptors, other receptors such as the trans-membrane G-protein coupled receptor (GPR30) have been suggested to bind estrogens [32, 33]. However, the functional role remains to be elucidated.

Bone cells express both estrogen receptors α and β [34-36] and estrogen is one of the important regulators of the balance between bone resorption and bone formation [37]. Estrogen receptors α and β are also expressed in chondrocytes in various animal species [38-41] and in humans [39, 42], indicating that cartilage is responsive to estrogens. In-vitro studies have indeed shown that estrogen affects proteoglycan synthesis [43], expression of matrix metalloproteinases [44, 45] and oxidative stress induced by reactive oxygen species [46]. However, the effects vary depending on the dose of estrogen applied. In addition to bone and cartilage, other tissues in the joint, such as ligaments and synovium, also express estrogen receptors [47, 48].

Thus, estrogen could affect the OA-process via bone, via cartilage, and/or via other tissues in the joint (figure 1.3). In this thesis, the pathways via cartilage and bone are studied.

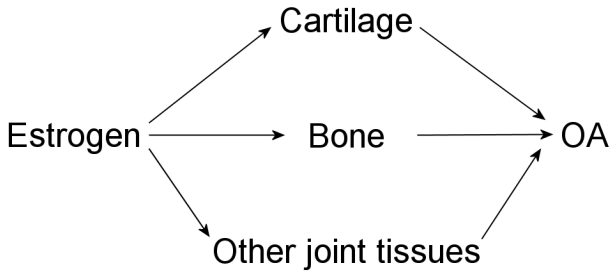

Figure 1.3: Possible routes via which estrogen affects OA.

Approach and outline of this thesis

Taken together, there are several indications that estrogens have an OA-protective effect, but the evidence is not conclusive, and the possible mechanism is poorly understood. In this thesis, the role of estrogens in OA is investigated at different levels, in order to obtain more insight into the potential protective effect of estrogens.

As already briefly mentioned, several animal studies have been performed investigating the effect of estrogen loss and estrogen treatment on OA. However, there is a lot of variation in experimental set-up, which may explain conflicting results. In **Chapter 2**, the current literature concerning the effects of ovariectomy and estrogen treatment on cartilage in animal models is reviewed in a systematic way.

When the estrogen receptors were discovered, mice lacking these estrogen receptors have been generated. In these mice, estrogen signalling is (partly) disturbed, which can

elucidate the importance of the separate estrogen receptors and the total estrogen signalling in all kinds of processes. The osteoarthritic process had not yet been studied in these mice. We investigated whether deletion of one or both estrogen receptors resulted in osteoarthritic changes in cartilage and subchondral bone of the knee, as is described in **Chapter 3**. Subchondral bone changes were studied using micro-computed tomography (micro-CT), which generates a three-dimensional dataset of the bone in a non-destructive way. Afterwards, the joints were sectioned for histology to study the cartilage changes.

Many, but not all postmenopausal women get OA [49], indicating that hormonal changes alone are not enough to cause OA. We hypothesize that estrogen depletion weakens the joint tissues and increases their susceptible to changes, but that an extra trigger is needed to develop osteoarthritic changes. In **Chapter 4**, this hypothesis is addressed by investigating the bone and cartilage changes in the knee joint of ovariectomized mice after a mild trigger that leads to OA-like changes. One group of mice received estrogen treatment in order to investigate whether estrogen could counteract the ovariectomy-induced changes. To study the role of bone changes, one group of ovariectomized mice received bisphosphonates, to inhibit the bone changes. The changes in bone architecture were studied using in-vivo micro-CT, which allows following changes over time in individual animals. In this chapter, the changes in bone and cartilage of the proximal tibia are described.

The knee consists of three bone structures: tibia, femur and patella (figure 1.4). Apart from the femorotibial compartment, the patellofemoral compartment is also often affected by OA. In **Chapter 5** the same hypothesis as in Chapter 4 is investigated using the same experimental set-up. This time the cartilage and bone changes in the patella are studied.

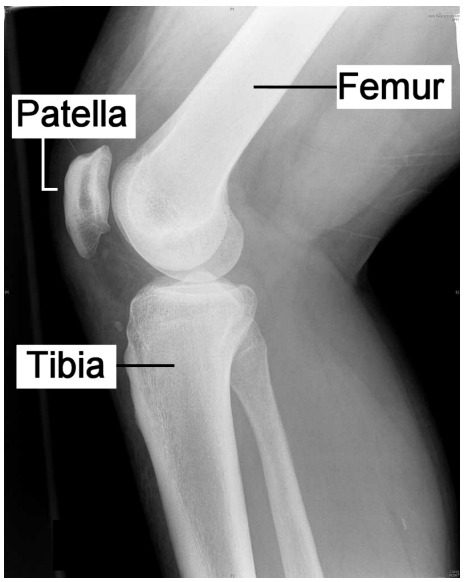

Figure 1.4: The knee joint consists of femur, tibia and patella. This X-ray image shows the lateral view of a human knee joint (adapted from [50]).

While animal studies provide a lot of insight in the osteoarthritic process of a joint, one cannot easily isolate the effects on one tissue. Cell or explant culture studies are more suitable for this. **Chapter 6** describes an in-vitro study focussing on the direct effects of estradiol on cartilage. Cartilage explants were cultured with and without estrogen, in the

absence and presence of the same osteoarthritis trigger as used in the in-vivo mouse model from Chapter 4 and 5. The effects on gene expression levels were studied.

The final chapter of this thesis discusses the findings of these studies and gives some directions for further research.

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Chapter 2

Animal models for osteoarthritis: the effect of ovariectomy and estrogen treatment. A systematic approach

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Summary

Objective

The prevalence of osteoarthritis (OA) increases dramatically in women after the age of 50. Animal models are used to study the effects of hormone depletion [by ovariectomy (OVX)] and estrogen treatment on OA. This review summarizes these animal studies, in order to get a better insight in the role of hormones on OA.

Methods

The literature was systematically reviewed until May 2007. The results were divided into two parts: the effect of OVX on cartilage, and the effect of estrogen treatment on cartilage. Only studies with an appropriate control group (e.g. sham-operated) were included.

Results and discussion

Eleven out of 16 animal studies showed that OVX resulted in cartilage damage. When only studies using sexually mature animals were included, we saw that 11 out of 14 studies showed a detrimental effect, indicating considerable evidence for a relation between cartilage degeneration and OVX in mature animals. The effect of estrogen treatment was inconclusive with only 11 out of 22 animal studies reporting a beneficial effect on cartilage, whereas all six studies administering selective estrogen receptor modulators (SERMs) after OVX described protective effects. The discrepancy between the studies may be caused by the large variation in experimental set-up. We suggested a list of quality criteria for animal models since standardisation of design and outcome parameters of animal experiments may help to compare different studies and to gain better insight in the role of hormones in the osteoarthritic process.

Introduction

Osteoarthritis (OA) is a degenerative joint disease, which results in structural changes in joint tissues, such as articular cartilage, subchondral bone and synovium. It can lead to a complete loss of articular surface as well as exposure of subchondral bone.

From epidemiological studies it is known that before the age of 50 the prevalence of OA is higher in men [1], but after the age of 50, the prevalence is higher in women [2]. The prevalence increases with age in both women and men, but in women, the prevalence of OA increases dramatically around the age of 50 [1, 3, 4]. This coincides with the start of menopause. Taken together, this suggests a link between OA and ovarian function, and suggests an OA-protective effect of estrogen. Estrogen receptors alpha and beta, through which estrogen signals, have been found in several tissues of the joint, including cartilage, bone, ligaments, and synovium [5-9]. This indicates that these tissues are responsive to estrogens. However, estrogen replacement therapy (ERT) showed contradictory effects on OA [10, 11].

Besides clinical studies, several animal models are being used to elucidate the role of estrogens in OA. Ovariectomy (OVX) is an established procedure to simulate the hormonal condition of postmenopausal women. It is often used as an osteoporosis model, but the osteoarthritic changes after OVX have been investigated as well. The role of estrogen is also studied by administrating estrogen or an estrogen antagonist to animals, with or without OVX. In this review we summarize animal studies investigating a role for OVX or estrogen treatment in the development of OA, in order to get a better insight in the pathogenesis of OA. Although all the tissues in the joint may be involved in the osteoarthritic process, we focus on articular cartilage in this review.

Methods

Relevant publications were searched in the PubMed database until May 2007. The following keywords were used: [(postmenopaus* OR post-menopaus* OR menopause OR OVX OR ovariectomy OR estradiol OR estrogen* OR female hormon* OR estrogen depletion) AND (osteoarthritis OR OA OR arthrosis OR osteoarthrosis OR degenerative joint disease OR cartilage)]. Additionally, reference lists of included articles were screened.

A study was eligible for inclusion if all of the following criteria were met: 1) original data from an own animal study, 2) articular cartilage was studied (excluding temporomandibular joint, intervertebral discs or growth plate) and 3) the article was written in English, German or Dutch.

We will summarize the studies in two parts: firstly, results showing the effect of OVX on the cartilage, and secondly, results showing the effect of treatment with estrogen or estrogen-antagonists on the cartilage. To study the effect of estrogen depletion by OVX, it is important to compare the data of OVX animals to those from sham-operated or non-operated control animals. Unfortunately, not all experimental set-ups included such a control group, for instance when the authors were only interested in the administration of estrogen or estrogen-antagonists after OVX. Only studies with a sham-operated or non-operated control group were included when discussing the effect of OVX. Study

characteristics for study population, treatment, and outcome measures were extracted as well as the direction of effect reported in the study.

Results

A total of 436 articles were identified initially. Of these, 22 met our selection criteria. After screening the reference lists of the selected studies and of review articles, another nine studies were included, bringing the total number of included studies to 31. The animals that were used in the selected studies include mice, rats, rabbits, miniature pigs, sheep, and cynomolgus monkeys (tables 2.1-2.3). Most studies investigated changes in the knee joint, but the parameters studied and the experimental set-up varied widely.

OVX effect

Sixteen animal studies describing the effect of OVX were included. These studies are summarized in table 2.1.

Detrimental effect

Eleven studies found an effect of OVX on the mechanical properties of the cartilage, its composition, or cartilage damage, like fibrillation or erosion of the cartilage surface to complete loss of cartilage.

The mechanical properties of the cartilage after OVX were tested in a sheep model by Turner et al [12]. They found that OVX animals had a lower aggregate modulus (a measure of compressive stiffness) and a lower shear modulus than sham-animals. According to Turner et al [12], this indicates a worsening of the mechanical properties. In contrast, an increase in stiffness was reported in two other studies. An increase in dynamic stiffness, a reduction in phase lag (reflecting a change in viscoelastic behaviour consistent with greater elasticity and reduced dissipation of shear forces), and locally an increase in shear modulus were described, again in an OVX sheep model [13]. An increase in cartilage stiffness was also seen in young OVX rabbits, along with an increase in thickness [14]. In that article it is discussed that the meaning of these changes in stiffness remains to be elucidated, and could be either beneficial or detrimental.

Apart from biomechanical measurements, compositional and histological changes were also studied in an OVX sheep model [13]. They observed thinning of the femoro-tibial cartilage, increased neovascularisation of the calcified cartilage layer, and higher histopathological scores, indicating a disturbance in the content and/or structural organization of the proteoglycan and collagen macromolecular assembly.

Changes in matrix composition were also studied in wrists of miniature pigs after OVX [15]. The cartilage contained more heavy sulphated glycosaminoglycans (GAG) (similar as seen with aging) in OVX animals than in control animals. The collagen content was affected as well: In OVX animals, type II collagen staining was negative to weak while in non-operated controls this was intermediate to strong.

Table 2.1: Description of the studies for OVX effect

Study	Animal (strain)	Treatment	Age	Number	Follow up	Joint	Measurement	Effect
Ma et al [22]	Mice (129S6/ SvEv)	Sham+DMM(m,f), OVX+DMM, ORX+DMM, ORX+DMM+E	6wk *	N=10	6,10,12 wk	Knee	Histology †	-
Oestergaard et al [18]	Rats (SD)	Sham, OVX, OVX+early E, OVX+late E	6 mo	N=10/11 /11/10	9 wk	Knee	Histology † CTX-II	-
Christgau et al [16]	Rats (SD)	Sham, OVX, OVX+E, OVX+SERM_low, OVX+SERM_high	6mo	N=12	9 wk	Knee	Histology † CTX-II	-
Hoegh-Andersen et al [17]	Rats (SD)	Sham, OVX, OVX+E, OVX+Serm_low, OVX+Serm_high	5/7 mo	N=10/12	9 wk	Knee	Histology † CTX-I, CTX-II	-
Dai et al [20]	Guinea pigs	Sham, OVX	32mo	N=5	0,6,12 wk	Knee	Histology, SEM, TEM	-
Calvo et al [21]	Rabbits (NwZ)	Sham, Sham+ACLT, OVX, OVX+ACLT	8mo *	N=6	22 wk	Knee	Histology †	-
Rasanen et al [14]	Rabbits (NwZ)	non-operated, OVX, Sham+E	21wk	N=5/9/8	22 wk	Knee	Mech properties, thickness	-
Claassen et al [15]	Miniature pigs (Gottingen)	Intact, OVX	21 mo	N=10 (?)	1 yr	Wrist	Histology, immuno	-
Cake et al [13]	Sheep (merino)	Non-operated, OVX	7yr	N=6	28-30 wk	Knee	Mech indentation, histology, immuno †	-
Parker et al [19]	Sheep (merino)	Non-operated, OVX, OVX+LMX, OVX+LMX+E	8-10 yr	N=6	3 mo	Knee	Histology †	-
Turner et al [12]	Sheep	Sham, OVX, OVX+E, OVX+2E	4-5 yr	N=9/13/ 10/4	1 yr	Knee	Mech properties, thickness	-
Hashem et al [26]	Rabbits (NwZ)	Sham, OVX, OVX+E, OVX+P, OVX+R, OVX+E+R, OVX+P+R, OVX+E+P+R	18wk	N=6-8	6 d	Knee	GAG content Collagen content	0
Chambers et al [25]	Mice (Str/ort)	Sham (m,f), OVX, ORX	3-6 wk	N=5/6/7	27 wk	Knee	Histology, immuno	0
Sokoloff et al [23]	Mice (DBA)	Intact (m,f), ORX, ORX+E, OVX	5wk	N=23/23 /23/13/ 19	15 mo	Knee	Gross examination	0
Silberberg et al [24]	Mice (C57Bl)	Sham, OVX (1m, 6m)	1,6 mo	N=32/45 /64	17,12 mo	Knee	Histology	0
Silberberg et al [27]	Mice (C57Bl)	Intact, OVX (6m, 12m)	6,12 mo	N=42/64 /53	6,12 mo	Knee	Histology	+/0

Note: All treatments used in a study are mentioned for completeness. However, in the discussion of the OVX effect, only OVX and control groups were included.

*Abbreviations in Table 2.1 (in alphabetic order): * OVX and OA induction not at same time point. Age is at time of OVX; † Blinded assessment reported; ACLT = anterior cruciate ligemant transection; d = days; DMM = destabilization of medial meniscus; E = estrogen treatment, see table 2.3 for details; f = female; GAG = glycosaminoglycans; immuno = immunohistochemistry; LMX = lateral meniscectomy; m = male; mech = mechanical; mo = months; NwZ = New Zealand; ORX = orchiectomy; P = progesterone treatment; R = relaxin treatment; SD = Sprague Dawley; SEM = scanning electron microscopy; SERM = selective estrogen receptor modulator, see table 2.3 for details; TEM = transmission electron microscopy; wk = weeks; yr = years; + = beneficial effect (lower incidence or severity of osteoarthritic changes); 0 = no effect; - = detrimental effect (higher incidence or severity of osteoarthritic changes); ? = not or not clearly described.*

The degradation of type II collagen can be studied by measuring the urinary CTX-II levels. OVX caused an increase in urinary CTX-II levels in rats, combined with increased surface erosion [16-18]. The cartilage damage on histology also increased after OVX in a sheep model [19] and in a guinea pig model [20]. A similar effect on cartilage damage was observed in rabbits after OVX combined with corticosteroids (to induce osteoporosis) [21]. OVX increased osteoarthritic abnormalities in non-operated knees, and also intensified the osteoarthritic features in knees where OA had been experimentally induced by anterior cruciate ligament transection (ACLT) [21]. This was also seen in a sheep model in which OVX was combined with lateral meniscectomy, where OVX decreased the proteoglycan content even more than after lateral meniscectomy alone [19]. In another model for surgically induced OA, namely destabilisation of the medial meniscus in mice, OVX increased the OA lesions as well [22].

No effect

Four of the included studies reported no effect of OVX on cartilage. OVX did not alter the OA severity score [23] or incidence [24] in mice. The hormone status [OVX or orchiectomy (ORX)] also did not appear to influence the development of OA or the distribution of cleaved aggrecans (neopeptides) in STR/ort mice [25], which have a high incidence of spontaneous OA. Some of these studies were performed with very young, sexually immature animals (table 2.1). In rabbits, OVX had no effects on GAG and collagen content in knee cartilage [26], although this study only looked at short term effects.

Beneficial effect

One study reported a beneficial effect of OVX on OA. OVX decreased the incidence of OA in mice, but only if it was performed at 6 months of age [27]. No significant effect was seen if mice were ovariectomized at 1 or 12 months of age [24, 27].

Estrogen treatment effect

In 22 studies, animals were treated with estrogen (table 2.2). In six studies, selective estrogen receptor modulators (SERMs) were administered (table 2.3).

Estrogen

Beneficial effect

Eleven out of 22 studies reported a beneficial effect of estrogen treatment on OA. Both in male (nonoperated or castrated) and in female mice the incidence of spontaneous OA was reduced after estradiol treatment [28-30]. The detrimental effects of OVX on the mechanical properties of cartilage were ameliorated by implantation of an estradiol pellet in ovariectomized sheep [12]. The changes in cartilage stiffness and thickness observed in rabbits after OVX returned to control levels when estradiol was administered [14]. Estradiol also inhibited the changes in CTX-II excretion and cartilage erosion in rats induced by OVX [16, 17]. The time of initiation was reported to be important, since delayed estrogen therapy was less effective in this model [18]. Ham et al [31] reported that long-term (3 years) estrogen treatment significantly reduced the severity of OA lesions in OVX cynomolgus monkeys. In the same animal model, ERT increased proteoglycan synthesis by articular chondrocytes [32]. The loss of proteoglycans seen after combined OVX and meniscectomy was mitigated by estrogen treatment in sheep [19].

Table 2.2: Description of the studies for estrogen effect. Abbreviations are explained in the legend of table 2.3

Study	Animal (strain)	Treatment	Estrogen	Admini-stration	Dosage	Frequency	Age	Number	Follow-up	Joint	Measurement	Effect
Silberberg et al [30]	Mice (C57Bl)	Intact(f), Intact+E (early, middle, late)	Alpha estradiol benzoate	Subcut	30 ug	Once/wk	1,6,12 mo	N=26/18/ 20/21	19,14,8 mo	Knee	Histology	+
Silberberg et al [29]	Mice (C57Bl)	Intact (m), ORX, ORX+E	Alpha estradiol benzoate	Subcut	33 ug	Once/wk	3wk	N=30/23/ 50	21mo	Knee	Histology	+
Silberberg et al [28]	Mice (C57Bl)	Intact (m), Intact (m)+E (early, middle, late)	Estradiol benzoate	Subcut	30 ug	Once/wk	1,6,12 mo	N=57/22/ 35/38	19,14,8 mo	Knee	Histology	+
Oestergaard et al [18]	Rats (SD)	Sham, OVX, OVX+early E, OVX+late E	17beta estradiol	Pellet subcut	0.25 mg/9wk	Continuously	6 mo	N=10/11	9 wk	Knee	Histology [b] CTX-II	+
Christgau et al [16]	Rats (SD)	Sham, OVX, OVX+E, OVX+Serm_low, OVX+Serm_high	17alpha ethinyl estradiol	Oral	0.1 mg/kg	5d/wk	6mo	N=12	9 wk	Knee	Histology [b] CTX-II	+
Hoegh-Andersen et al [17]	Rats (SD)	Sham, OVX, OVX+E, OVX+Serm_low, OVX+Serm_high	17alpha ethinyl estradiol	Oral	0.1 mg/kg	5d/wk	5/7 mo	N=10/12	9 wk	Knee	Histology [b] CTX-I, CTX-II	+
Rasanen et al [14]	Rabbits (NwZ)	non-operated, OVX, Sham+E	17beta estradiol	Oral	4 mg/day	Daily	21wk	N=5/9/8	22 wk	Knee	Mech properties, thickness	+
Parker et al [19]	Sheep (merino)	Non-operated, OVX, OVX+LMX, OVX+LMX+E	17beta estradiol	Skin patch	50 ug/d	Continuously	8-10 yr	N=6	3 mo	Knee	Histology [b]	+
Turner et al [12]	Sheep	Sham, OVX, OVX+E, OVX+2E	Pure crystalline estradiol	Implants	1.5 or 3 pg/d	Continuously	4-5 yr	N=9/13/ 10/4	1 yr	Knee	Mech properties, thickness	+
Ham et al [31]	Cyno-molgus monkeys	OVX, OVX+E	Conjugated equine estrogens	Oral	~0.625 mg/d	Daily	7-13 yr	N=60	3 yr	Knee	Histology [b]	+
Richmond et al [32]	Cyno-molgus monkeys	OVX, OVX+E	Conjugated equine estrogen	Oral	~0.625 mg/d	Daily	Adult	N=?	2 yr	Knee	Biochemistry (SO4 incorp)	+

Table 2.2 continued

Study	Animal (strain)	Treatment	Estrogen	Administration	Dosage	Frequency	Age	Number	Follow-up	Joint	Measurement	Effect
Ma et al [22]	Mice (129S6/ SvEv)	Sham+DMM(m,f), OVX+DMM, ORX+DMM, ORX+DMM+E	Estradiol	Pellet subcut	0.72mg/ 60d	Continuously	6wk [a]	N=10	2,4,8 wk	Knee	Histology [b]	0
Sokoloff et al [23]	Mice (DBA)	Intact (m,f), ORX, ORX+E, OVX	Diethylstilbesterol	Pellet subcut	1mg/9 mo	Continuously	5wk	N=23/23/ 23/13/19	15 mo	Knee	Gross examination	0
Ham et al [33]	Cyno-molgus monkeys	OVX, OVX+E	Conjugated equine estrogens	Oral	~0.625 mg/d	Daily	8-11 yr	N=30	3 yr	Knee	Biochemistry Immuno	0
Silberberg et al [34]	Mice (C57Bl)	Sham (m), Sham+E	Alpha estradiol benzoate	Subcut	120 ug	Twice/wk	6mo	N=4	1d, 1,2wk	Femoral head	Histology	-
Hashem et al [26]	Rabbits (NwZ)	Sham, OVX, OVX+E, OVX+P, OVX+R, OVX+E+R, OVX+P+R, OVX+E+P+R	Beta estradiol	Intra-muscular	20 ng/kg	Once (?)	18 wk	N=6-8	6 d	Knee	GAG content Collagen content	-
Tsai et al [37]	Rabbits (NwZ)	OVX, OVX+Elow, OVX+Ehigh	Estradiol benzoate	Intra-articular	0.06, 0.3 mg/kg	Once/3d	?	N=4/8/8	9/12 wk	Knee	Histology	-
Tsai et al [36]	Rabbits (NwZ)	OVX, OVX+E, OVX+Serm, OVX+E+Serm	Estradiol benzoate	Intra-articular	1 mg/kg	Twice/wk	Adult	N=3	3 wk	Knee	Histology	-
Rosner et al [35]	Rabbits (NwZ)	OVX+MMX, OVX+MMX+E, OVX+MMX+Serm	Estradiol valerate	Intra-muscular	0.175 mg/kg	3 d/wk	?	N=17	12 wk	Knee	Histology [b] Biochemistry	-
Rosner et al [38]	Rabbits (NwZ)	MMX(f), MMX+Elow, MMX+Ehigh	Estradiol valerate	Intra-muscular	0.4, 1.6 mg/kg	Once/2 wks	?	N=16/16/ 18	12 wk	Knee	Histology Biochemistry	-
Silberberg et al [39]	Mice (C57Bl)	Intact (m), Intact+E	Alpha estradiol benzoate	?	8 ug	Once/wk	New-born	N=22	3d, 1,2,4w	Femoral head	Electron microscopy	?
Silberberg et al [40]	Mice (C57Bl)	Intact (m), Intact+E	Alpha estradiol benzoate	Subcut	60 ug	Once	4wk	N=2	2,4,8,24h 2,3,7d	Femoral head	Electron microscopy	?

Table 3: Description of the studies for SERM effect

Study	Animal (strain)	Treatment	SERM	Administration	Dosage	Frequency	Age	Number	Follow-up	Joint	Measurement	Effect
Christgau et al [16]	Rats (SD)	Sham, OVX, OVX+E, OVX+Serm_low, OVX+Serm_high	Levormeloxifene	Oral	0.2 or 5 mg/kg	5d/wk	6mo	N=12	9 wk	Knee	Histology [b] CTX-II	+
Hoegh-Andersen et al [17]	Rats (SD)	Sham, OVX, OVX+E, OVX+Serm_low, OVX+Serm_high	#	Oral	0.2 or 5 mg/kg	5d/wk	5mo	N=10/12	9 wk	Knee	Histology [b] CTX-I, CTX-II	+
Tsai et al [36]	Rabbits (NwZ)	OVX, OVX+E, OVX+Serm, OVX+E+Serm	Tamoxifen	Intra-articular	0.35 mg/kg	Twice/wk	Adult	N=3	3 wk	Knee	Histology	+
Colombo et al [42]	Rabbits (NwZ)	MMX (m), MMX+Serm	Tamoxifen citrate	Oral	0.5 mg/kg	5 d/wk	?	N=8	5 wk	Knee	Histology [b]	+
Rosner et al [41]	Rabbits (NwZ)	MMX (f), MMX+Serm	Tamoxifen	Intra-muscular	0.5 mg/kg	Daily	Immature	N=15/20	12 wk	Knee	Histology [b] Biochemistry	+
Rosner et al [35]	Rabbits (NwZ)	OVX+MMX, OVX+MMX+E, OVX+MMX+Serm	Tamoxifen	Intra-muscular	1.17 mg/kg	3d/wk	?	N=17	12 wk	Knee	Histology [b] Biochemistry	+

#: cis3,4-7-hydroxy-3-phenyl-4-(4-(2-pyrrolidinoethoxy)phenyl)chromane

Abbreviations in Tables 2.2 and 2.3 (in alphabetic order):
[a] =OVX and OA induction not at same time point. Age is at time of OVX; ACLT=anterior cruciate ligemant transection; [b] = blinded assessment reported; d=days; DMM=destabilization of medial meniscus; E=estrogen treatment, see Table 3 for details; f=female; GAG=glycosaminoglycans; immuno=immunohistochemistry; LMX=lateral meniscectomy; m=male; MMX=medial meniscectomy; mo=months; NwZ=New Zealand; ORX=orchiectomy; P=progesterone treatment; R=relaxin treatment; SD=Sprague Dawley; SEM=scanning electron microscopy; Serm=selective estrogen receptor modulator, see Table 3 for details; subcut=subcutaneous; T=testosterone treatment; TEM=transmission electron microscopy; TMX=tamoxifen; wk=weeks; yr=years; ~= equivalent to dose for women of...; +=beneficial effect (lower incidence or severity of osteoarthritic changes); 0=no effect; -=detrimental effect (higher incidence or severity of osteoarthritic changes).

