




Effects of Anesthesia on Ozone-Induced Lung and Systemic Inflammation

Miranda L. Wilson^{1,3} · Jarl A. Thysell^{1,2} · Kristen K. Baumann¹ · Danny V. Quaranta^{1,2} · W. Sandy Liang¹ · Michelle A. Erickson^{1,2} 

Received: 16 December 2021 / Accepted: 18 January 2022 / Published online: 23 February 2022
This is a U.S. government work and not under copyright protection in the U.S.; foreign copyright protection may apply 2022

Abstract

Purpose Anesthetics are required for procedures that deliver drugs/biologics, infectious/inflammatory agents, and toxicants directly to the lungs. However, the possible confounding effects of anesthesia on lung inflammation and injury are under-reported. Here, we evaluated the effects of two commonly used anesthetic regimens on lung inflammatory responses to ozone in mice.

Methods We tested the effects of brief isoflurane (Iso) or ketamine/xylazine/atipamezole (K/X/A) anesthesia prior to ozone exposure (4 h, 3 ppm) on lung inflammatory responses in mice. Anesthesia regimens modeled those used for non-surgical intratracheal instillations and were administered 1–2 h or 24 h prior to initiating ozone exposure.

Results We found that Iso given 1–2 h prior to ozone inhibited inflammatory responses in the lung, and this effect was absent when Iso was given 23–24 h prior to ozone. In contrast, K/X/A given 1–2 h prior to ozone increased lung and systemic inflammation.

Conclusion Our results highlight the need to comprehensively evaluate anesthesia as an experimental variable in the assessment of lung inflammation in response to ozone and other inflammatory stimuli.

Keywords Ozone · Inflammation · Isoflurane · Ketamine · Xylazine · Atipamezole · Serum amyloid A

Introduction

Ozone (O₃) is a widespread air toxicant with well-established inflammatory effects in the lungs, but it also affects other organs, such as the liver, spleen, and brain. Approaches for selectively inhibiting lung inflammation may provide insight on the systemic effects of O₃ and could involve direct delivery of substances to the lungs through the trachea prior to O₃ exposure. Volatile anesthetics such as

isoflurane are frequently used for intratracheal instillations and other surgeries [1–3]. However, isoflurane can have anti-inflammatory effects [4, 5], which may be most potent in the respiratory tract since it is the first site of exposure. Injectable anesthetics such as ketamine/xylazine are also used for intratracheal instillations [6], but may also modulate inflammatory responses [7–10]. Currently, little is known about how anesthesia prior to O₃ exposure affects inflammatory responses. To test this, we exposed mice to isoflurane (Iso) or ketamine/xylazine followed by atipamezole reversal (K/X/A) prior to O₃. Conditions of anesthesia were designed to replicate those used for non-surgical intratracheal instillations of substances such as drugs or biologics that modulate effects of O₃ on the lungs in mice. The day after O₃ exposure, mice were evaluated for markers of systemic and pulmonary inflammation. We found that Iso and K/X/A modify lung inflammation in response to O₃.

✉ Michelle A. Erickson
mericks9@uw.edu

¹ Veterans Administration Puget Sound Healthcare System, 1660 S. Columbian Way, S-182, Seattle, WA 98108, USA

² Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Washington, 325 9th Avenue, Box 359755, Seattle, WA 98104, USA

³ Present Address: Department of Cell, Developmental, and Regenerative Biology, Icahn School of Medicine at Mount Sinai, 1 Gustave L. Levy Pl, New York, NY 10029, USA

Methods

Animal Use

Female CD-1 mice, age 10–12 weeks, (Charles River Laboratories, Malvern, PA, USA) were used for air and O₃ exposures. Females were used because they have more robust inflammatory responses to O₃. Mice were given food and water ad libitum and kept on a 12-h light/12-h dark cycle. Protocols were approved by the institutional animal care and use committee of the VA Puget Sound Healthcare System.

