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Inhalation of cigarette smoke induces structural changes in the murine knee joint via increased expression of TNF-alpha

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Abstract

Smoking is a major risk factor for cardiovascular, pulmonary, and musculoskeletal diseases, accelerating tissue degradation. It increases TNF-alpha expression, a cytokine with catabolic effects on cartilage, contributing to joint degeneration. This study evaluates smoking-induced TNF-alpha upregulation in osteochondral tissues, investigating the associated cellular and molecular mechanisms. Thirty-two male C57BL/6 mice (6–8 weeks old) were divided into four groups: Control, exposed to filtered air; Smoking, exposed to cigarette smoke (30 min/day, 5 days/week, for 45 consecutive days); Control + TNF-alpha inhibitor, exposed to filtered air and treated weekly with Adalimumab; and Smoking + TNF-alpha inhibitor, exposed to cigarette smoke and treated with Adalimumab. Femorotibial joints were subjected to histomorphometry, histology, ELISA (TNF-alpha, OPG, RANKL), and immunofluorescence (type I and II collagen). Smoking did not reduce cartilage area but led to decreased chondrocyte density, subchondral bone area, and collagen types I and II. TNF-alpha and RANKL levels increased in the Smoking group but were reversed/protected by Adalimumab, which restored cartilage and bone parameters, preventing further damage. These findings reinforce TNF-alpha as a therapeutic target in osteochondral diseases and highlight the relevance of anti-TNF therapies.

Keywords: Smoking; Type I Collagen; Type II Collagen; TNF-alpha Inhibitor.

Introduction

Osteochondral tissues support joint surfaces and include articular cartilage (the most superficial structure), subchondral, and trabecular bone [1]. Cartilage is an avascular tissue with limited cellular mitotic activity [2,3]. This tissue is composed of chondrocytes, a specific cell type embedded in an extracellular matrix (ECM) primarily consisting of type II collagen and proteoglycans [4]. The subchondral bone lies just beneath the cartilage and contains vascular channels that receive nourishment from the marrow tissue. This arrangement facilitates nutrient diffusion to the cartilage, thereby supporting its metabolism. Additionally, subchondral and trabecular bones are essential for the mechanical support of cartilage [1].

Tumor necrosis factor-alpha (TNF-alpha) is a proinflammatory cytokine that is upregulated in smokers and is implicated in the development and progression of joint diseases such as osteoarthritis [5]. This cytokine is

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recognized as a catabolic factor for cartilage [6–9], as it enhances the release of matrix metalloproteinases (MMPs) by synovial fibroblasts, resulting in cartilage degradation [6,9,10]. These events are primarily attributed to the inhibition of chondrogenesis and increased chondrocyte apoptosis [11]. In this context, several studies have investigated the effects of anti-TNF-alpha drugs on cartilage, mainly in ex vivo models, and observed a potential benefit by mitigating inflammatory responses [7,8,12,13].

It is important to note that the effects of smoking on joint diseases remain poorly understood, and there is no consensus regarding the mechanisms of joint damage resulting from smoking exposure. In osteoarthritis, clinical findings have been inconsistent: while some studies suggest that smoking increases the risk of arthritis development [14–17], others report the opposite [18,19].

We previously demonstrated that mice exposed to cigarette smoke showed increased expression of TNF-alpha in bone tissue samples, along with a concomitant reduction in type I collagen deposition. Given that type I collagen is the major component of the bone's organic matrix and is responsible for its tensile strength, compressive, and torsional properties [20], these findings underscore the structural changes induced by cigarette smoke in bone, which may partially explain the bone fragility observed in smokers in clinical studies [21,22].

Recognizing that TNF-alpha plays a significant role in various joint diseases [5,12,23], and that smoking induces upregulation of this chemokine, this study aims to evaluate the effects of TNF-alpha overexpression induced by smoking on osteochondral tissues, including cartilage, subchondral, and trabecular bone.

