Osteoarthritis and Cartilage 19 (2011) 466–473

Osteoarthritis and Cartilage

Oral salmon calcitonin reduces cartilage and bone pathology in an osteoarthritis rat model with increased subchondral bone turnover

R.H. Nielsen*, A.-C. Bay-Jensen, I. Byrjalsen, M.A. Karsdal

Nordic Bioscience A/S, Herlev Hovedgade 207, DK-2730 Herlev, Denmark

ARTICLE INFO

Article history:
Received 3 September 2010
Accepted 3 January 2011

Keywords:
Osteoarthritis
Oral salmon calcitonin
OARSI score
Meniscectomy
Ovariectomy
Rat model
Cartilage pathology

SUMMARY

Objectives: Traumatic osteoarthritis (OA) is possibly augmented by effects from loss of sex hormones. Salmon calcitonin is shown to reduce OA pathogenesis and bone resorption. We investigated the effects of oral salmon calcitonin treatment and ovariectomy on cartilage and bone pathology in a traumatic OA model.

Methods: Six groups with 10 7-month-old female Sprague Dawley rats each were subjected to bilateral meniscectomy (MNX), ovariectomy (OVX) or Sham surgery and treated for 8 weeks with oral salmon calcitonin (CT) or vehicle (V) in the following way: (1) Sham + V; (2) MNX + V; (3) MNX + CT; (4) OVX + V; (5) MNX/OVX + V; (6) MNX/OVX + CT. Weights were recorded weekly and CTX-II was measured in serum. At termination 56 days post-surgery, the right tibia was analyzed for changes in articular cartilage thickness, extent of cartilage damage and subchondral bone changes in predefined zones, as recommended in the novel OARSI histopathology score.

Results: The combined MNX/OVX model produced a significantly reduced cartilage thickness ($P = 0.033$) in the outer zone (Z1) of the tibial plateau and increased calcified cartilage damage ($P = 0.0004$) and serum CTX-II ($P = 0.003$). Addition of OVX to MNX significantly increased the width of matrix damage at the surface ($P = 0.025$) and 50% cartilage depth ($P = 0.004$). Treatment with oral salmon calcitonin counteracted the loss of cartilage thickness ($P = 0.055$), significantly reduced subchondral bone damage score ($P = 0.019$) and reduced the type II collagen degradation ($P = 0.009$).

Conclusions: Addition of ovariectomy augmented site-specific traumatic OA pathology, which was reduced by oral salmon calcitonin treatment. Treatments for OA might ideally affect both bone and cartilage.

Introduction

Osteoarthritis (OA) is a degenerative joint disease where progressive deterioration leads to destruction of joint structure and eventually loss of joint function.

Anterior cruciate ligament transection (ACLT) in dogs[1] and partial medial meniscectomy (MNX) and ACLT in rat[2,3] show progressive development of disease with initial loss of superficial chondrocytes and cartilage fibrillation, followed by type II collagen degradation and cartilage erosion. Subchondral bone sclerosis is visible at later stages of disease.

Ovariectomy abrupts estrogen production and increases bone and cartilage turnover. Ovariectomy alone has been shown to produce signs of OA in rats[4,5] and to increase OA pathology in traumatic OA models in the rabbit[6] and the mouse[7].

Recently, a novel histological scoring system was presented by the osteoarthritis research society international (OARSI) Histopathology Initiative[8]. The score recommends the use of quantitative and qualitative measures on various tissues to assess joint pathology.

Salmon calcitonin has been used for more than 30 years for the treatment of metabolic bone diseases[9]. The 32-amino-acid peptide hormone exerts a potent anti-resorptive effect by direct binding to osteoclasts[10–13] and directly affects chondrocytes[14,15]. When used in animal models of OA, nasal calcitonin was shown to reduce signs of OA in both articular cartilage[16,17] and subchondral bone[1]. Recently, a new oral formulation of salmon calcitonin was developed[15,18,19], which was demonstrated to be safe in clinical[20] and pre-clinical settings[15]. The carrier 5-CNAC is an enhancer of gastrointestinal peptide administration and is used to protect the salmon calcitonin from the acidic environment and facilitate

* Address correspondence and reprint requests to: R.H. Nielsen, Nordic Bioscience A/S, Herlev Hovedgade 207, DK-2730 Herlev, Denmark. Tel: 45-44-52-52-03; Fax: 45-44-54-88-88.
E-mail address: rhn@nordicbioscience.com (R.H. Nielsen).

1063-4584/$ – see front matter
doi:10.1016/j.joca.2011.01.008

uptake over the gastrointestinal epithelium without changing the biological function of the hormone[20,21].

The primary objectives of the current study were to investigate the effects of supplementary ovariectomy on pathology in a traumatic OA model, and to assess the effects of a preventive oral salmon calcitonin treatment. Recommendations from a novel histopathology score[8] were used to evaluate cartilage and bone pathology.

Methods

Animals

Sixty female Sprague Dawley rats, 7 months of age, were obtained from Taconic (Denmark). Animals were acclimatized, weighed and stratified into six groups of 10 animals per group and mean ± 95% confidence interval (95%CI) body weights from 309 ± 18 g to 314 ± 16 g. Animals were housed two by two in standard type III H cages with sawdust bedding and nesting material in a Scantainer-plus (Scanbur, Sweden). They were fed *ad libitum* with a standard diet (no. 1324, Altromin, Lage, Germany) and had access to Milli-Q water *ad libitum*.

Performance of bilateral partial medial meniscectomy, bilateral ovariectomy and Sham surgery

Rats were anaesthetized by a subcutaneous Hypnorm (Vetapharma, UK)/Dormicum (Roche, Switzerland) injection. Each animal received both knee surgery (either MNX or Sham surgery) and incision from the back (either OVX or Sham surgery). Both knees of each animal were subjected to the same kind of surgery, either partial medial meniscectomy (MNX) or Sham surgery (Sham). For performance of MNX, the skin over the knees was shaved and disinfected. The skin was penetrated and an insertion made medial to the patella, dislocating the patella laterally and providing access to the joint space. The anterior part of the medial meniscus was removed using a scalpel and surgical scissors. After relocation of the patella, the wound was closed with vicryl 5/0 (polyglactin 910)-braided absorbable suture (Ethicon, Edinburgh, UK). The skin was closed with staples (BD Biosciences, USA). Care was taken to keep the operation area moist with saline during the procedure. In Sham surgery, the meniscus was left untouched.