No effect

Three studies reported no effect of estrogen treatment. Collagen and proteoglycan content were not different in cartilage from estrogen treated and control cynomolgus monkeys [33]. In male castrated mice, estrogen treatment did not change the severity of spontaneous OA [23]. Also after surgically induced joint instability, estrogen treatment did not alter the OA severity in male castrated mice [22].

Detrimental effect

In contrast to the beneficial effects of estrogens, six studies have reported a detrimental effect of estrogens. An increase in degeneration and cell death after estradiol treatment in mice was reported [34]. Estrogen administration after OVX worsened the surgically induced cartilage damage in a rabbit model [35]. Also without surgically induced OA, Tsai and Liu [36] reported a similar effect. In their study, OVX together with a high dose of intra-articular estradiol administration caused degenerative changes. In a later study, Tsai and Liu [37] showed that a low dose of estradiol did not induce pathological changes, whereas a high dose again caused degenerative changes that progressed over time. In another rabbit model the proteoglycan synthesis was decreased in both non-operated normal knees and in post-meniscectomy osteoarthritic knees of estrogen treated animals [38]. A combination of hormones was used in a rabbit model. Treatment with estradiol, progesterone or relaxin, alone or in any combination led to a loss of cartilage collagen [26]. Estradiol alone or estradiol with relaxin showed the strongest loss of collagen.

Unclear effect

In two studies by Silberberg et al [39, 40], changes in ultrastructure of chondrocytes (organellar development) and fibrillarity of the matrix are described in very young (newborn) mice. However, one can only speculate whether these early developmental changes have a stimulating or mitigating effect on OA or represent the OA process at all at this age.

SERM

Beneficial effect

SERMs are a class of medication that act on the estrogen receptors. Their action can be either agonistic or antagonistic, depending on the tissue. In six out of six studies administering a SERM, a beneficial effect was found. The SERMs levormeloxifen and cis-3,4-7-hydroxy-3-phenyl-4-(4-(2-pyrrolidinoethoxy)phenyl)chromane inhibited the OVX-induced changes in CTX-II excretion and cartilage erosion in rats [16, 17]. The SERM tamoxifen was administered in four of the included studies. Tamoxifen had no effect in OVX rabbits, but OVX animals that received estradiol and tamoxifen had less cartilage damage than OVX animals receiving estradiol alone, suggesting that tamoxifen could counteract or inhibit the degenerative changes induced by estradiol [36]. Tamoxifen decreased the cartilage damage in a rabbit model with OVX and medial meniscectomy (MMX) [35]. Also without OVX, tamoxifen decreased the cartilage damage in this rabbit model [41]. Colombo et al [42] found that tamoxifen also offered protection in intact male rabbits. They hypothesized that the therapeutic effect of tamoxifen is not related to its anti-estrogenic properties, since its effect is similar in ovariectomized female [35] and intact male rabbits.

Discussion

In this review, we aimed to elucidate the effect of OVX and estrogen treatment on cartilage in animal models. In 11 animal models, OVX caused biomechanical, compositional or histological changes of the articular cartilage. Four studies reported no effect of OVX, and only one study observed less OA after OVX. This means that the evidence is inconclusive.

Exploring diversity in results

A possible explanation for the inconclusive effects is that there is a lot of variation in experimental set-up, such as animal species, duration of experiment, and age of the animals (table 2.1). Especially the use of sexually immature animals (e.g. mice younger than 5 weeks of age) may distort the effect of OVX or estrogen treatment. It is striking that immature animals were used in three of the studies that showed no OVX effect, whereas all animals were mature in the 11 studies that showed a detrimental effect. When we would exclude the data obtained with immature animals, 11 out of 14 studies showed a detrimental effect of OVX on cartilage, which makes the evidence considerably stronger. Therefore we would advice to use only mature animals in further experiments on the effect of OVX on OA.

The effect of estrogen treatment on OA was inconclusive, with 11 out of 22 studies reporting a beneficial effect and six studies even showing a detrimental effect. In contrast, the effect of estrogen-like substances or SERMs on OA is very consistent. Six out of six studies reported a beneficial effect on OA. In the estrogen treatment studies, there were again differences in experimental set-up. Apart from the variation in animal species, duration of experiment, and age of the animals (as mentioned above), the estrogen treatment studies also showed variation in the type of estrogen, dosage, and way of administration, which could affect the circulating levels. Unfortunately, most studies did not report circulating levels, which makes the comparison of the studies more difficult.

The difference between inconclusive effect for estrogen and a clear beneficial effect for SERMs may be caused by the different mechanisms or pathways via which SERMs act. In addition, the variation in experimental set-up is less in the SERM studies. For instance, in the SERM studies only two different animal species were used (rats and rabbits), whereas in the estrogen studies mice, rats, rabbits, sheep, and monkeys were used. Furthermore, one could speculate about a publication bias due to industry involvement in SERM supply. This would also explain the low number of SERM studies (six studies) compared to estrogen studies (18 studies). Although this may appear mainly in industry-related studies, we cannot exclude that there may also be a publication bias in non-commercial studies.

Another type of bias may be introduced when subjective outcome measurements such as scoring cartilage damage on histology were not performed blinded. When only including studies with reported blinded assessment of histology or with objective outcome measurements, there would be nine studies in which OVX caused detrimental articular cartilage changes, and only one study reporting no effect. Thus instead of being inconclusive, there would be strong evidence for a detrimental effect by OVX.

Possible working mechanism of estrogen

The molecular mechanism by which estrogen could act on chondrocytes cannot be extracted easily from the macroscopic parameters studied in most animal models. In-vitro experiments could give additional insight in these mechanisms. For instance, estrogens have been found to increase proteoglycan synthesis [43], and decrease matrix metalloproteinase-1 (MMP-1) mRNA levels [44] and nitric oxide production by chondrocytes [45]. However, other studies have reported a decrease in collagen and proteoglycan synthesis [46, 47]. The effect of estrogen on chondrocytes seems to be strongly dependent on the concentration that was used and further studies are needed to elucidate the molecular mechanisms.

OVX does not only change estrogen levels, but also progesterone (down), follicle stimulating hormone (up), luteinizing hormone (up), and testosterone (down). Besides, OVX is often accompanied with an increase in fat mass. This leads to increased levels of adipokines such as leptin. There is growing interest in the contribution of adipokines in several diseases and their role in OA is being investigated. For instance, Simopoulou et al [48] reported that leptin stimulates the expression of MMPs and interleukin-1 (IL-1), and thus may be important in OA development [49]. Body weight and hormone levels were not always mentioned in the included studies, so we cannot conclude on their contribution. The OVX-related changes in hormones and adipokines may play a role in the osteoarthritic changes observed after OVX, and may explain why estrogen treatment could not always reduce the OVX-induced changes.

The effect from estrogen on cartilage is probably not the only mechanism involved in the development of OA, since other tissues in the joints are also responsive to estrogen. Bone cells are known to express estrogen receptors [5, 50], and estrogen is one of the important regulators of the balance between bone formation and bone resorption [51]. Alteration in the subchondral bone remodelling, and subsequently, the bone structure may lead to changes in load distribution across the tibial plateaus. This may in turn cause cartilage damage. Subchondral bone changes have indeed been reported in several osteoarthritic models [52-55].

Synovial tissue also contains estrogen receptors [6,7], and estrogen may modulate the levels and activity of certain growth factors and cytokines produced by synovial cells. These cytokines play an important role in osteophyte formation [56]. Indeed, estrogen treatment increased the levels of components of the insuline-like growth factor (IGF) system (IGF-1, -2, IGF binding protein-1, -3) in monkey synovial fluid [57]. These proteins may originate from local tissues, including synovium, cartilage and bone.

Quality criteria for animal studies

Although the use of ERT in treatment of OA is controversial because of undesirable side effect (e.g. breast tumours), estrogen treatment and OVX in animal studies can be very useful to elucidate the pathways involved in OA. However, we found that there is a lot of variation in experimental set-up and data reporting, which mystifies the results of this review. There are no generally accepted criteria for the quality of animal studies to be included in a (systematic) review, like the criteria for clinical studies. We narrowed our review to studies selected on the in our opinion most important quality criterion, namely having an appropriate control group (sham-operated or non-operated control for OVX effect, vehicle of no administration for estrogen effect). However, one can think of

more criteria indicating high quality of an animal study. We suggest a list of criteria (table 2.4). Apart from the use of an appropriate control group, the study population (sex, age, strain and substrain of the animal) should be well described. As discussed above, age of the animal may distort the effect. Also the treatment should be clearly described. For instance in case of the estrogen treatment (or any other compound), it should be clear how the compound was administered, in which dosage, and at which frequency. Another issue of the experimental set-up is the sample size, which should be appropriate to achieve adequate statistical power. This depends on the type of measurement and the size of the expected effect. Justification of the number of animals used should be given. This could be done by means of a power calculation. In none of the included studies such a power calculation was mentioned. It is also important that the choice for the follow-up period is soundly based, since it should be long enough to see an effect. This may depend on the animal species, the treatment, and the research question. In addition, the outcome measurements should be validated. In case of scoring cartilage damage on histology various scoring systems are being used. Pritzker et al [58] proposed a scoring system with a wide application for both clinical and experimental OA assessment. It would increase comparability between studies if one such scoring system became the standard method. Some outcome measurements may be quite subjective (such as histology) whereas others are more objective (such as collagen content measured by absorbance). Especially in case of subjective measurements, it is important that the observer is blinded to the treatment, to avoid bias.

Since some of these criteria may be subject of debate, we did not apply them to our results. However, we did include information on these matters in tables 2.1-2.3. Applying these criteria may influence our results. We have already described the effect when taking into account the age at which OVX was performed or when including only blinded assessment.

Table 2.4: Suggested list of quality criteria for animal studies

Criterion	Description
Appropriate controls	Sham and/or vehicle treatment, matched in age, sex, and strain to experimental group. No historic controls.
Study population well described	Details on species, strain, substrain, sex, age, and initial body weight should be given.
Sample size appropriate	Large enough to achieve adequate statistical power.
Treatment well described	Details on administration of compounds (dosage, frequency, way of administration).
Follow-up period suitable	a. Long enough to expect changes. b. Details on drop-outs or lost samples should be given.
Reliable outcome measurements	Outcome measures validated or generally accepted.
Blinded outcome assessment	Especially for subjective measurements.

Apart from the criteria needed to evaluate the quality of a study and to exclude potential bias, additional information would help to interpret the data. For instance, circulating hormone levels and body weight may be of influence (as discussed above) and therefore interesting to know. This would facilitate subgroup analysis.

In conclusion, our review on the effects of OVX and estrogen treatment on cartilage in animal models showed inconclusive effects. However, the only criterion we applied was that appropriate controls were performed. We observed that some valuable criteria were not applied or not mentioned in the studies we evaluated. We now suggest a list of quality criteria for animal studies. Formulating and applying these quality criteria will improve the comparability of studies, facilitate the evaluation and thus be advantageous for reviews of literature and for animal studies in general.

Acknowledgements

We would like to thank Sonya Glasson for discussing the criteria for animal models.

Addendum

The literature search for this article was performed in May 2007. From May 2007 to April 2009, four new articles have been published which meet the inclusion criteria. A study by Luo et al [59] demonstrated that OVX in rats increased chondrocyte apoptosis and MMP13 protein expression. Estrogen treatment decreased these parameters. A study by Sondergaard et al [60] showed that OVX in rats led to more cartilage erosion and more serum CTX-II. Estrogen treatment reversed these effects. Bay-Jensen et al [61] reported that OVX in rats had a small effect on cartilage, which was detected by increased urinary CTX-II levels, but not by other type II collagen turnover markers. Estrogen treatment prevented the increase in CTX-II. Hart et al [62] observed unique alterations in cell metabolism after ovariohysterectomy in rabbits. However, it is not clear whether these alterations are beneficial or detrimental.

When these articles are added to those already described in this chapter, 14 out of 20 animal studies show a detrimental effect of OVX, and 14 out of 25 animal studies show a beneficial effect of estrogen treatment. The four additional articles do not change our conclusions.

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Chapter 3

Development of osteoarthritic features in estrogen receptor knockout mice

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Abstract

Objective

Estrogens are suggested to play a role in the development of osteoarthritis as indicated by the increased prevalence in women after menopause. We studied whether deletion of estrogen receptor (ER) α, β, or both in female mice results in cartilage damage, osteophytosis, and changes in subchondral bone of skeletally mature animals.

Methods

We studied knee joints of 6-month-old female ERα-/-, ERβ-/-, and (double) ERα-/-β-/- mice and their wild type (wt) littermates. The presence and size of osteophytes and osteoarthritic changes in cartilage were analysed using histology. Changes in subchondral plate and trabecular bone were studied using micro-CT.

Results

In ERα-/-β-/- mice, we observed an increase in number and/or size of osteophytes and thinning of the lateral subchondral plate. However, cartilage damage was not different from wt. In ERα-/- or ERβ-/- mice, no significant differences in cartilage damage score, osteophyte formation, or subchondral plate thickness were found.

The bone volume fraction of epiphyseal trabecular bone was unchanged in ERα-/- mice, increased in ERβ-/- mice, and decreased in ERα-/-β-/- mice.

Conclusions

We conclude that deletion of both estrogen receptors leads to increased osteophytosis, but deletion of one or both estrogen receptors does not lead to overt cartilage damage in 6-month-old mice.

Introduction

It has been suggested in literature that estrogen depletion plays a role in the onset of osteoarthritis (OA). The incidence increases with age in both women and men, but in women, the incidence of OA increases dramatically around the age of 50 [1,2], coinciding with the onset of menopause. Also in animal models a link between estrogen and OA has been found. In several animal models, ovariectomy leads to osteoarthritic changes [3], and estrogen replacement therapy reduces the cartilage degradation [4,5]. Estrogen acts via the estrogen receptor (ER), which has two isoforms: ERα and ERβ. Several studies have reported associations between polymorphisms in ERα and ERβ with OA [6-8]. Taken together, this argues for a role of estrogen in the development of OA.

The effect of estrogen on OA may be directly via the cartilage, since chondrocytes express both ERα and ERβ [9,10] and estrogens affect cartilage metabolism, as is shown in in-vitro studies [11,12].

Bone changes are also involved in OA [13-18], and ERs are expressed in osteoblasts [19-21] and osteoclasts [22,23]. Accordingly, estrogen may also affect OA via the bone. It is known that deletion of ERs in mice leads to an altered bone phenotype [24-26], although this has only been established for the metaphysis, diaphysis, or lumbar spine, but not for the epiphysis, which is in close contact with the cartilage and might have a role in the OA process [13].

We hypothesize that deletion of estrogen receptors in female mice leads to osteoarthritic changes in the articular cartilage, osteophyte formation and/or subchondral plate changes. Specifically, we investigated presence and extent of cartilage damage and osteophytes in the knee joints of female ERα-/-, ERβ-/-, and ERα-/-β-/- mice using histology and bone changes in the tibial epiphysis using micro-CT analysis.

Methods

Mice

Knee joints from female knockout (-/-) mice were obtained from two different stables. Schering-Plough (previously Organon) provided knee joints from ERα-/- mice, ERβ-/- mice, and their wild type (wt) controls (all n=6). Karolinska Institutet provided knee joints from wt (n=8), ERβ-/- (n=5), and ERα-/-β-/- mice (n=12). The mice were generated in a C57Bl/6 background. The ERα-/- mice were generated as described by Lubahn et al [27]. The ERβ-/- mice from Schering-Plough were generated as described by Rose et al [28]. The ERβ-/- mice from Karolinska Institutet were generated as described by Krege et al [29]. For the ERα-/-β-/- mice, the ERα gene was disrupted as described by Lubahn et al [27] and the ERβ gene was disrupted as described by Krege et al [29]. All mice were 6 to 7 months old. Approval was given by the local animal ethics committees of Schering-Plough and Karolinska Institutet, respectively, and animal care was in accordance with institutional guidelines.

Cartilage analysis

Knee joints were decalcified and embedded in paraffin. Frontal sections of knee joints were made (6 μm thick), which were stained with Haematoxylin-Eosin or Safranin-O.

Due to a technical problem, sections of one ERα wt mouse were lost. Both severity and extent of cartilage damage were scored with the grading and staging scoring system described by Pritzker et al [30]. The medial and lateral tibia compartments were scored blinded to the animals' identity. Each compartment can get a maximum score of 24. The scores of the two compartments were added, resulting in a maximum total score of 48. The average score of three sections (200 μm apart) per knee joint was calculated.

Osteophyte analysis

The presence or absence of osteophytes was scored in the histological sections. In each section, a value of 1 (osteophyte present) or 0 (no osteophyte) was assigned for medial and lateral tibia plateau. The average score of three sections per knee joint was calculated for medial and lateral tibia plateau, resulting in a score between 0.0 and 1.0 for each plateau (medial and lateral). A high score (closer to 1.0) means that an osteophyte was present in more sections, indicating a large osteophyte, or more (isolated) small osteophytes. A score of 1.0 means that an osteophyte was present in all sections.

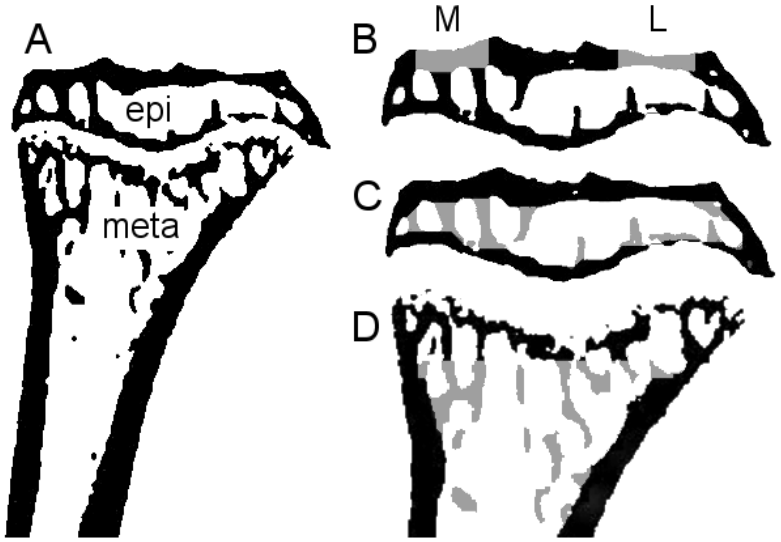

Figure 3.1: Regions that were analysed using micro-CT. A: Cross-sectional image of proximal tibia, including epiphysis (epi) and metaphysis (meta). B: Epiphysis with medial (M) and lateral (L) subchondral plate depicted in light grey. C: Epiphysis with trabecular bone depicted in light grey. D: Metaphysis with trabecular bone region depicted in light grey.

Bone analysis

Before decalcification for histology, the knee joints were scanned in a micro-CT scanner (Skyscan1072, Skyscan, Belgium) with isotropic voxel size of 8 μm. The reconstructed datasets were segmented using a local threshold algorithm [31]. Two regions of interest were selected in the tibia: the epiphysis and the metaphysis (figure 3.1). The metaphysis was studied to validate our findings with literature data. Both regions of interest were further divided into a cortical part and a trabecular part. In the cortical compartment of the epiphysis, a region of interest was selected at the middle of both the medial and lateral plateau with a width of 0.5 mm (in medial-lateral direction) and a length of 0.8 mm (in anterior-posterior direction), representing the subchondral plate. For

these regions the three-dimensional plate thickness was calculated. For the trabecular compartment in the epiphysis and metaphysis, bone volume fraction (BV/TV) and three-dimensional trabecular thickness (TbTh) were calculated.

Statistical analysis

A non-parametric test, the Mann-Whitney test, was used to compare data of the knockout mice with the corresponding group of wt mice. A p-value of <0.05 was considered significant.

Results

Cartilage and osteophytes

No difference in cartilage damage scores was found between knockout and wt mice (figure 3.2A). The cartilage damage was very mild ranging from 0.5 to 6.8 on a scale of 0-48. A representative image of cartilage damage is shown in figure 3.3A.

Small, mainly cartilaginous osteophytes were present at the outer edge of the medial tibial plateau. In ERα-/- and ERβ-/- mice the osteophyte score was not different from wt. However, in ERα-/-β-/- mice, more and/or larger osteophytes were present compared to wt mice (figure 3.2B). A representative image of osteophytosis is shown in figure 3.3B.

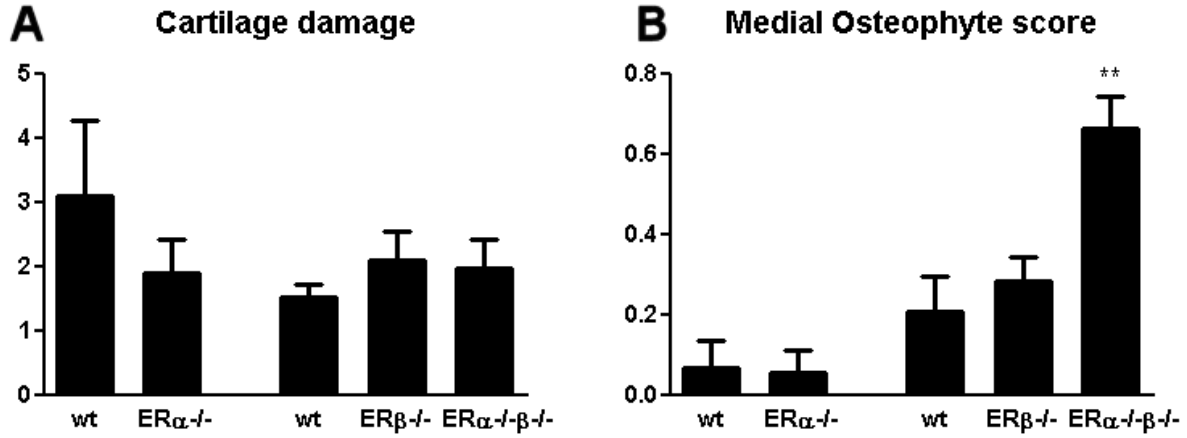

*Figure 3.2: Osteoarthritic parameters scored on histology in different groups of 6-month-old mice. A: Cartilage damage score of the tibia plateaus. B: Osteophyte score for medial tibia. Error bars indicate s.e.m., ** indicates $p<0.01$.*

Bone

In ERα-/- and ERβ-/- mice, no difference in subchondral plate thickness was observed when compared with wt mice (figure 3.4A and B). In ERα-/-β-/- mice, the subchondral plate was thinner (-10%) than in wt mice, but only at the lateral side (figure 3.4B).

In epiphyseal trabecular bone, BV/TV was unchanged in ERα-/- mice, increased in ERβ-/- mice and decreased in ERα-/-β-/- mice (figure 3.4C and table 3.1). TbTh was lower in ERα-/-β-/- mice than in wt mice.

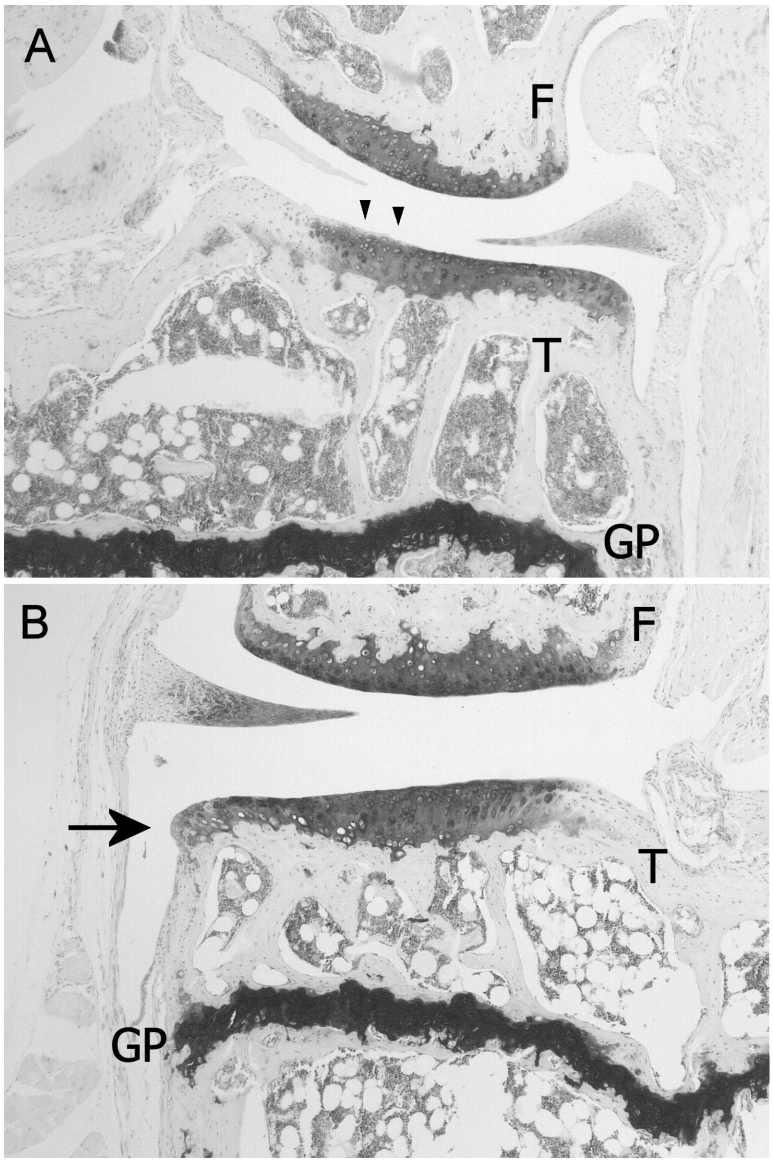

Figure 3.3: Representative histology images of knee joint of an ERα-/-β-/- mouse. A: Surface fibrillations and discontinuities at lateral tibia plateau (indicated by arrow heads). B: Osteophytosis at medial tibia plateau (indicated by arrow). F=femur, T=tibia, GP=growth plate.