Materials and Methods

Ethics Statement

Male C57BL/6 mice (6–8 weeks old), with an average weight of 25 grams, were obtained from the Central Animal Facility at the School of Medicine, University of São Paulo. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council Committee, updated 2011). The study was approved by the Institutional Review Board for Animal Studies at the University of São Paulo School of Medicine (Project Number 1847/2022).

Experimental Groups

The animals were assigned to four experimental groups, with eight animals in each group: Control Group (C): animals housed in the animal facility and exposed to filtered air (n = 8); Cigarette Smoke Group (S): animals exposed to cigarette smoke for 45 days (n = 8); Control and Inhibitor Treatment Group (C_Inhibitor): animals housed in the animal facility, exposed to filtered air, and receiving intraperitoneal injections of Adalimumab (10 mg/kg) once per week for 45 days (n = 8); Cigarette Smoke and Inhibitor Treatment Group (S_Inhibitor): animals exposed to cigarette smoke for 45 days and receiving intraperitoneal injections of Adalimumab (10 mg/kg) once per week during the same period (n = 8).

Cigarette Smoke Exposure Model

The animals were exposed to cigarette smoke for 30 minutes per day, 5 days per week, for 45 consecutive days. Exposure was conducted in an inhalation chamber—a plastic box with a capacity of 28 liters (approximately 40 × 27 cm at the base and 26 cm in height)—equipped with two air inlets: one for synthetic air and the other for cigarette smoke. A small fan positioned at the top of the chamber ensured uniform air distribution. Synthetic air was supplied at a flow rate of 2 L/min, while the second inlet provided a flow rate of 1.5 L/min, connected to a Venturi system that facilitated smoke suction from a lit cigarette into the chamber. This configuration-maintained carbon monoxide (CO) levels between 250 and 350 ppm. The animals were exposed to commercially filtered cigarettes (0.8 mg nicotine, 10 mg tar, and 10 mg CO per cigarette), and the mean carboxyhemoglobin concentration during exposures was maintained at 10 ± 1.3%. Each session used 10 cigarettes, while control groups were exposed to filtered air only (Figure 1) [24].

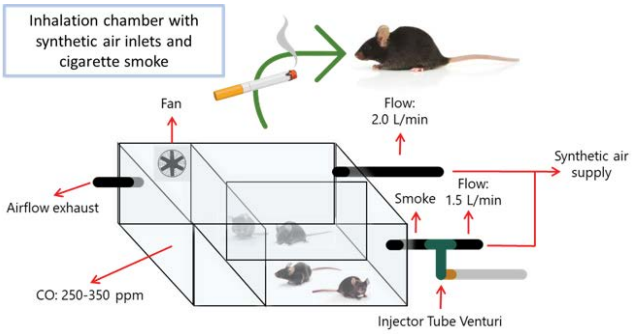

Figure 1: Inhalation chamber with synthetic air and cigarette smoke inlets.

Adalimumab Treatment

Animals treated with Adalimumab (Hyrimoz, Sandoz/Novartis) received intraperitoneal injections once weekly for 45 days, at a dose of 10 mg/kg [25].

Euthanasia for Tissue Collection

Upon completion of the exposure and treatment protocols, animals were euthanized via intraperitoneal injection of sodium thiopental (50 mg/mL). After anesthesia induction, a laparotomy was performed to section the inferior vena cava, enabling exsanguination. Tissue collection followed immediately.

The femorotibial joints were excised: the right tibiae were reserved for histomorphometry analysis, while the left tibiae

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were prepared for histological examination. The right femur, including articular cartilage, subchondral, and trabecular bone, was collected for tissue homogenization.

Histological Preparation

Following excision, the right femorotibial joints and left tibiae were fixed in 10% buffered formalin for 24 hours. Subsequently, specimens were decalcified in a 7% nitric acid solution for 3–4 days, rinsed in running water for 20 minutes, then washed with distilled water and placed again in 10% buffered formalin. The samples were dehydrated in 70% ethanol for two days and embedded in paraffin. Paraffin-embedded femorotibial joints were sectioned perpendicular to the articular surface to obtain histological slides of cartilage, subchondral, and trabecular bone. Serial sections of 4–5 µm thickness were cut with 50 µm spacing between them for application of histological staining, specifically hematoxylin and eosin (H&E) [26].