Anesthesia

Isoflurane was administered by first weighing all mice, followed by placing mice in an induction chamber filled with 4% isoflurane for 1.5 min and then transferring to a nose cone where 3% isoflurane was used to maintain deep anesthesia for 5 min, which approximates the maximum time that a skilled technician needs to complete an intratracheal injection. Control mice were placed in the empty anesthesia chamber with room air for 1.5 min and then returned to their home cage. 1–2 h or 23–24 h after isoflurane exposure, O₃ exposures began. Ketamine (80 mg/kg) and xylazine

(10 mg/kg) in sterile saline were administered by intraperitoneal injection (IP). 20–30 min after the injection, mice were given 1 mg/kg atipamezole IP to quickly counteract the effects of the anesthesia. Control mice were given IP saline. 1–2 h after the atipamezole injection, O₃ exposures began. All mice were numbered with a marker to track their identity, and mice were randomized so that the average time between anesthesia and air/ozone exposure was equivalent between groups. A schematic of O₃ and anesthesia regimens is shown in Fig. 1.

O₃ Exposure

O₃ exposures were conducted at 3 ppm for 4 h (10:00–14:00), which induce a robust inflammatory response without inducing respiratory distress [11]. For the exposure duration, 3–4 mice were housed in standard mouse cages with free access to water, but without food or bedding to prevent consumption of ozonated food or bedding materials. Up to four cages at a time were placed in a 30" × 20" × 20" polypropylene chamber where O₃ (3 ppm, chamber 1) or compressed dry air (chamber 2) was pumped into the chamber at equivalent rates. Anesthesia and O₃ exposures were replicated at least once to capture day-to-day variability, and results for each dose showed consistent trends. O₃ levels in the chambers were generated and regulated

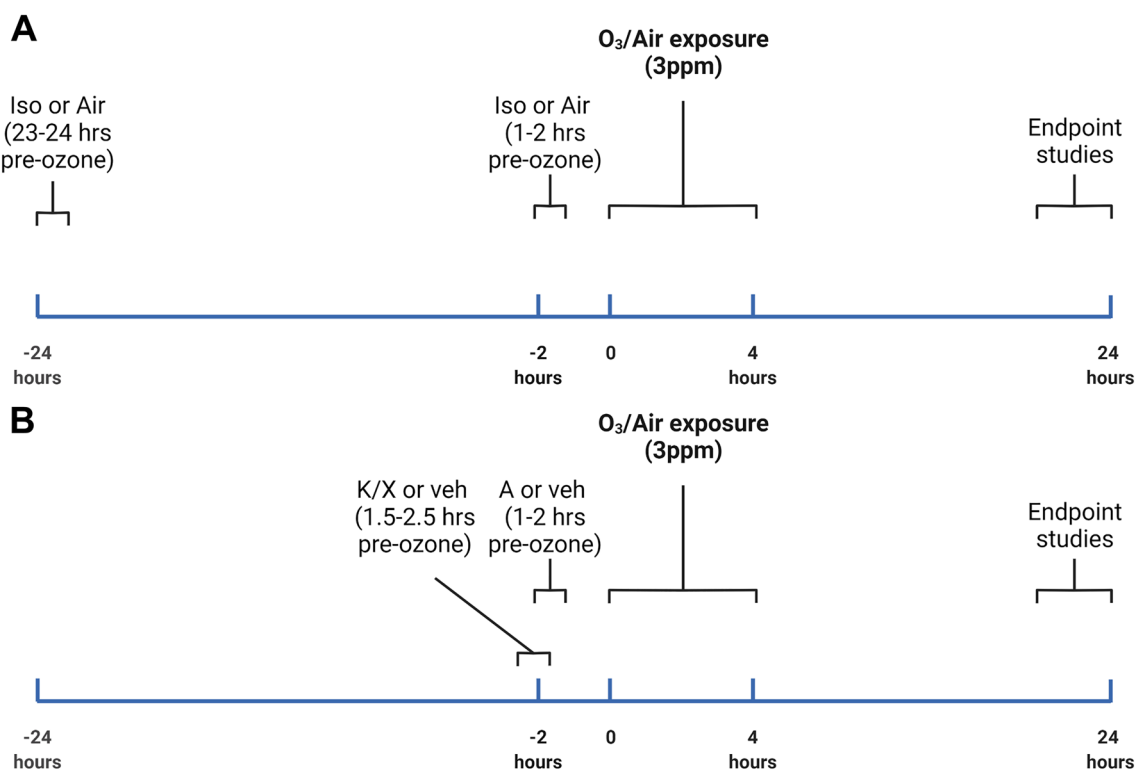


Fig. 1 Schematic of anesthesia/ozone (O₃) exposure protocols. **A** shows the exposure protocol for mice pre-exposed to isoflurane (Iso) or air control, and **B** shows the exposure protocol for mice pre-

exposed to Ketamine/Xylazine (K/X) and atipamezole (A) or saline vehicle. Created with Biorender.com

using an Oxycycler AT42 system (BioSpherix, Parish NY, USA). Prior to each experiment, the system was calibrated using a model 106-L O₃ detector (2B Technologies, Boulder, CO, USA), and O₃ levels were recorded from an inlet valve in one of the O₃-exposed mouse cages every 10 s for the duration of exposures. In all experiments, O₃ achieved its target concentration within 10 min, and levels were regulated within 10% of the target concentration (3 ppm ± 0.3 ppm) thereafter.

