

The determination of apoptosis rates on articular cartilages of ovariectomized rats with and without alendronate treatment

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Summary. Osteoporosis (OP) is a major health problem characterized by compromised bone strength. Osteoarthritis (OA) is a joint disease that progresses slowly and is characterized by breakdown of the cartilage matrix. Alendronate (ALN), a nitrogen-containing bisphosphonate (BIS), inhibits bone loss and increases bone mineralization, and has been used clinically for the treatment of OP. It is still controversial whether BIS is effective in inhibiting the progression of OA. Chondrocyte apoptosis has been described in both human and experimentally induced OA models. In our study we aimed to detect whether ALN could protect articular cartilage from degeneration and reduce apoptosis rates in experimentally OA induced rats. For this rats were ovariectomized (ovex), nine weeks after operation rats were injected 30 µg/kg/week ALN subcutaneously for six weeks. After six weeks articular cartilages were obtained. We did Safranin O staining and Mankin and Pritzker scorings to evaluate degeneration and investigated the expressions of p53, cleaved caspase 3, Poly ADP-ribose (PAR), Poly ADP-ribose polymerase 1 (PARP 1), and applied TUNEL technique to determine apoptotic rates. We found a significant decrease in glycosaminoglycan (GAG) amount and increased apoptosis which indicates damage on articular cartilages of ovex rats. GAG amount was higher and apoptosis rate was lower on articular cartilages of ALN treated ovex

rats compared to the ovex group. In contrary to studies showing that early ALN treatment has a protective effect, our study shows late ALN treatment has a chondroprotective effect on articular cartilage since we treated rats nine weeks after ovariectomy.

Keywords: Alendronate, Apoptosis, Articular cartilage, Osteoarthritis, Rat

Introduction

Osteoporosis (OP) is a major health problem characterized by compromised bone strength (Klein-Nulend et al., 2015). The number of people with OP increases as the population ages. Increasing numbers of patients with osteoporotic fractures may have a negative economic impact on society and on the quality of the lives of patients (Atik et al., 2010).

Bisphosphonates (BIS) have been widely used for

Abbreviations. ACLT, anterior cruciate ligament transaction; ALN, alendronate sodium; ATP, adenosine triphosphate; BIS, bisphosphonate; DNA, deoxyribonucleic acid; dUTP, deoxyuridine triphosphate; GAG, glycosaminoglycan; GTP, guanosine triphosphate; NAD, nicotinamide adenine dinucleotide; OA, osteoarthritis; OARSI, osteoarthritis research society international; OP, osteoporosis, Ovex, ovariectomized; PAR, Poly ADP-ribose; PARP, poly ADP-ribose polymerase; PBS, phosphate buffered saline; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick end-labeling.

the treatment and prevention of osteoporotic fractures. Alendronate (4-amino-1-hydroxy butyl ideneb phosphonate sodium salt) is a potent inhibitor of bone resorption, and was approved for use in the prevention of osteoporotic fractures by the USA Food and Drug Administration in 1995 (<http://www.fda.gov/forHealthProfessionals/Drugs/default.htm>). ALN which is a nitrogen containing BIS binds to hydroxyapatite mineral on bone resorption surfaces and inhibits osteoclast-mediated bone resorption although it is also known to regulate cell proliferation, differentiation, and gene expression in osteoblasts (Xiong et al., 2009).

OA is a joint disease that progresses slowly and is characterized by breakdown of the cartilage matrix, which may ultimately result in complete loss of the joint cartilage. Many factors contribute to the onset of OA, including both biochemical and biomechanical factors (Guilak, 2011; Lee et al., 2013). This disease is usually classified into primary or secondary OA. While injury or other predisposing factors lead to secondary OA, the prevalence of primary OA increases with age in both men and women, and the incidence in women increases dramatically after menopause. Current therapies predominantly target symptoms rather than providing prevention or curative treatment. ALN was reported to be chondroprotective in a rat anterior cruciate ligament transection (ACLT) model of osteoarthritis (OA) (Hayami et al., 2004; Shirai et al., 2011).

Chondrocyte apoptosis has been described in both human (Johnson et al., 2008; Chen et al., 2012; Sena et al., 2014), experimentally induced (Xu et al., 2014) and spontaneous animal models (Zamli et al., 2013, 2014) of OA, but its etiopathogenesis is uncertain. Apoptosis is a physiologic process that is vital in normal development, cell turnover, and homeostasis (Elmore, 2007). Different regulators of apoptosis are being studied for detection of apoptosis.

One of those regulators is tumor suppressor p53. It can promote apoptosis by several mechanisms. One of the proposed apoptosis mechanisms for p53 activity is via the activation of caspase. The p53 protein transcriptionally regulates Bax, a proapoptotic member of the Bcl-2 family. In response to apoptotic signals, Bax is redistributed from the cytosol to the mitochondria, where it causes a decline in mitochondrial membrane potential, followed by cytochrome *c* release and caspase activation (Amaral et al., 2010; Eriksson et al., 2012; Takada et al., 2013).

Caspase 3 is a crucial enzyme in the apoptotic process. Caspase 3 functions as effector or executioner caspase and is primarily responsible for the cleavage of poly ADP-ribose polymerase (PARP) during cell death that ultimately causes the morphological and biochemical changes seen in apoptotic cells (Elmore, 2007).