Evaluation of Articular Cartilage Thickness

Histological sections (4–5 µm) stained with H&E were analyzed to assess cartilage thickness using image analysis. Images were captured at 200x magnification and processed with Image-Pro Plus 6.0® software. Six vertical lines were drawn from the cartilage surface to the growth plate, starting from a central point and extending 250 µm to the right and left. The cartilage thickness (in µm²) was calculated as the arithmetic mean of these measurements.

Evaluation of Chondrocyte Density

Chondrocytes in H&E-stained articular cartilage sections were evaluated using the stereological point-counting method described by Gundersen et al. (1988) [26], with modifications. Image-Pro Plus 6.0 software was used for analysis. A reticulum comprising 100 orthogonally distributed points was superimposed on the image. A blinded observer analyzed 10 randomized fields at 400× magnification. Chondrocyte percentage (C%) was calculated using the formula:

$$C\% = (Pi/Ptc) \times 100$$

Where Pi represents the number of points intersecting stained cells, and Ptc the total number of points within the defined region from cartilage surface to growth plate. The final percentage was the average of all 10 fields and expressed as chondrocytes per µm².

Immunofluorescence of Type II Collagen in Cartilage

Tibial or femoral sections were mounted on polarized slides, deparaffinized in heated xylene, rehydrated through graded ethanol and water rinses. Antigenic sites were exposed via digestion with Chondroitinase ABC (Sigma-Aldrich) in 50 mM Trizma HCl, pH 8.0, containing 60 mM sodium acetate and 0.02% BSA for 2 hours at room temperature, followed by digestion with porcine gastric pepsin (8 mg/mL in 0.5N acetic acid) at 37°C for 30 minutes. After three 10-minute PBS washes, nonspecific sites were blocked with 5% BSA in PBS for 30 minutes.

Sections were incubated overnight at 4°C with rabbit polyclonal anti-type II collagen antibodies (1:100; Rockland Immunochemicals), followed by PBS-Tween 20 washes and 60-minute incubation at room temperature with Alexa 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen) in Evans Blue 0.006% in PBS.

Bone Trabecular Area and Trabecular Number Analysis

The trabecular bone area and number in tibial slides were analyzed using images captured with an Olympus BX-51 microscope equipped with a digital camera and scanning system (Oculus TCX). Image-Pro Plus 6.0 software was used for quantification.

Analysis followed the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee guidelines. Parameters measured included trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). All evaluations were performed blindly to eliminate bias.

Assessment of Type I Collagen Proportion in Trabecular Area

To assess type I collagen proportion in the tibial metaphysis, sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (pH 6.0) at 95°C. The sections were incubated with rabbit polyclonal anti-type I collagen antibody (1:50; Rockland Immunochemicals) for 12–18 hours at 4°C.

Following PBS-Tween washes, sections were incubated with Alexa 488-conjugated goat anti-rabbit IgG (1:200) in Evans Blue 0.006% for 60 minutes at room temperature. Samples were mounted with buffered glycerin.

Slides were examined under fluorescence microscopy (Olympus BX-51), and images captured using an Oculus TCX system. Quantification of type I collagen was performed with Image-Pro Plus 6.0 software, with results expressed as a percentage of the total trabecular area.

Enzyme-Linked Immunosorbent Assay

The left femoral bones were excised, and surrounding tissues were carefully removed. Samples were stored at −80°C until analysis. Homogenates were prepared to quantify TNF-alpha, RANKL, OPG, and NF-κB expression via ELISA, following manufacturer protocols (Bio-Techne, R&D Systems, USA).

Bone homogenization was performed using a manual device: femoral bones were frozen in dry ice and crushed with a manual pressure gun using three bursts, yielding a uniform homogenate suspended in 0.9% NaCl and stored at −80°C.