Blood Collection and Serum Amyloid A Measurement

22–24 h after the start of O₃ exposure (12:00–14:00), mice were re-weighed and deeply anesthetized with IP urethane (Millipore Sigma, St. Louis, MO, USA). Blood was collected from the abdominal aorta, allowed to clot for 30 min, and then placed on ice. Blood was centrifuged at 2500×g for 15 min and serum was collected, aliquoted, and frozen at –80 °C. Serum was diluted 1/10,000 for mice exposed to ozone and 1/50 for mice not exposed to ozone. Serum amyloid A (SAA) mouse duoset kits (R and D systems, Minneapolis, MN, USA) were used to quantify SAA in serum.

WLL and Pulmonary Inflammation Assessment

Whole-lung lavage (WLL) was performed on mice by perfusing and aspirating the lungs through the trachea three times with 1 mL sterile phosphate-buffered saline (3 mL total). WLL fluid was stored on ice and centrifuged at 200×g for 5 min at 4 °C. The supernatant was removed and 0.5 mLs of supernatant were reserved to resuspend the cell pellet. The remainder was frozen for measurement of total WLL protein, which was performed using a microBradford assay. Total cells were manually counted using a hemacytometer. Differential cell counts were performed on Hemacolor-stained cytocentrifuge preparations. Cell counts were performed using ImageJ, and at least 200 cells were counted to determine relative cell proportions.

Statistics

Statistical analysis was done with Prism 8.4.2 (GraphPad Software, San Diego, CA, USA). Data are reported as mean ± SD and analyzed by two-way ANOVA for main effects and interactions and Tukey's multiple comparisons test for comparisons of group means.

Results

Effects of Isoflurane and Ketamine/Xylazine/Atipamezole Anesthesia on Ozone-Induced Pulmonary Inflammation and Injury

We first determined whether O₃-induced responses in the lungs differed with Iso or K/X/A. Since drugs or other interventions to be administered intratracheally would ideally be given a short time before exposure to ozone to maximize their effects/minimize their metabolism, we evaluated anesthesia administration at 1–2 h prior to ozone exposure. We chose 1–2 h because this time frame allows the mice to recover from anesthesia and also reflects a time window that is typically sufficient for an administered drug to sufficiently activate/inhibit its biological target. A pilot study indicated that Iso given 1–2 h before O₃ inhibited inflammation, suggesting that this regimen is unsuitable for studying anti-inflammatory interventions for O₃ exposure. Therefore, we included a 24-h Iso pre-exposure in experimental replicates to determine whether the anti-inflammatory effects of Iso were sustained. The 24-h time point was chosen because the Iso and O₃ exposures could then occur at approximately the same time of day as the 1–2-h Iso pre-exposure group. We compared K/X/A to Iso 1–2-h pre-O₃ exposure to determine whether K/X/A is a suitable alternative anesthesia regimen to Iso (Schematics in Fig. 1A, B). Although the controls for ketamine were IP injected with saline, whereas Iso controls were not, we found that IP injections did not significantly influence the measured outcomes, and so we combined the Iso and Ketamine no anesthesia control groups for analysis. O₃ significantly increased WLL total cells, macrophages, and neutrophils except in the 1–2-h Iso group (Fig. 2A–C). We further show that mice exposed to K/X/A and O₃ had significantly increased WLL total cells, macrophages, and neutrophils vs. other O₃-exposed groups. O₃ significantly increased WLL protein in the no anesthesia group and 23–24-h Iso groups, but not in the 1–2-h Iso or K/X/A groups (Fig. 2D). The *F*-statistics and *p*-values for main effects and interactions are reported in Table 1.