Table 3.1: Trabecular bone changes in epiphysis and metaphysis. BV/TV: bone volume fraction; TbTh: trabecular thickness;. ↑ indicates significantly higher than wt; ↓ indicates significantly lower than wt; = indicates not significantly different from wt.

Epiphysis	BV/TV	TbTh
ERα-/-	=	=
ERβ-/-	↑	=
ERα-/-β-/-	↓	↓
Metaphysis		
ERα-/-	↑	↓
ERβ-/-	↑	=
ERα-/-β-/-	↓	↓

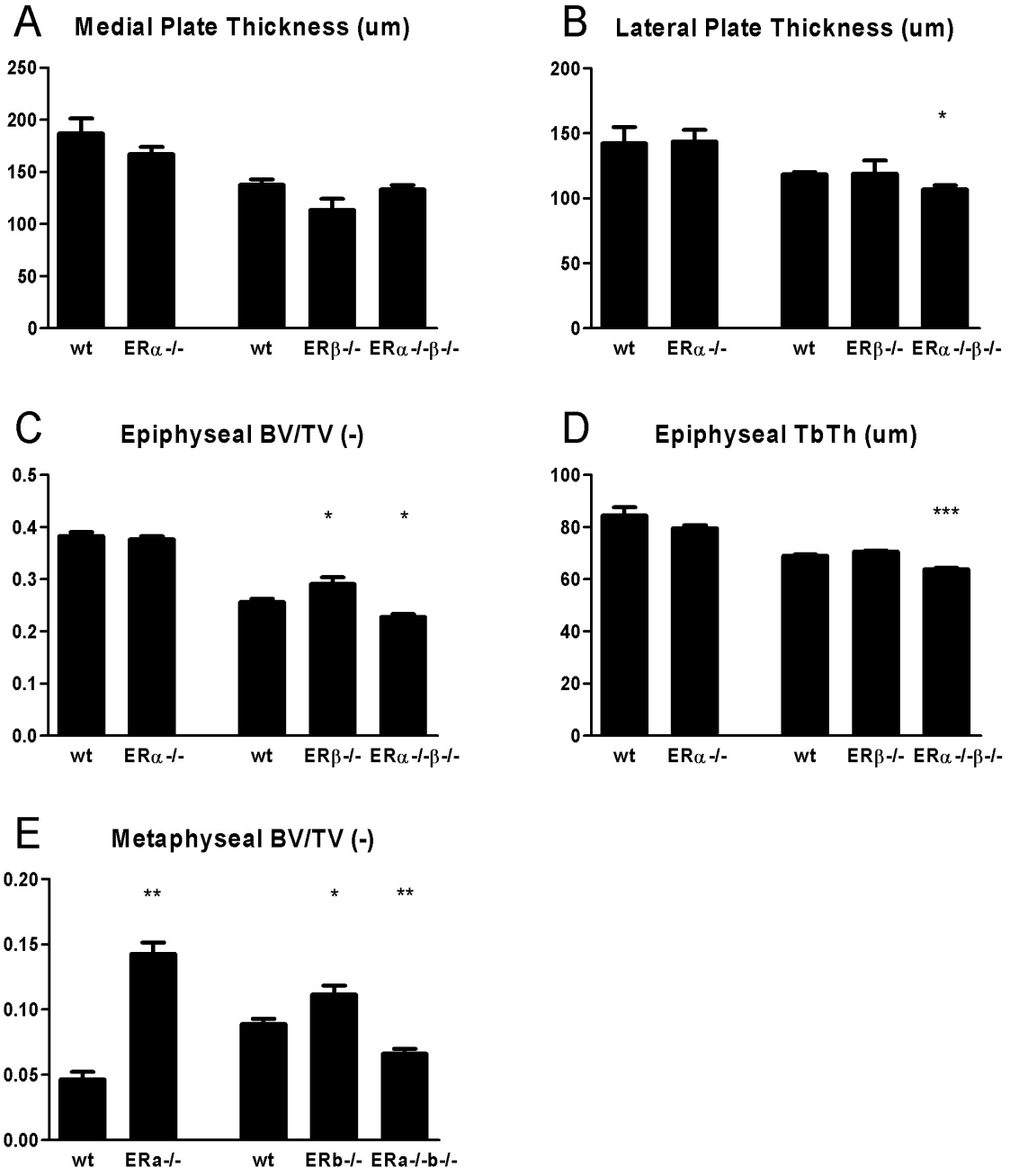

*Figure 3.4: Bone parameters calculated from microCT scans. A: Medial subchondral plate thickness. B: Lateral subchondral plate thickness. C: Epiphyseal bone volume fraction. D: Epiphyseal trabecular thickness. E: Metaphyseal bone volume fraction. Error bars indicate s.e.m., * indicates $p<0.05$, ** $p<0.01$, *** indicates $p<0.001$.*

In the metaphysis, BV/TV was far greater in ERα-/- than in wt mice (figure 3.4E) and TbTh was lower in ERα-/- than in wt mice (table 3.1). Also in ERβ-/- mice, metaphyseal BV/TV was higher than in wt mice (figure 3.4E), although the effect was smaller than seen in ERα-/- mice. In ERα-/-β-/- mice BV/TV and TbTh were lower than in wt mice. The changes in metaphyseal trabecular parameters are in agreement with previous reports [25,26].

Different stables

The wt mice corresponding to ERα-/- and ERβ-/- mice from Schering-Plough were significantly different from the wt mice corresponding to ERβ-/- and ERα-/-β-/- mice from Karolinska Institutet. The ERβ-/- mice were available from both stables, which allowed comparison between both groups. The effects of deleting ERβ relative to wt were not significantly different in mice from both stables. In figure 3.5 the epiphyseal bone volume fraction of ERβ-/- mice from both stables is shown. Since the number of animals from Karolinska Institutet was higher, we decided to present the ERβ-/- data only from this stable.

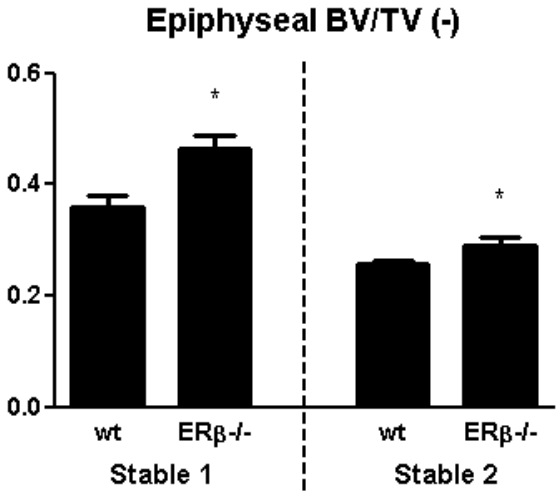

*Figure 3.5: Epiphyseal bone volume fraction of wt and ERβ-/- mice from the two different stables. Stable 1: Schering-Plough, both n=6; Stable 2: Karolinska Institutet, n=8 and n=5, respectively. Error bars indicate s.e.m., * indicates p<0.05.*

Discussion

In the present study, we investigated whether deletion of one or both estrogen receptors would lead to cartilage damage, osteophyte formation or subchondral plate changes. Only in double knockout mice (ERα-/-β-/-) increased osteophytosis and thinning of the lateral subchondral plate, both osteoarthritic features, were found. The cartilage damage was very mild in all groups (knockout and wt mice) and no difference in cartilage damage was found between knockout and wt mice at 6 months of age.

In single knockout mice, no difference in cartilage damage was found compared to wt. Deletion of one estrogen receptors may be compensated for by the other estrogen receptor. We anticipated increased cartilage damage in ERα-/-β-/- mice, as reduced estrogen signalling by ovariectomy is described to increase cartilage lesions in rats and monkeys [4,5,32,33] and in mice with surgically induced OA [34]. However, we observed no effect on cartilage damage. This may be explained by the fact that ER-knockout mice lack the ER since birth, whereas in the ovariectomy models estrogen is depleted at a later age. Another difference is that ovariectomy does not only lead to estrogen depletion, also progesterone levels decrease, and levels of follicle stimulating hormone and luteinizing hormone increase [35,36]. These hormones may also be involved in cartilage homeostasis.

We did see more or larger osteophytes in ERα-/-β-/- mice. A link between estrogen and osteophytes has been reported: Ovariectomy induced osteophyte formation in temporomandibular joints of rats [37] and polymorphisms in the ERα gene are associated with osteophytosis [6,38]. In our study, we observed no increased osteophytosis in the single knockout mice, indicative of a compensation mechanism.

We also analysed subchondral plate thickness, since this is the bone structure closest to the joint and changes in subchondral plate thickness have been observed in previous OA studies [15-18]. In ERα-/-β-/- mice lateral subchondral plate thickness was decreased, which is also observed at early time points in several osteoarthritic animal models [14,16,39]. This thinning is unlikely to reflect a general diminished cortical bone volume or thickness, because cortical bone volume in the metaphysis was unchanged and metaphyseal cortical thickness was even increased in our mice (data not shown). The increased osteophytosis and changed subchondral plate both indicate that there is some osteoarthritic development at the age of 6 months.

C57Bl/6 mice are known to develop OA spontaneously from the age of 6 months [40]. We used 6-months-old mice in this study, as waiting longer would increase the risk that development of spontaneous OA might overrule a potential acceleration of development of OA characteristics due to loss of estrogen signalling. We saw no pronounced cartilage damage in this study, although we cannot exclude that this may develop at a later age.

The values of most parameters were different between the groups of wt mice. This is most likely due to the different origin of the mice (different breedings, different stables). The ERβ-/- mice were available from both stables, which allowed comparison between ERβ-/- mice from both stables. We found that the effect of deleting ERβ relative to wt mice (e.g. increased bone volume fraction) was not different between stables, indicating that the observed effects were independent of the knockout strategy.

In addition to the epiphysis, we studied the trabecular bone changes in the metaphysis to validate our findings with literature data. The changes in metaphyseal trabecular parameters were in agreement with previous reports [25,26]. Deletion of ERs resulted in less pronounced changes in the epiphysis than in the metaphysis which is also seen after ovariectomy [17,41] and after ovariectomy with estrogen supplementation [17]. Apparently the epiphysis is responsive, but less sensitive to estrogen than the metaphysis, indicating that the estrogen-ER system in control of bone metabolism is different in metaphysis and epiphysis.

The bone changes in the single ER knockout mice differed from those in the double ER knockout mice and from what is seen after ovariectomy, as has been reported before. The effect of deleting one ER may be completely compensated by the other ER or by other receptors involved in the estrogen signalling, such as GPR30 [42]. Besides this, the serum estrogen levels are strongly increased in the ERα-/- mice, and the expression of ERα is increased in bones of ERβ-/- mice [26]. High doses of estrogen given to ovariectomized ERα-/- mice could inhibit the bone loss and even increase bone mineral density above intact level [43], suggesting that in the absence of ERα, high levels of estrogen can overcompensate the deletion of one ER.

In conclusion, deletion of both ER α and β increased osteophytosis but deletion of ER α and/or β does not lead to overt cartilage damage in 6-month-old mice.

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Chapter 4

Loss of estrogen increases susceptibility for osteoarthritic changes in articular cartilage, but not in subchondral bone

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A combined manuscript of Chapters 4 and 5 has been submitted for publication

Abstract

Objective

Estrogen depletion may play a role in onset or progression of osteoarthritis. We investigated in mouse knees whether estrogen depletion increases susceptibility of cartilage and bone for osteoarthritic changes by combining ovariectomy with an osteoarthritis trigger. Furthermore, we investigated the involvement of estrogen-induced bone changes in osteoarthritis development.

Methods

Female C3H/HeJ mice were divided into four groups: sham operated, estrogen depletion by ovariectomy (OVX), OVX with estradiol supplementation (OVX+E), and OVX treated with bisphosphonate (OVX+BP) to inhibit bone changes. In all mice, one knee was injected with iodoacetate (IA) to trigger cartilage degeneration, the contralateral knee was injected with saline. Bone changes were followed over time using in-vivo micro-CT; cartilage was analysed by histology at 12 weeks post-surgery.

Results

In sham mice, IA injection caused glycosaminoglycan depletion but no cartilage erosion or subchondral plate changes. Only in OVX mice, IA injection increased cartilage erosion. There was significant interaction between OVX and IA in their effect on cartilage erosion. Subchondral plate thickness decreased only in OVX+IA knees. No significant interaction between OVX and IA was found, but the effect of OVX and IA on subchondral plate thickness was additive. OVX+E and OVX+BP inhibited subchondral plate thinning and tended to diminish cartilage erosion. There was no spatial link between changes in bone and cartilage.

Conclusions

Estrogen depletion increases the susceptibility for osteoarthritic changes in articular cartilage, but not in subchondral bone. This provides further insight into postmenopausal osteoarthritis. No support for a direct causal link between bone and cartilage changes was found.

Introduction

It has been suggested in literature that estrogen depletion plays a role in onset or progression of osteoarthritis (OA). From epidemiological studies it is known that before the age of 50 men have a higher prevalence of OA than women [1], but after this age the prevalence is higher in women [2]. The prevalence increases with age in both men and women, but in women, it increases dramatically around the age of 50 [1,3,4], which coincides with menopause.

Also in animal models a link between estrogen and OA has been found. A number of animal studies have been performed to investigate the effect of estrogen depletion and estrogen replacement on articular cartilage (reviewed in [5]). In several animal models, ovariectomy leads to osteoarthritic changes, and estrogen replacement therapy reduces cartilage degradation [6-8].

Estrogen acts via the estrogen receptor (ER), which has two isoforms: ERα and ERβ. These receptors have been found in articular cartilage, bone, synovial tissue and ligaments [9-12], which may all be involved in OA. Several studies have reported associations between OA and polymorphisms in ERα and ERβ [13-15]. Also low serum estradiol levels have found to be associated with OA [16]. Taken together, this argues for a role of estrogen in the development of osteoarthritis.

The exact mechanism by which estrogen affects OA is not known. Apart from a direct effect of estrogen on cartilage, the bone may also be involved. Estrogen is known to affect bone metabolism and to regulate the balance between bone formation and resorption [17]. Subchondral bone changes have been reported in OA patients [18-20] and in animal models for OA [21-23]. It has been suggested that subchondral bone changes are important in the aetiology of OA [24]. Alteration in subchondral bone remodelling, and subsequently, in bone structure may lead to changes in load distribution. This may in turn cause or accelerate cartilage damage. Therefore, bone changes induced by estrogen depletion may play a role in OA development.

Although the prevalence increases after the age of 50, not all postmenopausal women get osteoarthritis [25], indicating that hormonal changes alone are not enough to cause OA. We hypothesize that estrogen depletion increases the susceptibility of tissues in the joint for changes, but that an extra trigger is needed to develop osteoarthritic changes. This concurs with the idea that OA is a multifactorial disease. We addressed this hypothesis by investigating bone and cartilage changes in the knee joint of ovariectomized mice and ovariectomized mice receiving estrogen replacement, combined with a mild osteoarthritis trigger induced by iodoacetate, an inhibitor of glycolysis that is widely accepted as model for osteoarthritis [26-29]. The role of bone changes was investigated by analysing ovariectomized mice that received bisphosphonates to inhibit bone changes in the absence or presence of the osteoarthritis trigger.

Methods

Animals

Female C3H/HeJ mice (Jackson, Bar Harbor, USA) were chosen because of their substantial bone loss after OVX [30]. They were housed in ventilated cages with 4 animals

per cage and were fed ad libitum. After 4 weeks of acclimatization, at the age of 12 weeks, the mice were randomly allocated to a treatment group (as explained below). After 12 weeks (at 24 weeks of age) the experiment was finished, and serum, knee joints and uteri were collected. The experiment was approved by the animal ethics committee.

Ovariectomy and estrogen supplementation

Estrogen depletion was induced by bilateral ovariectomy (OVX) in 8 animals. Another 8 animals underwent sham ovariectomy (Sham). A third group of 8 animals underwent bilateral ovariectomy and received estrogen supplementation by subcutaneous implantation of an estrogen pellet (Innovative Research of America, Sarasota, USA) (OVX+E). This pellet continuously released 17β-estradiol at a rate of 12 μg/day. As an analgesic, the animals received a subcutaneous injection of buprenorphine (Temgesic, 0.05 mg/kg body weight) before the operation. At the end of the operation, the mice received an intra-articular injection with 6 μl 0.5% iodoacetate (IA, Sigma-Aldrich) in one knee, and 6 μl saline (Sal) in the contralateral knee [26], resulting in the following experimental groups: Sham+Sal, Sham+IA, OVX+Sal, OVX+IA, OVX+E+Sal, OVX+E+IA.

During the experiment, five mice in the OVX+E group died, in weeks 7, 8 (2 mice), 10, and 12. Because the mouse that died in week 12 may have been dehydrated since its death, we decided not to include this animal for body weight and uterus weight, leaving only 3 mice available for these analyses at week 12. But the mouse that died in week 12 was included for bone and cartilage analyses, resulting in 4 animals in the OVX+E group at week 12.

Bisphosphonate treatment

To inhibit the bone changes, another group of 8 animals underwent bilateral OVX, and received a weekly intraperitoneal injection of alendronate dissolved in saline (2 mg/kg bodyweight, donated by Merck) (OVX+BP). These mice also received intra-articular injection with 6 μl 0.5% iodoacetate in one knee (OVX+BP+Sal) and 6 μl saline in the other knee (OVX+BP+IA).

Micro-CT analysis

Mice were scanned using an in-vivo micro-computed tomography (micro CT) [31,32] scanner (Skyscan 1076, Skyscan, Kontich, Belgium) at 9 μm voxel size . The mice were anaesthetized using a 5% isoflurane/oxygen mixture. The legs were stretched, taped to a polystyrene foam block and placed in a perspex holder. This way, both knee joints could be imaged at the same time, without interfering tissue of abdomen or tail and without needlessly radiating the abdomen. Mice were scanned every 3 weeks starting just prior to OVX or Sham operation (t=0).

Reconstructed grey-scale images were aligned visually using anatomical landmarks to get similar orientation for all knees. The scans were segmented using a local thresholding algorithm [33] and the proximal tibia was isolated (figure 4.1A). Using 3D data analysis software (CTAnalyzer, Skyscan) the tibial epiphysis was selected as region of interest for further analysis. Since we were only interested in bone changes occurring underneath the articular cartilage layer, care was taken not to include any osteophytes. The epiphysis was

further divided into a cortical (i.e. subchondral bone plate) and trabecular part [23], which were analysed separately using 3D-Calculator (freely available[34]).

In the cortical compartment of the epiphysis, regions of interest (0.5mm in medial-lateral direction, 0.7mm in anterior-posterior direction) were selected at the middle of both medial and lateral plateau, representing the subchondral plate [22] (figure 4.1B) to calculate three-dimensional thickness.

To describe the bone structure of the epiphyseal trabecular compartment (figure 4.1C) we calculated: bone volume fraction, describing the ratio of bone volume over tissue volume (BV/TV); structure model index (SMI), describing whether a bone structure is rod-like or plate-like [35]; connectivity density (ConnD), representing the number of trabecular connections in a given volume [36]; and three dimensional thickness (TbTh.) [37].

At the metaphysis a region of interest (1 mm high), containing only trabecular bone (figure 4.1D) was selected to calculate BV/TV and TbTh.

To follow bone changes over time within one mouse, datasets of week 0 and week 12 were matched by rotating and translating one data set with respect to the other [31,32]. Registration (matching) software was used, which automatically matches two datasets using a optimization criterion based on maximizing mutual information [38].

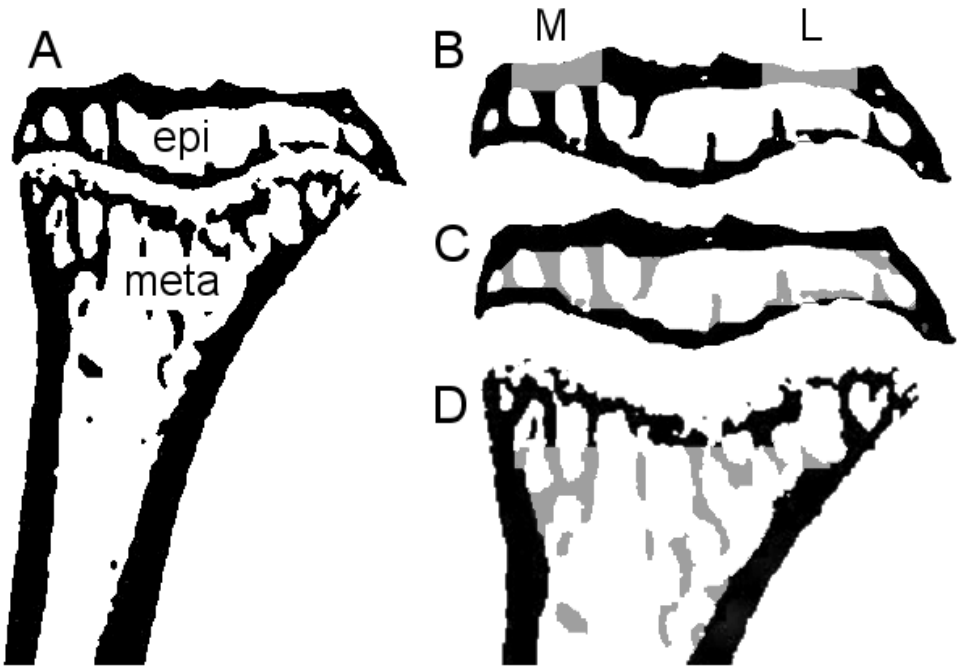

Figure 4.1: Regions that were analysed using micro-CT. A: Cross-sectional image of proximal tibia, including epiphysis (epi) and metaphysis (meta). B: Epiphysis with medial (M) and lateral (L) subchondral plate depicted in light grey. C: Epiphysis with trabecular bone depicted in light grey. D: Metaphysis with trabecular bone region depicted in light grey.

Cartilage and osteophyte analysis

Knee joints were fixed in 4% formalin, decalcified with EDTA and embedded in paraffin. Frontal sections (6 μm thick) were stained with Safranin-O. Severity and extent of cartilage erosion in medial and lateral compartments were scored by a blinded

observer with the grading and staging scoring system described by Pritzker et al [39]. Per knee the average of three sections (100 μm apart) was determined, giving a maximum score of 24. GAG depletion in medial and lateral tibial plateau was scored by rating maximal loss of Safranin-O staining (0 = no depletion, 1 = mild depletion, 2 = severe depletion except pericellular, 3 = severe depletion) multiplied by the percentage of the area affected, resulting in a maximum score of 300. Osteophytes were scored by assigning 0 for no osteophytes, 1 for cartilaginous osteophytes and 2 for (partly) calcified osteophytes.

Statistical analysis

Results are expressed as mean ± sem. A two-way ANOVA was performed to evaluate 1) whether there was interaction between the systemic treatment (Sham, OVX, OVX+E, or OVX+BP) and the IA injection, 2) whether the systemic treatment affected the results independent of IA injection, and 3) whether IA injection affected the results independent of the systemic treatment. In addition, data from multiple groups were compared using Kruskal-Wallis non-parametric analysis followed by Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired) as appropriate. The time-course data were compared using two-way repeated measures ANOVA. A p-value of <0.05 was considered significant.

Results

Effect of estrogen depletion and estrogen replacement

OVX mice weighed more than Sham mice at the end of the experiment (Sham: 21.9±0.6 gram; OVX: 25.1±0.3 gram, $p<0.05$) but no difference was found between OVX and OVX+E mice (OVX+E: 25.0±1.0 gram). The strong reduction in uterine weight in the untreated OVX group proved successful ovariectomy (Sham: 91.4±8.4 mg; OVX: 21.8±1.5 gram, $p<0.05$). Uterus weight of OVX+E mice (OVX+E: 189.6±33.5 mg) was increased compared to OVX and Sham mice.

The thickness of the subchondral plate, which is the bone structure closest to the cartilage, was followed over time by 3-weekly micro-CT scans. Medial subchondral plate thickness of the Sham+Sal, Sham+IA and OVX+Sal groups increased slowly over time, whereas in the OVX+IA group the medial plate thickness decreased in the first 3 weeks post-surgery, and stayed fairly constant afterwards, resulting in a lower value at 12 weeks post-surgery (figure 4.2A). At the lateral side, the decrease in subchondral plate thickness in the OVX+IA group the first 3 weeks post-surgery progressed until 12 weeks post-surgery. In contrast Sham+Sal, Sham+IA and OVX+Sal groups stayed fairly constant over time (figure 4.2B). Importantly, supplementation with estrogen partially (lateral) or completely (medial side) prevented the loss in subchondral plate thickness induced by OVX+IA (figures 4.2C and D). The time course of OVX+IA was significantly different from Sham+IA and OVX+E+IA, both for medial and lateral plate thickness.

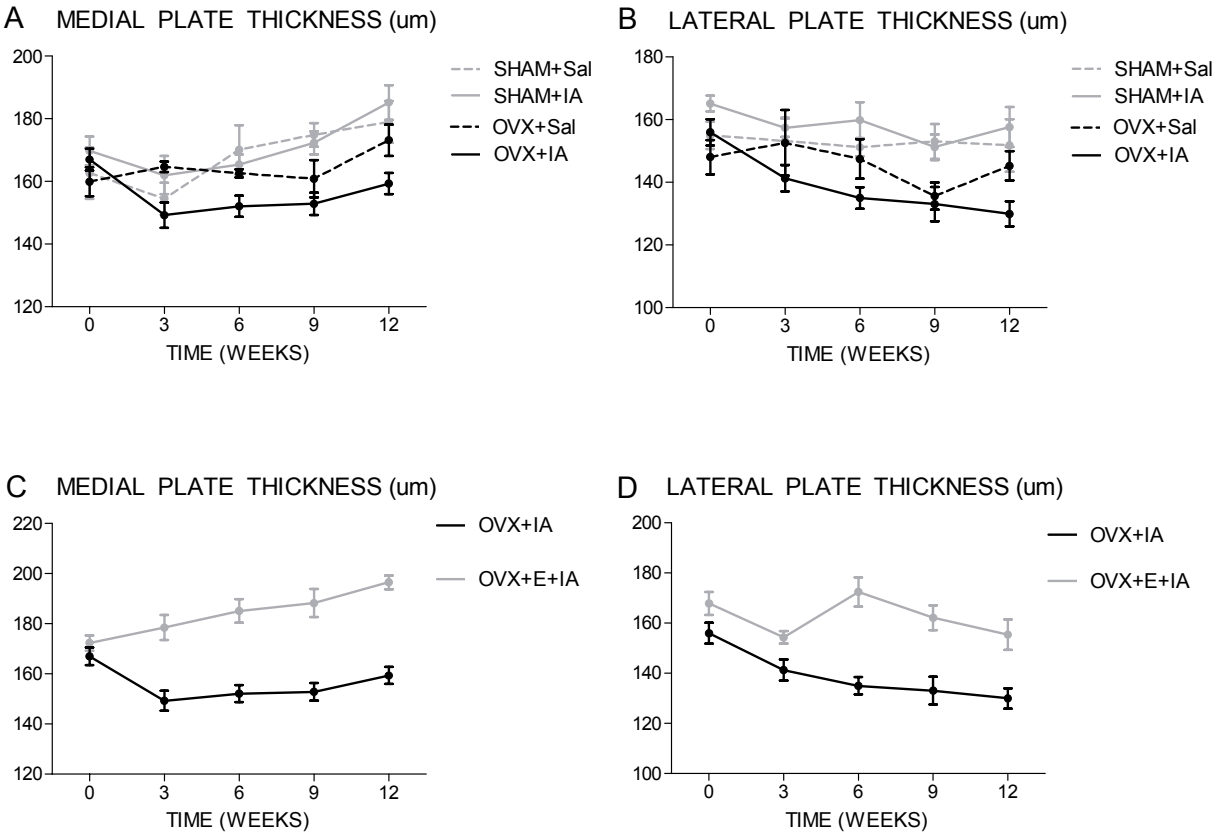

Figure 4.2: Time course of changes in subchondral plate thickness derived from in-vivo microCT analysis. Mice were scanned every three weeks. A and B: Sham and OVX groups. C and D: OVX+IA and OVX+E+IA groups. Both for medial and lateral plate thickness, the time course of the OVX+IA group was significantly different from that of the Sham+IA group and of the OVX+E+IA group, based on two-way repeated measures ANOVA.

