

Effects of Estrogen Level, Dietary Loading, and Aging on Types I, II, and X Collagen Expression and Structure of Rat Mandibular Condylar Cartilage

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Aims: To investigate how estrogen level, dietary loading, and aging affect cartilage structure and the expression of major collagens (types I, II, and X) in rat mandibular condylar cartilage (MCC). **Methods:** A total of 96 outbred Sprague Dawley female rats were randomly divided into two groups by ovariectomy (OVX) at 7 weeks old. One week later, the rats in each group were further divided into three subgroups on the basis of food hardness: hard food (diet board), normal food (pellet), and soft food (powder). The rats were sacrificed at the age of 5 or 14 months. The thickness of the fibrous, proliferative, and chondroblastic layers of the mandibular condylar cartilage were measured after toluidine blue staining. Immunohistochemical analysis was performed to evaluate the expression levels of types I, II, and X collagen. A linear regression model was used to investigate the main factors affecting changes in thickness and collagen expression. **Results:** The expression levels of types II and X collagen were decreased by ovarian estrogen deficiency and increased by dietary loading. Increased dietary loading was the main factor affecting an increase in thickness of the cartilage layers, while aging was the main factor affecting a decrease in thickness of the fibrous layer. A significant age-related increase was found in the expression of type I collagen. There was some degree of interaction between aging and dietary loading that affected the thickness of the chondroblastic layer and the expression of type X collagen. **Conclusion:** The physiologic level of estrogen plays a role in MCC development by promoting the expression of types II and X collagen. Dietary loading is essential to increase the expression of types II and X collagen, as well as the thickness of cellular layers, to maintain the integrity of the MCC. Aging seems to reduce the ability of the MCC to withstand occlusal loading. *J Oral Facial Pain Headache* 2020;34:331–340. doi: 10.11607/ofph.2636

Keywords: aging, collagen, dietary loading, estrogen, fibrocartilage, mandibular condylar cartilage

The TMJ, composed of the temporal fossa, articular disc, and mandibular condyle, is subjected not only to translational but also to rotational motion.¹ In comparison with other synovial joints containing hyaline cartilage, the TMJ is unique because the mandibular condylar cartilage (MCC) is classified as “fibrocartilage.” Fibrocartilage is characterized by its dual content of types I and II collagen.² Fibroblasts and type I collagen are mainly expressed in the superficial fibrous layer, while type II collagen (as well as differentiated chondrocytes) is present in the mature and hypertrophic layers, and type X collagen is mainly expressed in the hypertrophic layer in the MCC.³

Collagens form a 3D reinforcement structure to counteract mechanical forces.⁴ Type I collagen is universal in all vertebrates and is a key structural part of burden-bearing tissues, including ligaments, bones, tendons, and TMJs.⁵ With aligned type I collagen bundles, the superficial zone of the MCC displays a higher tensile modulus than hyaline cartilage and serves principally to transmit tensile loading. In contrast, the deeper zone provides resistance to compressive loading with type II collagen.⁶ It is reasonable to assume that resistance to compressive forces is important for weight-bearing joints. In addition, the MCC is involved in multidirectional forces and may therefore need both type I and type II collagen.^{7,8} Alterations in collagen molecular and/or structural

organization due to development, aging, and variation of loading influence the contributions of collagen to tissue function.⁹

The MCC is subjected to mechanical loading during masticatory function and plays a crucial role in TMJ function as a stress absorber.¹⁰ Stimuli evoked by mechanical loading are beneficial to the development, integrity, and maintenance of the MCC.^{11,12} Various food properties, such as hardness, consistency, and volume, lead to different levels of MCC loading.¹³ Loss of proteoglycans and type II collagen, as well as reduced cartilage cellular density, were observed in the MCC of mice due to abnormal loading induced by an anterior crossbite prosthesis.¹⁴ A difference in collagen and a higher protein content have been shown in the TMJ discs of male rats compared to female rats.¹⁵ Estrogen is involved in the etiology of TMJ degenerative disease via inducing pro-inflammatory cytokines, inhibiting the proliferation of chondrocytes, and decreasing proteoglycan content, but estrogen also exhibits type II collagen protective effects.^{16,17} Thus, it seems that estrogen has a dual effect on cartilage structure. Aging is considered to be a strong factor affecting the degradation of cartilage in all joints—in porcine condylar cartilage, collagens became highly crosslinked during artificial aging using ribose incubation.¹⁸

Studies on collagen content and its distribution in the condylar cartilage under different conditions have been reported.^{15,18} There is, however, no study about the combined effects of estrogen level, dietary loading, and aging on collagen expression in rat MCC. The aim of this experimental study was to investigate how estrogen level, dietary loading, and aging affect cartilage structure and the expression of major collagens (types I, II, and X) in rat MCC. The hypotheses were that each factor would have a specific effect and that there would be some degree of interaction among the factors affecting the expression of major collagens and structure of the MCC.