For OVX surgery, an incision centrally on the lower back provided access to both ovaries, which were removed. The wound was closed with vicryl 3/0 (polyglactin 910)-braided absorbable suture (Ethicon, Edinburgh, UK). For Sham OVX surgery, access to the ovaries was performed, but the ovaries were left intact. Rimadyl (Pfizer, Belgium), 5 mg/kg sc., was given once a day as analgesic for the following 4 days post-surgery to all animals. All procedures were approved by the Danish Animal Experiments Inspectorate.

Drug substance and concentration

5-CNAC (8-(N-2-hydroxy-5-chlorobenzoyl)-amino-caprylic acid) is a molecule based on the Eligen® technology from Emisphere[21] and was used as a carrier to protect the salmon calcitonin from degradation and to facilitate uptake from the gastrointestinal tract. The used calcitonin was recombinant salmon calcitonin. 5-CNAC and salmon calcitonin were provided by Novartis, Basel, Switzerland. The concentration of salmon calcitonin used in this experiment was decided from previous experiments[15].

Experimental design and weight recordings

The animals in the six groups were subjected to surgery and administered orally twice daily for 56 consecutive days post-surgery either with vehicle control (V) (150 mg/kg 5-CNAC) or oral salmon calcitonin (CT) (150 mg/kg 5-CNAC + 2 mg/kg salmon calcitonin) in the following way: (1) Sham + V; (2) MNX + V; (3) MNX + CT; (4) OVX + V; (5) MNX/OVX + V; (6) MNX/OVX + CT. Medication was administered by oral gavage of compounds dissolved in Milli-Q water to final concentrations of 30 mg/ml 5-CNAC and 0.4 mg/ml salmon calcitonin. Weights of the animals were recorded weekly and quantity of drug adjusted weekly according to new weight recordings.

After 56 days post-surgery the animals were euthanized and the knee joints were isolated. The right knee joints were used for histology, and those results are included in this manuscript. The left knee joints were reserved for other purposes.

Blood sampling, preparation of serum and measurement of CTX-II

Blood samples were drawn from overnight-fasted, CO_2/O_2-anaesthetized animals exactly 3 h after the morning dosing on day 21, 42 and 56 post-surgery. Baseline samples were obtained in the morning without prior dosing of compounds. Blood was collected from the eye orbital sinus into plain tubes and left to coagulate at room temperature for 30 min. Samples were centrifuged at 1500*g* for 10 min and all liquid was transferred to a new plain tube. Centrifugation was repeated at 1500*g* for 10 min and the serum was collected into a new plain tube and stored at −20°C until use. Concentration of CTX-II in the serum was measured using the Serum Pre-Clinical Cartilaps® ELISA (IDS Nordic, Herlev, Denmark) according to the manufacturer's instructions.

Preparation of tissues, sectioning and staining

The right knee joint was isolated at euthanasia, and the entire unopened knee was fixated in 4% formaldehyde in Dulbecco's phosphate buffered saline (DPBS, BioWhittaker, USA), pH 7.4. Subsequently, femur and tibia were carefully separated and the tibia decalcified in 15% ethylene diamine tetraacetic acid (EDTA), pH 7.4. The tibia was paraffin-embedded, cut anterio-posterior into 5 μm sections and mounted on glass slides. After deparaffinization and hydration, the sections were stained with Safranin O/Fastgreen and counterstained with hematoxylin.

Histology

The recommendations in the novel scoring system provided by the OARSI Histopathology Initiative were used for histological assessment[8]. Digital images were obtained using an Olympus BX-60 microscope and an Olympus camera. Using CorelDraw software (Corel, USA), the medial tibial plateau was divided into three zones of equal width with zone 1 (Z1) on the outside (medial edge of joint), zone 2 (Z2) in the central part of the medial plateau and zone 3 (Z3) on the inside (adjacent to the central cruciate ligaments) (Fig. 1). The ends of articular cartilage on the medial tibial plateau defined the outer borders.

The following procedure applies for the parameters non-calcified cartilage thickness, cartilage matrix loss width, subchondral bone plate thickness and calcified cartilage and subchondral bone damage score described below: from each knee, sections from three locations with 200 μm apart, from within the weight-bearing middle part of the plateau, were analyzed. From measurements on the three sections from each knee, a knee average was calculated.