Registration of the proximal tibia showed thinning of the medial and lateral subchondral plate and osteophyte formation in the OVX+IA mouse at 12 weeks post-surgery (figure 4.3). These changes were not observed in OVX+Sal knees. In OVX+E+IA knees, osteophytes had formed and medial plate thickness was increased due to endocortical apposition.

Since cartilage data are only available at 12 weeks post-surgery, we focused in greater detail on bone and cartilage changes at this time point. OVX significantly decreased medial and lateral subchondral thickness, independent of the presence or absence of IA injection. In the Sham group, IA injection had no effect on the subchondral plate thickness. In contrast, after OVX, IA injection decreased subchondral plate thickness, resulting in significantly lower subchondral plate thickness in the OVX+IA group than in the Sham+Sal, Sham+IA and OVX+Sal groups at medial and lateral side (figures 4.4A and B). However, no significant interaction was found by 2-way ANOVA.
Independent of the presence or absence of IA injection, estrogen supplementation after OVX significantly increased subchondral plate thickness. IA injection had no effect on subchondral plate thickness in the OVX+E group (figures 4.4A and B).

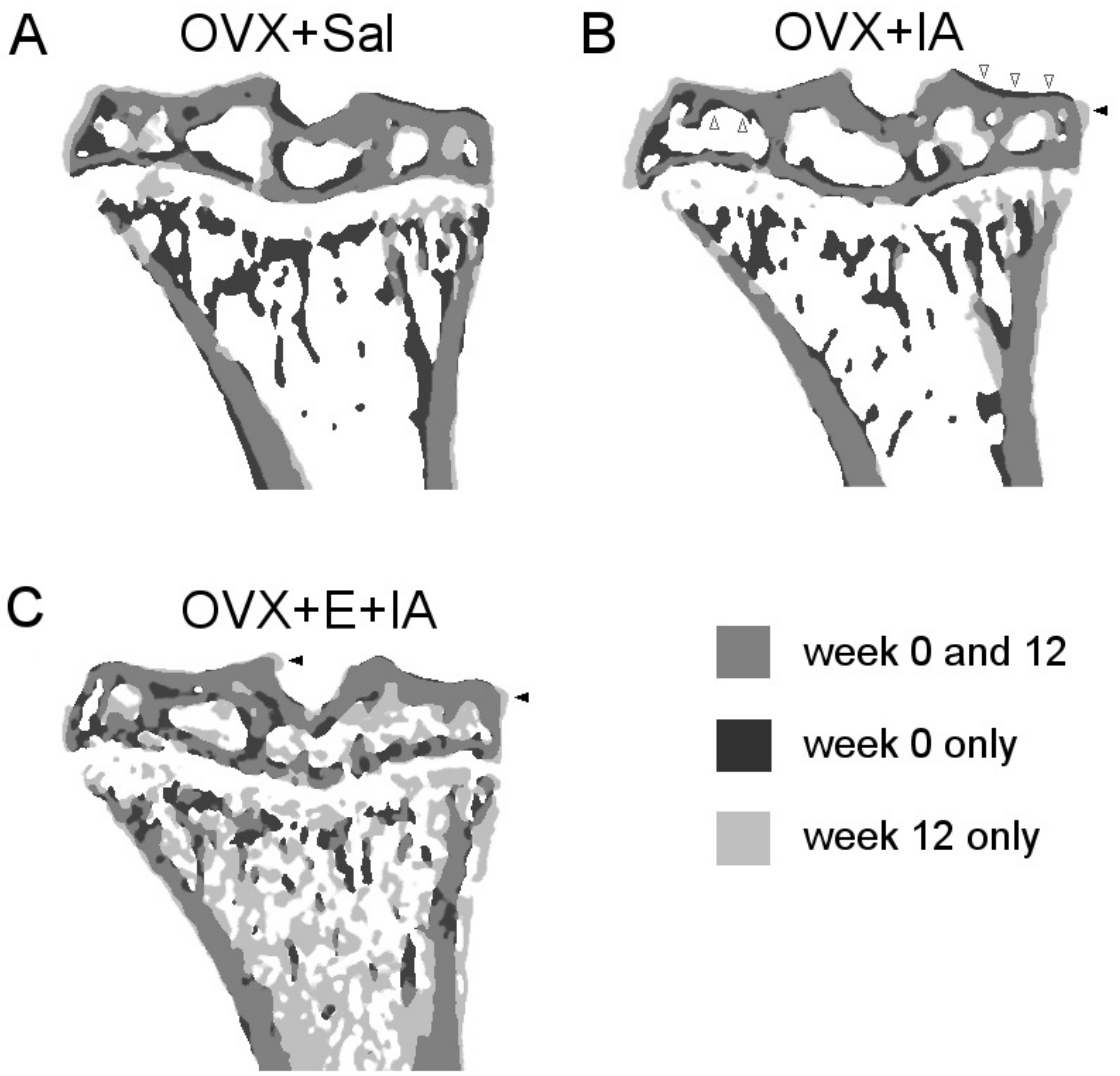

Figure 4.3: Overlaid registered longitudinal cross-section of proximal tibia scanned at week 0 (start of experiment) and week 12 for (A) OVX+Sal mouse, (B) OVX+IA mouse, and (C) OVX+E+IA mouse. Dark grey: present at both time points; Black: Only present at week 0 (i.e. resorbed in 12 weeks); Light grey: Only present at week 12 (i.e. newly formed in 12 weeks). Note the thinning of the subchondral plate (open arrow heads in B), loss of trabeculae in the metaphysis (both A and B), and the osteophyte formation at the medial epiphysis (closed arrow head in B and C).

In the epiphysis, OVX did not affect trabecular bone volume fraction (BV/TV) at 12 weeks post-surgery. The only difference in BV/TV was observed within the Sham group (Sham+Sal vs Sham+IA, figure 4.5A). Trabecular thickness (TbTh), structure model index (SMI) and connectivity density (ConnD) showed no difference among the four groups (figure 4.5B and data not shown).

Independent of IA injection, estrogen supplementation strongly increased BV/TV and ConnD, and decreased SMI compared with the OVX groups, while TbTh was the same in all groups (figures 4.5A and B, and data not shown). The increase in BV/TV and ConnD can also be observed in the registered images of 0 and 12 weeks post-surgery (figure 4.3).

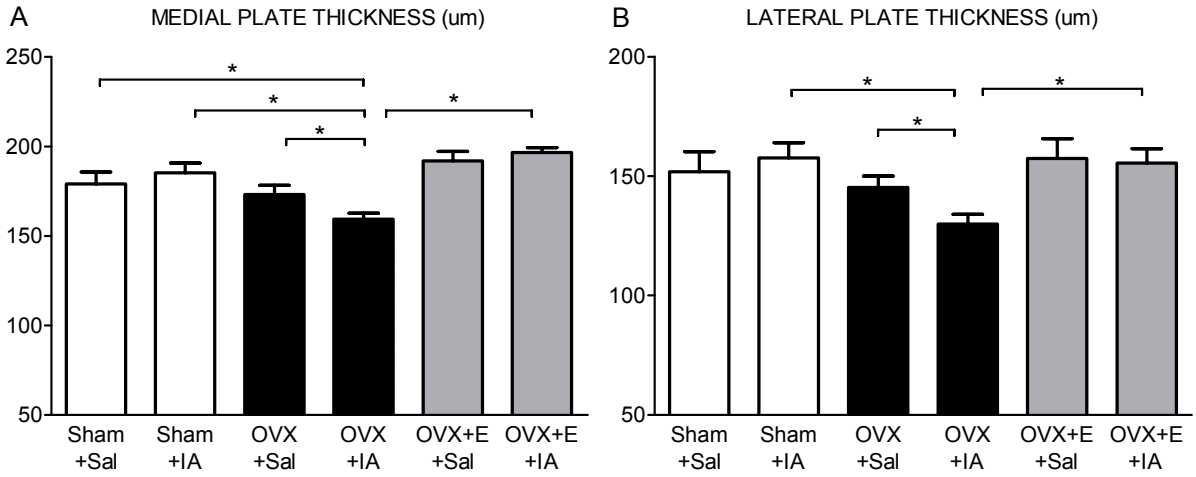

*Figure 4.4: Subchondral plate thickness derived from microCT analysis at 12 weeks post-surgery for Sham (n=8), OVX (n=7), and OVX+E (n=4) groups. A: Medial subchondral plate and B: Lateral subchondral plate. * indicates p<0.05 based on Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired).*

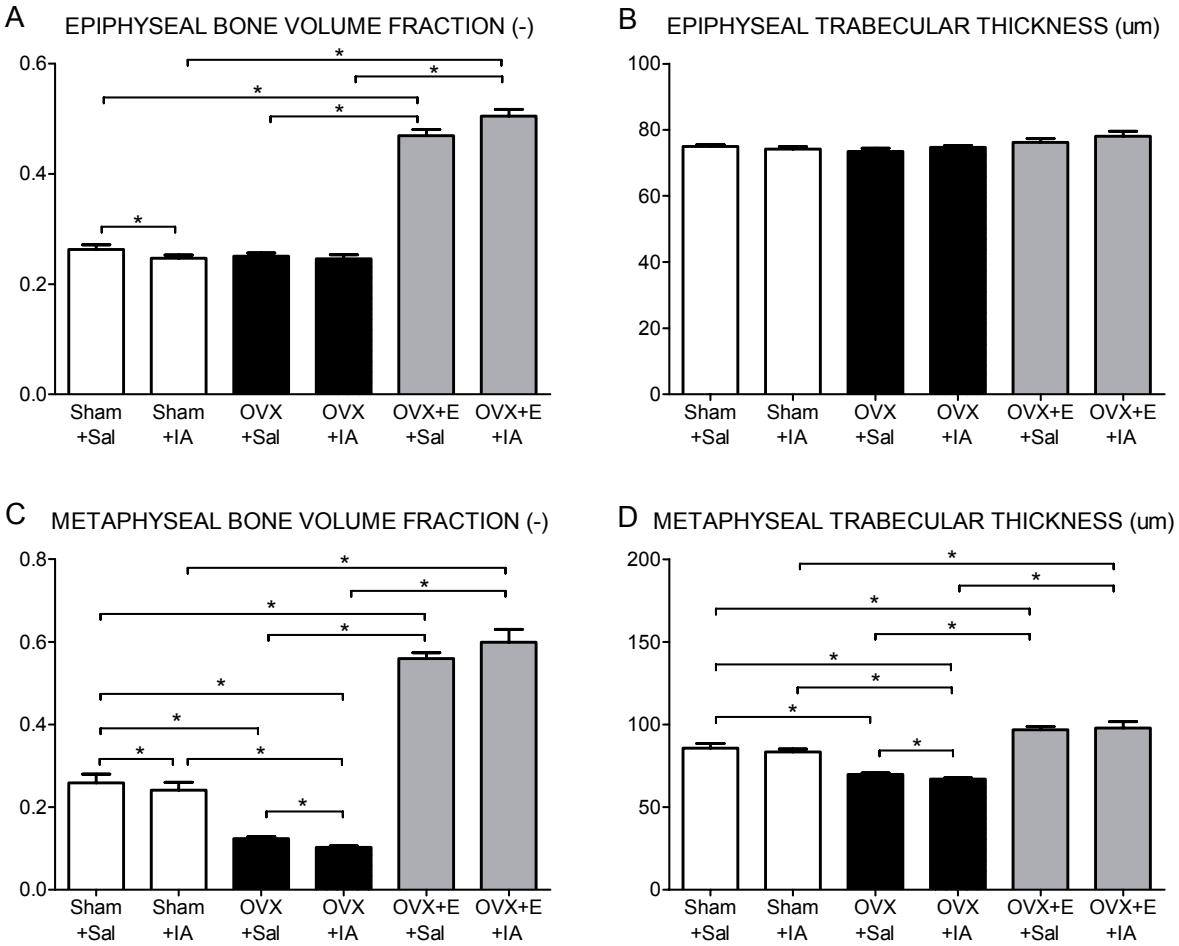

*Figure 4.5: Trabecular bone parameters derived from microCT analysis at 12 weeks post-surgery for Sham (n=8), OVX (n=7), and OVX+E (n=4) groups. A: Epiphyseal bone volume fraction, B: Epiphyseal trabecular thickness, C: Metaphyseal bone volume fraction, and D: Metaphyseal trabecular thickness. * indicates p<0.05 based on Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired).*

In contrast to the epiphysis, OVX caused a significant decrease in BV/TV and TbTh in the metaphysis (figures 4.5C and D), independent of IA injection. BV/TV was lower in IA-injected than in saline-injected legs in both Sham and OVX groups, whereas only in the OVX group TbTh was lower after IA injection.

As in the epiphysis, estrogen supplementation strongly increased BV/TV compared to the OVX group, independent of IA injection. However, in contrast to the epiphysis, estrogen supplementation also increased TbTh in the metaphysis (figures 4.5C and D). These changes in metaphyseal BV/TV are also clearly visible in the registered images of 0 and 12 weeks post-surgery (figure 4.3).

In none of the experimental conditions the metaphyseal cortical thickness was affected by IA injection (data not shown).

Although 6 µl 0.5% IA appeared to be a mild trigger (figure 4.6), IA significantly increased cartilage erosion and GAG depletion at the medial tibia plateau independent of Sham or OVX treatment. At the lateral tibia plateau, no significant effects were observed (data not shown). When studying the separate groups, IA injection did not lead to

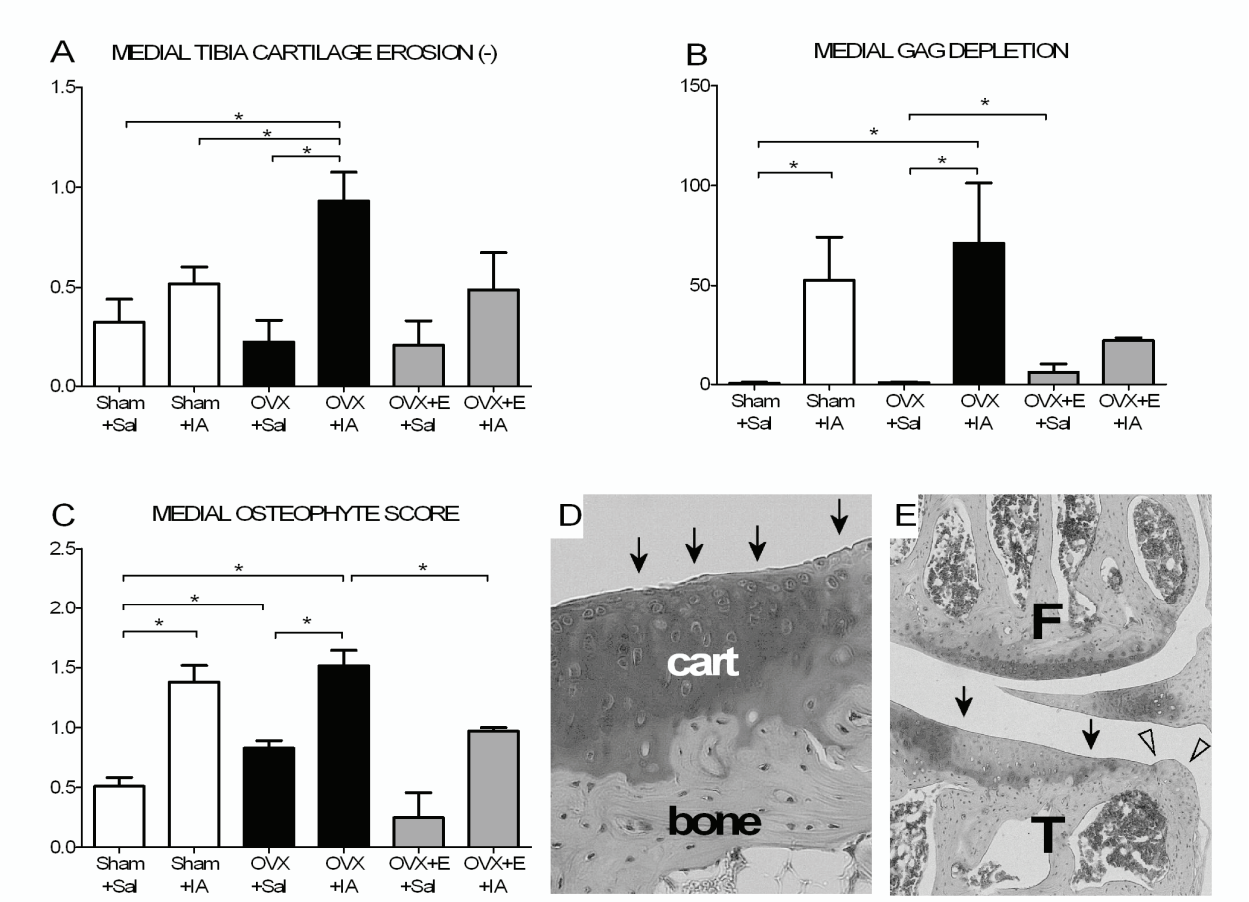

*Figure 4.6: Cartilage and osteophyte parameters derived from histology at 12 weeks post-surgery for Sham (n=8), OVX (n=7), and OVX+E (n=4) groups. A: Medial cartilage erosion, B: Medial GAG depletion, C: Medial osteophyte score. * indicates $p<0.05$ based on Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired). D: Representative image of cartilage erosion in medial tibia plateau. Arrows indicate surface fibrillation. E: Representative image of medial knee compartment with GAG depletion (between arrows) and osteophyte formation (indicated by open arrowheads).*

significant cartilage erosion in sham-operated mice. Also OVX alone (OVX+Sal) did not increase cartilage erosion or GAG depletion (figure 4.6A and B). Interestingly, cartilage erosion at the medial tibia plateau of OVX+IA knees was significantly higher than that in the Sham+Sal, Sham+IA, and OVX+Sal knees, which was also reflected by significant interaction between OVX and IA injection. For GAG depletion there was no interaction between OVX and IA injection.

Estrogen supplementation tended to limit cartilage erosion and GAG depletion caused by IA (figure 4.6A and B), although not significantly, which may be due to the low number of animals in this group (n=4).

IA injection significantly increased medial osteophyte score, independent of Sham or OVX treatment, resulting in severest osteophytes in both Sham+IA and OVX+IA (figure 4.6C). Estrogen supplementation significantly decreased osteophyte score, independent of IA injection (figure 4.6C). No significant effects on lateral osteophyte score were observed (data not shown).

Effect of bisphosphonate treatment

To study the effect of OVX on cartilage independent of bone changes we administered bisphosphonate (BP). Body and uterus weight of OVX+BP mice were not different from OVX mice (body weight: OVX: 25.1±0.3 gram, OVX+BP: 23.9±1.0 gram; uterus weight: OVX: 21.8±1.5 mg, OVX+BP 22.8±2.1 mg).

BP significantly increased lateral subchondral plate thickness, independent of IA injection. Subchondral plate thinning in OVX+IA knees was compensated by BP at the lateral (figure 4.7A), but not at the medial side (data not shown).

In the epiphysis, BV/TV and ConnD were increased in OVX+BP compared to OVX mice, independent of IA injection, while TbTh was similar in all groups (figure 4.7B and data not shown). SMI was decreased in OVX+BP+IA compared to OVX+IA knees.

Metaphyseal BV/TV (figure 4.7C) and TbTh were increased in the OVX+BP groups compared to the OVX groups, independent of IA injection. The OVX-induced loss in trabecular bone volume fraction was overcompensated by BP, resulting in higher values than the Sham groups. Metaphyseal cortical thickness was not affected by IA (data not shown).

At both medial and lateral side, BP tended to diminish cartilage erosions caused by IA albeit not significant (figure 4.7D). No effect on GAG depletion was observed. BP did not affect osteophyte formation.

Discussion

The current study demonstrates that loss of estrogens leads to increased susceptibility of articular cartilage for an osteoarthritis trigger. This observation corroborates clinical and epidemiological observations of increased OA incidence after menopause [1,3,4]. It also indicates the interplay between hormonal changes and external triggers in the aetiology of osteoarthritis.

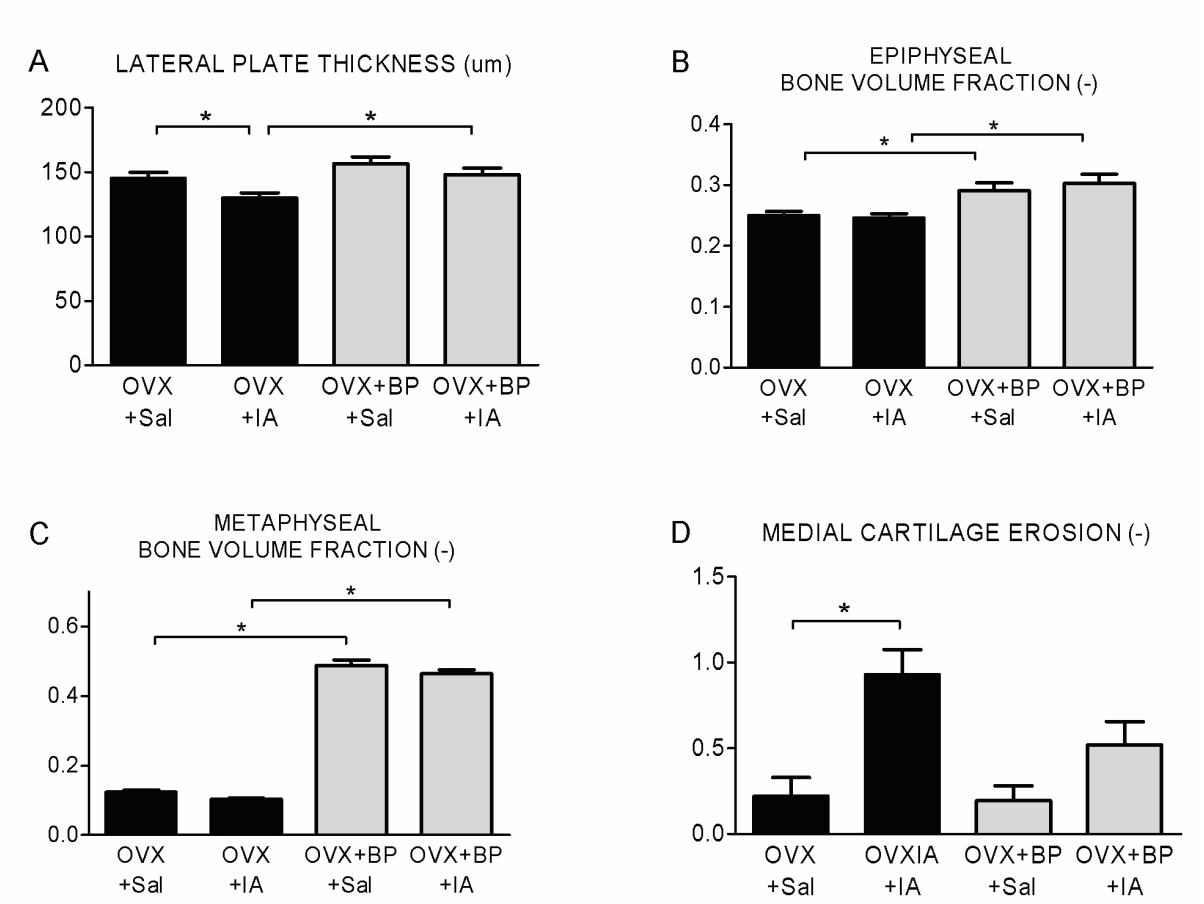

*Figure 4.7: Bone and cartilage parameters at 12 weeks post-surgery for OVX (n=7) and OVX+BP (n=8) groups. A: Lateral subchondral plate thickness, B: Epiphyseal bone volume fraction, C: Metaphyseal bone volume fraction, and D: Medial cartilage erosion. * indicates $p<0.05$ based on Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired)*

We aimed at applying a very mild OA trigger by injection of IA to test our hypothesis of a role for estradiol in the susceptibility for OA. It turned out that the trigger was indeed very mild and that after estradiol loss the changes in cartilage were indeed stronger, as reflected in the significant interaction between OVX and IA injection. However, still the changes were very mild which precludes observing a significant prevention of cartilage erosion after estradiol supplementation. Nevertheless, the direction of changes of all three cartilage parameters (erosion, GAG depletion and osteophytes) support the hypothesis that estradiol reduces the susceptibility to osteoarthritis in the tibia. New studies to support and confirm this hypothesis should aim at a stronger OA trigger to induce more cartilage erosion enabling to assess clear and significant effects of estradiol. Our data are supported by previous studies using more severe ways of OA induction in combination with OVX [8,40]. However, the effect of estrogen supplementation was not investigated in these studies [8,40]. Although several studies in other species reported increased cartilage erosion and decreased GAG content after OVX alone [6,8,41,42], in our study with mice we did not observe cartilage erosion after OVX alone. This suggests that loss of estrogens alone does not directly lead to changes in cartilage and bone in the tibia after 12 weeks. Longer follow-up may lead to increased cartilage erosion in the OVX group.