PARP 1 plays an important role in many cell death pathways. PARP 1 is the substrate of caspase 3. Activation of caspase 3 leads to cleavage of 116 kDa caspase substrate, PARP 1, into 89- and 24-kDa

fragments is considered as a marker of apoptosis (Luo and Kraus, 2012).

Terminal deoxynucleotidyl transferase (TdT) mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay detects apoptotic cells that undergo extensive DNA degradation during the late stages of apoptosis. The method is based on the ability of TdT to label blunt ends of double-stranded DNA breaks independent of a template (Elmore, 2007).

Animal models of OA have been used extensively for studying the pathogenesis of cartilage degradation as well as the efficacy of potential therapeutic interventions (Bendele, 2001). The observation that cartilage damage is increased by OP is consistent with data from previous studies indicating that the incidence and prevalence of OA are increased in postmenopausal women (Hussain et al., 2014). Ovariectomized (ovex) rats can simultaneously reproduce OP-like bone loss and OA-like cartilage lesions similar to those observed in human clinical cases and, thus, are being used as a combined model of postmenopausal OP and OA (Li et al., 2014; Yang et al., 2014). Ovariectomy also increased the severity of cartilage damage in surgically induced OA in animal models (Calvo et al., 2007; Bellido et al., 2010). In our study we hypothesized that ALN could prevent degeneration on articular cartilage of ovex rats by means of preventing apoptosis. To assess cartilage damage and determine whether ALN prevents cartilage degeneration we did Safranin O staining, then Mankin and Pritzker scorings. We also detected the expressions of apoptosis related proteins such as p53, cleaved caspase 3, PAR, PARP 1 and applied TUNEL.

Materials and methods

Animals

Thirty female Wistar rats with approximate average weight of 200 g (206.808±2.022 g) and age 12 weeks were used for our experiments. Ten of the rats without any operation and treatment were accepted as control group. Twenty of them were ovariectomized bilaterally to induce OA and OP. The ovariectomized rats were randomized into two groups nine weeks after ovariectomy. Ovex group: saline weekly for six weeks postoperatively; ovex+Aln group: Aln sodium 30 µg/kg/week for six weeks postoperatively. Rats without any operation and treatment were accepted as control group. Aln sodium 30 µg/kg/week (Fosamax[®]; Merck Pharmaceuticals, West Point, PA) the dose administered to rats is close to the clinical dose of ALN used for the treatment of osteoporosis in humans (70 mg/week). (Sama et al., 2004; Zamli et al., 2013). Rats were weighed weekly to determine appropriate drug dose and drug prepared 30 µg/kg dose was given subcutaneously to reduce interaction with food and in order to increase the bioavailability. After six weeks rats were sacrificed by giving high doses of anesthetic agents. After sacrifice of rats, the articular cartilages were

obtained. The experimental protocols were approved by the Animal Care and Usage Committee of Akdeniz University and were in accordance with the guidelines of the International Association for the Study of Pain.

Tissue processing

The articular cartilages were fixed by immersion in 10% formalin for 1 week. Then tissues were washed in tap water for 24 h and decalcified in 25% formic acid for 3 days. That process was followed by several washes under tap water for 24 h and then the tissues were neutralized in 0.35 M sodium sulphate solution for 3 days. Tissues were then washed in tap water for 24 h and this was followed by dehydration; immersion in 70%, 80%, 90% and 100% ethanol. After dehydration tissues were cleared in xylene and embedded in paraffin wax.

Histological analysis

Light microscopic evaluations

Formalin-fixed and paraffin-embedded samples were cut into 5 μ m sections and stained using Safranin O. Then Safranin O stained slides were evaluated using Zeiss Axioplan light microscope (Zeiss, Oberkochen, Germany), photographs of sections were taken with a digital camera (Diagnostic Instruments Inc. Spot Insight Qe, Sterling Heights, MI, USA).

Scoring for articular cartilage morphology

Scaling of cartilage degeneration was evaluated by Mankin and Pritzker's Osteoarthritis Research Society International (OARSI) score. Both Mankin and Pritzker cartilage morphology scoring systems were used (Pritzker et al., 2006; Moussavi-Harami et al., 2009) on photomicrographs of Safranin O stained samples that were generated by a light microscope attached computerized digital camera. The subcategories of the Mankin score are structure, tidemark integrity, proteoglycan staining, and cellularity. Pritzker's OARSI score is the multiplication of the grade and the stage of each sample. Grade is an index for the arthritis depth progression (severity). Stage is defined as the horizontal extent of cartilage involvement within one side of a joint compartment irrespective of the underlying grade. Score is defined as an assessment of combined arthritis grade and stage.

Immunohistochemistry

For immunohistochemical labeling, 5 μ m tissue sections were deparaffinized in xylene and sections were rehydrated through a decreasing gradient of ethanol (100%, 90%, 80% and 70% for 5 minutes, respectively). The antigen retrieval was achieved by overnight incubation of the slides in boric acid at 57°C. Boric acid was prepared with phosphate buffered saline (PBS)