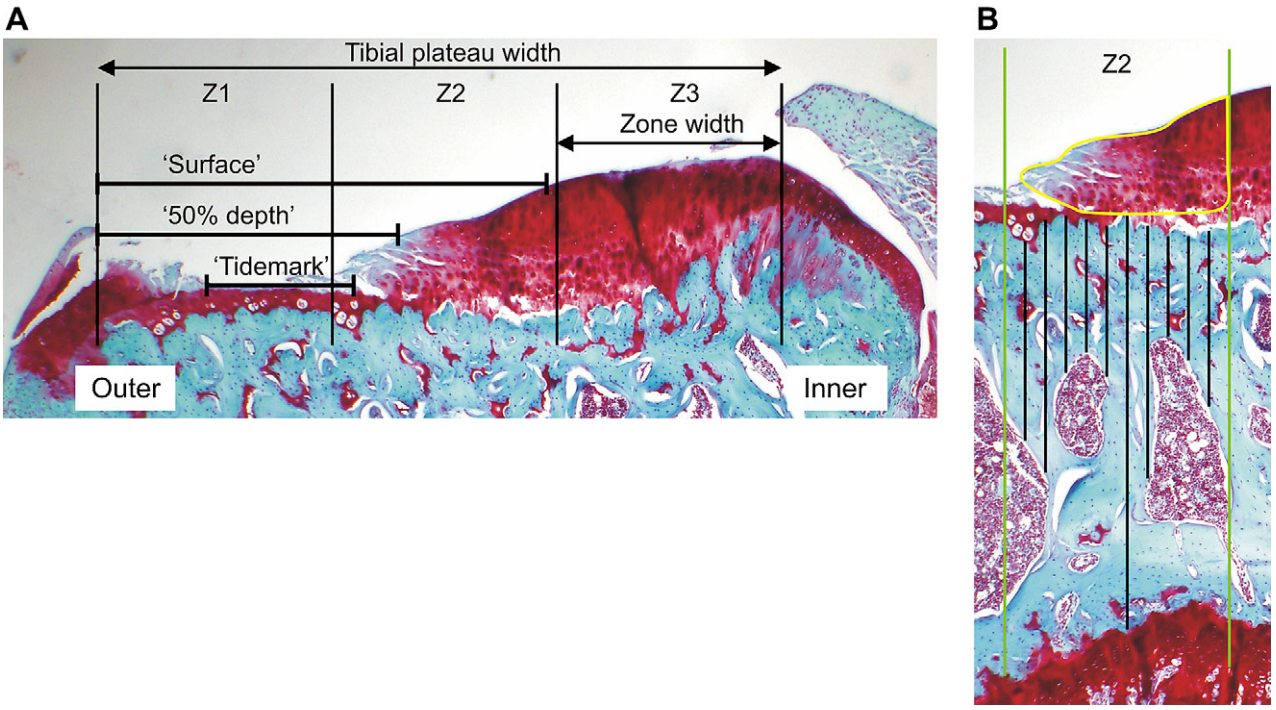

Fig. 1. Outline of measurements: (A) the medial tibial plateau was divided into three zones of equal width with zone 1 (Z1) on the outside (adjacent to the synovium), zone 2 (Z2) in the central part of the medial plateau and zone 3 (Z3) on the inside (adjacent to the central cruciate ligaments). Measurements for cartilage matrix loss width across the whole tibial plateau at "surface", "50% depth" and "tidemark" are indicated. (B) Yellow line: illustration of measurement of non-calcified cartilage area for calculation of the average cartilage thickness. Black lines: illustration of subchondral bone plate thickness measurements at 10 locations within Z2 on each section.

These individual knee averages were then used to calculate the group mean and 95%CI. Specimens were blinded to the reader.

Non-calcified cartilage thickness

The area of non-calcified articular cartilage was measured within zones Z1, Z2 and Z3, using ImagePro (Media Cybernetics, USA) [Fig. 1(B)]. Obtained area values were divided by the width of the individual zone to adjust for size variation between animals, thereby presenting the results as average cartilage thickness in micrometers.

Cartilage matrix loss width

The width of collagen matrix loss was measured in relation to the depth of full-thickness non-calcified cartilage matrix [Fig. 1(A)]. The widths of lesions were measured at any surface matrix loss ("Surface"), at 50% matrix loss compared to full-thickness matrix ("50% depth"), and 100% loss of non-calcified matrix, e.g., to the tidemark ("tidemark"). Each recording was divided by the tibial plateau width to adjust for size variation between specimens, thereby presenting the cartilage matrix loss width as percentage of the tibial plateau width.

Subchondral bone plate thickness

The length from the surface of the subchondral bone to the first margin of the bone marrow was measured at 10 locations within the zone Z2 on each section [Fig. 1(B)], and from those values a section-average was calculated. Three section averages from the three non-consecutive sections from each knee were used to calculate a knee average, and the knee averages were then used to calculate a group average and 95%CI. Values are shown in micrometers as mean ± 95%CI. The Z2 zone was used alone for measurements as the width of the tibial plateau in Z1 and Z3 often exceeded the width of the growth plate, and so those zones were not applicable for measurements.

Quantification of calcified cartilage and subchondral bone damage

Damage to the calcified cartilage and subchondral bone was scored using a numerical scale from 0 to 5, where 0 = no changes and 5 = increased basophilia at the tidemark, increased fragmentation at the tidemark and/or of the calcified cartilage, increased area of marrow mesenchymal changes, and collapse of articular cartilage into the epiphysis. The most severe lesion on each section was scored. Again, the knee-averages were used to produce a group mean and 95%CI.

Statistical analysis

Statistical evaluation was performed on carefully selected and independent parameters to avoid type 1 errors. Statistics was performed using SAS software (SAS, USA). Effects in the model system was assessed by comparisons between the groups: Sham + V, MNX + V, OVX + V and MNX/OVX + V. A Bartlett's test was performed to ensure homogeneity of variances in the groups, followed by an analysis of variance (ANOVA) to see if differences between group-means existed. If means were significantly different, a Dunnett's post-test was performed with the MNX/OVX + V group as reference group. By use of the Dunnett's post-test the statistical results were adjusted for multiple comparisons. *P*-values from the Dunnett's test are shown in *italic* in the figures. Direct effects from treatment with oral calcitonin were assessed using a Student's *t*-test between V-treated and CT-treated groups, e.g., MNX + V vs MNX + CT and MNX/OVX + V vs MNX/OVX + CT. *P*-values from the *t*-test are underlined in the figures.

Results

Number of observations included in the analysis

All 60 animals completed the study, but during sectioning for histology, one specimen from each of the Sham + V and MNX + V groups was lost. Thus, group means were calculated from 10 specimen means, except from histological parameters in the Sham and MNX groups, where group means were calculated from nine specimen means.

Body weights of the animals

Before the start of the experiment the animals were stratified into six groups with mean ± 95%CI body weights in grams from 309 ± 18 to 314 ± 16. At the end of the experiment the weights were (mean ± 95%CI in grams): Sham + V 314 ± 18; MNX + V 310 ± 14; MNX + CT 302 ± 20; OVX + V 354 ± 18; MNX/OVX + V 344 ± 16; MNX/OVX + CT 341 ± 26. Only the OVX + V group was significantly different from the Sham + V group at termination with a body weight increase of 12.7% ($P < 0.05$) (data not shown).

Histology

Representative pictures of tibial plateaus stained with Safranin O and Fast Green are shown in Fig. 2. The articular cartilage on Sham + V (A) and OVX + V (D) tibias was intact. In MNX + V (B) and MNX + CT (C), reduction in cartilage thickness was modest, but the extent of erosion at the cartilage surface was pronounced, and changes in the calcified cartilage were detectable. Widespread and profound cartilage erosion, and basophilia in the calcified cartilage, was observed on the tibias from the MNX/OVX + V group (E) on the outside of the plateau, whereas these changes appeared reduced in the MNX/OVX + CT group (F).

Width of the tibial plateau

The width of the tibial plateau from each section was measured (Fig. 1). Values were (mean ± 95%CI in μm): Sham + V 1791 ± 102; MNX + V 1872 ± 94; MNX + CT 1964 ± 74; OVX + V 1766 ± 490 MNX/OVX + V 1900 ± 38; MNX/OVX + CT 1923 ± 100. Statistical evaluation showed significant difference between the group-means and as a function of that, individual measurements of non-calcified cartilage areas and cartilage matrix loss width were corrected for the width of the individual zone or the tibial plateau, respectively.

Non-calcified cartilage thickness

The average non-calcified tibial cartilage thickness was quantified within each of the three zones Z1, Z2 and Z3 and results are shown in Fig. 3. In Z1, the cartilage thickness in the MNX/OVX + V group was significantly reduced by 49% ($P = 0.033$) compared to the Sham + V and by 43% ($P = 0.13$) compared to the MNX + V group. No effect was observed from OVX alone. As a function of salmon calcitonin treatment, the difference between MNX/OVX + V and MNX/OVX + CT was borderline significant ($P = 0.055$), whereas no difference was found between MNX + V and MNX + CT ($P = 0.87$). In Z2, cartilage thickness in the MNX/OVX + V was reduced by 23% ($P = 0.11$) and 16% ($P = 0.42$) compared to Sham + V and MNX + V, respectively. No significant effects were observed from calcitonin treatment.