We used slow-release estradiol pellets that led to a continuous non-cyclical estradiol level. This turned out to be a major challenge for the mice resulting in a loss of 5 out of 8 animals at the end of the experiment, limiting the power to detect significant changes. The cause of this unexpected death in the estradiol supplemented group is unclear. Data on this mortality effect in healthy, wild type mice is virtually absent. However, it has been shown that long-term estradiol supplementation causes death in an ovarian atrophy model in APPswe transgenic mice [43] as well as in a myocardial infarction model in C57BL/6J mice [44].

An important observation was that loss of estradiol didn't change epiphyseal bone characteristics. Intriguingly, subchondral plate thickness only decreased in the OVX situation when the osteoarthritis trigger IA had been applied. Although there was no significant interaction between OVX treatment and IA injection, the effect of both treatments added up in the OVX+IA mice, resulting in a significantly decreased subchondral plate thickness. This provides further support for a role of estradiol in the susceptibility for osteoarthritis and adds to the concept of the involvement of bone, specifically the subchondral bone plate, in osteoarthritis. Albeit an important support for this, the current data do not enable conclusions on cause or consequence.

The decrease in subchondral plate thickness is in line with findings from previous OA animal studies evaluating the early stage of the disease [21,23,45-47]. In some of these studies, this was followed by a later phase of plate thickening [21,46]. This also explains the discrepancy with sclerosis described in human studies [48-50], which often concern patients with late osteoarthritis, whereas our present study reflects a relatively early phase of osteoarthritis.

While loss of estradiol did not cause changes in epiphyseal bone, supplementation with estradiol strongly increased subchondral plate thickness and epiphyseal bone volume fraction. This contrasts observations at the metaphyseal level where both OVX (decrease in bone volume) and estradiol supplementation (increase in bone volume) had strong effects, suggesting that epiphyseal bone is sensitive but less dependent on circulating estradiol than metaphyseal bone. This might be explained by differences in estrogen receptor levels. However, no data are available yet to support this. An alternative explanation may be that local production of estradiol in bone [51-54] may be sufficient to maintain proper bone turnover in the epiphysis in the absence of circulating estradiol implicating that epiphyseal bone is more sensitive to estradiol or the local production is higher than in the metaphysis. This yet unknown difference between epiphyseal and metaphyseal bone in estradiol sensitivity or local production could be important for understanding the functional link between estradiol loss, bone turnover and development of osteoarthritis.

In an attempt to take this further we studied the impact of OVX on the IA effect on cartilage while the changes in bone would be blocked using BP. Generally, treatment with BP had a similar effect on epiphyseal bone after IA in the OVX condition as estradiol, i.e. increase in subchondral plate thickness and bone volume fraction and no effect on trabecular thickness. Interestingly, both at the medial and lateral side the cartilage erosion appeared to be less when OVX-IA mice were treated with BP. This is in line with reports that BPs may have an OA protective effect [55-57]. Whether BPs exert a direct effect on cartilage or whether this is due to the inhibited bone changes is not known.

The longitudinal bone data showed differences in bone dynamics for the medial and lateral side. At the medial side, the subchondral plate thickness increased (in Sham mice), while it stayed constant at the lateral side. Also the effects of treatment with BP or estrogen supplementation seemed to be side specific: BP treatment only exerted a significant effect at the lateral plate thickness, while estrogen supplementation increased both medial and lateral plate thickness. This indicates that at the medial subchondral plate bone formation dominates, while at the lateral subchondral plate bone resorption also contributes. These mice are relatively young (12 weeks at the start and 24 weeks at the end of the study) and may still be in a modelling phase in which the epiphyseal area of the joint is being restructured. In contrast to the subchondral plate changes, cartilage changes occurred predominantly at the medial side. A spatial link between changes in bone and cartilage seems to be absent, as also observed by Botter et al [22,23], indicating these may be two independent characteristics of osteoarthritis. Alternatively, the bone changes may result in an altered force distribution leading to cartilage erosion at a site distal from the bone changes.

IA injection not only had effect on epiphyseal bone but also affected metaphyseal bone, albeit mildly. It is unlikely that injection of IA has direct distant effects in the metaphyses. One possible explanation is that the IA injected leg was unloaded due to pain leading to bone loss. It is intriguing that the unloading effect would even enhance the already large decrease in bone volume due to OVX but it has been suggested that estrogen deficiency may exacerbate pain perception [58]. Unlike trabecular bone, cortical bone in the metaphysis was not changed by IA injection. This suggests that the changes in the subchondral plate (cortical bone) are not caused by unloading, but are an intrinsic part of the osteoarthritic pathological process.

In conclusion, this study supports the link between estradiol and development of osteoarthritis. Estrogen depletion increases susceptibility of articular cartilage for an osteoarthritis trigger. This provides further insight into postmenopausal osteoarthritis. The current data do not support a direct causal link between bone and cartilage changes. Finally, epiphyseal and metaphyseal bone have a different sensitivity to estradiol and the current data support notions that BP have an OA protective effect.

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Chapter 5

Loss of estrogen increases patellar cartilage damage and leads to early transient subchondral cortical thinning

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A combined manuscript of Chapters 4 and 5 has been submitted for publication

Abstract

Objective

Patella osteoarthritis (OA) is very common. Estrogen depletion may play a role in onset or progression of patella OA. We investigated in mouse patellae whether estrogen depletion increases susceptibility of articular tissues to osteoarthritic changes by combining ovariectomy with an osteoarthritis trigger. Furthermore, we investigated the involvement of estrogen-induced bone changes in patella OA development.

Methods

Female C3H/HeJ mice were divided into four groups: sham operated, estrogen depletion by ovariectomy (OVX), OVX with estradiol supplementation (OVX+E), and OVX treated with bisphosphonate (OVX+BP) to inhibit bone changes. In all mice, one knee was injected with iodoacetate (IA) to trigger cartilage degeneration, the contralateral knee was injected with saline. Patellar bone changes were followed over time using in-vivo micro-CT, and patellar cartilage was analysed by histology 12 weeks post-surgery.

Results

OVX increased patellar cartilage damage, independent of IA injection. There was no significant interaction between OVX and IA in their effect on cartilage. OVX and IA significantly decreased subchondral cortical thickness at week 3. There was no interaction between OVX and IA, but the effects on subchondral cortical thickness were additive in OVX+IA mice. Treatment with estradiol (OVX+E) inhibited patellar cartilage damage and cortical bone thinning, independent of IA. BP treatment (OVX+BP) inhibited patellar cartilage damage independent of IA, whereas only the combination of IA injection and BP treatment led to increased cortical thickness and bone volume fraction.

Conclusions

Estrogen depletion increased patellar cartilage damage and transiently enhanced subchondral cortical thinning, thereby supporting the link between estradiol and development of osteoarthritis.

Introduction

Osteoarthritis (OA) of the knee joint is a common, disabling, and expensive disease [1,2]. The knee is a complex joint consisting of three compartments: medial and lateral tibiofemoral joint (TFJ) compartments, and patellofemoral joint (PFJ). Although clinical studies of knee OA tended to focus on the tibiofemoral compartment alone, the patellofemoral compartment is often affected as well. An investigation in people with knee pain revealed that the most common radiographic pattern was combined TFJ and PFJ OA (40%), followed by isolated PFJ OA (24%)[3]. Isolated TFJ OA occurred in only 4% of subjects [3]. The patellofemoral joint is an important source of symptoms associated with knee OA, like pain, stiffness and disability [4].

McAlindon et al [5] studied the prevalence of PFJ OA in a community-based cohort aged over 55 years and reported that isolated PFJ OA was present more than twice as often in women than in men (24% of the women versus 11% of the men). This is in agreement with data from epidemiological studies in which the prevalence of OA (hand, knee and hip) is reported to be higher in women over 50 years of age than in men over 50 years of age [6]. It has been suggested that the decrease in estrogen levels occurring at menopause plays a role in the onset or progression of OA. This is plausible since tissues involved in the OA process, like cartilage, bone, synovium, ligament, and muscle, all express estrogen receptors (ERs) [7-14]. In addition, associations between OA and polymorphisms in ERα and ERβ have been reported [15-17] and low serum estradiol levels have found to be associated with OA [18]. The role of estrogen depletion and estrogen replacement therapy in OA has been studied in various animal models [19]. Several studies showed that ovariectomy leads to osteoarthritic changes, and estrogen replacement therapy reduces cartilage degradation [20-22]. However, so far OA of the patella has not been studied in relation to estrogen loss.

As OA is thought to be a multifactorial disease, we hypothesized that estrogen depletion increases the susceptibility of tissues in the patella for changes, but that an extra trigger is needed to develop osteoarthritic changes. We explored this hypothesis by investigating the bone and cartilage changes of the patella of ovariectomized mice with and without a mild osteoarthritis trigger. In addition, one group of mice received estrogen replacement after ovariectomy. The relationship with bone changes was investigated by analysing ovariectomized mice that received bisphosphonates to inhibit bone changes in the absence or presence of the osteoarthritis trigger.

Methods

Animals

Female C3H/HeJ mice, obtained from Jackson (Bar Harbor, USA), were housed in ventilated cages with 4 animals per cage and were fed ad libitum. After 4 weeks of acclimatization, at the age of 12 weeks, the mice were randomly allocated to a treatment group (as explained below). After 12 weeks, (at 24 weeks of age) serum was collected, after which the mice were killed and the knee joints and uteri were excised. The animal ethics committee gave approval for the experiment.

Ovariectomy and estrogen supplementation

Estrogen depletion was induced by bilateral ovariectomy (OVX) in 8 animals. Another 8 animals underwent sham ovariectomy (Sham). A third group of 8 animals underwent bilateral ovariectomy and received estrogen supplementation by subcutaneous implantation of an estrogen pellet (Innovative Research of America, Sarasota, Fl, USA) (OVX+E group). This pellet continuously released 17β-estradiol at a rate of 12 μg/day. As an analgesic, the animals received a subcutaneous injection of buprenorphine (Temgesic, 0.05 mg/kg body weight) before the OVX operation. At the end of the operation, the mice received an intra-articular injection with 6 μl 0.5% iodoacetate (IA, Sigma-Aldrich) in one knee, and 6 μl saline (Sal) in the contralateral knee [23], resulting in the following experimental groups: Sham+Sal, Sham+IA, OVX+Sal, OVX+IA, OVX+E+Sal, OVX+E+IA.

During the experiment, five mice in the OVX+E group died, in weeks 7, 8 (2 mice), 10, and 12. Because the mouse that died in week 12 may have been dehydrated since its death, we decided not to include this animal for body weight and uterus weight, leaving only 3 mice available for these analyses at week 12. But the mouse that died in week 12 was included for bone and cartilage analyses, resulting in 4 animals in the OVX+E group at week 12.

Bisphosphonate treatment

To inhibit bone changes, another group of 8 animals underwent bilateral OVX as described above, and received a weekly intraperitoneal injection of alendronate (2 mg/kg bodyweight, donated by Merck) (OVX+BP group). These mice also received intra-articular injection with 6 μl 0.5% iodoacetate in one knee and 6 μl saline in the other knee.

Micro-CT analysis

The mice were anesthetized using a 5% isoflurane/oxygen mixture and subsequently scanned in an in-vivo micro-computed tomography (micro CT) [24,25] scanner (Skyscan 1076, Skyscan, Kontich, Belgium) at a voxel size of 9 μm. Because knee joints are surrounded by soft tissue of the abdomen, the legs were stretched and fixed to a polystyrene foam block with non-woven tape and placed in a perspex holder. The mouse was then placed in a scanning bed, with the tail bend backwards towards the head. In this way, both knee joints could be imaged at the same time, without interfering tissue of abdomen or tail and without needlessly radiating the abdomen. The mice were scanned every 3 weeks starting just prior to OVX or sham operation, knee injections and start of treatments (t=0).

Reconstructed grey-scale images were rotated and aligned visually using anatomical landmarks to get a similar orientation for all knee joints. Next the scans were segmented, using a local thresholding algorithm [26]. Using 3D data analysis software (CTAnalyzer, Skyscan) the entire patella was selected as region of interest for further analysis (figure 5.1). The patella was further divided into a cortical part and a trabecular part (figure 5.1D), which were analysed separately.

In the cortical compartment of the patella, a region of interest was selected containing cortical bone in contact with the articular cartilage layer of the patella (subchondral cortical bone, figure 5.1E). The size of this region was 0.50 mm in medial-lateral

direction and 0.97 mm in proximal-distal direction. For this region, three-dimensional thickness was calculated.

For the trabecular compartment, bone volume fraction, which describes the ratio of bone volume over tissue volume (BV/TV), was calculated.

The presence or absence of osteophytes in microCT scans was scored. Being visible on microCT means that these osteophytes are (partially) calcified, otherwise they would not be visible.

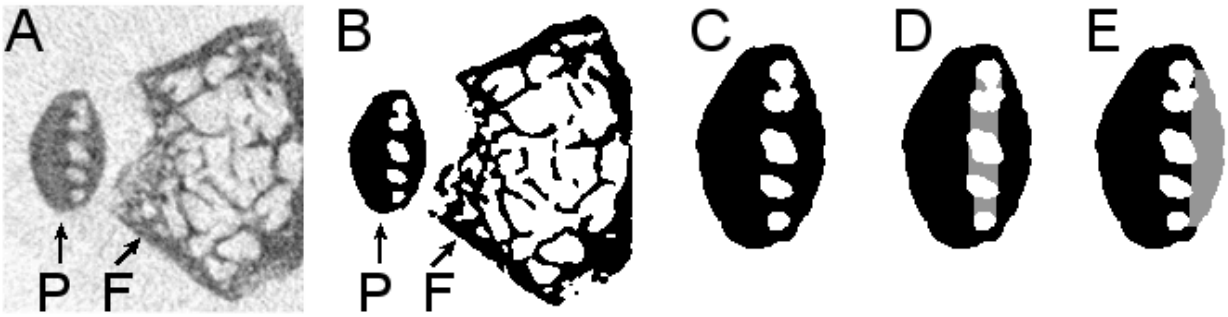

Figure 5.1: Processing microCT datasets illustrated by one cross section. A: Reconstruction and alignment. P indicates patella, F indicates femur. B: Segmentation. P indicates patella, F indicates femur. C: Isolation of patella. D: Separation of trabecular bone (grey) and cortical bone (black). E: Region of interest of subchondral cortical bone (in grey).

Cartilage and osteophyte analysis

Knee joints were fixed in 4% formalin, decalcified with EDTA and embedded in paraffin. Frontal sections of the knee joints were made (6 µm thick) which were stained with Safranin-O. Both severity and extend of cartilage damage in the patella was scored by a blinded observer using the grading and staging scoring system described by Pritzker et al [27]. Per knee joint the average score of three sections (100 µm apart) was calculated. Per patella a maximum score of 24 could be obtained. The presence or absence of osteophytes was scored. These osteophytes on histology can either be purely cartilaginous, which are not visible on microCT, or (partially) calcified

Statistical analysis

Results are expressed as mean ± sem. A two-way ANOVA was performed to evaluate 1) whether there was interaction between the systemic treatment (Sham, OVX, OVX+E, or OVX+BP) and the IA injection, 2) whether the systemic treatment affected the results independent of IA injection, and 3) whether the IA injection affected the results independent of the systemic treatment. In addition, data from multiple groups were compared using Kruskal-Wallis non-parametric analysis followed by Mann-Whitney (unpaired) or Wilcoxon signed rank tests (paired) as appropriate. The presence or absence of osteophytes was compared using a Fisher's exact test. A p-value of <0.05 was considered significant.

Results

Effect of estrogen depletion and estrogen replacement

OVX mice weighed more than Sham mice at the end of the experiment (Sham: 21.9±0.6 gram; OVX: 25.1±0.3 gram; $p<0.05$) but no difference in body weight was found between OVX and OVX+E mice (OVX+E: 25.0±1.0 gram). The strong reduction in uterine weight in the untreated OVX group measured at the end of the experiment proved the successful ovariectomy (Sham: 91.4±8.4 mg; OVX: 21.8±1.5 gram; $p<0.05$). Uterus weight of the OVX+E group (OVX+E: 189.6±33.5 mg) was increased compared to the OVX group and the Sham group.

Subchondral cortical bone

Both OVX treatment and IA injection significantly decreased the thickness of subchondral cortical bone at week 3, independent of the other treatment. Although there was no significant interaction between OVX treatment and IA injection, these two effects were additive in the OVX+IA mice, resulting in a decrease of 18% in subchondral cortical thickness at week 3 (figure 5.2A). This decrease was significantly different from the change in Sham+Sal and OVX+Sal mice. Subchondral cortical thickness of the OVX+IA mice progressively increased from week 3 onwards resulting in values similar to that of Sham+Sal, Sham+IA and OVX+Sal at week 12.

Supplementation with estrogen significantly increased the subchondral cortical thickness from week 3 till week 12, independent of IA injection. In the OVX+E+IA mice, estrogen supplementation prevented the IA-induced thinning of subchondral cortical bone at week 3 and resulted in a significantly increased subchondral cortical thickness at weeks 6, 9 and 12 (figure 5.2B).

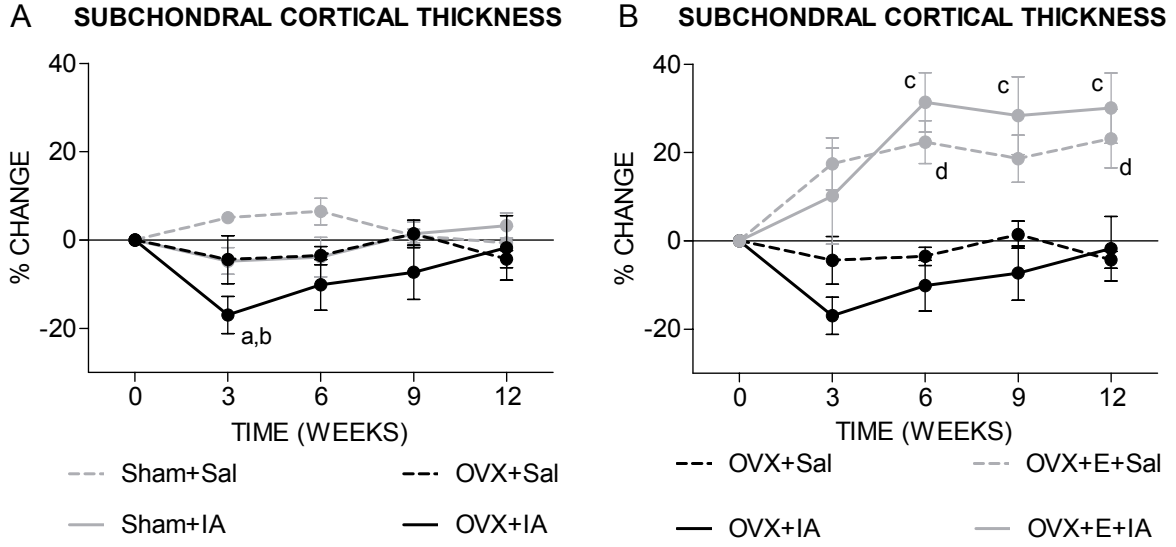

Figure 5.2: Time course of subchondral cortical thickness. Mice were scanned every 3 weeks in an in-vivo microCT scanner. A: Sham and OVX groups. Note that up to 9 weeks, Sham+IA and OVX+Sal curves are overlapping. a indicates $p<0.05$ for OVX+IA vs Sham+Sal according to Mann-Whitney test; b indicates $p<0.05$ for OVX+IA vs OVX+Sal according to Wilcoxon signed rank test (paired). B: OVX and OVX+E group. c indicates $p<0.05$ for OVX+IA vs OVX+E+IA; d indicates $p<0.05$ for OVX+Sal vs OVX+E+Sal, both according to Mann-Whitney test.

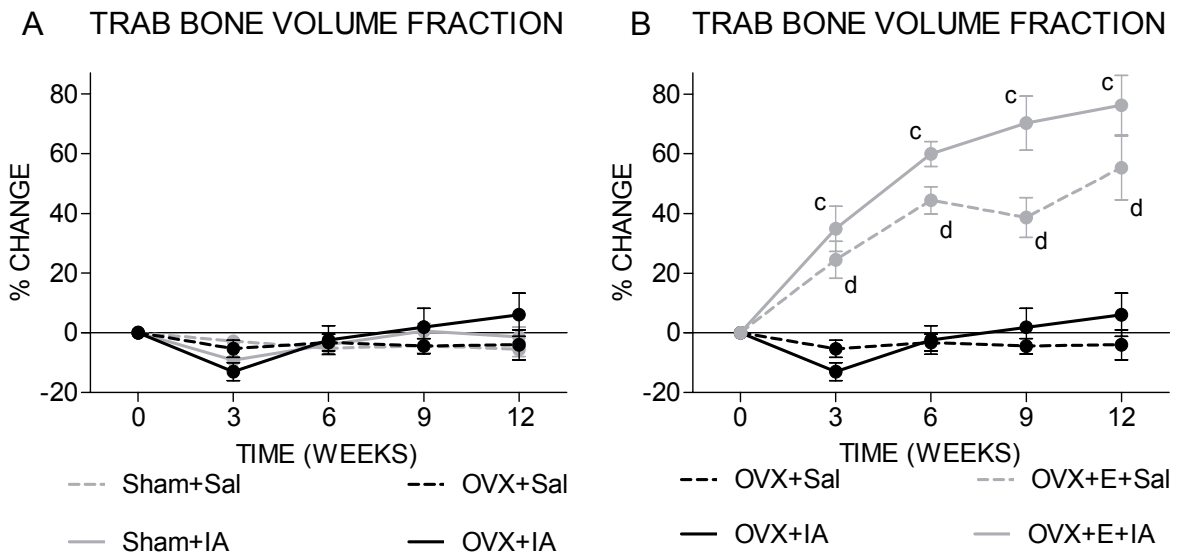

Figure 5.3: Time course of trabecular bone volume fraction, resulting from successive scans in in-vivo microCT scanner. A Sham and OVX groups. B: OVX and OVX+E groups. c indicates p<0.05 for OVX+IA vs OVX+E+IA; d indicates p<0.05 for OVX+Sal vs OVX+E+Sal, both according to Mann-Whitney test.

Trabecular bone

OVX treatment and IA injection did not significantly affect the trabecular bone volume fraction (BV/TV) (figure 5.3A).

In contrast, estrogen supplementation significantly affected BV/TV, independent of IA injection, resulting in a strongly increased BV/TV (figure 5.3B). The increase in BV/TV resulted from an increased trabecular bone volume as well as a decreased endocortical volume, as a consequence of increased subchondral cortical thickness.

Cartilage and osteophytes

Cartilage damage was assessed on histology (figure 5.4A) at the end of the experiment after 12 weeks. OVX treatment significantly affected the cartilage damage independent of IA injection, resulting in more severe cartilage damage in the OVX-groups than in the Sham-groups. The OVX+IA mice had the severest cartilage damage, which was significantly different from Sham+Sal (figure 5.4B).

Supplementation with estrogen significantly decreased the OVX-induced cartilage damage, independent of IA injection (figure 5.4B).

The presence of osteophytes at 12 weeks was evaluated both in histological sections (figure 5.4A) and in microCT scans (figure 5.4C), with the latter showing only calcified osteophytes. Two mice of the OVX+Sal group had osteophytes at the patella on histology only (table 5.1). All patellae of the OVX+IA group showed osteophytes both on histology and microCT. All of the OVX+E+IA patellae showed osteophytes on histology, but only half of them were visible on microCT scans.

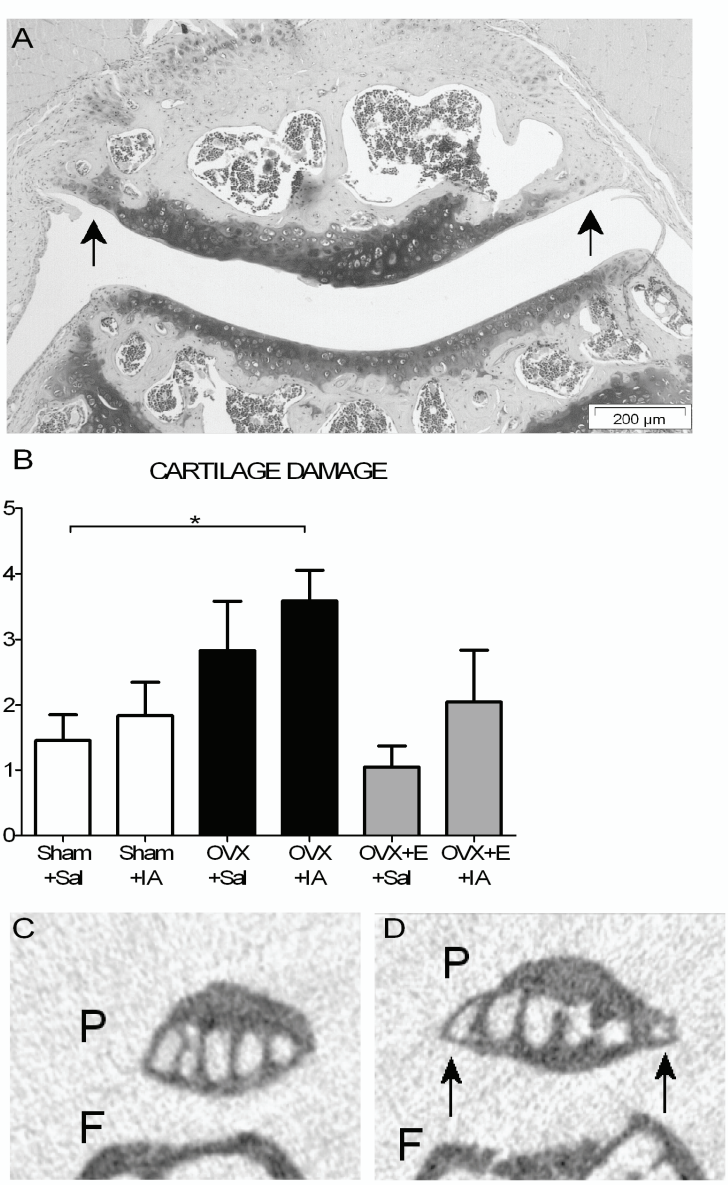

*Figure 5.4: Cartilage damage and osteophytosis at 12 weeks post-surgery for Sham, OVX and OVX+E groups. A: Cartilage damage and osteophytosis (arrows) on histology in an OVX+IA mouse. B: Cartilage damage scored on histology using Pritzker score. * indicates $p<0.05$ according to Mann-Whitney test. C: Patella without osteophytes on microCT image. P indicates patella, F indicates femur. D Patella with osteophytes (arrows) on microCT images in OVX+IA mice. P indicates patella, F indicates femur.*

Effect of bisphosphonate treatment

The effects of estradiol on the cartilage compartment may be indirect via estradiol effects on bone. To study the effect of OVX on cartilage independent of bone changes we examined the effect of OVX during bisphosphonate (BP) treatment. Body weight and uterus weight of the OVX+BP group were not different from the OVX group (body weight: OVX: 25.1±0.3 gram, OVX+BP: 23.9±1.0 gram; uterus weight: OVX: 21.8±1.5 mg, OVX+BP 22.8±2.1 mg).