Materials and Methods

Ethical Approval

This study protocol was approved by the Finnish National Project Authorization Board. All experimental procedures and animal care were in accordance with European Union Directive 2010/63/EU and Finnish legislation (Finnish Act on the Protection of Animals Used for Scientific or Educational Purpose [497/2013], Government Decree on the Protection of Animals Used for Scientific or Educational Purpose [564/2013]).

Animals and Housing

This study included 96 outbred specific pathogen-free Crl:CD(SD) female rats born in the Oulu Laboratory Animal Centre, University of Oulu, Finland. When the experiment started, the initial mean (SD) body weight of the rats was 221 (20 g).

This study was carried out in the conventional facility of the Oulu Laboratory Animal Centre. The temperature of the room where the animals were kept was $21 \pm 1^\circ\text{C}$, with ventilation rate 15 air changes per hour and relative humidity 40% to 60%. The illuminance was 350 lx at 1-m height. A fluorescent tube light was on from 07:00 to 18:00, and lights changed gradually from 06:00–07:00 and 18:00–19:00.

Ovariectomy

At 7 weeks of age, half of the rats underwent an ovariectomy (OVX). The OVX was performed under isoflurane anesthesia (IsoFlo vet, Orion Pharma). Buprenorphine (0.3 mg/mL, Vetergesic vet; Orion Pharma) and carprofen (50 mg/mL, Rimadyl vet; Zoetis) were administered as preemptive and post-operative analgesia, respectively, for 3 days.

Feeding

Before the experiment, all rats received the same pellet food ad libitum (2018C Teklad Global Rodent Diet, Envigo). From 8 weeks of age, rats were provided 2016S Teklad Global Rodent Diet ad libitum in one of three forms: free pellet (normal diet) in the wire lid hopper; diet board (hard diet); or powdered food (soft diet) in a cup on the cage floor. In the diet board group, the rats had to gnaw the aspen diet boards to reach the pellets that were tightly fit into the grooves of the boards.^{19,20}

Animal Model

A total of 96 rats were assigned into 12 groups ($n = 8$ each) on the basis of estrogen status (OVX or non-OVX), dietary loading (hard, normal, or soft food), and age (5 months or 14 months).

The dietary experiment started at 8 weeks of age (1 week after the OVX) and lasted 100 days (5-month-old group) or 360 days (14-month-old group). At the end of the experiment, rats were pre-anesthetized with isoflurane and euthanized by 100% carbon dioxide.

In case of welfare problems during the experiment, humane endpoints were applied. One rat from the non-OVX 14-month-old diet board group and two rats from the non-OVX 14-month-old powder group had to be sacrificed before termination of the experiment because of either a tumor or eye problem.

Sample Preparation

The craniums of the rats were routinely fixed in 4% formalin for 7 days immediately after decapitation and were decalcified with EDTA in a microwave oven at 37°C for 6 weeks. Each cranium was cut sagittally into the left and right parts to isolate the TMJ. The right parts were used in the present study. After dehydration (70%, 96%, and 100% alcohol), the samples were clarified in xylene and processed in paraffin in a vacuum oven. A series of sagittal sections of each TMJ sample were cut at 5 µm thick, after which the most central segments of the TMJ were affirmed by toluidine blue staining and arranged for further staining.

Histomorphometric Analysis

Three parts of the MCC were defined as reported previously,²⁰ and four layers were recognized: fibrous, proliferative, mature, and hypertrophic. The mature and hypertrophic layers were considered as the chondroblastic layer (Fig 1). The thicknesses of the fibrous, proliferative, and chondroblastic layers of the central MCC were measured.

Immunohistochemistry

After deparaffinization, sections were pretreated with 0.4% pepsin at 37°C for 60 minutes, and peroxidase-blocking solution (S2023, Dako) was applied to the sections at room temperature for 15 minutes. The sections were then incubated with primary antibodies at room temperature for 30 minutes and kept in a fridge at 4°C overnight. The primary antibodies were polyclonal rabbit type I collagen (1:3,000, Sigma-Aldrich), monoclonal mouse anti-chicken type II collagen (1:2,000, United States Biological), and monoclonal mouse type X collagen (1:100, BioCyc). Negative control slides were applied with antibody diluents (Dako).

The following day, the sections of type I and type II collagen were incubated with a labeled polymer (horseradish peroxidase rabbit solution, Dako) for 30 minutes. The sections of type X collagen were incubated with the secondary antibody anti-mouse immunoglobulin G (Vector Laboratories) and applied with ABC reagent (Vector Laboratories) for 30 minutes. All sections were then incubated with diaminobenzidine buffer (5 minutes for types I and II collagen, 10 minutes for type X collagen). After counterstaining with Mayer's hematoxylin (Histolab Products), the slides were dehydrated (96% and 100% alcohol), cleared with xylene, and coverslipped.