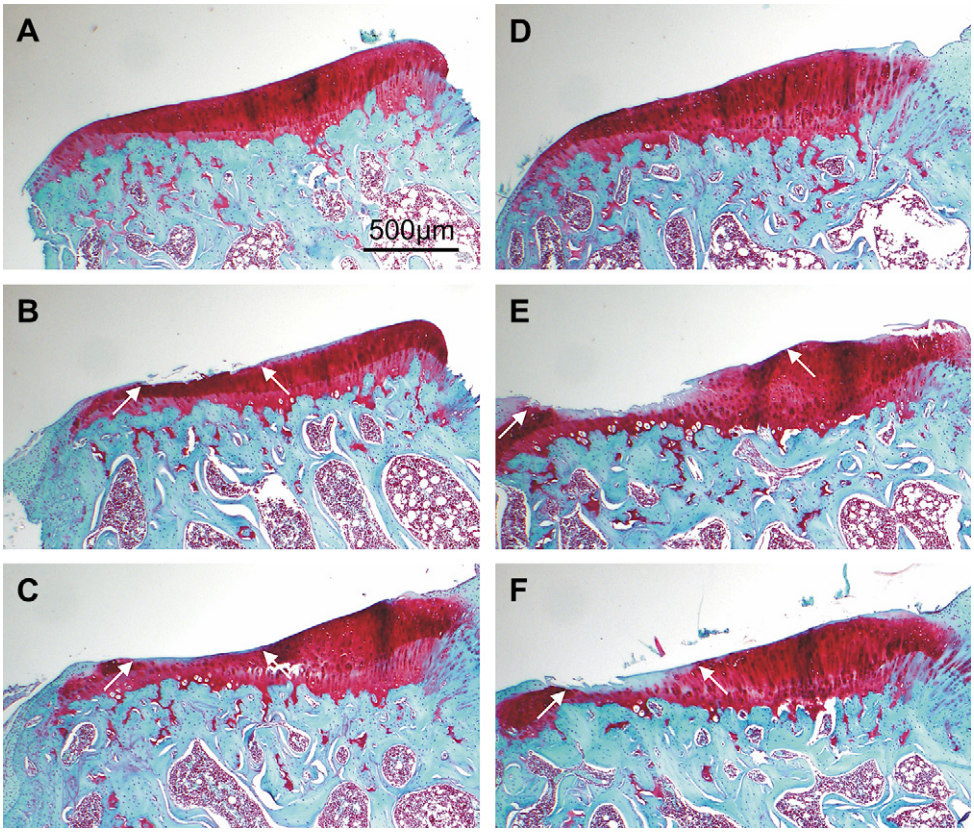

Fig. 2. Representative histological sections from the weight-bearing part of the medial tibial plateau. Pictures are from groups: Sham + V (A), MNX + V (B), MNX + CT (C), OVX + V (D), MNX/OVX + V (E) and MNX/OVX + CT (F). Sections were stained with Safranin O and Fast Green. The size bar of 500 μm (A) applies to all pictures. White arrows mark the borders of cartilage erosion.

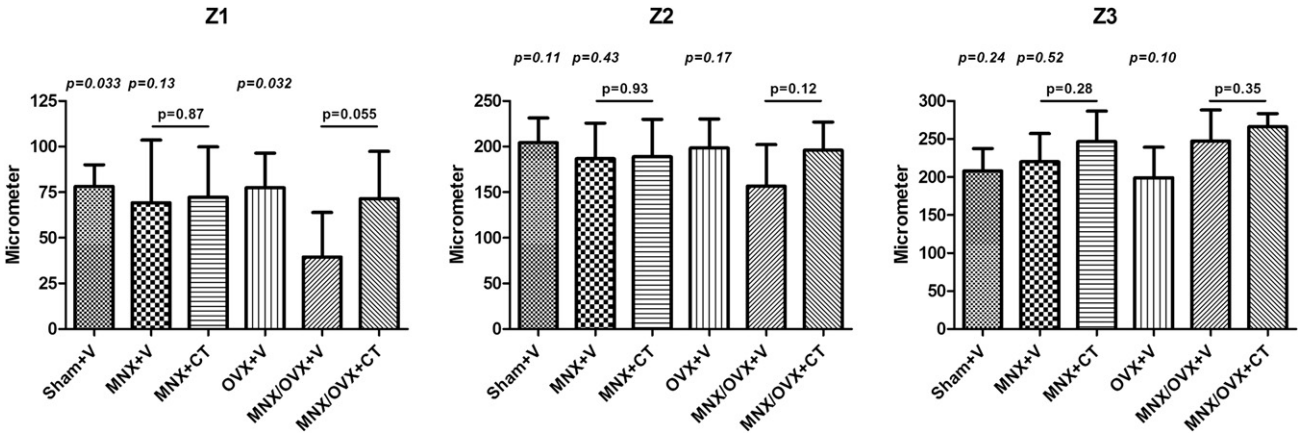

Fig. 3. Average non-calcified cartilage thickness in Z1, Z2 and Z3. Areas of non-calcified cartilage were measured within each zone and divided by the width of the zone to obtain average cartilage thickness. Values are presented as mean + 95%CI in micrometers. Z1: Sham + V (78.2 + 11.8), MNX + V (69.3 + 34.4), MNX + CT (72.3 + 27.5), OVX + V (77.5 + 18.9), MNX/OVX + V (39.5 + 24.3) and MNX/OVX + CT (71.5 + 25.8). Z2: Sham + V (204.4 + 26.9), MNX + V (186.7 + 38.9), MNX + CT (188.8 + 41.2), OVX + V (198.4 + 32.0), MNX/OVX + V (156.5 + 45.7) and MNX/OVX + CT (195.9 + 31.0). Z3: Sham + V (207.8 + 29.6), MNX + V (220.0 + 37.2), MNX + CT (146.6 + 40.0), OVX + V (199.1 + 40.1), MNX/OVX + V (247.3 + 40.8) and MNX/OVX + CT (266.0 + 17.5). *P*-values from the Dunnett's test with MNX/OVX + V as the reference group are shown in *italic*, *P*-values from the Student's *t*-test are underlined.

Measurements in Z3 indicated that cartilage thickness was slightly increased in all groups except the OVX + V, compared to Sham + V.