Table 5.1: Presence of osteophytes on histology and micro-CT.
Osteophyte presence was scored on histology and in micro-CT datasets of the patellae and presented as the number of patellae in which an osteophyte was visible on histology or on micro-CT. Identical italic character indicates significant difference between these conditions according to Fisher's exact test.

	Histology (# patellae with osteophyte / total # patellae)		Micro-CT (# patellae with osteophyte / total # patellae)	
Sham+Sal	1 / 8	*a*	0 / 8	*b*
Sham+IA	6 / 8	*a*	5 / 8	*b*
OVX+Sal	2 / 7		0 / 7	*c*
OVX+IA	7 / 7		7 / 7	*c, f*
OVX+E+Sal	0 / 4	*d*	0 / 4	
OVX+E+IA	4 / 4	*d*	2 / 4	
OVX+BP+Sal	0 / 8	e	0 / 8	
OVX+BP+IA	5 / 8	e	1 / 8	*f*

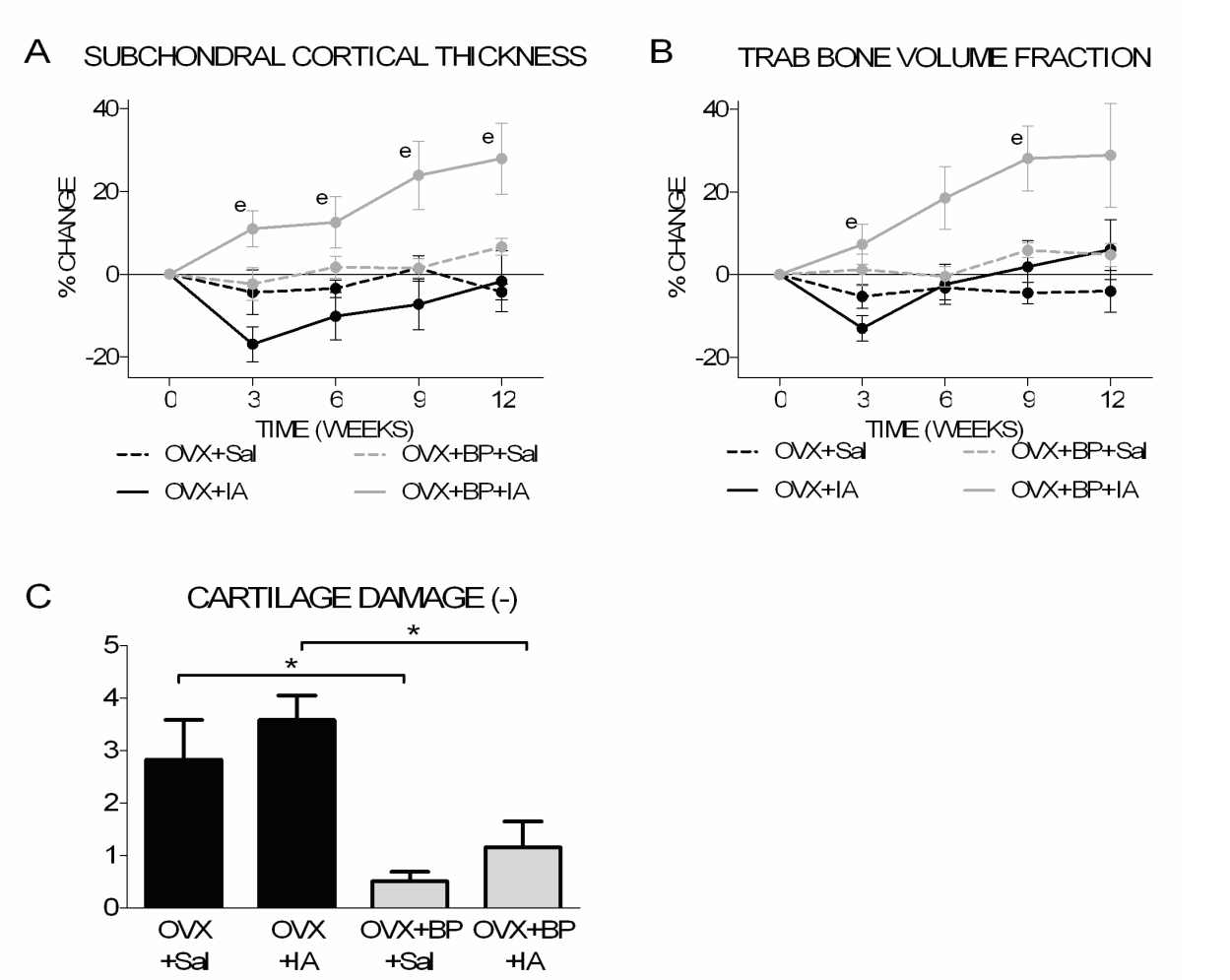

*Figure 5.5: Bone and cartilage parameters for OVX and OVX+BP groups. A: Subchondral cortical thickness. B: Trabecular bone volume fraction. C: Cartilage damage at 12 weeks post-surgery. e indicates $p<0.05$ for OVX+IA vs OVX+BP+IA according to Mann-Whitney test, * indicates $p<0.05$ according to Mann-Whitney test.*

Subchondral cortical bone

BP treatment significantly increased subchondral cortical bone, independent of IA injection. In OVX+BP+IA patellae, subchondral cortical bone was significantly thicker compared to OVX+IA patellae. In contrast, subchondral cortical bone of OVX+BP+Sal patellae did not increase (figure 5.5A).

Trabecular bone

In the trabecular bone, BP treatment significantly affected BV/TV, independent of IA injection. In OVX+BP+IA patellae BV/TV was increased, resulting in a significantly higher BV/TV than in OVX+IA patellae at week 3 and week 9. This resulted from an increase in bone volume. In contrast, the OVX+BP+Sal patellae did not show an increase in BV/TV (figure 5.5B).

Cartilage and osteophytes

BP treatment significantly affected cartilage damage, independent of IA injection, resulting in less cartilage damage in both OVX+BP+Sal and OVX+BP+IA (figure 5.5C).

In five out of eight OVX+BP+IA patellae osteophytes were seen on histology. Of these five patellae, only one had a visible osteophyte on microCT scans, which is significantly less than in the OVX+IA patellae where all patellae showed osteophytes on microCT scans (table 5.1).

Discussion

These results demonstrate that loss of estrogen leads to more severe cartilage damage in the patella and to early transient subchondral cortical thinning. Estrogen supplementation counteracts these effects. Thus, the loss of estrogen in postmenopausal women may explain the epidemiological observations that women over the age of 55 have higher prevalence of patellofemoral OA than men over 55 [5].

This study was designed to investigate the role of estrogen in the susceptibility of the patella for osteoarthritis, by combining a mild osteoarthritis trigger with estrogen depletion. While injection of a low dose of IA resulted in increased osteophytosis, it did not lead to more cartilage damage either in sham-operated or ovariectomized mice, indicating that estrogen depletion by OVX did not increase the susceptibility of patellar cartilage. However, OVX did increase the level of patellar cartilage damage and supplementation with estradiol after OVX abolished this effect. Our findings are in line with previous studies that reported more cartilage damage in the tibiofemoral compartments of the knee after OVX alone [20,22,28] or after OVX in combination with OA induction [22,29,30].

The bone changes were largest at 3 weeks in the OVX+IA patellae. Subchondral cortical thickness and trabecular bone volume fraction and thickness were decreased after 3 weeks. Whether these changes are caused by unloading of the patella (due to pain) or are caused by a biochemical response to IA remains unknown. The thinning of subchondral cortical bone is in accordance with previous reports where subchondral plate thinning in the tibia plateau was documented in the early stage of the disease in various

animal models for OA [31-34]. This early phase of thinning can be followed by a later phase of thickening [31,34].

OVX alone did not reduce trabecular bone volume in the patella, while OVX is known to decrease bone volume fraction in the metaphysis [35,36]. This indicates that the patellar trabecular bone responds differently from the metaphyseal trabecular bone and acts more similar to epiphyseal bone, which also has a weaker response to OVX than metaphyseal bone [37-39]. This may be due to differences in estrogen receptor levels or local estradiol production, although this currently remains speculative as no data regarding this are available.

In order to further investigate the relationship of bone changes with cartilage damage and osteophyte formation, we studied bone and cartilage changes in OVX mice in the absence or presence of BP. In this way we attempted to assess the impact of OVX on the IA effect on cartilage while the changes in bone would be blocked. Indeed, in the OVX+BP+Sal patellae no bone changes occurred. In contrast, treatment with BP after IA (OVX+BP+IA) strongly increased subchondral cortical thickness and trabecular bone volume fraction. Since this only occurred in IA injected knees, IA may have activated the bone turnover (increased activity of both osteoblasts and osteoclasts), of which the BP only reduced the osteoclastic resorption, resulting in a net increase. Interestingly, cartilage damage was less in both groups treated with BP (with saline and with IA), which is in line with reports suggesting that BPs may have an OA protective effect [40-42]. Since the decrease in cartilage damage occurred independent of the observed bone changes in both BP groups, BP may have a direct effect on cartilage. This precludes drawing definitive conclusions on the relation between bone changes and cartilage changes.

It has been suggested in literature that patella OA is distinct from tibiofemoral OA [4]. Previously, we have investigated cartilage and bone changes in the tibia of the mice in this experiment [39]. When comparing the changes in the patella to those in the tibia, we notice several differences between these compartments: First, cartilage damage is more severe in the patella than in the tibia, which is in line with previous reports [43,44]. Second, estrogen depletion increases cartilage damage in the patella, but increases only the susceptibility for cartilage damage in the tibia. Third, subchondral cortical bone became thinner in the first 3 weeks in both areas (patella and tibia) after OVX+IA, but in the tibia the thickness continued to be decreased, while in the patella the thickness subsequently increased to values similar to the Sham patellae. This may reflect different dynamics or a different mechanism in the patella, which is supported by differences in biochemical and mechanical properties of articular cartilage of patella and tibia[45-47], and by differences in biomechanics between the patellofemoral joint and the tibiofemoral joint [4].

In conclusion, estrogen depletion increases patellar cartilage damage. In addition, in the subchondral cortical bone there is an additive effect of estrogen depletion and the osteoarthritis trigger. Our study supports the link between estradiol and development of patella osteoarthritis and provides further insight into postmenopausal patellar osteoarthritis. Furthermore, the current data indicate that BP may have a direct effect on cartilage.

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Chapter 6

Estrogen modulates iodoacetate-induced gene expression in bovine cartilage explants

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Submitted for publication

Abstract

Objective

Estrogen loss may be involved in onset or progression of osteoarthritis. Estrogen receptors are present in chondrocytes, thus estrogen may exert effects directly on cartilage. However, studies on direct estrogen effects on cartilage are limited. We investigated in an in-vitro cartilage explant model whether estrogen prevents damage, or stimulates repair after damage induced by addition of iodoacetate (IA) as experimental model for osteoarthritis.

Methods

We used healthy bovine cartilage explants.

Prevention experiment: Explants precultured with or without estradiol (E) for three days were cultured with IA for four hours on day 0, and subsequently cultured as in preculture: with or without E. Explants were harvested at day 2 for gene expression analysis.

Repair experiment: At day 0 explants were cultured with IA for four hours and subsequently cultured with or without E. Explants were harvested at day 2, 10 and 14 for gene expression analysis.

Results

IA transiently down-regulated most genes tested, whereas VEGF was up-regulated on day 2. On day 14, TGFB1 and TGFB3 were up-regulated and MMP13 and VEGF down-regulated.

Estradiol affected gene expression of AGC1, MMP2, MMP14, TIMP2, TGFB2, and TGFB3.

Prevention experiment: Estradiol did not significantly affect IA-induced changes in gene expression (no significant interaction).

Repair experiment: Estradiol affected IA-induced changes in expression of COL2, MMP2, MMP3, MMP13, MMP14, TIMP2, TGFB2, TGFB3 and VEGF.

Conclusions

Estradiol affects expression of anabolic and catabolic genes in bovine cartilage explants and modulates the effects of IA. These effects of estradiol may be beneficial for cartilage maintenance and repair.

Introduction

Based on epidemiological studies and animal studies, it has been suggested that loss of estrogen is involved in the onset or progression of osteoarthritis (OA) [1-5]. In a previous study, we found that estrogen depletion in mice increased the susceptibility of articular cartilage to osteoarthritic changes [6]. In that model, the osteoarthritis trigger iodoacetate (IA), an inhibitor of glycolysis that is widely accepted as model for osteoarthritis [7-10], was injected intra-articularly to challenge the cartilage, in combination with ovariectomy (OVX) to induce estrogen depletion. The cartilage damage and subchondral bone changes were more severe in IA-challenged knees of OVX mice than in IA-challenged knees of sham-operated mice. Supplementation of estrogen tended to diminish the cartilage damage and subchondral bone changes. One possible explanation for these observations is that estrogen loss affects the subchondral bone tissue, since it is well known that bone cells express estrogen receptors [11,12] and that estrogen is an important regulator of bone metabolism [13]. Bone changes may play an important role in the onset or progression of OA, and thus estrogen may affect the OA process via the bone. However, a direct effect of estrogen on cartilage is also very well possible, since estrogen receptors alpha and beta, through which estrogen signals, have been found in cartilage [14]. Indeed, several in-vitro studies have reported effects of estradiol treatment on proteoglycan synthesis [15], damage induced by reactive oxygen species [16], nitric oxide production [17] and cytokine-induced matrix metalloproteinases production [18,19] in articular chondrocyte cultures. Apart from these monolayer cultures, cartilage explants have been cultured in the presence of estradiol. The advantage of explant cultures is that chondrocytes remain in their natural environment, surrounded by extracellular matrix and maintain their phenotype. The explant culture system thus approximates the in-vivo situation more closely than monolayer cultures. In cartilage explants stimulated by tumor necrosis factor α (TNFα) and oncostatin M, estradiol treatment was demonstrated to diminish type II collagen degradation [20]. However, in these explant and monolayer studies, only one time point (either < 48 hours[15-19] or 15 days[20]) was investigated while it has been demonstrated both in animal models [10,21,22] and in in-vitro cultures [23-25] that the response of cartilage to inflammatory or degenerative stimuli is a dynamic process.

The current study further explores the effects of estrogen in a cartilage explant model, providing more insight in the dynamic mechanism by which estrogen affects the cartilage metabolism. We formulated two hypotheses: 1) Estrogen may protect cartilage from an osteoarthritic impact, thus preventing major damage. 2) Estrogen may stimulate repair after osteoarthritic damage, thus facilitating better recovery.

In this study, we explored the effects of estrogen with and without iodoacetate on healthy bovine cartilage explants at several time points. Apart from the gene expression of the estrogen receptors, the expression of three classes of genes was investigated: matrix proteins; matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitor of metalloproteinases, TIMPs); and growth factors.

Materials & Methods

Explant preparation

Articular cartilage explants were obtained from metacarpophalangeal joints of calves aged 6–12 months. Explants were taken from the metacarpal side of the joint using a 6 mm diameter biopsy punch, and freed from the underlying bone by dissection with a scalpel. After dissection, explants from each joint were collected in a Petri dish. For each condition, explants were pooled from at least 4 different joints and cultured in a six-well plate. In each well, 3 explants (weighing each approximately 7,5 mg) were cultured with 3 ml Dulbecco's Modified Eagle Medium without phenol (DMEM high glucose; Gibco, Grand Island, NY, USA), containing 10% charcoal-treated fetal calf serum, 50 μg/ml gentamicin, 1.5 μg/ml fungizone and freshly added 25 μg/ml l-ascorbic acid-2-phosphate. This medium will be referred to as standard medium.

Culture conditions

To investigate the prevention hypothesis, the explants were pre-cultured for three days (day –3 till day 0), with either standard medium, or standard medium supplemented with 10^{-8} M estradiol (E medium, Sigma, St Louis, MO, USA). Estradiol was dissolved in 100% ethanol, and the final concentration ethanol was 0.0001% (which is used as vehicle for control conditions).

On day 0, the explants were washed with serum-free medium, and experimental medium was added. Experimental medium consisted of serum-free DMEM supplemented with 50 μg/ml gentamicin, 1.5 μg/ml fungizone, 10 μl/ml insulin-transferrin-selenium A supplement (ITS, BD Biosciences, Bedford, MA, USA), freshly added 25 μg/ml l-ascorbic acid-2-phosphate, and either vehicle (veh), or 10^{-8} M estradiol (E), and either in the presence or absence of 25 μM mono-iodoacetate (IA, Sigma, St Louis, MO, USA). This resulted in the following four conditions in the prevention experiment: I) P_Ctrl (no estradiol, no IA); II) P_IA (no estradiol, with IA); III) P_E (with estradiol, no IA); and IV) P_E+IA (with estradiol, with IA). After 4 hours of incubation with these conditions, the explants were washed with DMEM and for the remaining culture period the culture was continued as in the preculture period with either standard medium, or E medium. Medium was refreshed 24 hours before harvesting. Explants were harvested at day 0: 0h (end of preculture, before IA addition), and day 2 (48 hours after the start of IA addition).

To investigate the repair hypothesis, the pre-culture period in standard medium was equal for all explants. After pre-culture for three days the explants were washed on day 0 with serum-free medium, and experimental medium was added. The following four conditions were used in this repair experiment: I) R_Ctrl (no estradiol, no IA); II) R_IA (no estradiol, with IA); III) R_E (with estradiol, no IA); and IV) R_IA+E (with estradiol, with IA). After 4 hours of incubation with these conditions, the explants were washed with DMEM and the culture was continued for the remaining culture period with either standard medium, or E medium, as described above. Medium was refreshed every three days and 24 hours before harvesting. Explants were harvested at day 0: 0h (end of preculture, before IA addition), day 2 (48 hours after the start of IA addition), day 10, and day 14.

Incubation protocols are depicted schematically in figure 6.1. Coding for the experimental conditions as depicted in figure 6.1 will be used throughout this chapter.

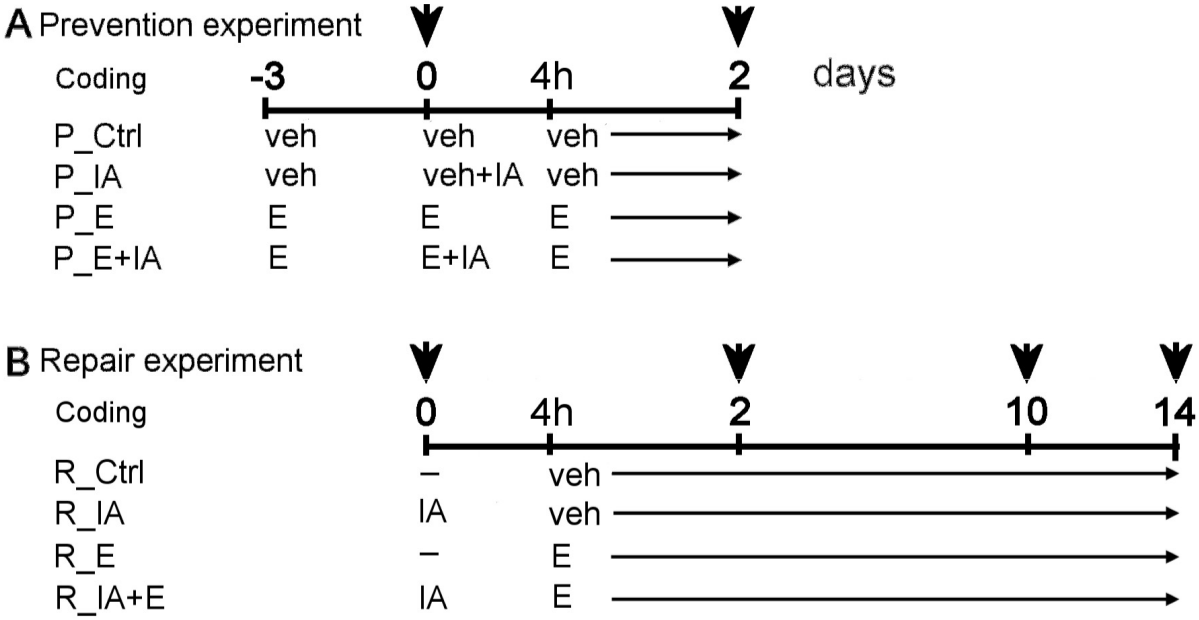


Figure 6.1: Schematic representation of experiments to test preventive and repair effects of estradiol. A: Prevention experiment. Cartilage explants were pre-cultured for 3 days, either with standard medium with vehicle (veh) or estradiol medium (E). On day 0, the explants were treated with IA, or no IA, for 4 hours. After these 4 hours, culture was continued with standard medium with vehicle (veh) or with estradiol medium (E). Explants were harvested at day 0 (before IA addition) and day 2, indicated by arrowheads. B: Repair experiment. Cartilage explants were pre-cultured for 3 days with standard medium. On day 0, explants were treated with IA, or no IA (indicated with -), for 4 hours. After these 4 hours, explants were incubated with standard medium with vehicle (veh) or with estradiol medium (E). Explants were harvested at day 0 (before IA addition), day 2, 10 and 14, indicated by arrowheads. The coding of the conditions is used throughout this chapter.

Gene expression analysis

At harvesting, an inner core (4 mm diameter) of each explant was punched out to exclude tissue at the edge of the explant that was damaged by preparing the explants. We know from previous studies that cells at the edge of the explant die due to damage [26]. The cores of four explants were pooled to form one sample. The cores were snap-frozen in liquid nitrogen. For each condition, at least 6 samples were collected. The frozen cartilage was processed using a Mikro-Dismembrator S (B. Braun Biotech International GmbH, Melsungen, Germany). RNA was extracted using RNA-Bee (TEL-TEST, Inc; Friendswood, TX, USA) according to manufacturer's guidelines and subsequently precipitated with isopropanol. RNA was further purified using RNeasy Micro Kit (Qiagen, Venlo, The Netherlands) with on-column DNA digestion. Total RNA content was determined spectrophotometrically (NanoDrop® ND1000, Isogen Life Science, The Netherlands) and 500 ng total RNA of each sample was reverse transcribed into complementary DNA (cDNA) using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). Taq-Man® and SybrGreen™ assays were performed on an

ABPrism 7000 system (Applied Biosystems, Foster City, CA, USA). The studied genes and their primer sequences can be found in table 6.1 (at the end of this chapter, page 99). 3-Phosphoglycerate kinase (PGK), ribosomal protein L15 (RPL15) and actin gamma (ACTG1) were used to calculate a combined housekeeper index [27] for normalization.

Data analyses

The mRNA expression relative to the housekeeper index was calculated by the equation $2^{-\Delta CT}$ (ΔCT = CT of target gene minus CT of housekeeper index). An analysis of variance (ANOVA) was performed followed by a tukey's post hoc test to compare data from two treatments. Interaction between IA addition and estradiol treatment was tested with a two-way ANOVA. Differences were considered significant when $p<0.05$. For graphical display purpose, expression levels of control conditions were set to 1 and experimental conditions were plotted relative to controls, resulting in a fold change.

Results

ESR1 was expressed in bovine cartilage explants, indicating that estrogens can indeed have an effect. ESR1 gene expression was effected by the treatment conditions: Addition of IA (IA vs Ctrl) significantly down-regulated ESR1 on day 2 (5.2 fold) and day 10 (2.1 fold) after IA addition, but not on day 14. Treatment with estradiol significantly down-regulated ESR1 on day 2 in both prevention and repair experiment (1.5 and 2.7 fold, respectively), but no significant effect on ESR1 was observed on day 10 and day 14. Treatment with estradiol did not significantly affect the IA-induced effect on ESR1 gene expression.

mRNA expression levels of MMP9 and ESR2 were very low under all conditions (CT-value > 35). Therefore these genes were not further analysed.

Effect of IA addition

For analyses of the effect of IA addition on gene expression, the control conditions of the prevention and repair experiments (P_Ctrl and R_Ctrl) were combined as well as the IA conditions (P_IA and R_IA).

Two days after the 4 hours IA addition, most genes studied were significantly down-regulated (COL2, AGC1, MMP2, MMP13, MMP14, TGFB1, TGFB2, TGFB3). In contrast, VEGF was strongly up-regulated, on average 8.2 times the expression in the control condition (figure 6.2A).

On day 10 after IA addition, MMP2 was still significantly down-regulated, while TIMP1, TIMP2, TGFB1, and VEGF were significantly up-regulated. The expression of the other genes was not significantly different from control on day 10 (figure 6.2B).

On day 14 after IA addition TGFB1 and TGFB3 were significantly up-regulated and MMP13 was significantly down-regulated. In contrast to previous days, at day 14 VEGF expression was significantly down-regulated (figure 6.2C).

From these analyses it is clear that following IA addition there is a dynamic temporal and directional expression of each of the genes studied. This is illustrated for AGC1, MMP2, TIMP1, TGFB1 and VEGF in figure 6.2D.