Effects of Iso and K/X/A Anesthesia on Ozone-Induced Systemic Inflammation and Weight Loss

We next determined whether the effects of anesthesia extended to markers of ozone-induced systemic inflammation and behavioral effects. We have previously shown that the acute-phase protein SAA is consistently increased in blood following a 3 ppm exposure to O₃ and

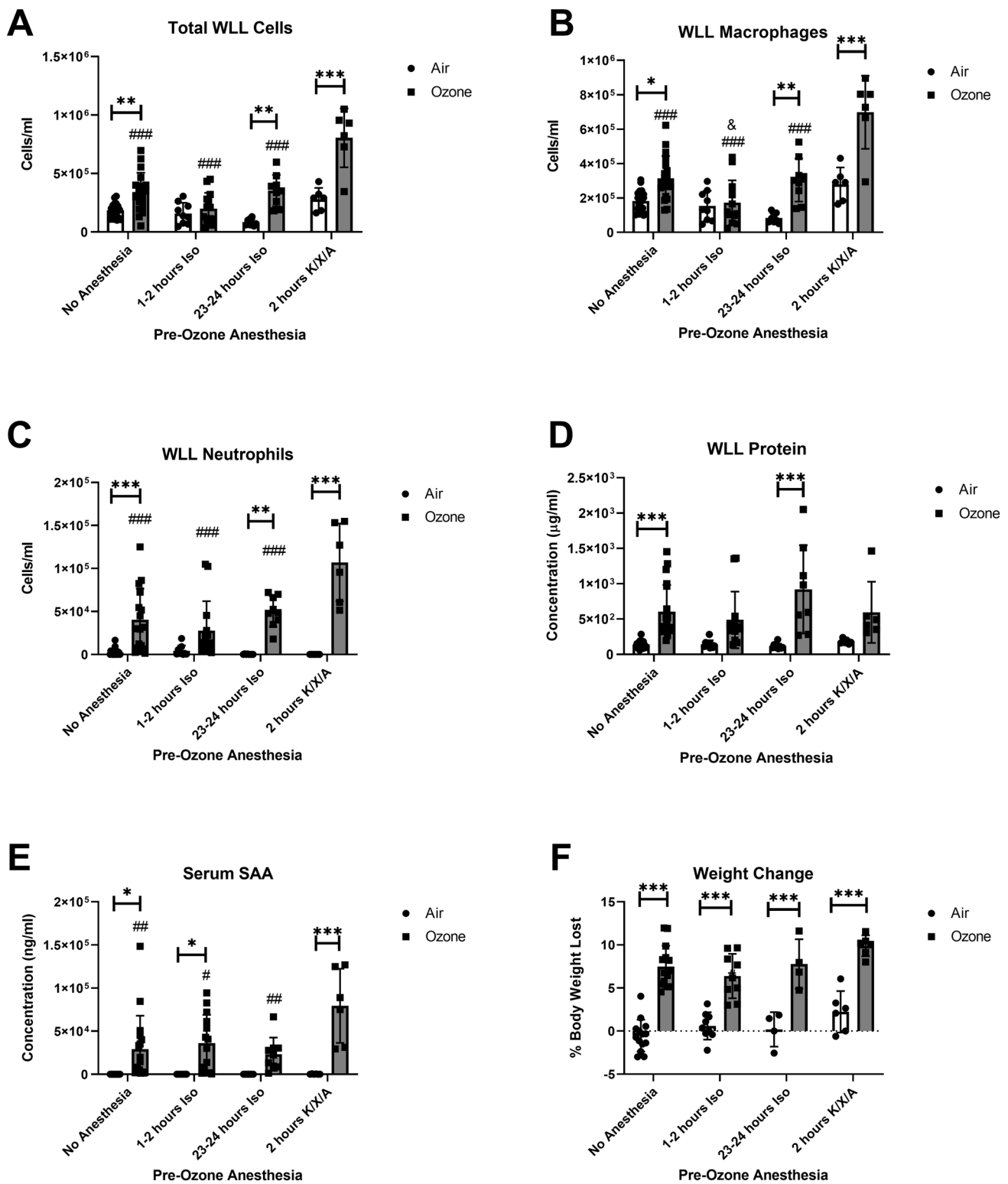


Fig. 2 Effects of isoflurane (Iso) and ketamine/xylazine/atipamazole (K/X/A) on inflammatory (A–C) and vascular damage (D) markers in the lung, blood levels of serum amyloid A (SAA, E), and weight loss (F). In all panels, $n=6-20$ per group. $***p < 0.001$,

