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Short Title: Exposure to second-hand smoke exacerbates osteoarthritis

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Abstract

Osteoarthritis (OA), formerly understood to be a result of passive wear, is now known to be associated with chronic inflammation. Cigarette smoking promotes systemic inflammation and has been implicated in increased joint OA incidence in some studies, though the recent observational data on the association are contradictory. We hypothesize that second-hand smoke (SHS) treatment will increase the incidence of OA in a mouse model that has been subjected to a surgical destabilization of the medial meniscus (DMM). To test this hypothesis, we applied either SHS treatment or room air (RA) to mice for 28 days post-DMM surgery. Histopathology findings indicated that the knees of SHS mice exhibited more severe OA than their control counterparts. Increased expression of matrix metalloprotease-13 (MMP-13), an important extracellular protease known to degrade articular cartilage, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), an intracellular effector of inflammatory pathways, were observed in the SHS
group. These findings provide greater understanding and evidence for a detrimental role of cigarette smoke on OA progression and systemic inflammation.

Key words: Second-hand smoke, osteoarthritis, DMM
Introduction

Osteoarthritis (OA) is a progressive degenerative disease that affects joint homeostasis. It is characterized by chronic pain, structural damage to articular cartilage and underlying subchondral bone and loss of joint function. In 2013, it was estimated in the Global Burden of Disease that over 242 million (Cross et al., 2014) people worldwide suffer from symptomatic knee and hip OA, with incidences increasing each year. While OA has traditionally been thought of as a wear-and-tear disease, more recently OA has been found to be closely related to chronic systemic inflammation (Berenbaum, 2013). For example, obese individuals have a higher occurrence of OA in the hand and hip vs. the knee (Visser et al., 2014), thus helping to dispel the wear and tear model. Thus, OA is clearly not a simple wear and tear disease. Many other serious diseases such as insulin resistance, hypertension, and Alzheimer’s disease are closely related to systemic inflammation and are strong risk factors for the development of OA (Puenpatom and Victor 2009; Ferreira et al., 2014; Caillon and Schiffrin, 2016; Wang et al., 2018). Additionally, biomarkers associated with OA (TGF-β1, HTR-A1, DDR2, MMP13 and NF-κB) are linked to inflammation (Larkin et al., 2013; Yessica Eduviges et al., 2020; Martinez-Nava et al., 2020).

Individuals who smoke cigarettes exhibit local and systemic inflammation (Zhang et al., 2002; van der Vaart et al., 2004; Madani et al., 2018). Starting in lung tissues, cells exhibit a local inflammatory response (Reynolds et al., 2011). This response is, at least in part, dependent on the receptor for advanced glycation end-products (RAGE) (Wood et al., 2014) in resident lung tissue. This pulmonary response to smoke then leads to the release of extracellular vesicles that systemically carry pro-inflammatory cytokines to every tissue in the body (Feller et al., 2018). Systemic inflammation resulting from the elaboration of pro-inflammatory mediators may be a
major reason that smoking is a risk factor for many serious diseases (Ambrose and Barua, 2004; Greene and Loeser, 2015; Straub and Schradin, 2016).

The effects of cigarette smoke on OA have not yet been completely elucidated. A more complete understanding may provide additional insights into the mechanisms involved in OA generally, as well as the relationship between OA and chronic inflammation. In several observational studies, smoking has been seen to correlate with higher and lower incidence of OA, depending on the population and the methodologies. In a Korean cross-sectional study, a weak association was found between indirect smoking and higher OA (Kang et al., 2016). Conversely, researchers in America found a small protective effect among heavy smokers compared to their nonsmoking counterparts, after adjusting for covariates such as age, sex, and weight (Felson et al., 1989). Likewise, other researchers have observed through cohort or meta-analysis studies that smoking protects against joint OA (Kong et al., 2017). Although avascular, the knee joint is responsive to system wide physiological changes (Sokolove and Lepus, 2013). Environmental factors also play a role as in the case of rheumatoid arthritis (RA), in which case cadmium (Cd) exacerbates the homeostasis imbalance in cartilage (Reyes-Hinojosa et al., 2019). Furthermore, Cd can negatively affect the presence of essential elements such as zinc (Zn), iron (Fe), manganese (Mn), nickel (Ni) and chromium (Cr) in cartilage and this possibly favors cartilage degeneration through the decrease of the extracellular matrix of cartilage including proteoglycans and glycosaminoglycans (Martinez-Nava et al., 2020). It is important to take into account that tobacco contains 1-2 µg/g of Cd. Therefore, it is an important source of exposure to this toxic metal and may account for the effect of tobacco on the progression of OA.