Immunohistochemical Analysis

The MCC was viewed with a microscope at ×10 magnification (Leica, Leitz) and estimated utilizing ImageJ software (National Institutes of Health), as previous-

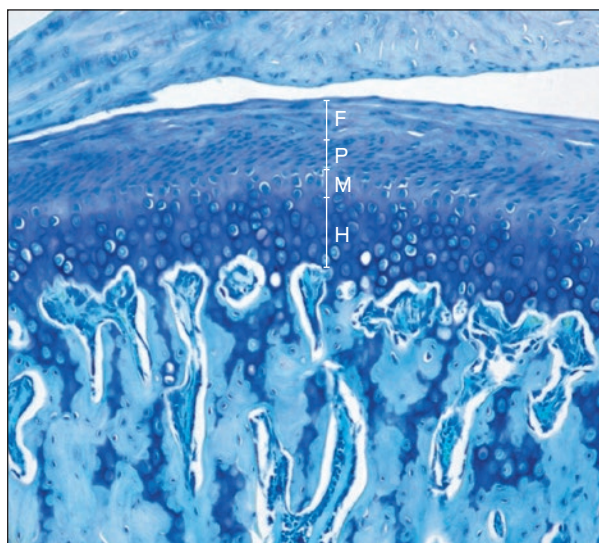


Fig 1 Four layers of the central region of rat condylar cartilage (20× magnification): F = fibrous; P = proliferative; M = mature; H = hypertrophic (M+H = chondroblastic).

ly described.²⁰ Immunostaining against the antibody was imaged by adjusting the color threshold, at which point the positive-stained zone was estimated. Color filters (hue, saturation, and brightness) were applied and adjusted to illustrate the area of immunostaining against the antibody. The positively stained area was thereafter measured. The percentage of collagen positive-stained area was determined by dividing the value of the collagen positive-area by the value of the whole area of the MCC.

Statistical Analysis

Linear regression models for the fibrous, proliferative, and chondroblastic layer thicknesses and for types I, II, and X collagen were performed to investigate the factor effects of the independent variables (estrogen level [OVX or non-OVX], dietary loading [diet board, pellet, or powder], aging [5 months old or 14 months old]) and two-way interaction terms.

The following comparisons of groups for analysis of variance (ANOVA) were based on these linear regression models. Independent-sample *t* test was performed for type I collagen, and Tukey-Kramer multiple comparison test was used as a post hoc test to make comparisons between groups for types II and X collagen and for thickness. If the variances of groups were unequal, Tamhane T2 test was used. *P* < .05 was deemed to indicate statistical significance. Analyses were conducted using SPSS version 24 (IBM). Data acquisition and analysis were performed blinded. Repeated measurements were done by the same investigator, and intraclass correlation coefficients (ICCs) were 0.888 for the fibrous layer thickness, 0.905 for the proliferative layer thick-

Table 1 Results of Linear Regression (*P*) for Fixed Effects of Estrogen Level, Dietary Loading, and Aging on Layer Thickness and Collagen Expression

Factor	Thickness			Expression		
	Fibrous layer	Proliferative layer	Chondroblastic layer	Type I collagen	Type II collagen	Type X collagen
Estrogen level	.59	.98	.72	.054	.048	.010
Dietary loading	< .001	< .001	< .001	.27	< .001	< .001
Aging	.003	.34	.19	< .001	.12	.87

Independent variables were estrogen level, dietary loading, and aging, adjusted for two-way interaction terms; dependent variables were collagen type and layer thickness.

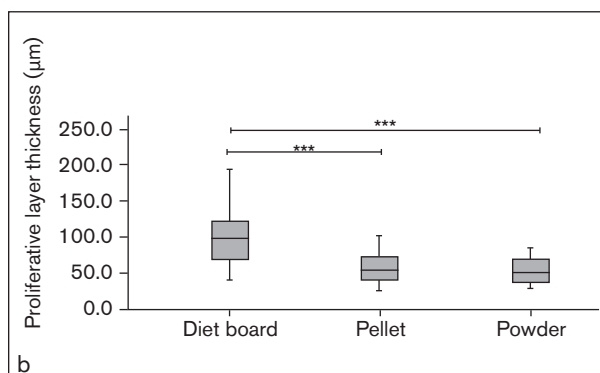
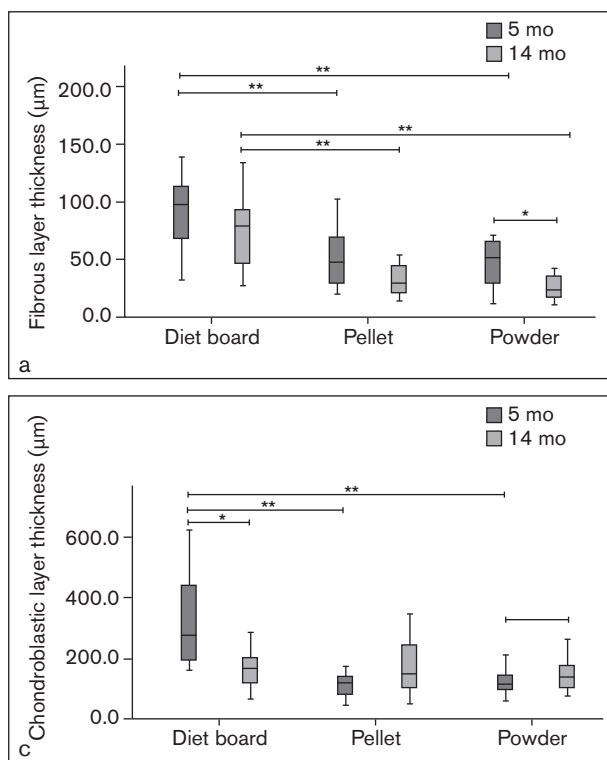