Cartilage matrix loss width

The extent of cartilage matrix loss across the tibial plateau was assessed by quantification of the length of erosion at cartilage surface, 50% cartilage depth and at the tidemark. Results are shown in Fig. 4. No erosion was measured on any of the sections from the Sham + V and OVX + V groups. The effect from OVX was evident when comparing MNX + V to the MNX/OVX + V; extent of damage at the surface ($P = 0.023$) and 50% cartilage depth ($P = 0.0067$) was significantly increased and extent of damage at the level of the tidemark ($P = 0.13$) was two-fold increased. Although treatment with calcitonin did not produce significant effects, the reduced extent of damage at the surface ($P = 0.58$), 50% depth ($P = 0.33$) and tidemark ($P = 0.10$), indicated a more potent effect from treatment on the deeper layers of the cartilage.

Subchondral bone plate thickness

The subchondral bone plate thickness was assessed by measuring the length from the surface of the subchondral bone to the first margin of the bone marrow in the zone Z2. Results are shown in Fig. 5. Neither surgery nor treatment with calcitonin seemed to produce any difference between groups, and mean thickness values for all groups were found in the small range between 312 and 353 μm.

Calcified cartilage and subchondral bone damage

The join MNX/OVX surgery produced a highly significant increase in the damage score compared to Sham + V ($P = 0.0004$) and OVX + V ($P < 0.0001$), and borderline significant to MNX + V ($P = 0.054$) (Fig. 6). A significant effect from calcitonin treatment was observed in the MNX/OVX model ($P = 0.033$), but not with MNX alone ($P = 0.68$).

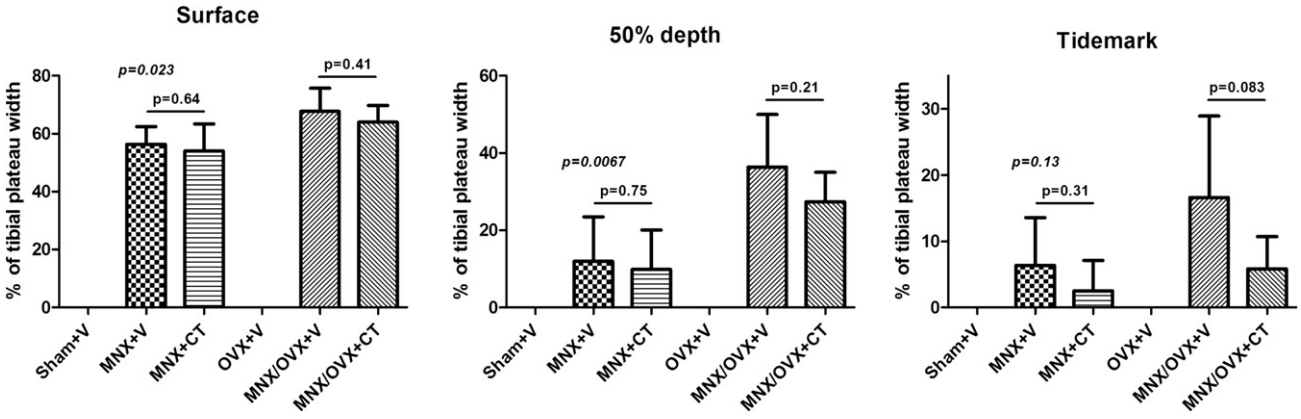

Fig. 4. Cartilage matrix loss width across the tibial plateau. Length of erosion at surface, 50% depth and tidemark were measured and divided by the plateau width. Sham + V and OVX + V groups did not show any erosion. Values are presented as mean + 95%CI in percent of the plateau width. Surface: Sham + V (0.0 + 0.0), MNX + V (56.4 + 6.1), MNX + CT (54.1 + 9.3), OVX + V (0.0 + 0.0), MNX/OVX + V (67.7 + 8.0) and MNX/OVX + CT (64.0 + 5.8). 50% depth: Sham + V (0.0 + 0.0), MNX + V (12.0 + 11.5), MNX + CT (9.87 + 10.2), OVX + V (0.0 + 0.0), MNX/OVX + V (36.4 + 13.6) and MNX/OVX + CT (27.4 + 7.6). Tidemark: Sham + V (0.0 + 0.0), MNX + V (6.37 + 7.2), MNX + CT (2.53 + 4.6), OVX + V (0.0 + 0.0), MNX/OVX + V (16.6 + 12.3) and MNX/OVX + CT (5.85 + 4.9). *P*-values from the Dunnett's test with MNX/OVX + V as the reference group are shown in *italic*, *P*-values from the Student's *t*-test are underlined.

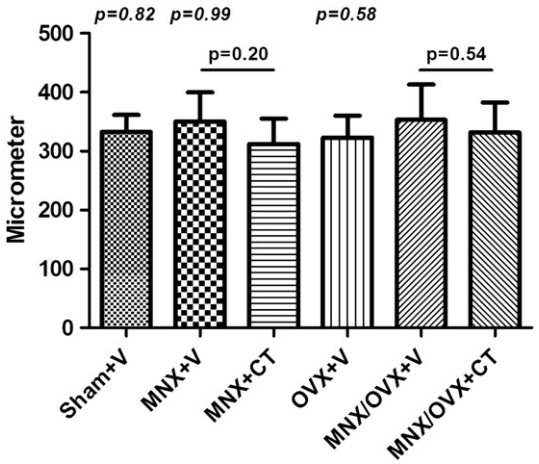

Fig. 5. Subchondral bone plate thickness. The average distance between upper subchondral bone surface and underlying marrow lumen was measured in Z2. Values are presented as mean + 95%CI in micrometers: Sham + V (332.9 + 28.6), MNX + V (350.3 + 49.7), MNX + CT (312.4 + 42.8), OVX + V (323.1 + 36.9), MNX/OVX + V (353.3 + 59.9) and MNX/OVX + CT (331.8 + 50.9). *P*-values from the Dunnett's test with MNX/OVX + V as the reference group are shown in *italic*, *P*-values from the Student's *t*-test are underlined.

Type II collagen degradation

The type II collagen degradation was assessed by measuring the concentration of CTX-II in the serum (Fig. 7). Compared to Sham + V, CTX-II was significantly increased in the OVX + V group at 3 weeks ($P = 0.003$) and 6 weeks ($P < 0.001$) after surgery, and in the MNX/OVX + V group 3 weeks after surgery ($P = 0.009$). CTX-II levels in the MNX + V, MNX + CT and MNX/OVX + CT were not different from the Sham + V group at any time point.