Therefore, we hypothesize that pulmonary exposure to second-hand smoke will disrupt joint tissue homeostasis and exacerbate the degradation of articular cartilage in the knee.
Materials and methods

**Mouse Model and Induction of OA**

Female mice of C57BC6 background \( n = 14 \) were obtained at 8 weeks of age, at which time the medial meniscus was destabilized to induce OA through a surgical transection of the medial meniscotibial ligament according to previously reported protocol (Larkin et al., 2013).

**Smoke Administration**

Mice were divided into room air group (RA; \( n = 7 \)) and second-hand smoke group (SHS; \( n = 7 \)). Commencing three days after surgical transection, SHS mice were given a second-hand smoke treatment using a nose-only delivery system (Scireq Scientific, Montreal, Canada) for 30 minutes each weekday for 4 weeks. For a given treatment, smoke was generated from 3 Kentucky 3R4F research cigarettes. Each treatment was administered according to previous protocol (Wood, Winden et al. 2014). RA mice were handled similarly but with room air in the apparatus.

**Running Wheel Cages**

Mice were housed in cages equipped with running wheels that allow for and record voluntary activity. Running wheel data is a valuable way for researchers to assess physical performance and mental well-being of mice (Novak, Burghardt et al. 2012). Both SHS and RA mice were held in individual cages which allowed data for each animal to be gathered independently. Activity was measured in running wheel cages. Mice were housed in a running wheel cage beginning at the
initiation of smoking treatment, three days after DMM surgery and remained until sacrifice 28 days later.

_Tissue Fixation & Preparation_

Knee tissues were harvested and fixed overnight in 4% paraformaldehyde. Knees were decalcified using a formic acid solution that was changed every other day for the first week and every three days for the second week, ending only after a negative ammonium oxalate test. The knees were then processed using an automatic tissue processor (ThermoFisher Scientific, MA, USA) and embedded in paraffin wax with the anterior tibial surface flush with the cutting plane.

_Histological Analysis_

Tissues sections were stained using Safranin-O and Fast Green to visually determine histological integrity. Photographs of each knee joint were taken at 10X and 20X magnifications using a light microscope equipped with a digital camera (Olympus America Inc., Center Valley, PA, USA). The articular cartilage from each stained slide was analyzed using both OARSI (Pritzker, Gay et al. 2006) and modified Mankin (Mankin et al., 1971) scoring systems to quantify the pathological state of each joint. In OARSI scoring, a score of 0 represents unaltered articular cartilage and 6 represents severe OA. The modified Mankin system uses a scale of 0-14, with 0 being unaltered cartilage. Tissue scoring was performed by 3 researchers who were blinded to the treatment given, and scores were averaged for each joint.
IHC Staining and Scoring

Immunohistochemical staining was performed on serial sections of the right knee joint from each mouse. Distinct slides were stained with antibodies against TGF-β1, NF-κB, HTRA1, DDR2, or MMP-13. First, each slide was deparaffinized and blocked using 5% bovine serum albumin for 30 minutes. Primary antibodies against TGF-β1 (ab92486) (Abcam, Cambridge, MA, United States), NF-κB (ab16502) (Abcam, Cambridge, MA, United States), HTRA-1 (ab38611) (Abcam, Cambridge, MA, United States), DDR-2 (SC-8989) (Santa Cruz Biotechnology, Santa Cruz, CA, United States), and MMP-13 (AB8120) (Chemicon, Temecula, CA, United States) were used. All antibodies were diluted 1:200, except for MMP-13 which was diluted 1:100. Slides were incubated overnight at 4°C. Samples were rinsed with PBS and incubated with a goat anti-rabbit biotinylated secondary antibody. Slides were rinsed again with PBS and incubated with an avidin/biotin ABC mix (Vectastain elite ABC Kit). After another PBS rinse, peroxidase substrate (Vector Labs, NovaRED) was added to produce a colored product, followed by Fast Green staining. Negative controls were prepared by staining without the addition of primary antibody. Photographs of slides were taken as described above. Counting of stained cells vs. total cells was performed using ImageJ by a blinded investigator (NIH, Bethesda, MD, United States).

Statistical Analysis

Statistical Analyses were performed by the Statistics Department at Brigham Young University through the SAS software using a mixed-models analysis of variance (ANOVA) with a post-hoc t-test. The dependent variables were the running wheel.
Results

Running Wheel

After housing all mice in a running wheel cage for four weeks, we quantitatively compared physical activity between SHS and RA groups. Figure 1 shows average daily totals for each treatment group. All animals displayed a period of low activity immediately following DMM surgery prior to rebounding to normal activity for a mouse after the surgery. We did not find a statistical difference between animals in the SHS or control groups. Similar voluntary activity between the groups demonstrates that voluntary active mobility of SHS mice was not significantly affected by SHS treatment, and that all animals experienced similar pain levels, as increased pain typically causes mice to run significantly less.