Fig 2 Box plots illustrating thickness (μm) of the (a) fibrous, (b) proliferative, and (c) chondroblastic layers in the different age and diet groups. **P* < .05. ***P* < .01. ****P* < .001.

ness, 0.967 for the chondroblastic layer thickness, 0.944 for type I collagen, 0.979 for type II collagen, and 0.997 for type X collagen.

Results

The thickness of the fibrous layer was significantly associated with dietary loading and aging, but not with estrogen level (Table 1). In both age groups, the thickness was significantly higher in the diet board rats than in both the pellet and the powder rats. In the powder group, the thickness was significantly higher in the 5-month-old rats than in the 14-month-old rats. There were no significant differences within the diet board and pellet groups (Fig 2a).

The thickness of the proliferative layer was significantly associated with dietary loading, but not with estrogen level or aging (Table 1). The thickness was

higher in the diet board rats than in both the pellet and the powder rats (Fig 2b).

The thickness of the chondroblastic layer was significantly associated with dietary loading, but not with estrogen level or aging (Table 1). There was some degree of interaction between aging and dietary loading. In the 5-month-old group, the thickness was higher in the diet board rats than in both the pellet and the powder rats, but there were no significant differences in the 14-month-old group. In the diet board group, the thickness was higher in the 5-month-old rats than in the 14-month-old rats, and there were no significant differences within the pellet or the powder groups (Fig 2c).

Staining of type I collagen was strongly expressed in the fibrous and proliferative layers of the MCC in all groups (Figs 3a and 3b). The expression of type I collagen was significantly associated with aging, but not with dietary loading or estrogen level (Table 1). Type I

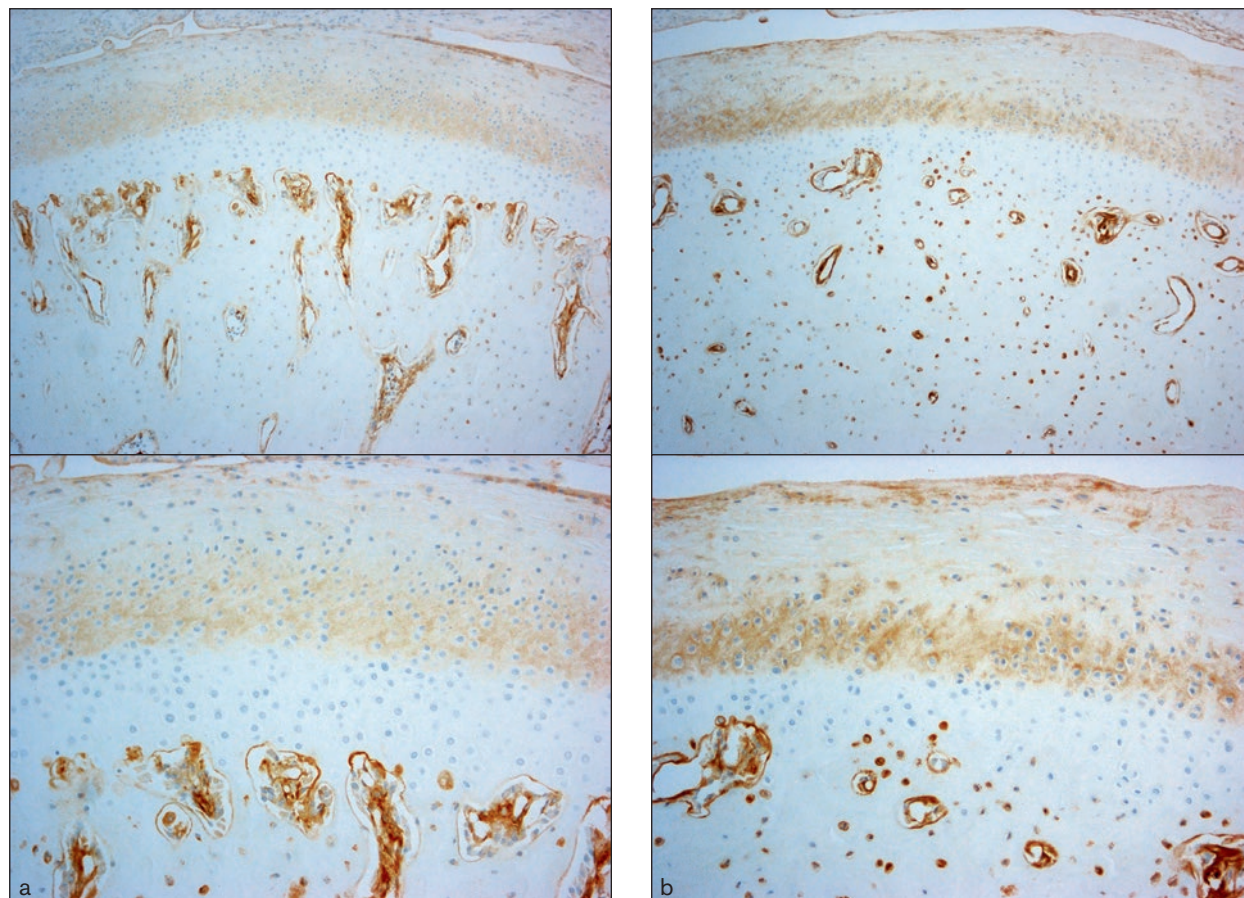
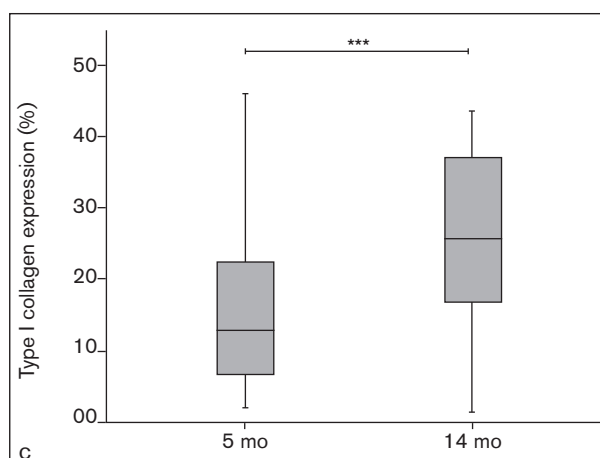