Discussion

We have investigated the effects of partial medial meniscectomy, ovariectomy and oral salmon calcitonin treatment on non-calcified articular cartilage thickness, cartilage matrix loss width, subchondral plate thickness, calcified cartilage and subchondral bone damage,

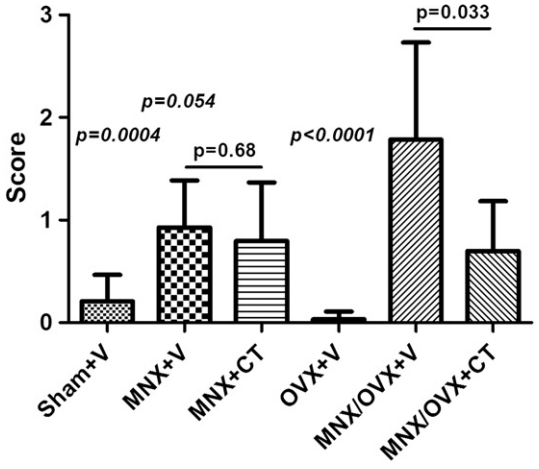

Fig. 6. Calcified cartilage and subchondral bone damage. On each section, the most severe lesion within the calcified cartilage and subchondral bone was assigned a score from 0 to 5, where 0 represents no damage and 5 represents severe damage. Values are presented as mean + 95%CI: Sham + V (0.21 + 0.26), MNX + V (0.93 + 0.46), MNX + CT (0.80 + 0.57), OVX + V (0.033 + 0.075), MNX/OVX + V (1.78 + 0.95) and MNX/OVX + CT (0.70 + 0.48). *P*-values from the Dunnett's test with MNX/OVX + V as the reference group are shown in *italic*, *P*-values from the Student's *t*-test are underlined.

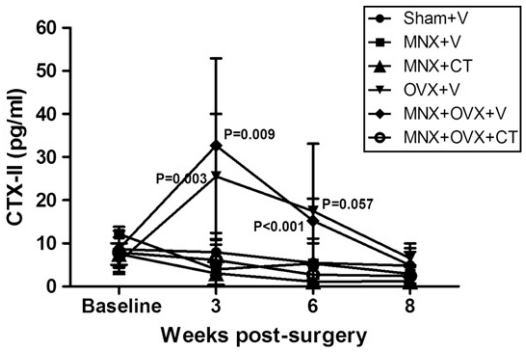

Fig. 7. Concentration of c-terminal telopeptide of type II collagen (CTX-II) measured in serum. Serum was obtained at indicated time points and measured for the concentration of CTX-II. Values are shown as mean + 95%CI (pg/ml). The shown *P*-values are from Student's *t*-test.

and type II collagen degradation in a traumatic OA model without and with increased bone turnover. We found that (1) OA pathology was site-specific and developed on the outer part of the tibial plateau, (2) addition of OVX surgery to MNX surgery increased OA pathology, (3) the MNX/OVX model produced a significant reduction in cartilage thickness and a significant increase in cartilage matrix loss width, calcified cartilage and subchondral bone damage score and type II collagen degradation and (4) treatment with oral salmon calcitonin counteracted cartilage erosion and extent of matrix loss and significantly reduced type II collagen degradation and damage to the calcified cartilage and subchondral bone.

The current data show that OVX surgery augmented OA pathology inflicted by traumatic injury, as earlier studies have indicated[6,7]. The extent of cartilage damage was significantly increased by addition of OVX, and both cartilage loss and damage to the calcified cartilage were pronounced. The response produced from MNX alone was smaller than expected, however this does not question the additive effect of OVX surgery. Importantly, significant cartilage loss in the combined model (MNX/OVX) was found only on the outer 1/3 of the medial plateau (Z1, adjacent to the synovium). This was as expected[8], as the outer zone (Z1) is subject to more mechanical forces compared to the central (Z2) and inner (Z1) zones. Unilateral knee surgery as a model is most common, but we have no reason to believe that the present use of bilateral MNX has changed the course of OA, compared to other studies using unilateral surgery[8].

We found that OVX surgery alone produced no cartilage pathology in evaluated parameters, which at first glance contradicts previous findings stating that the OVX model in rats is a validated model of postmenopausal OA[4,15,22]. The discrepancy, however, is partly due to the fact that the present study includes only extent of cartilage matrix loss, whereas previous studies only investigated changes in the superficial layer of the articular cartilage (fibrillation)[4]. We found that serum CTX-II was not elevated from MNX surgery alone, which could be explained by the moderate cartilage erosion observed. A likely reason could also be that cartilage erosion induced by traumatic injury alone releases only small amounts of the CTX-II marker. This is supported by the observation that even a much worsened erosion of the articular non-calcified cartilage in the MNX/OVX + V compared to the OVX + V group did not give rise to a marked difference in serum CTX-II concentrations between these groups. This indicates that CTX-II could also be released from the calcified cartilage, and that a metabolism-mediated CTX-II release potentially is able to disguise CTX-II released from the articular cartilage. As this study illustrates, the use of old animals way past the age of skeletal maturity can facilitate a marked distinction in biomarkers produced from surgery or intervention. We were not able to detect

any morphological change to the subchondral bone, likely because sclerotic bone changes do not occur at later stages of the disease[8].

Increased body weight due to OVX surgery was considered of no importance to OA severity in this study, as the OVX + V group, showing the largest weight increase, produced no signs of OA pathology. However, obesity could potentially worsen traumatic injury in other settings.

Other investigations suggest that OA could be linked to an altered metabolic state affecting the systemic level of bone turnover, as the prevalence of OA in women is shown to increase dramatically around the age of 50, coinciding with the start of menopause and the loss of sex hormones[23–27]. Induction of experimental osteoporosis by ovariectomy in monkeys[5,28], rabbits[6] and rodents[22] has also shown that estrogen depletion results in increased bone turnover, emergence of OA symptoms and aggravation of OA progression. However, distinct effects of ovariectomy arising from subchondral bone changes vs direct effects of estrogen loss on cartilage health have not been dissociated by anyone so far.

The present study is the first to show that an oral formulation of salmon calcitonin is able to reduce OA cartilage pathology in a traumatic OA rat model with increased bone turnover. These chondroprotective effects of oral calcitonin are in general alignment with those published previously using various non-oral formulations of calcitonin both *in vitro* and *in vivo*[1,14,15,18,19,29].