containing 2.1% boric acid (0149654; Merck, Darmstadt, Germany). After rinsing 3 times in PBS, endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methanol for 15 min and three rinses in PBS. For the reduction of nonspecific background staining, the sections were incubated with Ultra V Block (TA-125UB; Lab Vision Corp, Fremont, CA, USA) for 10 minutes at room temperature. Rabbit polyclonal anti-p53 (Santa Cruz-sc6243), rabbit polyclonal anti-cleaved caspase 3 (9664S; Cell signaling), mouse monoclonal anti-PAR (ALX-804-220-R100; Enzo Life Sciences), rabbit polyclonal anti-PARP 1 (AB 6079; Abcam) were applied as 1:500 dilution overnight at 4°C in a humidified chamber. Next day the sections were washed three times for 5 minutes with PBS and then incubated with biotinylated secondary antibodies (BA-9200; 1:400, Biotinylated Goat Anti-Mouse IgG Antibody Vector Laboratories) for anti-PAR antibody and (BA-1000; 1:400, Biotinylated Goat Anti-Rabbit IgG Antibody Vector Laboratories) for anti-p53, anti-cleaved caspase 3, anti-PARP 1 antibodies for 45 minutes then with LabVision UltraVision Large Volume Detection System: anti polyvalent horseradish peroxidase (HRP) complex (TP-125-HL; Thermo Scientific,) for 30 minutes at room temperature. The sections were washed three times for 5 minutes with PBS, and antibody complexes were visualized after incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB, D4168; Sigma-Aldrich, St. Louis, MO, USA) resulting in a brown precipitate. Sections were counterstained with Mayer Hematoxylin (Merck 1092490500, NJ, USA) to enable visualization of nuclei then dehydrated (rinsing the slides with 70%, 80%, 90% and 100% ethanol for 5 minutes, respectively) and mounted in Kaisers glycerin gelatin (Merck; OB514196, NJ, USA). Negative control sections for each antibody were performed by replacing the primary antibody with normal rabbit IgG (sc2027; Santa Cruz Biotechnology), and mouse IgG (sc2025; Santa Cruz Biotechnology), for all samples at the same concentrations as the primary antibodies. In all cases no signal was observed. Immunohistochemistry was performed on three independent samples with identical results. After slides were evaluated using Zeiss Axioplan light microscope (Zeiss, Oberkochen, Germany), photographs of sections were taken with a digital camera (Diagnostic Instruments Inc. Spot Insight Qe, Sterling Heights, MI, USA).

TUNEL staining

Apoptosis in articular cartilages was detected by enzymatic labeling of DNA strand breaks by using TUNEL. Paraffin sections of 5- μ m thickness from articular cartilage tissues were cut and placed on slides covered with poly-l-lysine, and after drying, the slides were left in the incubator at 45°C overnight and at 60°C for 1 hour. After deparaffinization and rehydration, slides were washed twice in PBS for 5 minutes.

After incubation of slides with the permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 minutes at 4°C and washing twice with PBS for 5 minutes, the labeling reaction was performed by using 50-μL of TUNEL reagent for each sample, except negative control, in which reagent without enzyme was added and incubated for 1 hour at 37°C. After PBS washings, slides were incubated with converter reagent for 30 minutes at 37°C. After washing, color development for localization of cells containing labeled DNA strand breaks was performed by incubating the slides with Fast Red substrate solution for 10 minutes. Labeling with TUNEL was conducted with a In Situ Cell Death Detection kit (1684809; Roche, Mannheim, Germany) and was performed according to the manufacturer's instructions.

H-SCORE and semi-quantitative evaluations

The evaluations of the immunohistochemical and TUNEL stainings of control, ovex and ovex+Aln groups were done utilizing H-SCORE (Acar et al., 2012). Briefly, stained sections were evaluated using an Axioplan light microscope (Zeiss, Oberkochen, Germany) with a special ocular scale. From each three randomly selected slides, five different fields at 200X magnification were evaluated for the analysis of immunohistochemical staining of the antibodies. The staining was scored in a semiquantitative fashion that included the intensity of specific staining in sections. The evaluations were recorded as percentages of positive-stained cells of all types for both proteins of four intensity categories: 0 (none), 1+ (weak, but detectable above control), 2+ (distinct), 3+ (intense). For each tissue, an H-SCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining ($H\text{-SCORE} = \sum S_i P_i$), where i is the intensity score and P_i is the corresponding percentage of the cells). The H-SCORE values were graphed.

Two observers blinded to the experimental groups performed the H-SCORE, and interindividual variation was 8%. The distributions of positively immunoreactive cells in all experimental groups were also determined semiquantitatively (0 = negative, (+) = weak positive, + = positive, ++=strong positive) and presented in Table 1.

Statistical analysis

Statistical analyses of p53, cleaved caspase 3, PAR, PARP 1, TUNEL H-SCORE; Mankin and Pritzker scores were evaluated by Kruskal Wallis followed by Mann-Whitney U test, Bonferroni correction was used. Probability values of less than 0.05 were considered significant; values are presented as mean±SEM. The interplay between cartilage histology and TUNEL H-SCORE was determined via Spearman's correlation analysis. All statistical analyses were performed using SPSS 18 (Quarry Bay, Hong Kong).

Results

Light microscopic evaluation of articular cartilages of control, ovex and ovex+Aln rats

Safranin O staining, which shows glycosaminoglycans (GAG), enabled us to determine whether the articular cartilage was damaged. In the control group, cartilage was thick and whole and can be seen as a red zone (Fig. 1a,b). In the ovex group, cartilage was slightly to moderately damaged (Fig. 1c,d). In the ovex+Aln group cartilage was thicker compared to the ovex group which means ALN prevented damage on articular cartilage (Fig. 1e,f).