Fig 3 Immunohistochemical staining of the central part of the cartilage for type I collagen in (a) 5-month-old and (b) 14-month-old rats at $\times 10$ (top) and $\times 20$ (bottom) magnification. (c) Box plot illustrating type I collagen expression (% of area stained positive) in the different age groups. $***P < .001$.



collagen was significantly higher in the 14-month-old rats than in the 5-month-old rats (Fig 3c).

Staining of type II collagen was strongly expressed in both the mature and hypertrophic layers of the MCC in all groups (Figs 4a to 4f). The expression of type II collagen was significantly associated with dietary loading and with estrogen level, but not with aging (Table 1). In the non-OVX group, the expression of type II collagen was significantly higher in the diet board rats than in the powder rats (Fig 4g).

Staining of type X collagen was strongly expressed in the hypertrophic layer of the MCC in all

groups (Figs 5a to 5f). The expression of type X collagen was significantly associated with dietary loading and with estrogen level, but not with aging (Table 1). There was some degree of interaction between aging and dietary loading. In the 5-month-old group, the expression of type X collagen was significantly higher in non-OVX diet board rats than in the pellet and the powder rats of both estrogen levels. In the 5-month-old group, the expression was significantly higher in the OVX diet board rats than in the OVX pellet and OVX powder rats. There were no significant differences in the 14-month-old group (Fig 5g).