Calcitonin is approved as anti-resorptive intervention for treatment of osteoporosis. The hormone binds directly to receptors on osteoclasts to reduce their resorptive activity[10–13] and chondrocytes display reduced matrix metalloproteinase (MMP) expression and activity, as well as decreased type II collagen degradation when exposed to very small concentrations of salmon calcitonin[14]. Furthermore, calcitonin is shown to promote proliferation of isolated articular chondrocytes and to stimulate proteoglycan synthesis[30].

Badurski *et al.*, were the first to demonstrate that salmon calcitonin reduced cartilage erosion and protected against cartilage glycosaminoglycan loss in experimental OA[31]. Recently, Manicourt and colleagues used the dog ACLT model and showed that calcitonin reduced subchondral bone resorption and trabecular thinning, which were speculated to be crucial factors in the reduction of cartilage erosion. In addition, the authors concluded that direct effect of calcitonin on the articular cartilage was present, as they observed a significant increase in proteoglycan content in the non-operated knees[1]. Clinical trials have confirmed that an oral formulation of salmon calcitonin and 5-CNAC is safe to use and preliminary evidence of chondroprotective effects of calcitonin in clinical settings has been presented in both postmenopausal and OA patients[1,20,29,32]. Hence previous studies indicate that effects from salmon calcitonin treatment could be both bone and cartilage anti-catabolic and cartilage anabolic, however, curative effects need to be shown in long-term clinical trials.

The role of the subchondral bone in OA pathology is heavily debated, and an interesting question is how anti-resorptive treatment alone would affect the course of disease. Alendronate, a bisphosphonate, was used to inhibit bone degradation in a traumatic OA rat model, and was reported to improve cartilage pathology[3]. However, CTX-II levels in serum showed that alendronate directly affected cartilage metabolism, and effects cannot be accredited to impact on subchondral bone alone.

The fact that calcitonin treatment was not able to reduce OA pathology when MNX surgery was performed alone could be explained by the modest response from the model, as this leaves only a little window for treatment. The MNX/OVX model, however, produced a larger window for treatment, and here oral salmon calcitonin significantly reduced the damage in the calcified cartilage and subchondral bone. Cartilage loss from Z1 was borderline significantly reduced when compared directly to the corresponding control group. An inclusion of more animals in this study would have reduced the variance in the groups and likely would have overcome the borderline significance.

OVX surgery introduced a metabolic component into the system. We speculate that depletion of estrogens from ovariectomy increased the activity of catabolic enzymes[22] and that orally administered salmon calcitonin inhibited MMP-mediated type II collagen degradation[15], thereby maintaining the structural strength of the cartilage matrix to resist increased mechanical load induced from meniscectomy.

The present investigations used relevant parameters from the novel OARSI Histopathology Initiative score[8]. The score successfully applies user-friendly quantitative and qualitative measures to evaluate pathology, however, weighing the contribution of the different parameters to compute a total joint score could be problematic, and the parameters could be evaluated separately with advantage. Perhaps the most descriptive parameter would be quantification of lost cartilage, as this would mimic a longitudinal study design. For that the original articular cartilage surface must be estimated. However, as the cartilage damage in the present study was non-focal, with erosions extending primarily to the outer of the tibial plateau, the estimation of original articular cartilage surface was found to be not possible in this experiment. For this rat model, we recommend no less than three non-consecutive sections from each knee to be analyzed, considering the variation that was found in measurements.

In conclusion, our findings demonstrate that loss of cartilage thickness and extent of cartilage damage is augmented when ovariectomy is added to meniscectomy. Oral salmon calcitonin treatment counteracted OA pathology in cartilage and bone, and might prove curative effect in clinical trials.

Role of the funding source

Grant support was provided by “The Danish Agency for Science, Technology and Innovation, Ministry of Science, Technology and Innovation”. The grant provider had no involvement in the experiment or manuscript. The experiment had no study sponsor.

Contributions

Conception and design: Rasmus H. Nielsen, Morten Karsdal.

Analysis and interpretation of data: Rasmus H. Nielsen, Anne-Christine Bay-Jensen, Inger Byrjalsen.

Drafting of the article: Rasmus H. Nielsen, Anne-Christine Bay-Jensen.

Revision of the article: Rasmus H. Nielsen, Anne-Christine Bay-Jensen, Morten Karsdal.

Final approval of the version to be submitted: All authors.

Conflict of interest

Rasmus H. Nielsen, Anne-Christine Bay-Jensen, Inger Byrjalsen and Morten A. Karsdal are employees at Nordic Bioscience A/S. Morten A. Karsdal own stock in Nordic Bioscience A/S.

Acknowledgements

The authors would like to acknowledge the work of our technical colleagues Henrik Simonsen, Trine Overgaard and Christina Hansen at Nordic Bioscience, Denmark for their efforts in the animal facilities and laboratory. No professional writing assistance was used on this manuscript.

References

1. Manicourt DH, Altman RD, Williams JM, Devogelaer JP, Druetz-Van Egeren A, Lenz ME, *et al*. Treatment with calcitonin suppresses the responses of bone, cartilage, and synovium in