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Samples were loaded onto ELISA plates pre-coated with specific capture antibodies. A biotin-labeled secondary antibody was added, followed by streptavidin polymer to amplify binding. TMB substrate was used for visualization, resulting in a blue color that turned yellow upon reaction termination. Absorbance was measured at 450 nm using a microplate reader (SpectraMax i3, Molecular Devices, USA).

Statistical Analysis

Statistical analyses were conducted using SigmaStat (SPSS Inc., Chicago, IL, USA). Normality was assessed with the Shapiro–Wilk test. For normally distributed data, two-way ANOVA was applied to evaluate the effects of multiple factors. Results are expressed as mean ± standard deviation, with $p \leq 0.05$ considered statistically significant. Non-parametric data were analyzed using appropriate tests.

Results

Effects of TNF-alpha Inhibitor treatment on cartilage, subchondral, and trabecular bone tissues in the experimental model of cigarette smoke exposure

No significant reduction in cartilage area was observed in the groups exposed to cigarette smoke (Smoking and Smoking-Inhibitor) when compared to the control groups (Control and C_Inhibitor) (Figure 2A). In contrast, analysis of chondrocyte density revealed a significant reduction in the Smoking group compared to all other groups, indicating that administration of the TNF-alpha inhibitor reversed/protected this effect in the S_Inhibitor group (Figure 2B).

Immunostaining demonstrated a significant decrease in type II collagen expression in the articular cartilage of the Smoking group compared to the other groups. This effect was reversed/protected following TNF-alpha inhibitor administration, as observed in the S_Inhibitor group (Figure 2C).

Regarding subchondral bone area, a significant reduction was observed in both groups exposed to cigarette smoke (Smoking and S_Inhibitor) compared to the control groups (Control and C_Inhibitor). However, subchondral bone area was significantly higher in the S_Inhibitor group compared to the Smoking group, indicating partial recovery/protection following TNF-alpha inhibitor treatment (Figure 3A). Moreover, type I collagen expression in the subchondral area of the tibial bone was significantly reduced in the Smoking group compared to all other groups. This reduction was reversed/protected in the S_Inhibitor group (Figure 3B).

Similarly, a significant reduction in trabecular area was found in the Smoking and S_Inhibitor groups compared to the Control and C_Inhibitor groups (Figure 4A). Additionally, a significant reduction in type I collagen in the trabecular bone was observed in the Smoking group relative to the other groups, and this effect was reversed/protected by TNF-alpha inhibitor administration in the S_Inhibitor group (Figure 4B).

Smoking increased TNF-alpha and RANKL expression, which was reversed/protected by TNF-alpha inhibitor treatment in the experimental model of cigarette smoke exposure

A significant increase in TNF-alpha expression was observed in osteochondral tissue homogenates from the Smoking group compared to the other groups. This increase was reversed/protected in the S_Inhibitor group following TNF-alpha inhibitor treatment (Figure 5A). Similarly, RANKL expression was significantly elevated in the cartilage homogenates of the Smoking group compared to the other groups and was also reversed/protected in the S_Inhibitor group (Figure 5B). In contrast, no significant differences in OPG expression were observed between the experimental groups (Figure 5C).