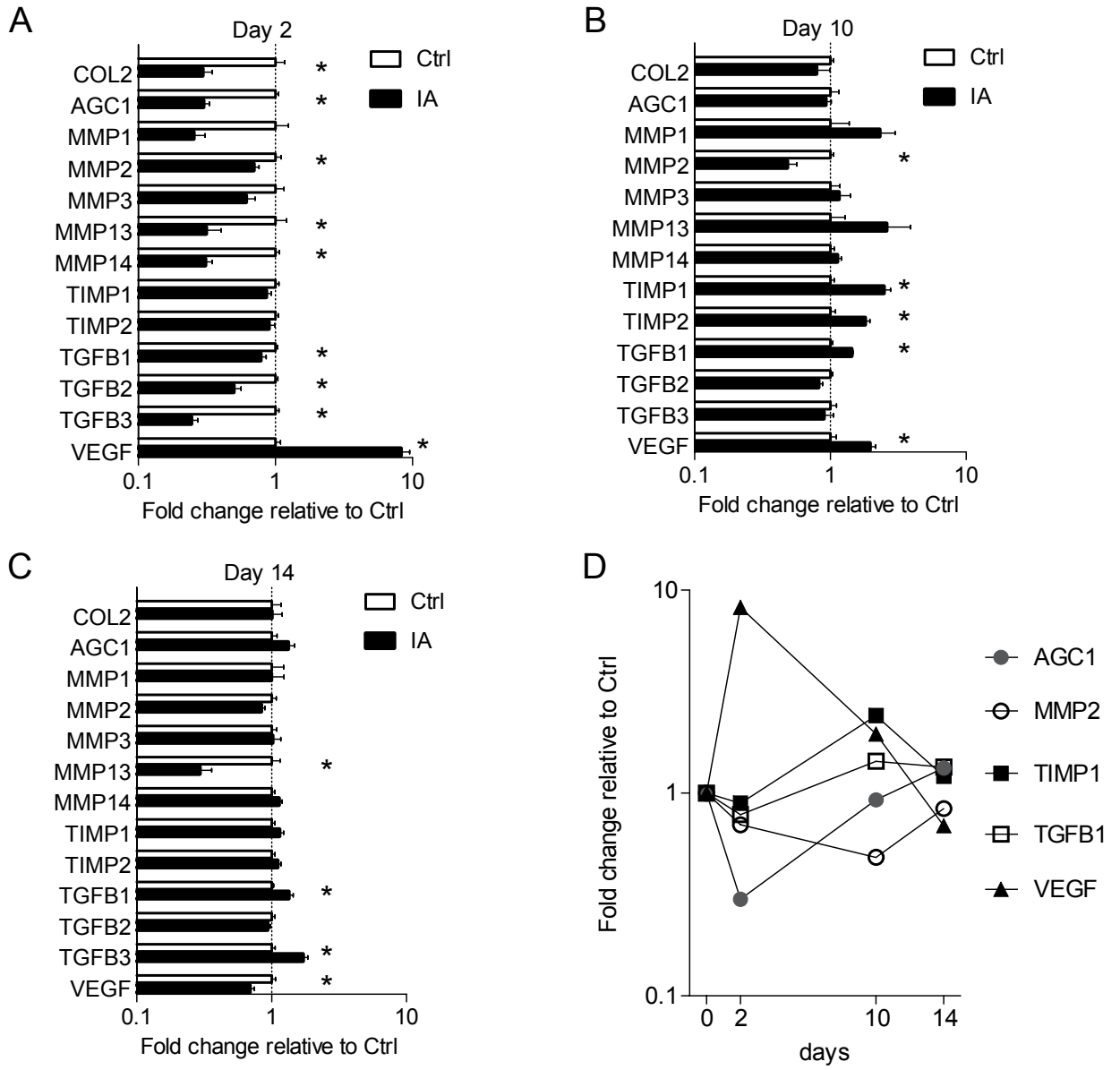


*Figure 6.2: Effect of IA addition depicted as fold change relative to Ctrl. * indicates significant differences between Ctrl and IA. Error bars indicate sem. A: day 2, n=12; B: day 10, n=6; C: day14, n=12. D: Time course of IA effect illustrates dynamic temporal and directional expression of AGC1, MMP2, TIMP1, TGFB1, and VEGF.*

Effect of estradiol treatment

Prevention experiment: In the prevention experiment, Ctrl explants were compared with explants cultured in the presence of estradiol. On day 0, after three days of pre-culture, no significant differences in gene expression between P_E and P_Ctrl explants were found. On day 2, estradiol treatment significantly down-regulated AGC1, MMP2, TIMP2, and TGFB2 (figure 6.3A).

Repair experiment: Compared to the control condition (R_Ctrl) estradiol treatment (R_E) significantly down-regulated MMP2, MMP14, TIMP2, TGFB2, and TGFB3 on day 2 (figure 6.3B). On day 10 of incubation estradiol treatment resulted in up-regulation

of AGC1, MMP14 and TGFB3. Interestingly, two of these genes, MMP14 and TGFB3, were down-regulated at day 2, while the third gene, AGC1, was not regulated at the early stage (figure 6.3C). On day 14, none of the studied genes were significantly up- or down-regulated in the R_E explants (figure 6.3D).

These analyses show that estradiol treatment results in a dynamic pattern of gene expression. This is illustrated for COL2, AGC1, MMP2, MMP13, and TGFB3 in figure 6.3E.

Prevention experiment: testing preventive effect of estradiol

Effect of estradiol treatment on IA challenged explants

In order to investigate the hypothesis that estradiol may prevent the osteoarthritic impact caused by IA, we tested for interaction between IA addition and estradiol treatment. No interaction was found for any of the genes tested, indicating that estradiol did not affect the impact of IA.

Repair experiment: testing repair effect of estradiol

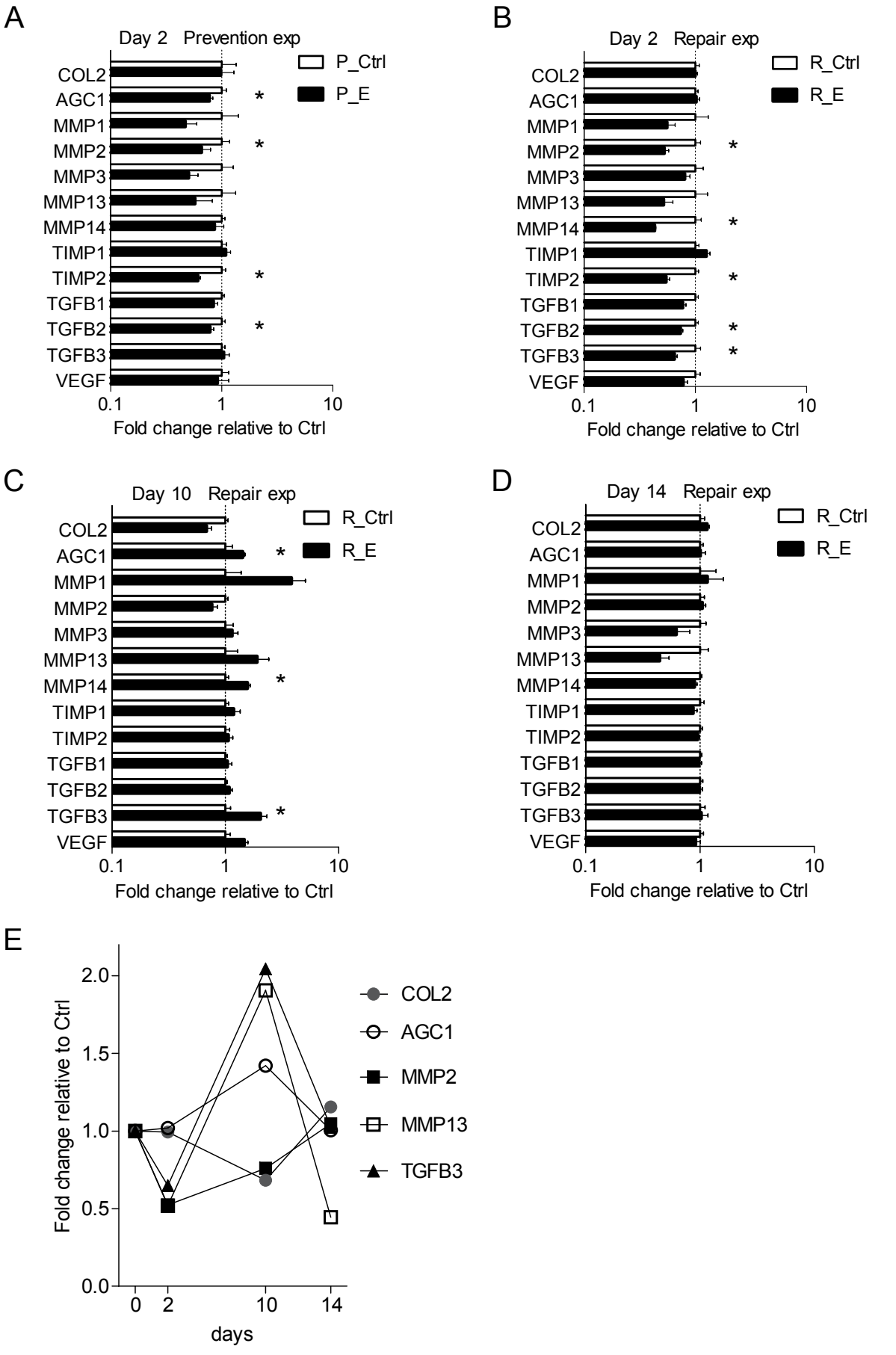
Effect of estradiol treatment on IA challenged explants

In order to investigate the hypothesis that estrogen may stimulate repair after IA, we tested for interaction between IA addition and estradiol treatment. The observed interactions were dependent on the day of incubation: On day 2 there was significant interaction between IA addition and estradiol treatment for MMP2, MMP14, TIMP2, TGFB2 and TGFB3 (figure 6.4A). The decrease in expression of these genes induced by IA addition was weakened or even extinguished by estradiol treatment.

On day 10, VEGF was the only gene showing significant interaction between IA addition and estradiol treatment (figure 6.4B). The increase in VEGF gene expression induced by IA was absent in the presence of estradiol.

On day 14, there was significant interaction between IA addition and estradiol treatment for COL2, MMP2, MMP3, and MMP13 (figure 6.4C). In the absence of estradiol, IA addition had no effect on COL2 and MMP3, but in the presence of estradiol, IA addition resulted in an increase in expression of COL2 and MMP3. For MMP13, the decrease in gene expression induced by IA was abolished in the presence of estradiol.

*Figure 6.3 on next page: Effect of estradiol treatment depicted as fold change relative to Ctrl. * indicates significant differences between P_Ctrl and P_E or between R_Ctrl and R_E. Error bars indicate sem. A: Day 2 of prevention experiment, B: Day 2 of repair experiment, C: Day 10 of repair experiment, D: Day 14 of repair experiment. E: Time course of E effect in repair experiment illustrates dynamic temporal and directional expression of COL2, AGC1, MMP2, MMP13, and TGFB3.*


A
Day 2 Prevention exp
P_Ctrl
P_E
COL2
AGC1
MMP1
MMP2
MMP3
MMP13
MMP14
TIMP1
TIMP2
TGFB1
TGFB2
TGFB3
VEGF
*
0.1
1
10
Fold change relative to Ctrl
B
Day 2 Repair exp
R_Ctrl
R_E
C
Day 10 Repair exp
D
Day 14 Repair exp
E
Fold change relative to Ctrl
2.0
1.5
1.0
0.5
0.0
0
2
10
14
days

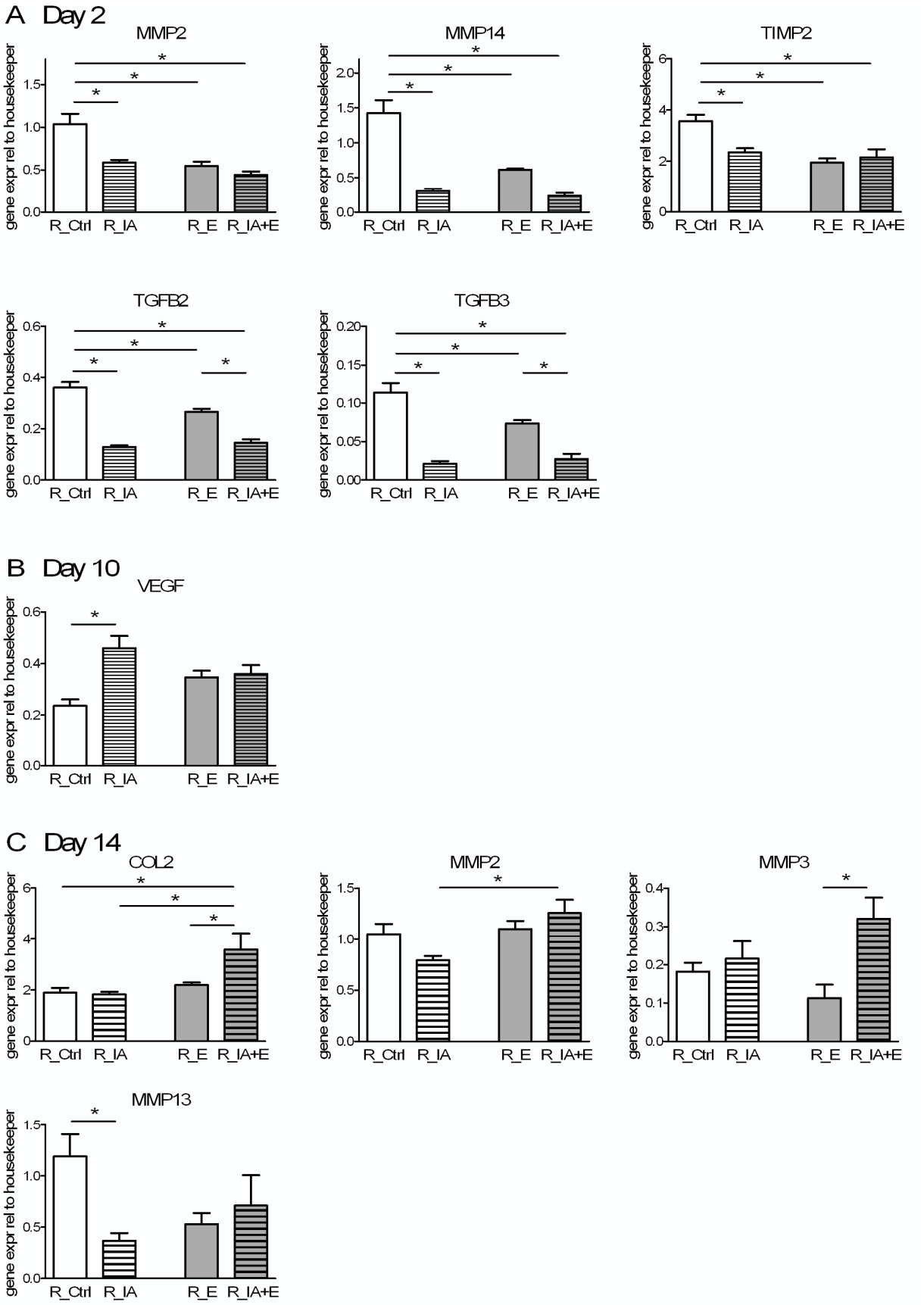


*Figure 6.4: Expression of genes in repair experiment with significant interaction between estradiol treatment and IA addition. A: Day 2, B: Day 10, C: Day 14. Gene expression relative to housekeeper index, * indicates significant difference. Error bars indicate sem.*

Discussion

In the present study we demonstrated that treatment of cartilage explants with estradiol regulated the expression of all three groups of genes we studied: matrix components, MMPs and TIMPs, and growth factors, indicating an effect on cartilage metabolism. In addition, experiments were performed in which the cartilage explants were challenged with IA either after estradiol treatment (prevention experiment), or prior to estradiol treatment (repair experiment). We demonstrated that treatment with estradiol modulated the changes in gene expression induced by IA in the repair experiment, but not in the prevention experiment.

Our first hypothesis was that estrogen protects the cartilage from the impact of IA, either by reducing the vulnerability of cartilage or by countering the induction of changes. This was explored in the prevention experiment. Pre-culture with estradiol had no significant effect on the IA-induced gene expression. Apparently, estradiol did not alter the setting of the cartilage in such a way that it is protected from IA-induced transcriptional changes. This argues against a preventive effect of estradiol in osteoarthritis. The pre-culture period of three days before IA addition may have been too short to achieve a protective effect of estradiol. However, this seems unlikely as estradiol acts via its receptor, which is a nuclear receptor that directly affects gene transcription, and transcriptional effects of estradiol in cartilage have been observed after as early as 24 hours [28]. Moreover, it has been demonstrated that pre-culture for 24 hours with estradiol modulated insulin-induced type II collagen production [29] and that estradiol treatment affected proteoglycan synthesis [15], nitric oxide production [17] and cytokine-induced MMP production [18,19] within 48 hours in articular chondrocyte cultures. The absence of transcriptional interaction between estradiol pre-treatment and IA we observed indicates that estradiol and IA act via different pathways. However, this does not necessarily implicate that there is no functional interaction at protein level.

In contrast to the prevention experiment, we did find an effect of estradiol in the experiments, in which we explored the hypothesis that estrogen stimulates repair. Estradiol affected the IA-induced changes of several genes, both anabolic and catabolic (figure 6.4). Apart from MMP2, which was affected on days 2 and 14, different genes were regulated on days 2, 10 and 14, indicating a dynamic temporal effect. The patterns of expression of the genes that showed interaction were different on day 2 than on day 14. On day 2, the IA-induced changes were weakened in the presence of estradiol. This was mainly caused by the estradiol effect on explants cultured in the absence of IA (R_Ctrl vs R_E), while the same level of expression was observed in the explants cultured in the presence of IA (R_IA vs R_IA+E, see figure 6.4). In contrast, on day 14, IA addition alone and estradiol treatment alone had no effect on COL2, MMP2 and MMP3, while IA addition in the presence of estradiol resulted in higher expression levels of these genes than IA alone or estradiol alone. This may indicate a repair response and may be a secondary response to estradiol, as this response does not occur on days 2 and 10 in our experiment. These data clearly show the impact of estradiol on IA-induced gene expression in cartilage but the consequences for protein levels and the resulting balance between anabolic and catabolic processes will define whether the effects are beneficial and needs additional studies.

The changes we observed in MMP and TIMP gene expression are in line with findings in previous studies. In our study we observed that estradiol treatment regulated MMP2, MMP3, MMP13, MMP14, and TIMP2 after IA addition. Richette et al [18] found that in cultured rabbit articular chondrocytes, estradiol modulated interleukin-1β-induced expression of MMP3 and MMP13 in a biphasic manner. They also reported no effect of estradiol on TIMP1 and aggrecan, similar to our findings. However, our findings on MMP1 (no effect of estradiol) are different from findings in literature. Richette et al observed that estradiol modulated interleukin-1β-induced expression of MMP1, which contrasts our findings. Another study [19] reported that after stimulation with TNFα, MMP1 protein expression but not gene expression was suppressed by estradiol in isolated human articular chondrocytes. These studies used monolayer cultures and a different compound to challenge the cartilage than in our study, which may explain the difference in results. In addition they investigated only one (early) time point (20 hours [18] and 48 hours [19]). We showed that the effects are very dynamic, which emphasizes the importance of using multiple time points in order to get better insight in the different phases that occur after challenging cartilage.

Pre-treatment with estradiol for three days did not regulate any genes on day 0, before IA addition. On day 2, five studied genes were down-regulated by estradiol in the repair experiment. These effects were extinguished or even reversed on day 10, when three studied genes were regulated. At day 14, none of the studied genes was affected by estradiol treatment. This may indicate that chondrocytes adapt to the presence of estradiol. The adaptation may be regulated via the estrogen receptor, as we observed down-regulation of the ESR1 gene expression on day 2. Down-regulation of estrogen receptor gene expression by estrogen has also been reported for a breast cancer cell line [30]. However, on day 10 and day 14, ESR1 gene expression was not down-regulated. This suggests that (an) alternative mechanism(s) are involved, such as altered estradiol metabolism.

Addition of IA resulted in dynamic regulation of gene expression. After the initial changes at day 2, the expression of most genes recovered to normal values after 14 days. However, despite the short (4 hours) challenge with IA the expression of a few genes was still altered after 10 and 14 days. These late changes may reflect a recovery or repair process, as is also observed in animal models, where intra-articular injection of IA results in early inhibition of proteoglycan synthesis followed by a recovery [10,21,31]. The increased expression of TGFB1 and TGFB3 are supportive for such a repair response, as TGFβ has been demonstrated as important growth factor in cartilage repair [32].

Addition of IA caused down-regulation of almost all studied genes on day 2, including several MMPs. Also at later time points, none of the studied MMPs were up-regulated. This may be counter-intuitively, as MMPs have been reported to be up-regulated in human OA [33,34] and in the rat IA-induced model for OA [34]. However, the sequence of events from very early to late OA is unknown. It may be that our data reveal an early phase (hours to days) whereas the increase in MMPs as reported in animal models and humans may reflect a later phase of the disease process (weeks, months, or in human even years). Moreover, in animal models and human OA other tissues may also be involved. Up-regulation of MMPs may result from an inflammatory response, and not from a direct effect of IA on cartilage metabolism.

VEGF showed an exceptional response pattern to IA addition. While almost all studied genes were down-regulated on day 2, VEGF was strongly up-regulated. VEGF has been suggested to be important in cartilage degeneration. It is produced by OA chondrocytes, but not by normal chondrocytes [35-37]. In addition, mechanical stress induced VEGF expression in bovine cartilage disks [38]. VEGF does not only control angiogenesis, but also induces catabolic mediators such as MMPs and interleukin-1 and -6. [39]. However in our study, we did not observe significantly increased levels of MMPs after IA addition. Although different triggers may challenge the cartilage in a different way, resulting in different responses [40], VEGF expression may be a general response of cartilage to a challenge.

The possible beneficial effect of estrogen on cartilage is seen in several animal models, in which induction of OA is combined with ovariectomy and/or with estrogen replacement therapy [41-43]. However, in these in-vivo models other tissues in the joint, such as bone, synovium, ligament and muscle may contribute the OA protective effects of estrogen, as these tissues also express estrogen receptors [11,12,44-47]. The current study with cartilage explants contributes to the knowledge of estrogen effects directly on cartilage.

In conclusion, estradiol affects the expression of both anabolic and catabolic genes in bovine articular cartilage explants, and modulates the effects of iodoacetate. This provides insight into the mechanism underlying the observations in human studies and animal experiments in which estrogen loss is associated with cartilage degeneration and supports the notion for a role of estradiol in cartilage maintenance and repair.

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Table 6.1: Accession numbers and primer sequences of studied genes.

Target/primer		Accession No.	Oligonucleotide sequence (5' - 3')
BtESR1	Fw	NM_001001443	CAGGGAGCTGGTACACATGA
	Rv		ATGCCTTCCACACATTTTCC
BtESR2	Fw	NM_174051	GACTCACCTGCTGAATGCTG
	Rv		TGACGTGAGACAGGAGCATC
BtAGC1	Fv	NM_173981	AATTACCAGCTACCCTTCACCTGTA
	Rv		TCCGAAGATTCTGGCATGCT
	P		AGGGCACAGTGGCCTGCGGA
hCOL2	Fv	NM_001113224	CCGGTATGTTTCGTGCAGCCATCCT
	Rv	NM_001001135	GGCAATAGCAGGTTCACGTACA
	P		CGATAACAGTCTTGCCCCACTT
BtMMP1	Fw	NM_174112	TGGAGCAATGTCACACCCTTG
	Rv		TTGTCACGATGATCTCCCCTG
BtMMP2	Fw	NM_174745	TTCGACGGCATCTCTCAGATC
	Rv		TGTTCGCCAGATGAATCGG
BtMMP3	Fw	XM_586521	CACTCAACCGAACGTGAAGCT
	Rv		CGTACAGGAACTGAATGCCGT
BtMMP9cs	Fw	NM_174744	GACCAGGACAAGCTCTACGG
	Rv		AGGAAGGTGAAGGGGAAGAC
BtMMP13	Fw	NM_174389	TCTTGTTGCTGCCCATGAGT
	Rv		GGCTTTTGCCAGTGTAGGTGTA
BtMMP14	Fw	NM_174390	GGACTGTCCGGAATGAGGATCT
	Rv		TTGGAATGCTCAAGGCCCA
BtTIMP1	Fw	NM_174471	TCCCTGGAACAGCATGAGTTC
	Rv		TGTCGCTCTGCAGTTTGCA
BtTIMP2	Fw	NM_174472	CCAGAAGAAGAGCCTGAACCA
	Rv		TGATGTTCTTCTCCGTGACCC
BtTGFb1	Fw	XM_592497	CTGCTGAGGCTCAAGTTAAAAGTG
	Rv		CAGCCGGTTGCTGAGGTAG
BtTGFb2	Fw	NM_001113252	GCCGAGTTCAGAGTCTTTCGTTT
	Rv		GCGCTGGGTTGGAGATGTTA
BtTGFb3	Fw	NM_001101183	AGGAGCACAATGACCTGACC
	Rv		TCTCCACTGAGGACACGTTG
BtVEGF	Fw	NM_174216	TATGTGCTGGCTTTGGTGAG
	Rv		ATTTTCAAGCCGTCCTGTGT
BtACTG1	Fw	NM_001033618	TTACAACGAGCTGCGTGTGG
	Rv		TGGCAGGAGTGTTGAACGTC
BtRPL15	Fw	NM_001077866	CACAAGTTCCACCACACTATTGG
	Rv		TGGAGAGTATTGCGCCTTCTC
BtPGK	Fw	NM_001034299	CTGGACAAGCTGGATGTGAA
	Rv		AACAGCAGCCTTGATCCTCT

Chapter 7

General discussion

For several decades, people have been intrigued by the role of estrogen in osteoarthritis (OA). As described in Chapter 1, observations from epidemiological studies, animal studies, and cell culture studies have indicated that estrogen could have an OA-protective effect. However, some results are conflicting, and the mechanism by which estrogen would protect against OA is still unclear. The aim of this thesis was to get a better insight into the role of estrogen in osteoarthritis, with focus on cartilage and bone. Animal studies and in-vitro studies were chosen to provide this insight. We aimed at answering the following questions: 1) What is the evidence from literature on animal models about the effect of estrogen loss and estrogen treatment on cartilage? 2) Does deletion of estrogen receptors, and thereby blocking the estrogen signalling, lead to osteoarthritic changes? 3) Does loss of estrogen increase the susceptibility of tissues in the joint for osteoarthritic changes? 4) Can estrogens protect the cartilage from damage, and if so, does this happen by limiting damage, or by stimulating repair?

In the current chapter the most important findings are highlighted and discussed in relation to current scientific knowledge. In addition, suggestions for future research will be discussed.

Cartilage

One characteristic of OA that is often studied in animal models is degeneration of articular cartilage. Evaluation of the literature revealed that 11 out of 16 animal studies reported an adverse effect of estrogen loss (ovariectomy) on cartilage damage (Chapter 2). There is a lot of variation in experimental set-up of the studies evaluated in Chapter 2, such as animal species, duration of experiment, and age of the animal. Especially this last parameter may be of importance when studying hormonal effects. When excluding the studies using sexually immature animals, the evidence became stronger, with 11 out of 14 studies showing a detrimental effect of estrogen loss on cartilage. Animal species may also explain the variation in results. For instance, in studies using mice [1-5] the results were not consistent, whereas all the evaluated studies using rats [6-8] showed a detrimental effect of ovariectomy on cartilage. However, a recent study [9] (presented at a conference, not included in Chapter 2) repeated the experiment with ovariectomy in rats, but could not reproduce the results of previous rat studies [6-8]. Thus instead of species differences, other aspects of the experimental set-up may explain the variation in results. For instance, the authors of the three articles showing a detrimental effect of OVX in rats were all affiliated to the same institution (Nordic Bioscience), using the same animal facility. Details in animal care (e.g. food, cage size) may be very important and may affect the outcome. In addition to animal species and animal care, the strain of the animals may affect the results. The mouse studies examined different strains (129S/SvEv, DBA, STR/ort, C57Bl, or C3H/Hej) and it is known that OVX-induced bone loss varies among mouse strains [10]. However, whether the response in cartilage is also strain dependent is unknown.