the early stages of canine experimental osteoarthritis and significantly reduces the severity of the cartilage lesions. Arthritis Rheum 1999;42:1159–67.
2. Stoop R, Buma P, van der Kraan PM, Hollander AP, Billinghurst RC, Meijers TH, *et al*. Type II collagen degradation in articular cartilage fibrillation after anterior cruciate ligament transection in rats. Osteoarthritis Cartilage 2001;9:308–15.
3. Hayami T, Pickarski M, Wesolowski GA, McLane J, Bone A, Destefano J, *et al*. The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model. Arthritis Rheum 2004;50:1193–206.
4. Hoegh-Andersen P, Tanko LB, Andersen TL, Lundberg CV, Mo JA, Heegaard AM, *et al*. Ovariectomized rats as a model of postmenopausal osteoarthritis: validation and application. Arthritis Res Ther 2004;6:R169–80.
5. Ham KD, Loeser RF, Lindgren BR, Carlson CS. Effects of long-term estrogen replacement therapy on osteoarthritis severity in cynomolgus monkeys. Arthritis Rheum 2002;46:1956–64.
6. Calvo E, Castaneda S, Largo R, Fernandez-Valle ME, Rodriguez-Salvanes F, Herrero-Beaumont G. Osteoporosis increases the severity of cartilage damage in an experimental model of osteoarthritis in rabbits. Osteoarthritis Cartilage 2007;15:69–77.
7. Ma HL, Blanchet TJ, Peluso D, Hopkins B, Morris EA, Glasson SS. Osteoarthritis severity is sex dependent in a surgical mouse model. Osteoarthritis Cartilage 2007;15:695–700.
8. Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative – recommendations for histological assessments of osteoarthritis in the rat. Osteoarthritis Cartilage 2010;18(Suppl 3):S24–34.
9. Cranney A, Tugwell P, Zytaruk N, Robinson V, Weaver B, Shea B, *et al*. Meta-analyses of therapies for postmenopausal osteoporosis. VI. Meta-analysis of calcitonin for the treatment of postmenopausal osteoporosis. Endocr Rev 2002;23:540–51.
10. Chambers TJ, Moore A. The sensitivity of isolated osteoclasts to morphological transformation by calcitonin. J Clin Endocrinol Metab 1983;57:819–24.
11. Suzuki H, Nakamura I, Takahashi N, Ikuhara T, Matsuzaki K, Isogai Y, *et al*. Calcitonin-induced changes in the cytoskeleton are mediated by a signal pathway associated with protein kinase A in osteoclasts. Endocrinology 1996;137:4685–90.
12. Shyu JF, Shih C, Tseng CY, Lin CH, Sun DT, Liu HT, *et al*. Calcitonin induces podosome disassembly and detachment of osteoclasts by modulating Pyk2 and Src activities. Bone 2007;40:1329–42.
13. Sorensen MG, Henriksen K, Schaller S, Henriksen DB, Nielsen FC, Dziegiel MH, *et al*. Characterization of osteoclasts derived from CD14+ monocytes isolated from peripheral blood. J Bone Miner Metab 2007;25:36–45.
14. Sondergaard BC, Wulf H, Henriksen K, Schaller S, Oestergaard S, Qvist P, *et al*. Calcitonin directly attenuates collagen type II degradation by inhibition of matrix metalloproteinase expression and activity in articular chondrocytes. Osteoarthritis Cartilage 2006;14:759–68.
15. Sondergaard BC, Oestergaard S, Christiansen C, Tanko LB, Karsdal MA. The effect of oral calcitonin on cartilage turnover and surface erosion in an ovariectomized rat model. Arthritis Rheum 2007;56:2674–8.
16. Behets C, Williams JM, Chappard D, Devogelaer JP, Manicourt DH. Effects of calcitonin on subchondral trabecular bone changes and on osteoarthritic cartilage lesions after acute anterior cruciate ligament deficiency. J Bone Miner Res 2004;19:1821–6.
17. El Hajjaji H, Williams JM, Devogelaer JP, Lenz ME, Thonar EJ, Manicourt DH. Treatment with calcitonin prevents the net loss of collagen, hyaluronan and proteoglycan aggregates from cartilage in the early stages of canine experimental osteoarthritis. Osteoarthritis Cartilage 2004;12:904–11.
18. Karsdal MA, Byrjalsen I, Riis BJ, Christiansen C. Investigation of the diurnal variation in bone resorption for optimal drug delivery and efficacy in osteoporosis with oral calcitonin. BMC Clin Pharmacol 2008;8:12.
19. Karsdal MA, Byrjalsen I, Riis BJ, Christiansen C. Optimizing bioavailability of oral administration of small peptides through pharmacokinetic and pharmacodynamic parameters: the effect of water and timing of meal intake on oral delivery of Salmon Calcitonin. BMC Clin Pharmacol 2008;8:5.
20. Karsdal MA, Byrjalsen I, Henriksen K, Riis BJ, Lau EM, Arnold M, *et al*. The effect of oral salmon calcitonin delivered with 5-CNAC on bone and cartilage degradation in osteoarthritic patients: a 14-day randomized study. Osteoarthritis Cartilage 2010;18:150–9.
21. Malkov D, Angelo R, Wang HZ, Flanders E, Tang H, Gomez-Orellana I. Oral delivery of insulin with the eligen technology: mechanistic studies. Curr Drug Deliv 2005;2:191–7.
22. Oestergaard S, Sondergaard BC, Hoegh-Andersen P, Henriksen K, Qvist P, Christiansen C, *et al*. Effects of ovariectomy and estrogen therapy on type II collagen degradation and structural integrity of articular cartilage in rats: implications of the time of initiation. Arthritis Rheum 2006;54:2441–51.
23. Mouritzen U, Christgau S, Lehmann HJ, Tanko LB, Christiansen C. Cartilage turnover assessed with a newly developed assay measuring collagen type II degradation products: influence of age, sex, menopause, hormone replacement therapy, and body mass index. Ann Rheum Dis 2003;62:332–6.
24. Oliveria SA, Felson DT, Reed JI, Cirillo PA, Walker AM. Incidence of symptomatic hand, hip, and knee osteoarthritis among patients in a health maintenance organization. Arthritis Rheum 1995;38:1134–41.
25. Cauley JA, Kwoh CK, Egeland G, Nevitt MC, Cooperstein L, Rohay J, *et al*. Serum sex hormones and severity of osteoarthritis of the hand. J Rheumatol 1993;20:1170–5.
26. Spector TD, Perry LA, Jubb RW. Endogenous sex steroid levels in women with generalised osteoarthritis. Clin Rheumatol 1991;10:316–9.
27. Sowers MF, Hochberg M, Crabbe JP, Muhich A, Crutchfield M, Updike S. Association of bone mineral density and sex hormone levels with osteoarthritis of the hand and knee in premenopausal women. Am J Epidemiol 1996;143:38–47.
28. Ham KD, Carlson CS. Effects of estrogen replacement therapy on bone turnover in subchondral bone and epiphyseal metaphyseal cancellous bone of ovariectomized cynomolgus monkeys. J Bone Miner Res 2004;19:823–9.
29. Manicourt DH, Azria M, Mindeholm L, Thonar EJ, Devogelaer JP. Oral salmon calcitonin reduces Lequesne's algofunctional index scores and decreases urinary and serum levels of biomarkers of joint metabolism in knee osteoarthritis. Arthritis Rheum 2006;54:3205–11.
30. Franchimont P, Bassleer C, Henrotin Y, Gysen P, Bassleer R. Effects of human and salmon calcitonin on human articular chondrocytes cultivated in clusters. J Clin Endocrinol Metab 1989;69:259–66.
31. Badurski JE, Schwamm W, Popko J, Zimnoch L, Rogowski F, Pawlica J. Chondroprotective action of salmon calcitonin in experimental arthropathies. Calcif Tissue Int 1991;49:27–34.
32. Bagger YZ, Tanko LB, Alexandersen P, Karsdal MA, Olson M, Mindeholm L, *et al*. Oral salmon calcitonin induced suppression of urinary collagen type II degradation in postmenopausal women: a new potential treatment of osteoarthritis. Bone 2005;37:425–30.