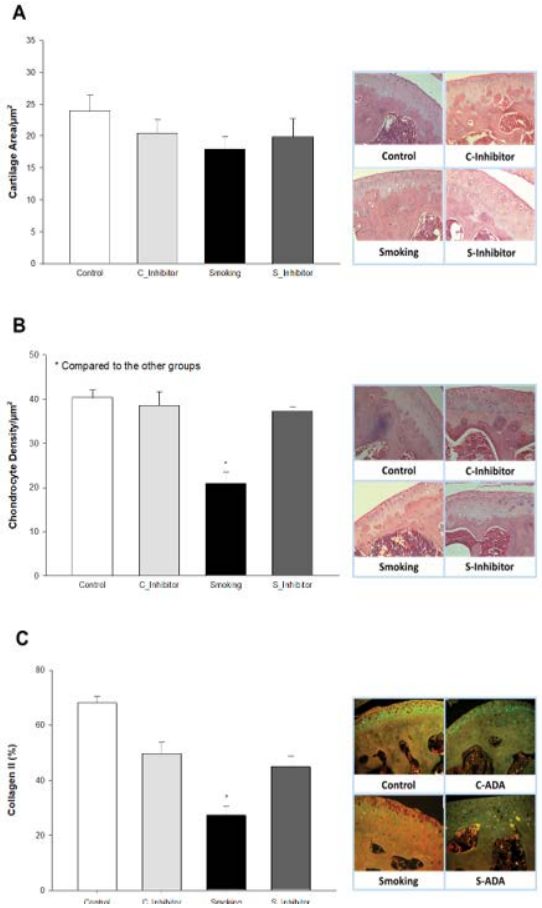

Figure 2: Effect of TNF-alpha inhibitor on cartilage in the cigarette smoke ex-posure model. Cartilage area (A), chondrocyte density (B), and type II collagen expression in the articular cartilage of the Smoke (n = 8), S_Inhibitor (n = 8), Control (n = 8), and C_Inhibitor (n = 8) groups. A significant decrease in chon-drocyte density and type II collagen was observed in the Smoking group com-pared to the Control and C_Inhibitor groups. Data are presented as mean ± SD; $p \leq 0.05$.

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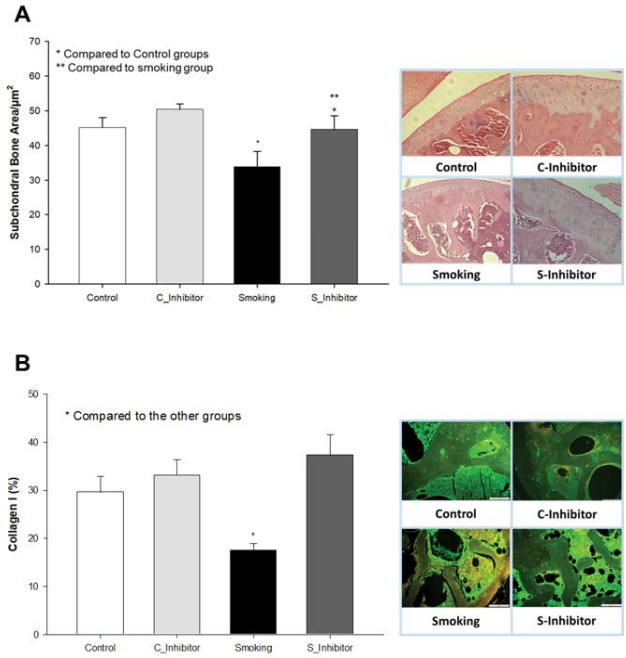

Figure 3: Effect of TNF-alpha inhibitor on subchondral bone area in the cigarette smoke exposure model. (A) A significant decrease in subchondral bone area was observed in the Smoking group (n = 8) compared to the Control (n = 8) and C_Inhibitor (n = 8) groups, with a significant increase in the S_Inhibitor group (n = 8) compared to the Smoking group; $p \leq 0.05$. (B) Type I collagen expression in the subchondral tibial bone area showed a significant decrease in the Smoking group (n = 8) compared to all other groups. Data are presented as mean ± SD; * $p \leq 0.05$.