Immunohistochemical findings

p53 immunolocalization on articular cartilages of control, ovex and ovex+Aln rats According to p53 immunohistochemistry in the control group there was weak positive p53 immunoreactivity in deep zone and positive p53 immunoreactivity in surface and transitional zones (Table 1, Fig. 2a). In the ovex group although p53 immunoreactivity was positive in surface zone as in the control group, it was strongly positive in transitional and deep zones. (Table 1, Fig. 2b). In the ovex+Aln group p53 immunoreactivity was similar to the control group (Table 1, Fig. 2c). Our H-SCORE results showed that p53 immunoreactivities were higher in the ovex and the ovex+Aln groups compared to the control group. The differences between the control and the ovex groups ($p=0.015$) and the control and the ovex+Aln groups ($p<0.001$) were statistically significant (Table 2, Fig. 2d).

Table 1. Semi-quantitative scoring of p53, cleaved caspase 3, PAR, PARP 1 and TUNEL immunostaining intensities on articular cartilages of control, ovex and ovex+Aln groups.

Groups	p53	Cleaved caspase3	PAR	PARP1	TUNEL
Articular Cartilage Zones					
Control					
Surface zone	+	+	(+)	(+)	+
Transitional zone	+	+	(+)	(+)	(+)
Deep zone	(+)	(+)	0	0	0
Calcification zone	0	0	0	0	0
Ovex					
Surface zone	+	+	+	+	++
Transitional zone	++	++	+	+	+
Deep zone	++	++	0	0	(+)
Calcification zone	0	0	0	0	0
Ovex+Aln					
Surface zone	+	+	+	+	+
Transitional zone	+	+	+	+	+
Deep zone	(+)	0	0	0	(+)
Calcification zone	0	0	0	0	0

0= negative, (+)= weak positive, +=positive, ++=strong positive; ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized.

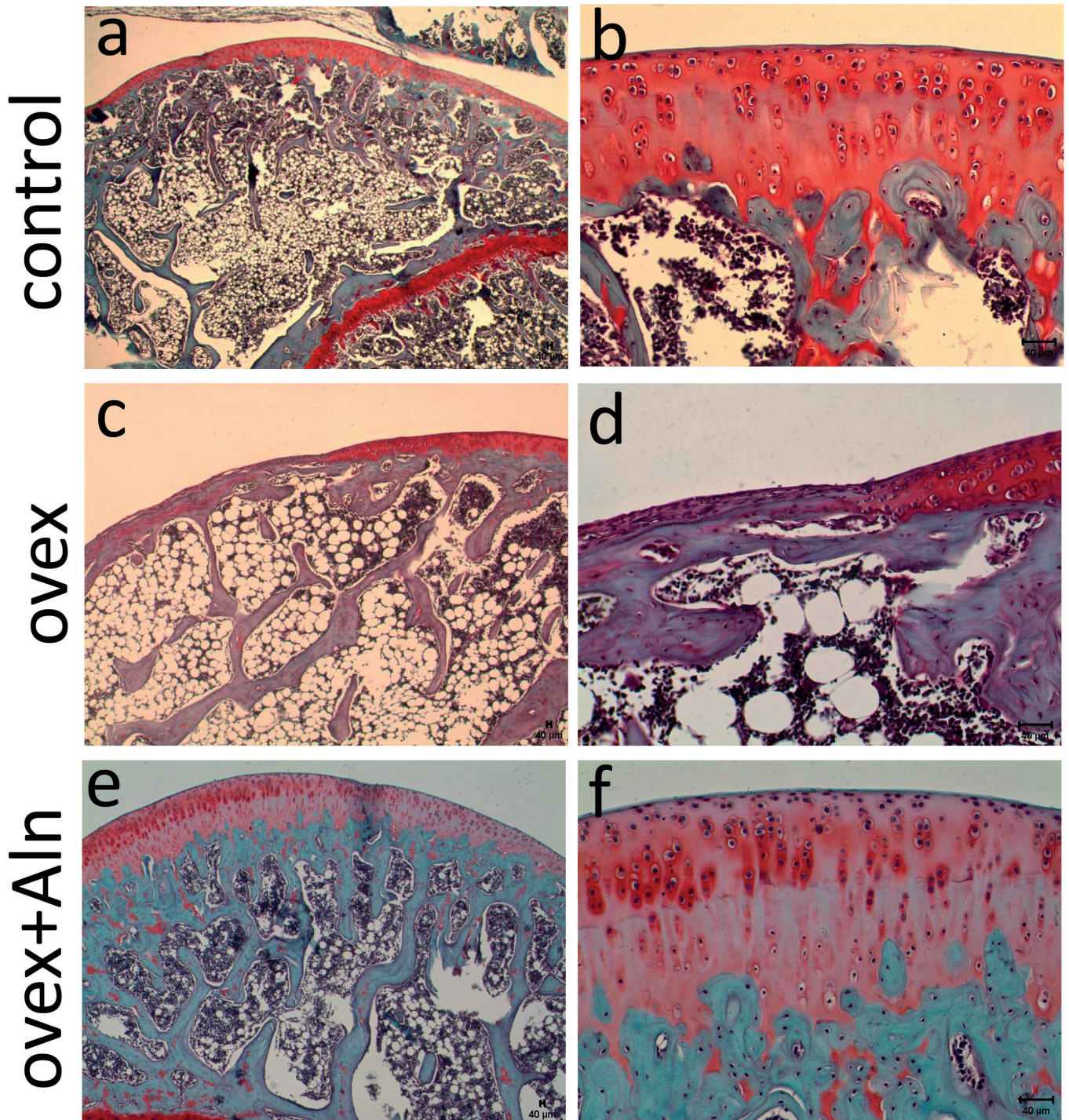


Fig. 1. Safranin O staining on articular cartilages of control, ovex and ovex+Aln rats. Compared to the control group (**a, b**) glycosaminoglycan amount was decreased in the ovex group (**c, d**); in the ovex+Aln group glycosaminoglycan (GAG) amount was higher compared to the ovex group (**e, f**): ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized. Scale bars: a, c, e, 224 μm ; b, d, f, 30 μm .