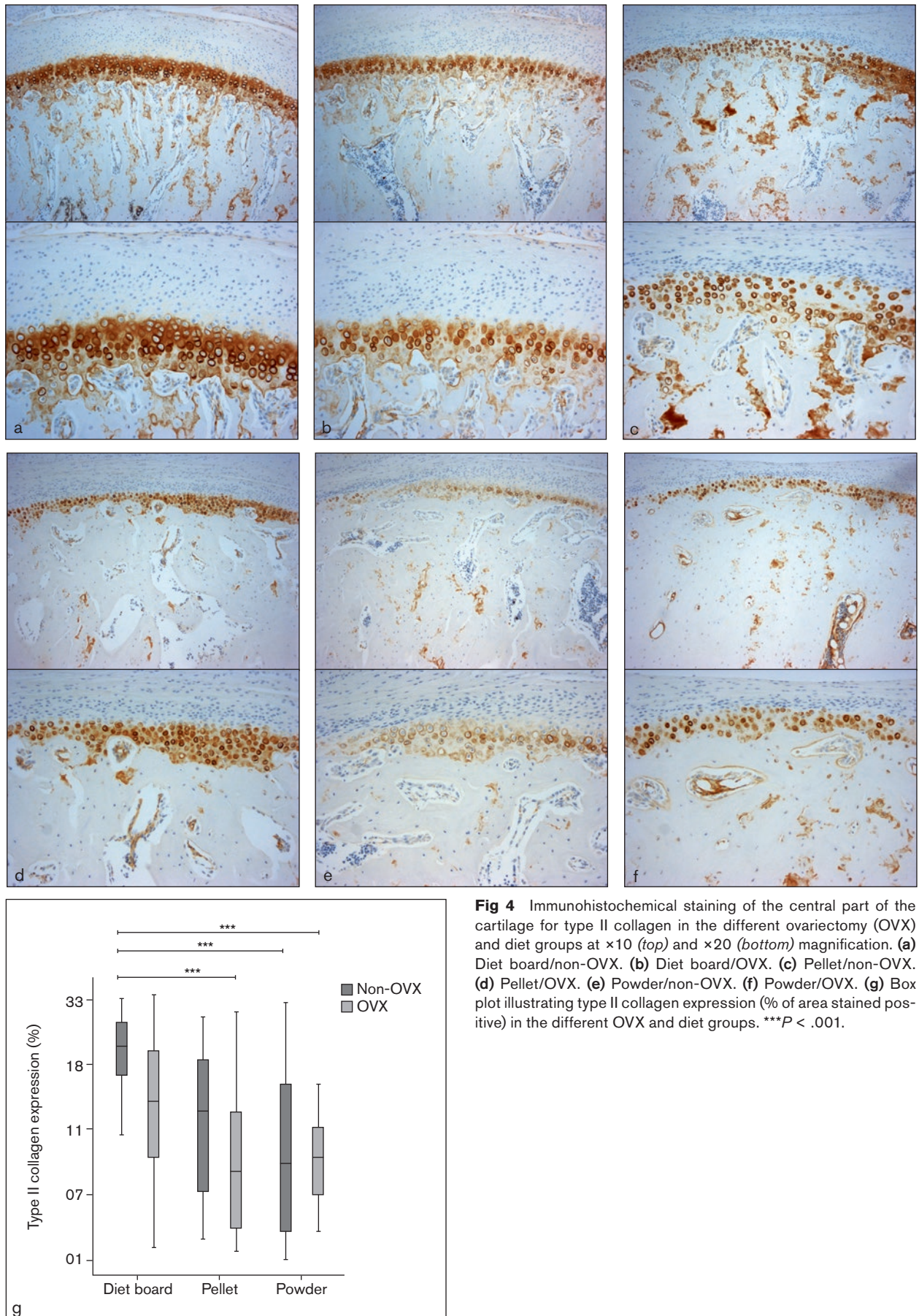


Fig 4 Immunohistochemical staining of the central part of the cartilage for type II collagen in the different ovariectomy (OVX) and diet groups at $\times 10$ (top) and $\times 20$ (bottom) magnification. (a) Diet board/non-OVX. (b) Diet board/OVX. (c) Pellet/non-OVX. (d) Pellet/OVX. (e) Powder/non-OVX. (f) Powder/OVX. (g) Box plot illustrating type II collagen expression (% of area stained positive) in the different OVX and diet groups. $***P < .001$.