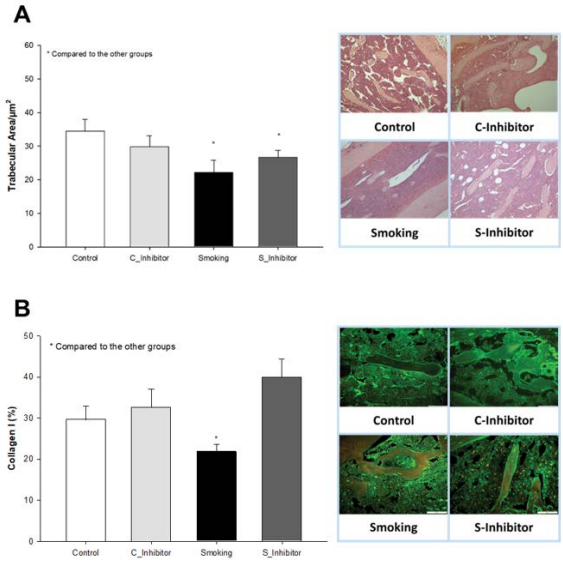

Figure 4. Effect of TNF-alpha inhibitor on trabecular bone area in the cigarette smoke exposure model. (A) A significant decrease in trabecular area was found in the Smoking (n = 8) and S_Inhibitor (n = 8) groups compared to the Control (n = 8) and C_Inhibitor (n = 8) groups; $p \leq 0.05$. (B) Type I collagen expression in the trabecular area of the tibial bone showed a significant decrease in the Smoking group (n = 8) compared to all other groups. Data are presented as mean ± SD; * $p \leq 0.05$.

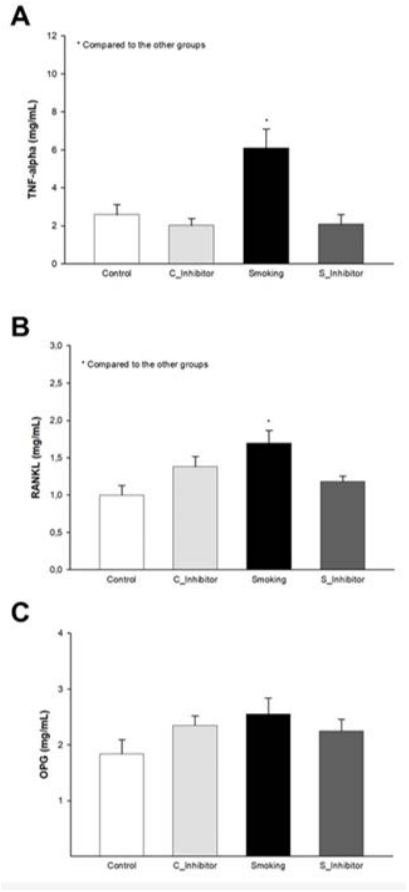

Figure 5: Expression of TNF-alpha, RANKL, and OPG in osteochondral tissue homogenates in the cigarette smoke exposure model. A significant increase in TNF-alpha (A) and RANKL (B) expression was observed in the Smoking group (n = 8) compared to the Control (n = 8), C_Inhibitor (n = 8), and S_Inhibitor (n = 8) groups. No significant differences were observed in OPG (C) expression among the experimental groups. Data are presented as mean ± SD; $p \leq 0.05$.

Discussion

In this study, we demonstrated that exposure to cigarette smoke induces a significant increase in TNF-alpha expression in osteochondral tissue homoge-nates, accompanied by structural alterations in cartilage, subchondral, and tra-becular bone. The administration of a specific TNF-alpha inhibitor, Adalimumab, effectively reversed/protected these alterations, underscoring the pivotal role of this cytokine in mediating the deleterious effects of cigarette smoke on oste-ochondral tissues.

In cartilage, cigarette smoke exposure resulted in a decreased density of chondrocytes, accompanied by reduced type II collagen deposition. Additionally, we observed a reduction in the subchondral and trabecular bone areas, concom-itant with diminished type I collagen deposition.

The decrease in type I collagen within subchondral and

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trabecular bone suggests increased fragility in these tissues, potentially compromising their mechanical support and the nutritional supply to the overlying cartilage [27].

Although no significant reduction in total cartilage area was observed, the decline in chondrocyte density and type II collagen deposition indicates com-promised cartilage composition. These changes may impair the tissue's capacity to absorb mechanical loads, thereby diminishing the functional integrity of the joint.