Cleaved caspase 3 immunolocalization on articular cartilages of control, ovex and ovex+Aln rats

In the control group cleaved caspase 3 immunoreactivity was weak positive in deep zone and positive in surface and transitional zones (Table 1, Fig.

2e). In the ovex group cleaved caspase 3 immunoreactivity was positive in surface zone and strong positive in transitional and deep zones (Table 1, Fig. 2f). In the ovex+Aln group cleaved caspase 3 immunoreactivity was similar to the control group; it was positive in surface and transitional zones. But there

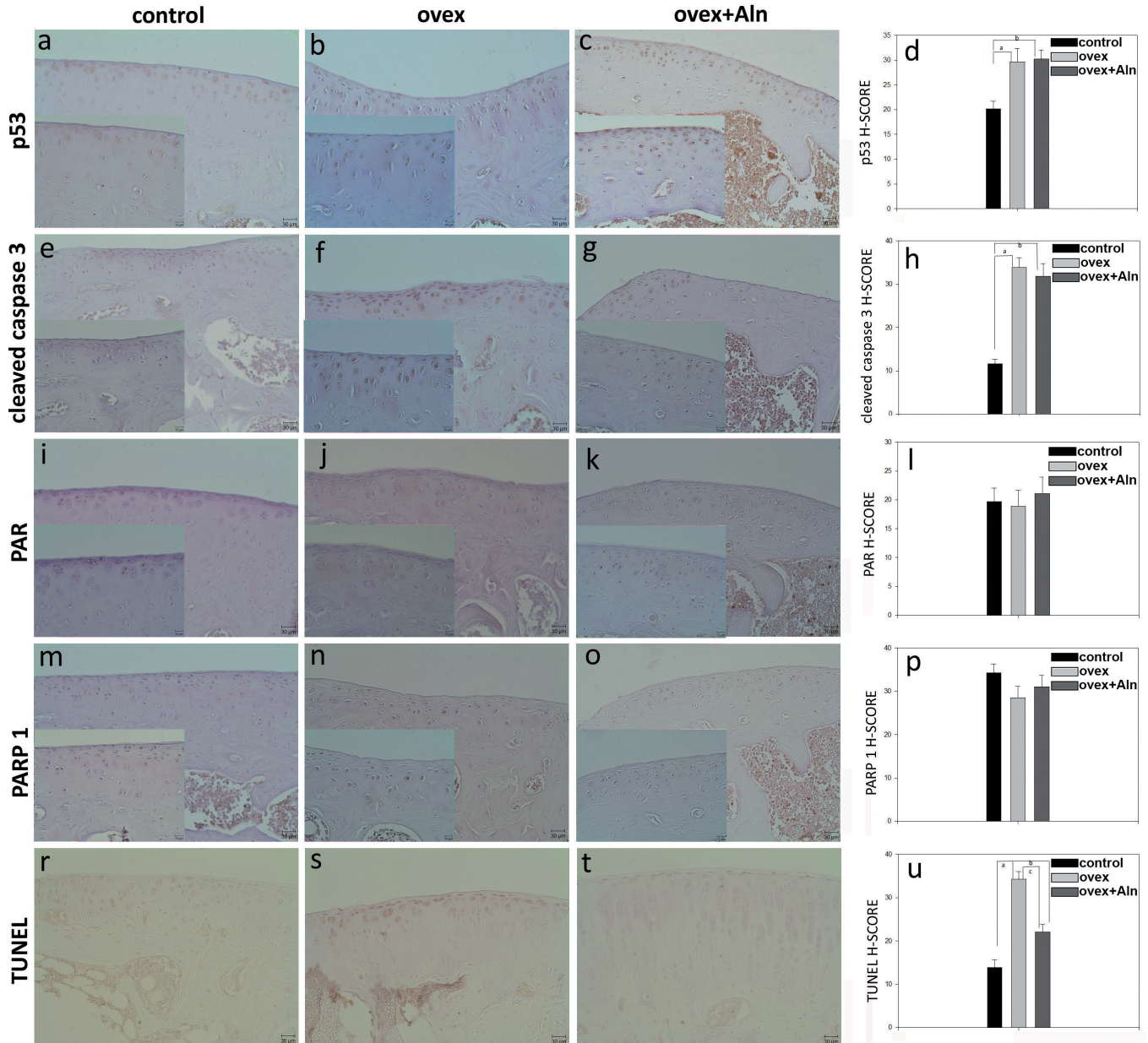


Fig. 2. Immunohistochemistry and H-SCORE graphs of p53 (a-d), cleaved caspase 3 (e-h), PAR (i-l), PARP1 (m-p) and TUNEL (r-u) on articular cartilages of control, ovex and ovex+Aln rats. There are inserts on the lower left corners of each picture. ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized. On p53 H-SCORE graph a, $p=0,015$ between control and ovex; b, $p<0,001$ between control and ovex+Aln. On cleaved caspase 3 H-SCORE graph a, $p<0,001$ between control and ovex; b, $p<0,001$ between control and ovex+Aln. On TUNEL H-SCORE graph a, $p<0,001$ between control and ovex; b, $p<0,001$ between control and ovex+Aln; c, $p<0,001$ between ovex and ovex+Aln. Scale bars: a-c, e-g, i-k, m-o, r-t, 30 µm; inserts, 15 µm.