Histological Analysis

Using a Safranin-O/Fast Green stain, we analyzed the right knee of each animal to determine OA progression. We utilized both OARSI and modified Mankin scoring criteria to quantify joint degradation, the results of which are shown in Figure 2. Both SHS and RA mice displayed joint damage as a result of DMM surgery. Through OARSI scoring, we found no degradative difference in SHS mice compared to control (Figure 2A). However, using modified Mankin scoring, we observed increased joint tissue degradation in SHS mice compared to RA controls (Figure 2A, p < 0.05). Articular cartilage of SHS mice displayed decreased proteoglycan levels, increased chondrocyte clustering, and apical fibrillation compared to RA controls (Figure 2B).
**OA Biomarkers**

We chose five canonical biomarkers of OA: TGF-β1, HTR-A1, DDR2, MMP-13, and NF-κB. Figure 3 shows a graph of the expression profiles for each biomarker. We observed reduced TGF-β1 expression (p < 0.05) and increased NF-κB1 and MMP-13 expression (p < 0.05) in the SHS group compared to RA controls. No differences were observed in HTR-A1 and DDR2 expression in SHS knee chondrocytes. Increased MMP-13 expression indicates activation of pro-inflammatory pathways, leading to more rapid cartilage degradation.

**Discussion**

Traditionally, understanding the effect of cigarette smoking on OA has been elusive. The controversy over whether smoking is detrimental or beneficial to joint health overall has been due to a reliance on observational research alone. In the present study, we report that SHS exposure exacerbates the progression of OA in a murine DMM model. This conclusion is supported by modified Mankin score data and differential TGF-β1, NF-κB and MMP-13 expression, as seen in Figures 2 and 3. Mankin scoring is useful in determining joint tissue histopathology. The criteria consider important aspects of joint homeostasis including cartilage erosion, chondrocyte periphery staining, chondrocyte spatial arrangement, and proteoglycan staining. Because many factors are involved in maintaining cartilage health, Mankin scoring is a valuable tool for evaluating joint health. OARSI scoring is similarly valuable, but it only compares joint tissues on a structural level while not delving into metabolic stress effects. Higher Mankin scores among SHS knees indicate inflamed and distressed chondrocytes, no longer able to maintain the extracellular matrix and avoid
apoptosis. No difference in OARSI scores were observed, indicating that no structural differences were present between SHS and RA knees. In the SHS mice, physical activity measured by the running wheel cages led to the observation that behavior indicative of greater pain was not indicated compared to the RA mice.

The HTRA1-DDR2-MMP-13 axis is commonly assessed in the study of OA. We tested for differential expression along this axis to determine OA status. MMP-13, an important matrix metalloproteinase (Li et al., 2017), showed elevated expression in SHS mice compared to control. This provides a likely explanation for the increased tissue damage supported by the modified Mankin scores. MMP-13 and elevated Mankin scores suggest that OA is more progressive as a result of smoke treatment. In addition to SHS-mediated increases in MMP-13 expression, NFκB was another biomarker increased in SHS mice. Interestingly, both are associated with inflammation (Goldring and Otero, 2011). SHS chondrocytes tended to express HTRA1 and DDR2 less than RA controls, but this difference was not significant. These observations do not disprove the possibility that MMP-13 was upregulated by additional pathways. Pro-inflammatory pathways that increase MMP-13 expression in mice include: RAGE (Larkin et al., 2013), NF-κB, p38, JNK, and TNF-α (Rose and Kooyman, 2016). More research is needed to understand to what extent parallel and/or divergent pathways are involved in increasing the expression of this important metalloprotease.

In lung tissue, cigarette smoking increases RAGE activation, which culminates in greater expression of pro-inflammatory NF-κB (Reynolds et al., 2011). Individuals who smoke cigarettes exhibit local and systemic inflammation (Zhang et al., 2002, van der Vaart et al., 2004; Madani et al., 2018). Smoking has been shown to explain the development of more than 35% of positive cases of rheumatoid arthritis and the relative risk of developing RA persisted 15 years after people
stopped smoking (Hutchinson, 2015). This suggests that heavy metals resulting from the low
temperature combustion of tobacco may remain in the body long-term exerting biological activity.