Our findings corroborate previous in vitro studies demonstrating that ciga-rette smoke impairs chondrocyte viability and promotes apoptosis [28,29]. Fur-thermore, our results provide a more comprehensive understanding of how cig-arette smoke alters cellular metabolism and the composition of distinct oste-ochondral tissues, offering mechanistic insights into clinical evidence linking smoking with the exacerbation of osteoarthritis [14,16,30] and chronic inflam-matory joint diseases [31].

The adverse effects of smoking on bone metabolism have been extensively documented, particularly in the context of osteoporosis, where smoking has been associated with decreased bone mineral density. In this context, we have previ-ously shown that smoking-induced upregulation of TNF-alpha is associated with trabecular bone loss and alterations in its organic matrix composition [20,27,32]. These structural bone changes have been attributed, at least in part, to enhanced bone resorption by osteoclasts and increased apoptosis of osteoblasts [32].

In the present study, the reduction in subchondral and trabecular bone area, along with decreased type I collagen, may be attributed to the concurrent increase in TNF-alpha and RANKL levels in osteochondral tissues. TNF-alpha binds to RANKL receptors, promoting the fusion of pre-osteoclasts into multinucleated cells. The subsequent interaction between RANKL and its receptor RANK is essential for the differentiation of these cells into mature osteoclasts, which are responsible for bone resorption [33].

The reversal of structural alterations in osteochondral tissues following TNF-alpha inhibition reinforces the critical role of this cytokine as a therapeutic target in joint diseases. This is particularly relevant given the established con-tribution of cigarette smoking to the progression of these pathologies.

Conclusions

In conclusion, our results demonstrate that cigarette smoke exposure leads to structural alterations in multiple osteochondral tissue components by increasing TNF-alpha expression. These findings support clinical evidence associating smoking with the worsening of joint diseases. Furthermore, our data highlight the therapeutic relevance of TNF-alpha inhibitors in preventing or reversing these structural changes, reinforcing their potential as treatment strategies in oste-ochondral disorders.

Author contributions

A.F.C: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writ-ing—original draft preparation, writing—review and editing, visualization, su-pervision, project administration and funding acquisition; A.F.d.S.: Methodology and software; A.R.M.: Methodology; F.M.d.A.: Methodology and validation; A.L.P.: Software; C.U.d.S.: Methodology; C.d.N.d.S.: Methodology; V.D.R.d.S.: Methodology and software; D.L.: Methodology and investigation; A.P.B.: Methodology, writing—review and editing, validation and project administra-tion; A.P.P.V.: Methodology, validation, data curation and supervision; W.R.T.: Conceptualization, validation, methodology, data curation and supervision; I.d.F.C.T.: Conceptualization, methodology, software, validation, formal anal-ysis, investigation, resources, data curation, writing—review and editing, visu-alization, supervision, project administration and funding acquisition; F.D.T.Q.S.L.: Conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft prepa-ration, writing—review and editing, visualization, supervision, project admin-istration and funding acquisition. All authors have read and agreed to the pub-lished version of the manuscript.

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Institutional review board statement

All animals received care in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council Committee, updated 2011). The study was approved by the Institutional Review Board for Animal Studies at the University of São Paulo School of Medicine (Project Number 1847/2022).

Informed consent statement: Not applicable.

Data availability statement

The data generated during this study are available upon request from the corresponding author (F.D.T.Q.S.L.)

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Conflicts of interest

The authors declare no conflicts of interest, financial or otherwise, including honoraria, educational grants, membership, consultancies, stock ownership, expert testimony, patent licensing, or personal/professional affiliations that could be perceived to influence the subject matter or materials discussed in this manuscript.

Abbreviations

The following abbreviations are used in this manuscript:

ECM	Extracellular Matrix
TNF-alpha	Tumor Necrosis Factor-alpha
MMP	Matrix Metalloproteinases
CO	Carbon Monoxide
H&E	Hematoxylin and Eosin
BSA	Bovine Serum Albumin
TMB	Tetramethylbenzidine

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