Alendronate, ovariectomized rats and articular cartilage

was no cleaved caspase 3 immunoreactivity in deep zone (Table 1, Fig. 2g). According to our H-SCORE results cleaved caspase 3 immunoreactivities were higher in the ovex and the ovex+Aln groups compared to the control group. Differences between the control and the ovex groups ($p<0.001$) and the control and the ovex+Aln groups ($p<0.001$) were statistically significant (Table 2, Fig. 2h).

PAR immunolocalization on articular cartilages of control, ovex and ovex+Aln rats

PAR immunoreactivity in the control group was weak positive in surface and transitional zones (Table 1, Fig. 2i). PAR immunoreactivity in surface and transitional zones of articular cartilages of the ovex (Table 1, Fig. 2j) and the ovex+Aln groups (Table 1, Fig. 2k) were positive. There was no statistically significant difference between any of the groups for PAR H-SCORE (Table 2, Fig. 2l).

PARP 1 immunolocalization on articular cartilages of control, ovex and ovex+Aln rats

PARP 1 immunoreactivity had a similar distribution and intensity pattern like PAR immunoreactivity. It was weak positive in surface and transitional zones in the control group (Table 1, Fig. 2m). There was positive PARP 1 immunoreactivities in surface and transitional zones of the ovex (Table 1, Fig. 2n) and the ovex+Aln (Table 1, Fig. 2o) groups. PARP 1 H-SCORE did not represent a statistically significant difference between any of the groups (Table 2, Fig. 2p).

TUNEL results

TUNEL staining was positive in surface and weak positive in transitional zones in the control group (Table 1, Fig. 2r). In the ovex group TUNEL staining was strong positive in surface zone, positive in transitional zone and weak positive in deep zone (Table 1, Fig. 2s). In the ovex+Aln group TUNEL intensity was positive in surface and transitional zones; and it was weak positive in deep zone. (Table 1, Fig. 2t). According to our H-SCORE results TUNEL intensity was higher in the ovex group compared to the control group and this difference was statistically significant ($p<0.001$). TUNEL intensity increase in the ovex group was reversed by ALN treatment. TUNEL intensity was less in the ovex+Aln group compared to the ovex group, the difference between the ovex and the ovex+Aln group was

Table 2. Descriptive statistical data of H-SCORE values of p53, cleaved caspase 3, PAR, PARP 1 immunohistochemistry and TUNEL staining on articular cartilages of control, ovex and ovex+Aln groups.

	H-SCORE values		
	Control	Ovex	Ovex+Aln
p53	20.200±1.472	29.660±2.723	30.250±1.799
Cleaved caspase3	11.640±1.064	33.860±2.286	31.840±2.930
PAR	19.720±2.325	18.920±2.794	21.060±2.890
PARP1	34.300±1.975	28.568±2.630	31.034±2.690
TUNEL	13.886±1.758	34.250±1.792	22.080±1.792

Values are presented as mean ± SEM. ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized.

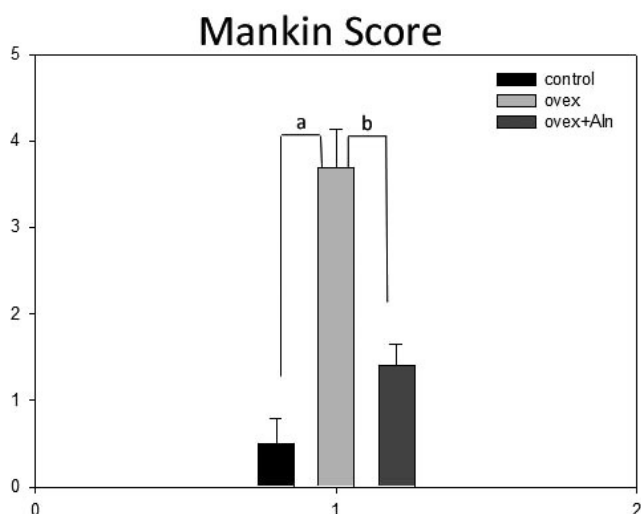


Fig. 3. Mankin scores on the control, ovex and ovex+Aln groups. ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized. a, $p=0,016$ between control and ovex; b, $p=0,095$ between ovex and ovex+Aln

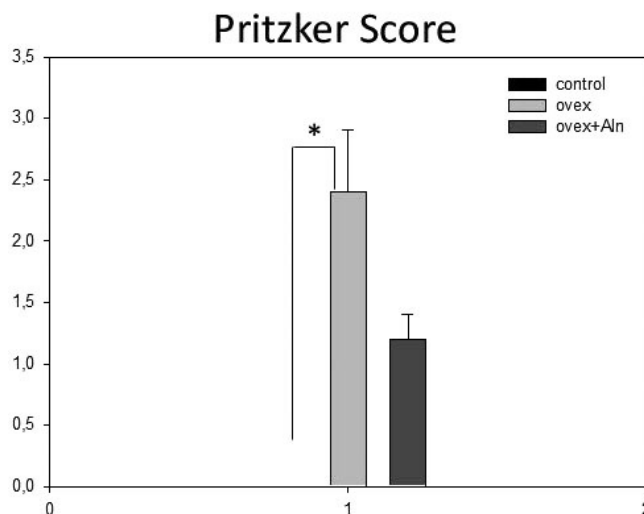


Fig. 4. Pritzker scores on the control, ovex and ovex+Aln groups. ovex: ovariectomized, ovex+Aln: alendronate treated ovariectomized. * shows $p=0,016$.

statistically significant ($p < 0.001$) (Table 2, Fig. 2u).