In our own animal model, ovariectomy increased cartilage damage in the patella (Chapter 5) but not in the tibia (Chapter 4). However, ovariectomy in combination with a chemical inducer of OA (iodoacetate) increased the cartilage damage in the tibia (Chapter 4). Another model we used to elucidate the role of estrogen and its receptors is the

estrogen receptor knockout mouse, lacking estrogen receptor α, β, or both. In 6-month-old knockout mice, cartilage damage was very mild and not different from wild-type mice (Chapter 3). For both the ovariectomy model and the knockout mice, the chosen time point of evaluation was rather early. More severe cartilage damage may develop at a later time point, although spontaneous OA occurring at later age [11] may then overrule the OA induced by loss of estrogen signalling.

The evaluation of literature also revealed that the effect of estrogen treatment was inconclusive with only 11 out of 22 animal studies reporting a beneficial effect on cartilage. Also in these studies, there was a lot of variation in experimental set-up. In our animal model (Chapters 4 and 5), estrogen treatment after OA induction with iodoacetate reduced the cartilage damage in the patella. In the tibia a similar trend was observed, although this was not statistically significant, most likely because the unexpected death of mice in the estrogen treatment group limited the chance to detect significant changes. In our bovine cartilage explant cultures (Chapter 6), estrogen treatment affected the expression of both anabolic and catabolic genes, including genes encoding for matrix constituents, genes encoding for matrix metalloproteinases (MMPs) and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), and genes encoding for growth factors such as transforming growth factor β (TGFβ). When the explants were cultured in the presence of estrogen after iodoacetate (IA) addition, estrogen modulated the IA-induced changes of anabolic and catabolic genes. Although these changes may be beneficial for cartilage maintenance and repair, their exact meaning remains to be elucidated.

Osteophytes

Besides cartilage degradation, development of osteophytes at the rims of joints or vertebrae is a second hallmark of osteoarthritis. Osteophytosis was increased in the tibia of both ovariectomized mice (without OA induction) and double knockout mice (ERα-/-β-/-). In both models, estrogen signalling is strongly reduced. In contrast, in the single knockout mice (ERα-/- and ERβ-/-), these changes were not observed. This suggests a compensation mechanism in these mice, as is also described for ERα and ERβ in bone metabolism [12]. A link between estrogen and osteophytes has been previously been suggested: Ovariectomy-induced osteophyte formation in temporomandibular joints of rats [13] and polymorphisms in the ERα gene are associated with osteophytosis [14,15].

Osteophyte formation closely resembles the process of endochondral ossification [16], as also occurs in the growth plate during growth. An increase of estrogen during puberty suppresses the endochondral ossification and promotes fusion of the growth plate [17,18]. Lack of estrogen signalling in ovariectomized mice and in double knockout mice (ERα-/-β-/-) may stimulate endochondral ossification, and thereby stimulate osteophytosis.

Bone

As described in Chapter 1, OA is also characterized by changes in subchondral bone, which can be divided into subchondral plate and subchondral trabecular bone. We observed thinning of the subchondral plate in both the tibia and the patella of the

ovariectomized mice, but only when OA was induced by iodoacetate. The thinning was counteracted by estrogen treatment. Subchondral plate thinning was also seen in the double knockout mice (ERα-/-β-/-). The decrease in subchondral plate thickness is in line with previous observation in several animal models for osteoarthritis at early time points [19-24]. However, the thinning seems to contrast the subchondral sclerosis which is often observed in human OA [25-27] and which is considered to be a hallmark of OA. This contrast is most likely explained by differences in disease state: Our observations concern early stage OA, whereas most human studies concern late stage OA. Indeed, in some animal studies an early phase of thinning was followed by a later phase of thickening [24,28].

The trabecular bone in the epiphysis and in the patella was not affected by ovariectomy either with or without OA induction by iodoacetate, and the trabecular changes in the estrogen receptor knockout mice were small in the epiphysis. In contrast, the trabecular bone in the tibial metaphysis strongly decreased both in the ovariectomized mice and in the double knockout mice. Thus, the response of trabecular bone to estrogen signalling loss is different in the tibial metaphysis than in the epiphysis and the patella. This may be due to differences in estrogen receptor levels, although no data are available. However, the epiphyseal and patellar trabecular bone do respond to estrogen, as estrogen treatment in the ovariectomized mice resulted in a strong increase in trabecular bone volume.

The estrogen-driven bone changes may influence the effect seen on cartilage. In an attempt to block bone changes, bisphosphonates were given to the ovariectomized animals. However, this treatment did not only prevent the thinning of the subchondral plate, but also led to an increase in trabecular bone volume in the tibial epiphysis as well as in the patella. The patellar cartilage damage was less in the bisphosphonates treated group, and a similar trend was seen in the tibial cartilage damage. It remains unclear whether the decreased cartilage damage was due to bisphosphonate effects on the subchondral plate, on the trabecular bone, or perhaps on the cartilage itself. Thus from this experiment, it could not be concluded whether the bone changes have a role in the initiation or progression of the cartilage damage.

Broader perspective

In this thesis, we focused on the effects of estrogen on cartilage and bone in relation to OA. Apart from changes in these two tissues, synovial inflammation, ligament laxity, and muscle weakness are also involved in OA pathology and these changes may contribute to OA progression. Estrogen receptors are expressed in these tissues [29-32], which indicates that they are responsive to estrogens, and thus estrogen may exert its possible OA-protective effects via these tissues as well.

In addition, estrogen has several systemic effects, which will also affect the local environment in the joint. For instance, loss of estrogen increases fat mass. This results in increased levels of adipokines such as leptin, which may in turn stimulate the expression of matrix metalloproteinases (MMPs) and interleukin 1 (IL-1) [33]. Loss of estrogen also increases T-cell production [34], which results in increased cytokine production, such as

tumor necrosis factor α (TNFα) [35]. These changes may contribute to the development of OA.

After menopause and ovariectomy, the decrease in estrogen levels is accompanied by changes in circulating levels of other hormones: progesterone and testosterone decrease, follicle stimulating hormone and luteinizing hormone increase. These hormones may affect the joint tissues as well, but they are barely studied.

The knee joint was the joint of interest in Chapters 3, 4 and 5. However, OA can also affect other joints, like the hip, shoulder, and joints in the hand. Whether the findings are also applicable to other joints is not clear. Different effects of estrogen replacement therapy on hip and knee OA in women have been reported, with a clearer protective effect for hip OA than for knee OA [36,37]. These differences may be explained by a different physical challenge (e.g. in terms of weight-bearing), or regional differences in the distribution of estrogen receptors α and β, although no data are available.

Suggestions for future research

As with most scientific research, the experiments described in this thesis raise several new questions. In Chapter 3, the estrogen receptor knockout mice showed no overt osteoarthritic changes in cartilage and subchondral bone. Only 6-month-old mice were used, which raises the question whether more pronounced osteoarthritic changes would develop in older estrogen receptor knockout mice. Or would there still be no clear osteoarthritic changes in these older mice? If the answer to the last question were yes, this would be in line with the idea that reduced estrogen signalling alone is not enough to cause OA, but may increase the susceptibility of the cartilage to changes, as was found in Chapter 4. An experiment in which OA was induced in estrogen receptor knockout mice would elucidate this. Another point of concern is the elevated estrogen level in the ERα-/- mice, which mystifies the findings in these mice because the deletion may be (over)compensated by the other receptor. Ovariectomizing these mice will avoid increased estrogen levels. Alternatively, the estrogen receptors could be deleted in only one tissue, for instance only in cartilage, or only in bone. In this way, elevated estrogen levels will not occur, and more interestingly, the specific effects on one tissue can be studied. This would help answering the question whether estrogen exerts its OA protective effect via cartilage or via bone.

In the mouse model in which ovariectomy was combined with iodoacetate (Chapters 4 and 5), cartilage damage was present, but fairly mild. This trigger was chosen to be mild in order to study the effects of estrogen depletion on the susceptibility for OA, without overruling the estrogen effects. A stronger OA trigger (higher concentration iodoacetate, or a different OA model) and/or longer follow-up may increase cartilage damage. The effects of estrogen treatment and bisphosphonate treatment, which were not significant in the tibia study, may then reach significance.

In this thesis cartilage and bone were studied. The effect of ovariectomy on other tissues could also be investigated. For instance, do the mechanical properties of ligaments and muscles deteriorate after ovariectomy? In addition, gene expression in joint tissues from ovariectomized mice (or rats) could be compared to that of sham-operated animals

in order to get more insight into the molecular mechanism that are affected by estrogen loss.

In both the ovariectomized mice and the estrogen receptor knockout mice, the knee joint was studied. However, even within the knee joint, we found different effects of ovariectomy (tibia vs patella, as discussed in Chapter 5). This complicates extrapolating our findings to other joints. Additional experiments are needed to evaluate the effect of loss of estrogen (signalling) on other joints, such as hip, ankle or lumbar spine. For these joints too, it may be better to evaluate also at later time points.

The genes that were studied in the explant study included matrix genes, genes involved in degradation (MMPs) and their inhibitors (TIMPs), growth factors, and the estrogen receptors. This list could be extended, for instance with pro-inflammatory and anti-inflammatory cytokines. Estrogen is known to affect the production of cytokines in osteoblasts [38]. Will estrogen affect cytokine production in chondrocytes, and possibly shift the balance between pro- and anti-inflammatory cytokines?

In the explant study, estrogen treatment modulated the effects of iodoacetate, but would this also be the case for an other osteoarthritis trigger? A different trigger may challenge the cartilage in a different way [39], and perhaps estrogen affects this other challenge differently. Examples of other triggers are interleukin-1, TNFα, oncostatin M, and retinoic acid.

The explant experiments were performed with healthy bovine articular cartilage. Ideally, these experiments were performed with healthy human cartilage. However, this is very hard to obtain. Osteoarthritic instead of healthy human articular cartilage is more available, and this could be used to investigate the effect of estrogen on additionally induced damage, instead of on initial damage.

An important difference between our explant cultures and the in-vivo situation is mechanical loading. If loss of estrogen increases the susceptibility of cartilage for damage induced by mechanical loading, this will not be picked up in the explant study. In addition to the chemical triggers, the cartilage could be challenged mechanically.

Another difference with the in-vivo situation is the absence of other tissues. For answering our question whether estrogen would directly affect cartilage, the use of cartilage explants was a logical choice. However, the interaction with other tissues, e.g. with bone or synovium, may be very important. Molecules released from other tissues may diffuse into the cartilage. By culturing synovium, bone tissue, or bone cells, the effect of estrogen on the release can be investigated. In addition, conditioned medium from these tissues can be added to a cartilage explant culture. This may provide more information on the role of other tissues in OA.

Concluding remarks

Loss of estrogen (or estrogen signalling) induced only minor cartilage changes. However, it increased the susceptibility of the tibial cartilage to osteoarthritic changes. A combination of circumstances seems to be needed to initiate OA. This corroborates with the idea that OA is a multifactorial disease, in which estrogen may affect the joint, via cartilage, bone, ligament, synovium, muscle, adipokines, cytokines, immune response, pain perception and other yet unknown mechanisms. The contribution of estrogen in each

of the separate mechanisms may be small, but together these estrogen effects, in combination with other contributors like genetic predisposition, activity level, loading pattern, food intake, et cetera, can make the difference between a healthy and OA joint.

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Summary

Samenvatting

Summary

Osteoarthritis (OA) is one of the most frequently occurring disorders of the musculoskeletal system, leading to joint pain and disability. Although all tissues in the joint can be affected, the focus of this thesis is on changes in bone and cartilage. In the introductory **Chapter 1**, the composition and the degradation process of cartilage were described. In addition, bone changes that occur in OA and their role in the disease process were discussed. Evidence from epidemiological studies, animal studies, and cell culture studies suggests that estrogen may have an OA-protective effect, but the possible mechanism is poorly understood. Both cartilage and bone are responsive to estrogen, and therefore the OA-protective effect of estrogen may act via both tissues. In this thesis, the role of estrogen in OA was investigated at different levels, in order to obtain more insight in the protective effects of estrogen.

In **Chapter 2**, the current literature concerning the effects of hormone depletion (by ovariectomy) and estrogen treatment on cartilage in animal models was reviewed in a systematic way. Eleven out of 16 animal studies showed that ovariectomy results in cartilage damage. The effect of estrogen treatment was inconclusive with only 11 out of 22 animal studies reporting a beneficial effect on cartilage. There was a large variation in experimental set-up, such as animal species, age, and duration of experiment, which may influence the results. For instance, when only studies using sexually mature animals were included, we saw that 11 out of 14 studies showed a detrimental effect of ovariectomy, indicating considerable evidence for a relation between ovariectomy and cartilage degeneration in mature animals. We formulated a list of quality criteria for animal studies, similar to criteria for clinical studies. Applying these criteria will improve the comparability of studies and will facilitate evaluations.

Estrogen receptor knockout mice were studied in **Chapter 3** to investigate the role of estrogen signalling in the osteoarthritic process. We hypothesized that deletion of one or both estrogen receptors would result in osteoarthritic changes in cartilage and subchondral bone. Deletion of both receptors led to osteophytosis and thinning of the subchondral plate, both osteoarthritic features. However, deletion of one or both estrogen receptors (α and/or β) did not lead to overt cartilage damage.

In **Chapters 4 and 5**, ovariectomy (or sham operation) in mice was combined with an osteoarthritis trigger (or a saline injection). In **Chapter 4**, changes in cartilage and bone of the proximal tibia were described. The tibial cartilage damage was highest in the ovariectomized mice with the osteoarthritis trigger. Subchondral plate thinning occurred only in this group, and not after ovariectomy alone, or after the osteoarthritis trigger alone. Estrogen treatment and treatment with bisphosphonate counteracted the decrease in subchondral plate thickness and tended to diminish cartilage damage. These results demonstrated that estrogen depletion increases the susceptibility of cartilage for an osteoarthritis trigger

In **Chapter 5,** cartilage and bone of the patella was studied. Patellar cartilage damage was highest in the ovariectomized mice, independent of the presence or absence of the osteoarthritis trigger. The early thinning of subchondral cortical bone was strongest in ovariectomized mice that received the osteoarthritis trigger. Estrogen treatment and treatment with bisphosphonate counteracted the decrease in subchondral plate thickness

and diminished cartilage damage. Thus, estrogen depletion increased patellar cartilage damage, but did not increase its susceptibility.

The effects of estrogen on cartilage without interference of surrounding tissues were investigated in a culture study of bovine cartilage explants, as described in **Chapter 6**. Estrogen treatment regulated the expression of several genes, both anabolic and catabolic. When estrogen was added to the explant culture after challenging the explants with iodoacetate, estrogen modulated the iodoacetate-induced changes. These effects may be beneficial for cartilage repair. This study also provided more insight in the effects of the osteoarthritis trigger iodoacetate on cartilage

In **Chapter 7** the most important findings of the previous chapters were discussed in relation to each other and in the context of current scientific knowledge. In addition, suggestions for future experiments were given. In conclusion, the results in this thesis support the idea that OA is a multifactorial disease, in which the contribution of estrogen may be small. However the estrogen-induced changes, together with changes caused by other contributors, can result in osteoarthritis.

Samenvatting

Artrose is een van de meest voorkomende aandoeningen aan het bewegingsapparaat en leidt tot gewrichtspijn en invaliditeit. Hoewel alle weefsels in het gewricht aangedaan kunnen zijn, ligt de focus van dit proefschrift op veranderingen in bot en kraakbeen. In het inleidende **Hoofdstuk 1** werden de samenstelling en het afbraakproces van het kraakbeen beschreven. Daarnaast werden de botveranderingen die optreden bij artrose en hun rol in het ziekteproces uiteengezet. Er zijn aanwijzingen uit epidemiologische studies, dierstudies en celkweekstudies die wijzen op een artrose-beschermend effect van oestrogeen. Het mogelijke mechanisme wordt echter onvolledig begrepen. Zowel kraakbeen als bot reageren op oestrogeen, dus het artrose-beschermende effect van oestrogeen kan via beide weefsels werken. In dit proefschrift is de rol van oestrogeen in artrose onderzocht op verschillende niveaus, met als doel meer inzicht te krijgen in de beschermende effecten van oestrogeen.

In **Hoofdstuk 2** werd de huidige literatuur betreffende de effecten van hormoondepletie (dmv ovariëctomie) en oestrogeenbehandeling op kraakbeen in diermodellen besproken op een systematische manier. Elf van de 16 dierstudies toonden aan dat ovariëctomie tot kraakbeenschade leidt. Het effect van oestrogeenbehandeling was onduidelijk, met slechts elf van de 22 dierstudies die een positief effect op kraakbeen lieten zien. Er was grote variatie in de experimentele opzet, zoals verschillen in diersoort, leeftijd en experimentduur, die wellicht de resultaten beïnvloeden. Wanneer bijvoorbeeld alleen de studies werden meegenomen waarin geslachtsrijpe dieren werden gebruikt, zagen we dat in elf van de 14 studies ovariëctomie een negatief effect had. Dit duidt op een aanzienlijk bewijs voor een relatie tussen ovariëctomie en kraakbeenschade in volwassen dieren. We hebben een lijst met kwaliteitscriteria voor dierstudies opgesteld, identiek aan de criteria voor klinische studies. Het toepassen van deze criteria zal er toe leiden dat studies beter vergeleken kunnen worden en makkelijker te evalueren zijn.

Oestrogeenreceptor-knockout muizen werden bestudeerd om de rol van oestrogeensignalering in het artrose proces te onderzoeken. Dit is beschreven in **Hoofdstuk 3**. We hadden als hypothese gesteld dat het verwijderen van één of beide oestrogeenreceptoren zou leiden tot artrotische veranderingen in kraakbeen en subchondraal bot. Het verwijderen van beide receptoren leidde inderdaad tot osteofytvorming en verdunning van de subchondrale plaat, wat beide artrotische kenmerken zijn. Echter, het verwijderen van één of twee oestrogeenreceptoren (α en/of β) resulteerde niet in duidelijke kraakbeenschade.

In **Hoofdstuk 4 en 5** werd ovariëctomie (of een sham-operatie) in muizen gecombineerd met een artrose-trigger (of een controle injectie met fysiologisch zout). In **Hoofdstuk 4** zijn de veranderingen in kraakbeen en bot van de proximale tibia beschreven. De kraakbeenschade in dit gebied was het hoogst in de muizen die zowel ovariëctomie als de artose-trigger hadden gekregen. Verdunning van de subchondrale plaat trad enkel op in deze groep, en niet na alleen ovariëctomie, of alleen de artrose-trigger. Oestrogeenbehandeling en behandeling met bisfosfonaat ging de verdunning van de subchondrale plaat tegen en de kraakbeenschade neigde af te nemen. Kortom, oestrogeendepletie maakte het kraakbeen gevoeliger voor een artrose-trigger.

In **Hoofdstuk 5** werd het kraakbeen en bot van de patella bestudeerd. De kraakbeenschade in de patella was het hoogst in de muizen die ovariëctomie hadden

ondergaan, onafhankelijk van het al dan niet toedienen van de artose-trigger. Verdunning van de subchondrale cortex was het sterkst in de muizen die zowel ovariëctomie als de artrose-trigger hadden gekregen. Oestrogeenbehandeling en behandeling met bisfosfonaat ging de verdunning van de subchondrale plaat tegen en de kraakbeenschade nam af. Dus, oestrogeendepletie verergerde de kraakbeenschade in de patella, maar verhoogde niet de gevoeligheid van het kraakbeen.

Zoals beschreven in **Hoofdstuk 6**, zijn de effecten van oestrogeen op kraakbeen onderzocht in een kweekstudie met runderkraakbeen-explants. Hierdoor kon verstoring van omringende weefsels worden uitgesloten. Oestrogeenbehandeling bleek de expressie van zowel anabole als katabole genen te reguleren. Wanneer oestrogeen werd toegevoegd aan de explantkweek nadat een artrose-trigger was toegediend, beïnvloedde oestrogeen de veranderingen veroorzaakt door de artrose-trigger. Deze effecten kunnen gunstig zijn voor het kraakbeenherstel. Deze studie gaf ook meer inzicht in de effecten van de artrose-trigger joodacetaat op kraakbeen.

In **Hoofdstuk 7** werden de belangrijkste bevindingen uit voorgaande hoofdstukken besproken in de context van de huidige wetenschappelijke literatuur. Daarnaast werden enkele suggesties voor verder onderzoek gegeven. Concluderend kan gesteld worden dat de resultaten van dit proefschrift het idee ondersteunen dat artrose een multifactoriële aandoening is, waarin de bijdrage van oestrogeen wellicht gering is. Echter, de veranderingen die door oestrogeenverlies veroorzaakt zijn, kunnen samen met andere veranderingen leiden tot artrose

Dankwoord

Curriculum vitae

PhD portfolio

List of publications

Dankwoord

Op deze plaats wil ik graag iedereen bedanken die, op welke manier dan ook, heeft bijgedragen aan het tot stand komen van dit proefschrift. Een aantal mensen wil ik in het bijzonder noemen:

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Als laatste natuurlijk Sjoerd: Bedankt voor al je steun en liefde!

Curriculum vitae

Yvonne Hendrika Sniekers werd op 11 juli 1980 geboren in Heel. In 1998 behaalde zij haar VWO diploma aan Scholengemeenschap Sint Ursula te Horn, waarna zij Biomedische Technologie studeerde aan de Technische Universiteit Eindhoven. Als onderdeel van haar studie liep Yvonne stage aan de University of Oxford, waar zij onderzoek deed naar het zuurstofgebruik van kraakbeencellen. Hierna begon zij aan haar afstudeerproject dat zich richtte op het bepalen van diffusiecoëfficiënten in de groeischijf. In februari 2004 rondde Yvonne haar studie cum laude af.

In juli van datzelfde jaar startte zij als promovendus bij de afdeling Orthopedie en de afdeling Inwendige Geneeskunde van het Erasmus Medisch Centrum Rotterdam. Zij werd begeleid daar Prof. dr. ir. Weinans, Prof. dr. Van Leeuwen en Dr. Van Osch. De resultaten van ruim 4 jaar onderzoek zijn beschreven in dit proefschrift.

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student:	Yvonne Sniekers
Erasmus MC Departments:	Orthopaedics
	Internal Medicine
Research Schools:	MUSC (Musculoskeletal Science Center)
	Molmed
PhD period:	July 2004 – February 2009
Promotors:	Harrie Weinans
	Hans van Leeuwen
Supervisor:	Gerjo van Osch

1. PhD training

In depth courses, seminars, workshops

- Laboratory animal science	2004
- MUSC (Musculoskeletal Science Center) retreat	2004, 2006
- Biomedical English Writing and Communication	2005
- Classical methods for data-analysis (NIHES)	2005
- Research Integrity	2006
- Animal imaging	2008

Presentations on conferences

Podium presentations

- ORS meeting, San Diego, USA	2007
- Skyscan user meeting, Brugge, Belgium	2007
- NVCB meeting, Zeist, The Netherlands	2008

Poster presentations

- ECTS meeting, Prague, Czech Republic Awarded ECTS travel grant	2005
- ORS meeting, Chicago, USA	2006
- OARSI meeting, Prague, Czech Republic	2006
- OARSI meeting, Fort Lauderdale, USA	2007
- OARSI meeting, Rome, Italy	2008

Other presentations

- Science day Orthopaedics, podium presentation	2005
- Osteoarthritis research discussion of Departments of General Practice, Rheumatology, Orthopaedics, Internal Medicine	2005
- Research discussion at Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht	2005
- Science days Internal Medicine, poster presentation	2006, 2008
- Molmed day, poster presentation	2006
- Research discussions at Department of Orthopaedics (13x)	2004-2009
- Literature discussions at Department of Orthopaedics (12x)	2004-2009

- Research discussions at Department of Internal Medicine (8x) 2004-2008
- Literature discussions at Department of Internal Medicine (3x) 2004-2008
- Research and literature discussions of Endocrinology group at Department of Internal Medicine (2x) 2004-2008

Research meetings attended

- Research discussions at Department of Orthopaedics (weekly) 2004-2009
- Literature discussions at Department of Orthopaedics (weekly) 2004-2009
- Research discussions at Department of Internal Medicine (weekly) 2004-2008
- Literature discussions at Department of Internal Medicine (monthly) 2004-2008
- Research and literature discussions of Endocrinology group at Department of Internal Medicine (2-weekly) 2004-2008
- Osteoarthritis research discussion of Departments of General Practice, Rheumatology, Orthopaedics, Internal Medicine (2 monthly) 2005-2008
- Osteoarthritis, Estrogens and Fat research discussion of Departments of General Practice, Rheumatology, Orthopaedics, Internal Medicine (2 monthly) 2007-2008

2. Teaching activities

Supervising practicals and excursions

- VWO scholieren week 2006, 2007

Supervising student internships

- Internship student Biomedical Engineering, TU/e, 3 months 2007
- Internship student Veterinary Medicine, UU, 2 months 2008

Lecturing

- "Computed Tomography" at course Biomedical Research Techniques, Molmed 2008

List of publications

Publications based on studies described in this thesis

- **YH Sniekers**, H Weinans, SM Bierma-Zeinstra, JPTM van Leeuwen, GJVM van Osch.
  Animal models for osteoarthritis: the effect of ovariectomy and estrogen treatment – a systematic approach.
  Osteoarthritis & Cartilage 16(5):533-541, 2008

- **YH Sniekers**, GJVM van Osch, AGH Ederveen, J Inzunza, JA Gustafsson, JPTM van Leeuwen, H Weinans.
  Development of osteoarthritic features in estrogen receptor knockout mice.
  Accepted for publication in Osteoarthritis & Cartilage, 2009

- **YH Sniekers**, GJVM van Osch, H Weinans, JPTM van Leeuwen.
  Estrogen is important for maintenance of cartilage and subchondral bone in a murine model of knee osteoarthritis.
  Manuscript under review

- **YH Sniekers**, GJVM van Osch, H Jahr, H Weinans, JPTM van Leeuwen.
  Estrogen modulates iodoacetate-induced gene expression in bovine cartilage explants.
  Manuscript under review

Other publications

- **YH Sniekers**, CC van Donkelaar.
  Determining diffusion coefficients in inhomogeneous tissues using fluorescence recovery after photobleaching.
  Biophys J 89(2):1302-1307, 2005

- **YH Sniekers**, F Intema, FPJG Lafeber, GJVM van Osch, JPTM van Leeuwen, H Weinans, SC Mastbergen.
  A role for subchondral bone changes in the process of osteoarthritis; a micro-CT study of two canine models.
  BMC Musculoskeletal Disorders 9:20, 2008

- F Intema, **YH Sniekers**, H Weinans, ME Vianen, SA Yocum, AM Zuurmond, J de Groot, FPJG Lafeber.
  Subchondral bone changes in two canine models for osteoarthritis.
  Manuscript under review

Valete et plaudite!