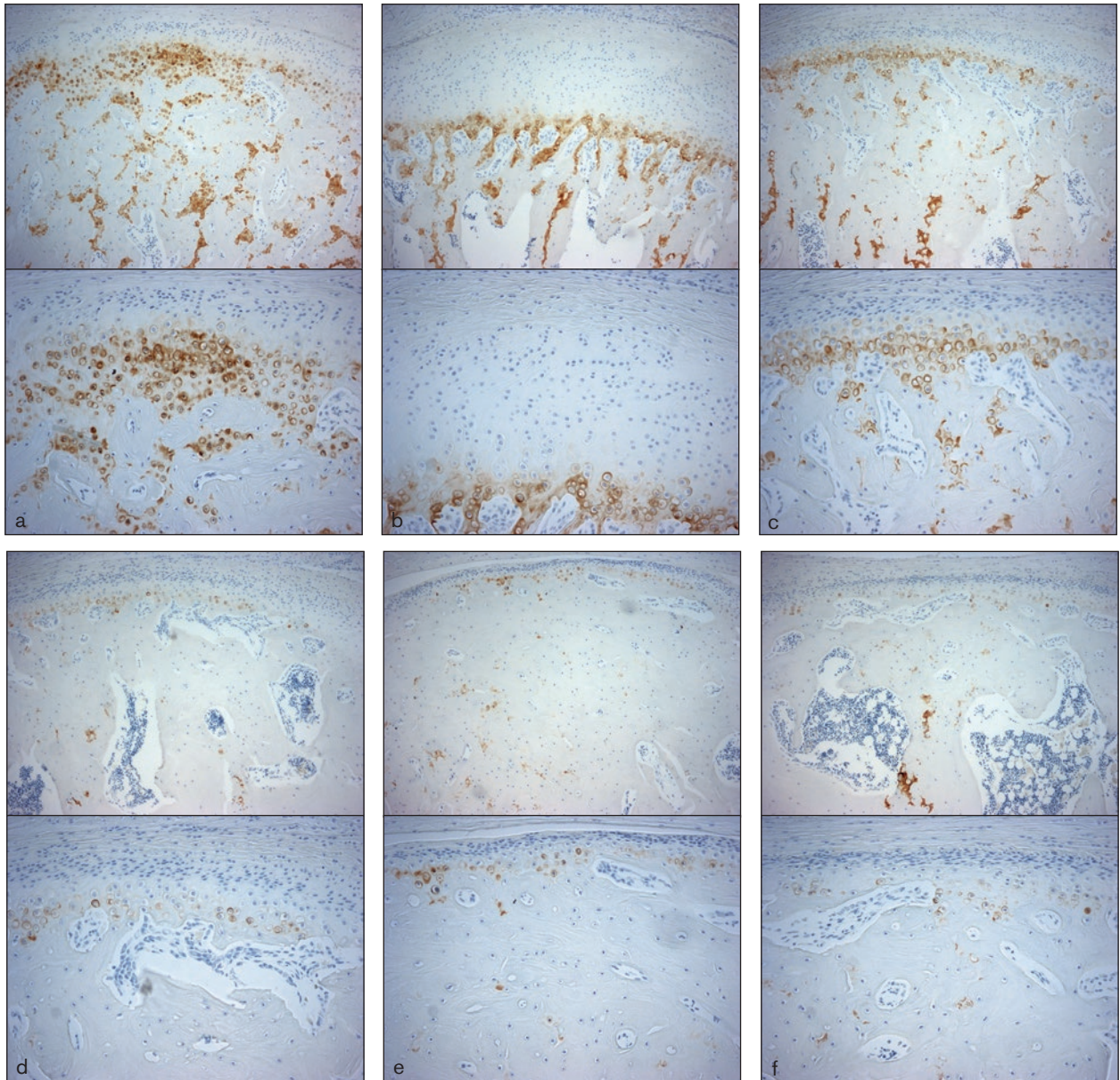
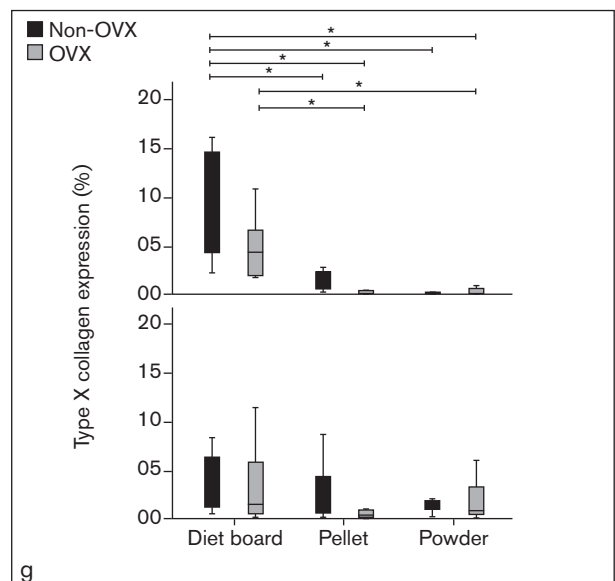


Fig 5 Immunohistochemical staining of the central part of the cartilage for type X collagen in the different ovariectomy (OVX) and diet groups at 10× (top) and 20× (bottom) magnification in the 5-month age group. (a) Diet board/non-OVX. (b) Diet board/OVX. (c) Pellet/non-OVX. (d) Pellet/OVX. (e) Powder/non-OVX. (f) Powder/OVX. (g) Box plot illustrating type X collagen expression (% of area stained positive) in the different age (5-month [top] and 14-month [bottom]), OVX, and diet groups. **P* < .05.



Discussion

The present study showed that estrogen level was a major factor affecting changes in type II and type X collagen expression, and that absence of ovarian estrogen lead to lower expression of both collagens while estrogen level did not affect the thickness of the cellular layers. A strong association between female sex and TMD has been shown in epidemiologic studies.^{21,22} Serum level of estradiol is reported to be significantly higher in TMD patients than in healthy controls, suggesting that estrogen participates in the pathophysiology of TMD.²³ The present results concerning the association between type II collagen and estrogen level are in accordance with the study by Oestergaard et al, which pointed out that a significant increase in type II collagen breakdown was accompanied by estrogen deficiency.²⁴ Additionally, it has also been found that products of type II collagen degradation increase in OVX rats, which accelerates joint arthritis.²⁵ Type X collagen, synthesized by hypertrophic chondrocytes and associated with mature cartilage, is thought to facilitate the process of mineralization and mark the onset of endochondral ossification.²⁶ Estrogen has been shown to induce maturation of the hypertrophic cartilage.²⁷ Estrogen receptors ER α and ER β have been found in mandibular condylar chondrocytes and subchondral bone, indicating that the mandibular cartilage is a target for estrogen.²⁸ In physiologic conditions, regulation of type II and type X collagen generation has been identified with changes in ER α expression and the proportion of ER α /ER β in chondrogenic cell line ATDC5.²⁹ Orajärvi et al have shown that hormonal changes after OVX can regulate the expression of ER α and type X collagen in the MCC of female rats.^{30,31} These findings suggest that OVX causes the changes in active estrogen receptors that lead to the different expression levels of types II and X collagen. Furthermore, changes in estrogen level have been found to be associated with pathologic subchondral bone findings in TMJ osteoarthritis.^{32–34}