Cartilage degeneration scaling

According to our Safranin O staining cartilage of the control group was intact. Ovex group articular cartilages showed degenerative changes compared to the control group articular cartilages. Ovex group had significantly greater cartilage surface irregularity than the control group. The cartilage lesions consisted of different degrees of Safranin O staining loss, surface fibrillation, vertical clefts, decreased number and degeneration of the chondrocytes. The cartilage lesions were local. The difference in cartilage damage between the control and the ovex groups was significant according to Mankin and Pritzker scores. Both Mankin and Pritzker scores were significantly higher in the ovex group than in the control group (respectively, $p = 0.016$ and $p = 0.016$) (Figs. 3, 4). Mankin scores were 0.5 ± 0.289 , 3.7 ± 0.436 and 1.4 ± 0.245 for control, ovex, ovex+Aln groups respectively. And Pritzker scores were 0 ± 0 , 2.4 ± 0.510 and 1.2 ± 0.2 for control, ovex, ovex+Aln groups respectively. This demonstrated that the OA model was successfully developed in the ovex group. The slight to moderate cartilage damage that was noted in the ovex group was repaired at different levels in the ovex+Aln group. ALN was found to have significantly inhibited GAG loss and the severity of cartilage degradation in ovex+Aln group. The difference between ovex and ovex+Aln group was statistically significant for Mankin score ($p = 0.002$) but not for Pritzker score ($p = 0.095$). Although ALN did not completely prevent cartilage damage, it was partially chondroprotective in ovariectomy model, as determined by histologic analysis. When we did Spearman test to see whether there was a correlation between the cartilage histology and TUNEL H-SCORE there was not a significant relation for Mankin score ($r = 0.234$, $p = 0.135$). However for Pritzker score there was a statistically significant, positive, moderate correlation: Pritzker score increased in the ovex group ($r = 0.472$, $p = 0.002$).

Discussion

OA is the most common disorder of the joint, causing joint pain and dysfunction in affected patients. At the cellular level, OA is characterized by a loss of tissue cellularity and extracellular matrix (ECM) damage. Chondrocytes are the resident cells found in cartilage tissue and are responsible for both synthesis and turnover of the ECM; therefore, maintaining the health of chondrocytes is an important factor for preventing articular cartilage degeneration (Takayama et al., 2014).

A positive correlation has been shown between the degree of severity of OA and the number of apoptotic chondrocytes in both experimentally induced OA in rabbit cartilage (Hashimoto et al., 1998b) and human OA cartilage (Hashimoto et al., 1998a). Chondrocytes

maintain the dynamic equilibrium between production of the extracellular matrix and its enzymatic degradation. Loss of this balance in favor of catabolic events results in the loss of articular cartilage seen in OA. Thus, chondrocyte viability is essential for maintaining the integrity of articular cartilage, and reduced cellularity (attributable to either necrosis or apoptosis) may predispose the aging individual to matrix degeneration and may be associated with the onset and/or progression of OA.

Although ALN is being used clinically for the treatment of OP it is still controversial whether BIS is effective in inhibiting the progression of OA. Some investigators reported that BIS suppressed subchondral bone resorption and prevented cartilage degeneration (Hayami et al., 2004) whereas others reported that BIS treatment aggravated cartilage degeneration despite greater subchondral bone volume and thickness (Ding et al., 2008). The mechanism by which BIS exerts its effects is unclear. However, most studies show that BIS increases periarticular bone volume and bone mineral concentration (Myers et al., 1999; Hayami et al., 2004; Ding et al., 2008; Nishitani et al., 2009). Since ALN is a potent inhibitor of osteoclastic bone resorption, ALN may reduce articular cartilage damage by preventing periarticular bone loss and preserving subchondral architecture. A possible mechanism by which ALN attenuates OA progression is that ALN may prevent the expression of cartilage degradation factors from the surrounding tissues and maintain cartilage stiffness by protecting the structure of the extracellular matrix and chondrocytes (Shirai et al., 2011).

A study by Yatsugi et al. (2000) suggested the possible involvement of p53 in chondrocyte apoptosis and cartilage destruction (Yatsugi et al., 2000). Immunohistochemical analyses of p53 in the articular cartilage of patients with rheumatoid arthritis and OA showed increased staining of p53 in chondrocytes with apoptotic morphology. Consistent with the literature we also found an increased p53 expression in the ovex group, but in the ovex+Aln group there was not a statistically significant decrease. ALN treatment was unable to reduce increased p53 expression in the ovex+Aln group.