$**p < 0.01$, $*p < 0.05$, air vs. O₃. $###p < 0.001$, $##p < 0.01$, $#p < 0.05$, vs. K/X/A + O₃ group. $&p < 0.05$ vs. No Anesthesia + O₃. Group mean comparisons were carried out using Tukey's multiple comparisons test

Table 1 Two-way ANOVA analysis results showing percentages of variation by factor (main effects, interactions) and *F*-statistics

Figure	Anesthesia	Exposure	Interaction
2A—total WLL cells	28.97%; <i>F</i> (3,82)=22.15, <i>p</i> <0.0001	29.96%; <i>F</i> (1,82)=68.73, <i>p</i> <0.0001	12.79%; <i>F</i> (3,82)=9.780, <i>p</i> <0.0001
2B—WLL macrophages	31.77%; <i>F</i> (3,82)=23.49, <i>p</i> <0.0001	25.59%; <i>F</i> (1,82)=56.74, <i>p</i> <0.0001	11.72%; <i>F</i> (3,82)=8.662, <i>p</i> <0.0001
2C—WLL neutrophils	9.766%, <i>F</i> (3,82)=6.535, <i>p</i> =0.0005	44.88%; <i>F</i> (1,82)=90.08, <i>p</i> <0.0001	11.82%; <i>F</i> (3,82)=7.907, <i>p</i> =0.0001
2D—WLL total protein	2.776%; <i>F</i> (3, 81)=1.309, <i>p</i> =0.2773	33.06%; <i>F</i> (1, 81)=46.76, <i>p</i> <0.0001	3.888%; <i>F</i> (3,81)=1.833, <i>p</i> =0.1478
2E—serum SAA	7.296%, <i>F</i> (3,81)=3.668, <i>p</i> =0.0156	35.35%; <i>F</i> (1, 81)=53.31, <i>p</i> <0.0001	7.204%; <i>F</i> (3,81)=3.622, <i>p</i> =0.0165
2F—weight change	7.784%; <i>F</i> (3, 58)=6.412, <i>p</i> =0.0008	52.74%; <i>F</i> (1, 58)=130.3, <i>p</i> <0.0001	1.903%; <i>F</i> (3,58)=1.567, <i>p</i> =0.2070

For Fig. 2D, one sample was excluded because of apparent blood contamination. For Fig. 2E, one sample was excluded because not enough serum was recovered to complete the assay. For Fig. 2F, body weights were not measured in one experimental replicate and so 23 samples are missing

its upregulation occurs in the absence of changes in other pro-inflammatory cytokines in blood [12]. Weight loss is a measurable outcome of sickness behavior, which can be induced by O₃. Anesthesia has no effect on O₃-induced weight loss based on the post hoc mean comparisons of the ozone groups (Fig. 2F); however, there was a significant main effect of treatment that explained 5.172% of the total variation (Table 1). O₃ significantly increased SAA levels in all groups except for mice exposed to Iso 23–24 h prior (Fig. 2E). SAA levels induced by O₃ were significantly higher in mice pre-anesthetized with K/X/A vs no anesthesia or Iso.

Discussion

We found that isoflurane inhibits O₃-induced pulmonary inflammation if given 1–2 h prior to exposure, and this effect diminishes by 24 h. Isoflurane induces anesthesia via potentiation of inhibitory γ -aminobutyric acid type A (GABA A) receptors and inhibition of glutamatergic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-d-aspartate (NMDA) receptors [13]. Isoflurane also has anti-inflammatory activities in a variety of rodent models [4], although the mechanisms are not well defined. It has been shown that activation of GABA A receptors and inhibition of NMDA receptors have anti-inflammatory effects on immune cells [14–16], suggesting that the anti-inflammatory activities of Iso could be mediated through direct actions on leukocytes. In contrast to Iso, K/X/A increased O₃-induced inflammatory responses to O₃. Anti-inflammatory/protective effects through NMDA receptor antagonism have been described for ketamine and ketamine/xylazine combinations [9]. Whereas xylazine is a α 2-adrenergic receptor agonist and potentiates anesthesia, atipamezole is an antagonist and leads to rapid anesthesia reversal within about 20 min [17]. The data on pro- vs. anti-inflammatory activities of the

α 2 adrenergic receptor are equivocal; however, a recent study showed that atipamezole may promote or prolong inflammation through its action via a α 2-independent mechanism [18]. It is also possible that anesthetics affect the amount of leukocyte trafficking to the lungs by altering the cytokine and chemokine profiles that are induced by O₃. Acute exposures to O₃ upregulate cytokines and chemokines such as CCL11, IL-6, G-CSF, CXCL10, and CXCL1 in a dose-dependent and cell type-specific manner [19]. Cytokines, such as TNF- α , IL-1, and IL-6, chemokines, such as CXCL1, CXCL10, and CCL7, and chemokine receptors such as CCR2 have been shown to contribute to leukocyte trafficking to the lungs in response to O₃ [20–25]. Future work is needed to delineate the immunomodulatory mechanisms of Iso and K/X/A.