Biochemical and biomechanical factors are essential to TMJ growth, and masticatory loading is considered a vital functional stimulation for TMJ remodeling.³⁵ It has been reported that type II collagen is an indispensable collagenous component in articular cartilage and is crucial for chondrocytes to develop and mature.³⁶ Under loading, progenitor cells located in the proliferative zone of the MCC are activated to undergo proliferation and differentiate into chondrocytes, with expression of aggrecan and type II collagen.³⁷ In the present study, rats in the diet board–feeding group needed to gnaw the wood to get food, forcing the rat's mandible to follow the incisal guidance in the sagittal direction.¹⁴ It can be assumed that during incision,

the loading on the TMJs of diet board rats was higher than on the TMJs of the pellet and the powder rats. Physiologic loading has been reported to induce higher expression of type II collagen, while type II collagen is degenerated by overloading.³⁸ Increased thickness of the central part of the MCC was observed in the diet board rats.²⁰ Here, the thickness of the cartilage layers of the MCC were higher in the diet board rats than in both the pellet and the powder rats. Additionally, the expression of type II collagen was increased by the diet board, providing more evidence that diet board feeding physiologically increases MCC loading during incision and is therefore a useful model to study the effects of increased loading on the MCC of rodents.³⁹ As previously reported, mechanical loading increases the thickness of the condylar cartilage, the number of cells, and the expression of types II and X collagen.⁴⁰ Polur et al have reported that decreased loading induces inhibition of chondrogenesis of condylar cartilage.⁴¹ It is evident that sufficient dietary loading promotes MCC development and maintains the integrity of the MCC by increasing the expression of types II and X collagen.

It has been reported that the prevalence of TMJ osteoarthritis is higher in the elderly population, and the repair capability of articular cartilage is reduced with increasing age.⁴² The results of the present study showed that type I collagen expression was higher in the older rats (14 months) than in the younger rats (5 months). The results are in agreement with Orajärvi et al, who showed that 26-month-old male diet board rats had a higher type I collagen expression in the MCC than 15-month-old male diet board rats.³⁹ Type I collagen is located on the most superficial layer in bundles that run tangentially to the MCC surface so the MCC is able to have more resistance against shear stress.⁴³ Chondrocytes are known to initially synthesize type I collagen and produce type II collagen later.⁴⁴ After injury, fibrocartilage-like tissue containing type I collagen is formed in the superficial zone of the hyaline cartilage and has limited ability to repair itself.^{3,45} Therefore, the higher expression of type I collagen observed in the MCC of aging rats could be a response to the decreased thickness of the fibrous layer brought on by age. Even though appropriate thickness is necessary for the condylar cartilage to withstand multidirectional forces, decreased thickness of the MCC and reduced expression of matrix metalloproteinase-8 by aging have been reported.²⁰ Here, in the older-age rats, the thickness of the chondroblastic layer and the expression of type X collagen in the MCC were more significantly affected by some degree of interaction between aging and dietary loading compared to the younger rats. Gradual loss of the cartilage matrix, as well as low extracellular matrix turnover and thin cartilage, could be re-

lated to the fact that aging, in addition to female sex, is one primary factor for TMJ osteoarthritis.⁴⁶ More research, however, will be needed to study the effect of estrogen on subchondral bone, as well as the influence of selective estrogen receptor modulators, as therapeutic modalities in TMJ osteoarthritis.

Conclusions

The physiologic level of estrogen plays a role in the development of the MCC by promoting the expression of types II and X collagen. Dietary loading is essential to increase the expression of types II and X collagen, as well as the thickness of cellular layers to maintain the integrity of the MCC. Aging seems to reduce the ability of the MCC to withstand occlusal loading.

Highlights

- Physiologic level of estrogen is beneficial for the MCC.
- Dietary loading is essential to maintain the integrity of the MCC.
- The expression of type I collagen is positively related to age.

Acknowledgments

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J.Y.: data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; S.L.: design and data acquisition, critically revised the manuscript; E.M., M.T.: data acquisition and analysis, critically revised the manuscript; P. Pesonen.: analysis, critically revised the manuscript; H.M.V.: design, critically revised the manuscript; S.S.: design, critically revised the manuscript; A.R., P. Pirttiniemi: data conception, design, and interpretation, critical revision of the manuscript.

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