Li et al. (2012) reported high levels of basal cleaved caspase 3 and PARP in OA chondrocytes in primary cultures of OA patients' chondrocytes and concluded that OA chondrocytes have an apoptotic background (Li et al., 2012). Also Takada et al. (2011) denoted that the percentage of cleaved caspase 3 positive cells was significantly higher in severe OA cartilage than in mild OA cartilage (Takada et al., 2011). In our study we also found an increased expression of cleaved caspase 3 in the ovex group. There was a decrease for cleaved caspase 3 expression in the ovex+Aln group compared to the ovex group but this decrease wasn't statistically significant. The correlation between caspase 3 activation and cartilage degeneration supported the idea that chondrocyte apoptosis is involved in the pathology of

cartilage degeneration.

Sena et al. (2014) investigated apoptosis in human chondrocytes isolated from fractured calcaneal osteochondral fragments after culture of those cells via immunofluorescence and Western blotting of cleaved caspase 3, PARP 1 (Sena et al., 2014). They found a significant increase of the apoptotic process in fractured specimens compared with control ones as shown by increased expressions of cleaved caspase 3 and PARP 1. The percentage of patients that develop OA after a joint trauma is very high (Sanders et al., 1993; Thomas et al., 2010; Gurkan et al., 2011). According to our immunohistochemical findings expression levels and immunostaining patterns of PAR and PARP 1 were similar in control, ovex and ovex+Aln groups. There was not a statistically significant change for PAR and PARP 1 between the groups.

TUNEL which detects DNA fragmentation is an established method for detecting DNA fragments. Using in situ TUNEL staining of OA knee cartilage, Blanco and colleagues (Blanco et al., 1998) found, on average, 6% cell death, while similar studies by Hashimoto et al. (1998a) and Heraud et al. (2000) showed 22.3% and 18%, respectively. Sharif et al. (2004) investigated apoptosis rates by p53, caspase 3 and TUNEL stainings in knee and hip cartilage samples of humans with OA. They indicated that at any point in time, 2-3% of chondrocytes in OA cartilage died by apoptosis, compared with 0.6% in non-arthritic cartilage, and that an increased level of apoptosis corresponded to reduced cellularity in the cartilage matrix (Sharif et al., 2004). These discrepancies may be attributable to topographic variations in apoptosis as well as slight differences in the TUNEL protocols used. Franciozi et al. (2013) also showed an increased TUNEL staining in articular cartilage of rats in which OA was generated by strenuous running (Franciozi et al., 2013). We also found increased TUNEL staining in the ovex group. Stained TUNEL positive cell number was decreased in the ovex+Aln group with a statistically significant difference. TUNEL positive cell rates were approximately 13%, 34% and 22% in the control, ovex and ovex+Aln groups, respectively. These discrepancies may be attributable to differences in the TUNEL protocols used. But since we treated sections of all groups in the same way differences seen between groups is reasonable. Among the apoptosis markers we used, TUNEL staining showed the most obvious decrease in the the ovex+Aln rats.

We observed a decrease of GAG in articular cartilages of the ovex group by Safranin O staining. Studies showed that decreased GAG can trigger increased caspase 3 activity, suggesting that compromised matrix can induce chondrocyte apoptosis (Thomas et al., 2007; Otsuki et al., 2008; Almonte-Becerril et al., 2010; Rosenzweig et al., 2012). According to this increased apoptosis rates in the ovex group could be triggered by GAG decrease. Decrease in the amount of GAG was recovered in articular cartilages of the ovex+Aln group.

Cell death in OA has received substantial

consideration in the literature. The aim of our study was to determine apoptosis rates and whether ALN inhibits articular cartilage degeneration in a rat ovex model of OA, although ALN is generally thought to inhibit osteoclastic bone resorption. For this we did Safranin O staining and determined apoptosis through activation of caspase-3, accumulation of p53, and cleavage of the DNA repair enzyme PARP 1 on articular cartilages of ovariectomized rats following ALN treatment. Our results demonstrated a significant decrease of GAG amount and increased cell death in the articular cartilage of ovex rats. We also indicated ALN had a chondroprotective effect in our experimental rat model of OA as reflected in lower Mankin and Pritzker's scores.

There are some studies in the literature reporting injection of ALN just after ovariectomy (early) or some time after ovariectomy (late) in rats. Significantly different outcomes from early and late ALN treatment in ovex rats suggest that further clinical studies are necessary to evaluate the importance of timing of treatment with anti-resorptive agents for improved management of post-menopausal OA. However findings in human studies using BIS and other anti-resorptive agents in OA treatment have also been contradictory (Hayami et al., 2004; Neogi et al., 2008; Shirai et al., 2011). In one of the ovex rat studies Zhu et al. (2013) showed early ALN treatment completely prevented both subchondral bone loss and cartilage surface erosion induced by ovariectomy. Although late ALN treatment also inhibited subchondral bone loss and significantly reduced cartilage erosion in the ovex rats, these tissues did not completely recover even after 10-weeks of ALN treatment (Zhu et al., 2013). In another study done by the same group they found that early ALN treatment (intra-articular) completely inhibited cartilage thickening and effectively improved the subchondral bone in rats following ovex, while late ALN treatment showed no effects (Chen et al., 2014). Our ALN treated ovex rat group mimics late ALN treatment group that researchers used in the literature since we started injecting ALN nine weeks after ovariectomy. In contrast to these studies our study proves that late ALN treatment had a protective effect on articular cartilage.

The mechanism of increased apoptosis rates in cartilages of human or animal models with OA has not been fully elucidated. Since OA is a disease that reduces the quality of life and current therapies predominantly target symptoms, rather than providing prevention or curative treatment, more detailed studies are required to explain the pathophysiology of OA.

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Alendronate, ovariectomized rats and articular cartilage

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