Our results are the first to show that isoflurane exposure prior to O₃ inhibits pulmonary inflammation, whereas K/X/A exposure has an enhancing effect. These results complement other recent works that have investigated anesthesia's effects on pulmonary inflammation in response to ozone and other pro-inflammatory agents [5, 16, 26] and effects of anesthetics on the recovery of cells from WLL [27]. Although the scope of our study is limited in that we did not define a full-time course of anesthesia's effects, our results highlight that brief, acute anesthetic regimens can alter inflammatory responses to O₃. We conclude that anesthetics are important experimental variables whose evaluation is necessary for studies requiring their use prior to exposure to O₃ or other pathological stimuli.

Author Contributions All authors contributed to the collection and analysis of data, editing of manuscript drafts, and approved the final version of the manuscript. MAE, JAT, KKB, and MLW contributed to the study conception and design and manuscript preparation.

Funding This study was funded by the National Institutes of Health (R21ES029657, MAE) and the Veterans Administration (MAE).

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval The use of mice on this study was approved by the VA Puget Sound Healthcare System IACUC, protocol 0924. Human subjects were not used on this study.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

1. Driscoll KE et al (2000) Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci* 55:24–35. <https://doi.org/10.1093/toxsci/55.1.24>
2. Morales-Nebreda L et al (2014) Intratracheal administration of influenza virus is superior to intranasal administration as a model of acute lung injury. *J Virol Methods* 209:116–120. <https://doi.org/10.1016/j.jviromet.2014.09.004>
3. Lawrenz MB, Fodah RA, Gutierrez MG, Warawa J (2014) Intubation-mediated intratracheal (IMIT) instillation: a noninvasive, lung-specific delivery system. *J Vis Exp*. <https://doi.org/10.3791/52261>
4. Lee YM, Song BC, Yeum KJ (2015) Impact of volatile anesthetics on oxidative stress and inflammation. *Biomed Res Int* 2015:242709. <https://doi.org/10.1155/2015/242709>
5. Lacher SE, Johnson C, Jessop F, Holian A, Migliaccio CT (2010) Murine pulmonary inflammation model: a comparative study of anesthesia and instillation methods. *Inhalation Toxicol* 22:77–83. <https://doi.org/10.3109/08958370902929969>
6. Helms MN, Torres-Gonzalez E, Goodson P, Rojas M (2010) Direct tracheal instillation of solutes into mouse lung. *J Vis Exp*. <https://doi.org/10.3791/1941>
7. Tan S, Wang Y, Chen K, Long Z, Zou J (2017) Ketamine alleviates depressive-like behaviors via down-regulating inflammatory cytokines induced by chronic restraint stress in mice. *Biol Pharm Bull* 40:1260–1267. <https://doi.org/10.1248/bpb.b17-00131>
8. Chen MH et al (2018) Rapid inflammation modulation and antidepressant efficacy of a low-dose ketamine infusion in treatment-resistant depression: a randomized, double-blind control study. *Psychiatry Res* 269:207–211. <https://doi.org/10.1016/j.psychres.2018.08.078>
9. Erdem MK, Yurdakan G, Yilmaz-Sipahi E (2014) The effects of ketamine, midazolam and ketamine/xylazine on acute lung injury induced by alpha-naphthylthiourea in rats. *Adv Clin Exp Med* 23:343–351. <https://doi.org/10.17219/acem/37124>
10. Szelenyi J et al (2000) Opposite role of alpha2- and beta-adrenoceptors in the modulation of interleukin-10 production in endotoxaemic mice. *NeuroReport* 11:3565–3568. <https://doi.org/10.1097/00001756-200011090-00032>
11. Kierstein S et al (2008) Ozone inhalation induces exacerbation of eosinophilic airway inflammation and hyperresponsiveness in allergen-sensitized mice. *Allergy* 63:438–446. <https://doi.org/10.1111/j.1398-9995.2007.01587.x>