Systemic inflammation induced by cigarette smoking results in part from RAGE signaling,
which upregulates NF-κB in chondrocytes. NF-κB expression leads to an increase in matrix
metalloproteinases, including MMP-13 (Vincenti and Brinckerhoff, 2002). SHS mice exhibited a
marked increase in NF-κB expression as compared to RA mice, which may further explain the
observed increased in MMP-13 expression.

TGF-β1 is involved in many cellular pathways; however, its effect on knee OA is unclear.
As has been observed, TGF-β1 expression can follow an inverse trend to HTRA1 (Larkin et al.,
2013). Many have observed the chondrogenic role for TGF-β1 (Bauge et al., 2014), arguing that
it is initially upregulated to promote tissue homeostasis. Importantly, this observation is consistent
with our discovery that TGF-β expression is lower in SHS mice. TGF-β1 levels are high in an
effort to heal and recover, while MMP-13 is generally released in response to the metabolic stress
itself. Competing pathways like these are common in complex biological systems. More
investigation is required to further understand the pro- or anti-inflammatory effects of differential
expression of TGF-β1 in response to SHS treatment.

As has been discussed, smoking increases local inflammation in several joint tissues, as
well as in lung tissue and smooth muscle cells. The RAGE pathway has been implicated in this
pro-inflammatory response (Reynolds et al., 2011). A RAGE knockout model exhibits reduced
MMP-13 expression as well as increased TGF-β1 in joint tissues (Matias, Mecham et al. 2016).
RAGE targeting reveals an intriguing connection between RAGE, MMP-13, TGF-β1, and NF-κB
that supports our current observations. Typically, we would predict a level of synergy between
these and other pathways, but it appears that cigarette smoking does not exhibit a pro-inflammatory
response through the HTRA1-DDR2-MMP-13 pathway directly. Further investigations are needed to reveal other pathways that may be involved in the observed biomarker level differences.

While the animals’ physical activity was similar, SHS mice exhibited increased OA progression compared to RA mice, as indicated by the increased Mankin scores and upregulated MMP-13. Through these observations, we report that SHS threatens tissue homeostasis in the knee joint. The toxicity of cigarette smoking has been well established, and smoking is accordingly broadly discouraged. We reiterate that any perceived benefit of smoking, demonstrated in past or current research is far outweighed by the health risks it imposes. As determined by this study, we report that, contrary to some previously reported literature, smoking has a deleterious effect on joint health.

Limitations and Future Direction

It was not deemed humane to perform DMM surgery on both knees simultaneously such that one might be used for gene expression analysis and the other for histology/immunohistochemistry. We recognize that mice are physiologically different than humans and the direct comparison of this study to human observational studies is uncertain. We have not yet analyzed an exhaustive list of markers for OA or inflammation. Now that we have characterized the histological differences between SHS and RA treatments in DMM mice, a logical next step would be to collect knee tissues for actual gene expression analyses. These could include additional pathways associated with inflammation and RAGE signaling.
**Ethics Statement**

This study was performed in strict accordance with the recommendations in the U.S. Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved Brigham Young University Institutional Animal Care and Use Committee (IACUC) protocol 130601. The BYU IACUC is overseen and approved with oversight by the U.S. Office of Laboratory Animal Welfare. Animals were housed according to IACUC recommendations. Methods of euthanasia used were carbon dioxide inhalation followed by cervical dislocation, or anesthesia induced by ketamine/xylazine followed by transcardial perfusion. Humane endpoints were strictly observed, and every effort was made to minimize suffering. Animal pain and discomfort was monitored using the Brigham Young University IACUC required Pain and Distress Monitoring scale, which conforms to the guidelines of the American Veterinary Medical Association.

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**Conflict of Interest**

The authors have no conflict of interest to disclose.
Figure 1: Average daily activity by RA and SHS mice. After DMM surgery and concurrent with treatment, all mice were housed in individual wheel cages that measure daily activity. SHS mice did not run more than RA mice on average (p > 0.05).
Figure 2: Articular cartilage was analyzed using OARSI and Mankin scoring systems. A. OARSI scoring revealed no significant difference while Mankin scoring showed a significant difference, with SHS mice displaying more progressed OA. B. Safranin-O staining revealed slightly more progressed OA in SHS mice. * = p < 0.05.
Figure 3: IHC staining for common OA biomarkers. A. Cell counting was used to quantitatively compare RA and SHS mice. TGF-β1, NF-κB and MMP-13 levels were differentially expressed in SHS mice. B. Sample images from both RA and SHS mouse tissues. * = p < 0.05; ** = p < 0.01
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