12. Erickson MA et al (2017) Serum amyloid A: an ozone-induced circulating factor with potentially important functions in the lung-brain axis. *FASEB J* 31:3950–3965. <https://doi.org/10.1096/fj.201600857RRR>
13. Michelson NJ, Kozai TDY (2018) Isoflurane and ketamine differentially influence spontaneous and evoked laminar electrophysiology in mouse V1. *J Neurophysiol* 120:2232–2245. <https://doi.org/10.1152/jn.00299.2018>
14. Reyes-Garcia MG, Hernandez-Hernandez F, Hernandez-Tellez B, Garcia-Tamayo F (2007) GABA (A) receptor subunits RNA expression in mice peritoneal macrophages modulate their IL-6/IL-12 production. *J Neuroimmunol* 188:64–68. <https://doi.org/10.1016/j.jneuroim.2007.05.013>
15. Bhandage AK et al (2018) GABA regulates release of inflammatory cytokines from peripheral blood mononuclear cells and CD4(+) T cells and is immunosuppressive in type 1 diabetes. *EBioMedicine* 30:283–294. <https://doi.org/10.1016/j.ebiom.2018.03.019>
16. Wang Y et al (2016) N-methyl-D-aspartate receptor activation mediates lung fibroblast proliferation and differentiation in hyperoxia-induced chronic lung disease in newborn rats. *Respir Res* 17:136. <https://doi.org/10.1186/s12931-016-0453-1>
17. Mees L et al (2018) Faster emergence behavior from ketamine/xylazine anesthesia with atipamezole versus yohimbine. *PLoS ONE* 13:e0199087. <https://doi.org/10.1371/journal.pone.0199087>
18. Hu J et al (2018) Dexmedetomidine prevents cognitive decline by enhancing resolution of high mobility group box 1 protein-induced inflammation through a vagomimetic action in mice. *Anesthesiology* 128:921–931. <https://doi.org/10.1097/ALN.0000000000002038>
19. Tovar A et al (2020) Transcriptional profiling of the murine airway response to acute ozone exposure. *Toxicol Sci* 173:114–130. <https://doi.org/10.1093/toxsci/kfz219>
20. Bhalla DK, Reinhart PG, Bai C, Gupta SK (2002) Amelioration of ozone-induced lung injury by anti-tumor necrosis factor-alpha. *Toxicol Sci* 69:400–408. <https://doi.org/10.1093/toxsci/69.2.400>
21. Cho HY, Zhang LY, Kleeberger SR (2001) Ozone-induced lung inflammation and hyperreactivity are mediated via tumor necrosis factor-alpha receptors. *Am J Physiol Lung Cell Mol Physiol* 280:L537–546. <https://doi.org/10.1152/ajplung.2001.280.3.L537>
22. Johnston RA, Schwartzman IN, Flynt L, Shore SA (2005) Role of interleukin-6 in murine airway responses to ozone. *Am J Physiol Lung Cell Mol Physiol* 288:L390–397. <https://doi.org/10.1152/ajplung.00007.2004>
23. Park JW et al (2004) Interleukin-1 receptor antagonist attenuates airway hyperresponsiveness following exposure to ozone. *Am J Respir Cell Mol Biol* 30:830–836. <https://doi.org/10.1165/rcmb.2003-0373OC>
24. Michalec L et al (2002) CCL7 and CXCL10 orchestrate oxidative stress-induced neutrophilic lung inflammation. *J Immunol* 168:846–852. <https://doi.org/10.4049/jimmunol.168.2.846>
25. Francis M et al (2017) Editor's highlight: CCR2 regulates inflammatory cell accumulation in the lung and tissue injury following ozone exposure. *Toxicol Sci* 155:474–484. <https://doi.org/10.1093/toxsci/kfw226>
26. Yuki K et al (2021) Anesthetics isoflurane and sevoflurane attenuate flagellin-mediated inflammation in the lung. *Biochem Biophys*

- Res Commun 557:254–260. <https://doi.org/10.1016/j.bbrc.2021.04.045>
27. Tighe RM, Birukova A, Yaeger MJ, Reece SW, Gowdy KM (2018) Euthanasia- and lavage-mediated effects on bronchoalveolar measures of lung injury and inflammation. *Am J Respir Cell Mol Biol* 59:257–266. <https://doi.org/10.1165/rcmb.2017-0357OC>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.