Optimizing anesthesia and delivery approaches for dosing into lungs of mice

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Abstract

Microbes, toxins, therapeutics and cells are often instilled into lungs of mice to model diseases and test experimental interventions. Consistent pulmonary delivery is critical for experimental power and reproducibility, but we observed variation in outcomes between handlers using different anesthetic approaches for intranasal dosing into mice. We therefore used a radiotracer to quantify lung delivery after intranasal dosing under inhalational (isoflurane) versus injectable (ketamine/xylazine) anesthesia in C57BL/6 mice. We found that ketamine/xylazine anesthesia resulted in delivery of a greater proportion (52±9%) of an intranasal dose to lungs relative to isoflurane anesthesia (30±15%). This difference in pulmonary dose delivery altered key outcomes in a model of viral pneumonia, with mice anesthetized with ketamine/xylazine for intranasal infection with influenza A virus developing worse lung pathology and more consistently losing body weight relative to control animals randomized to isoflurane anesthesia. Pulmonary dosing efficiency through oropharyngeal aspiration was not affected by anesthetic method and resulted in delivery of 63±8% of dose to lungs, and a non-surgical intratracheal dosing approach further increased lung delivery to 92±6% of dose. We conclude that anesthetic approach and dosing route can impact pulmonary dosing efficiency. These factors should be considered when planning and reporting studies involving delivery of fluids to lungs of mice.

Introduction

Studies investigating lung infections, lung injury, allergic airway inflammation, lung fibrosis, lung cancer, and lung stem cell biology often require delivery of experimental agents to lungs of mice. Administration routes for bolus dosing of fluids into lungs include intranasal (i.n.) dosing, intratracheal (i.t.) dosing and dosing through oropharyngeal aspiration (o.a.). Choice of dosing route is an important decision in study design as experimental outcomes can be altered by the quantity of dose delivered to lungs or to extrapulmonary tissues. Different dosing routes also vary in anesthetic requirements, invasiveness, and technical difficulty.

To guide experimental approach, a previous study assessed the effect of various factors including type of anesthetic on the distribution of i.n. doses into BALB/c mice. This study concluded that either injectable (Avertin) or inhaled (isoflurane, halothane) anesthetics resulted in similar delivery to lungs (1). Since this influential report, several factors have changed. Safety concerns have led to a decline in use of both Avertin and halothane (2, 3). Increased availability of knockouts and transgenics on the C57BL/6 (B6) background has led to B6 mice becoming the most widely used laboratory strain. Additionally, minimally invasive approaches for dosing via o.a. and i.t. routes have been developed which can more efficiently deliver fluids to lungs relative to i.n. dosing (4–6).

In our previous work we noticed that i.n. doses passed more readily into the nasal sinuses in studies where B6 background mice were anesthetized with ketamine/xylazine compared to experiments in which mice were anesthetized with isoflurane (7, 8), but we did not know whether pulmonary deposition of dose differed depending on anesthetic used during dosing. To guide future studies using these mice and anesthetics, we therefore measured the effect of anesthetic approach on i.n. delivery of fluid to lungs of B6 mice. As we have used and refined o.a. and i.t. methods, we also measured dose distribution using these administration routes.

We found striking effects of both anesthetic approach and dosing route on the efficiency of pulmonary delivery. Our results will be useful to guide design of experiments with improved reproducibility, with potential to reduce the number of mice needed to produce clear results from experiments involving dosing of fluids into lungs.

Methods

Animals

63 C57BL/6 background mice (Jax #000664) were housed at the UCSF Parnassus Laboratory
64 Animal Resource Center specific pathogen-free facility. Both male and female mice were used
65 at ages 6-14 weeks. Mice were kept on a 12-hour light-dark cycle. Protocols were approved by
66 the UCSF Institutional Animal Care and Usage Committee.

67 *Anesthesia*

68 Mice were anesthetized either by inhalation of isoflurane (4% in oxygen) or by intraperitoneal
69 (i.p.) injection with ketamine (70 mg/kg) and xylazine (15 mg/kg) in normal saline. For fluid
70 dosing to lungs, it is important not to overdose ketamine/xylazine as higher doses can cause
71 asphyxiation from aspirated fluid. For terminal anesthesia prior to collection of lung samples,
72 mice were euthanized with ketamine (100 mg/kg) and xylazine (40 mg/kg) prior to
73 exsanguination.

74 *Intranasal dosing*

75 Gel-loading pipette tips (Sorenson #13810) were used to introduce 50 µl of dose dropwise into
76 the posterior opening of one nare. Mice were held upright for 20 seconds after dosing to allow
77 aspiration of dose.

78 *Tracking radiolabeled albumin doses*

79 For quantitative and visual tracking of inoculum we used 50 µl of phosphate-buffered saline
80 containing ^{125}I-albumin (0.25 mg/ml, ~2.5 KBq/ml, Jeanatope, Iso-Tex Diagnostics, Inc.) and
81 Evans blue dye (1 mg/ml). Organ samples were collected 10 minutes after mice were dosed.
82 Dose distribution was measured using a gamma counter (Packard 5000 series) against three
83 standards containing 100% of injected dose.

84 *Influenza A virus infection model*

85 Mice were infected by the i.n. route with 50 plaque-forming units (p.f.u.) of influenza A virus
86 (A/PR/8/34 H1N1) propagated with Madin-Darby canine kidney (MDCK) cells. For propagation,
87 MDCK cells cultured in minimal essential medium (MEM) supplemented with 10% FBS and
88 penicillin/streptomycin in a humidified incubator at 37ºC and 5% CO_2 were infected and cultured
89 for 72h. The supernatant containing the virus was then collected and stored at -80ºC. Infectious
90 virus was quantified by culturing dilutions of the viral stock with MDCK cells in a 6-well plate for
91 1 hour, followed by addition of an overlay of 1.2% Avicel RC-581 in MEM, culture for 72 hours,
92 formalin fixation and staining with crystal violet for p.f.u. determination (9). Viral stocks were

93 diluted in sterile PBS at 4ºC prior to inoculation. Mice were dosed at zeitgeber time (ZT) 3-5 and
94 handled under biosafety level 2 conditions.

95 *Bronchoalveolar lavage analysis*

96 After terminal anesthesia and exsanguination, lungs were collapsed by opening the diaphragm
97 and tracheal insertion of 20G stub needles. A 1 ml syringe containing 1 ml of phosphate-
98 buffered saline was then washed in and out of the lungs three times to recover bronchoalveolar
99 lavage (BAL) fluid. BAL cells were counted using a LUNA-II automated cell counter (Logos
100 Biosystems) and BAL supernatant total protein was measured using a Pierce total protein assay
101 (Thermo Scientific, #23225).

102 *Dosing by oropharyngeal aspiration*

103 As previously described, anesthetized mice were placed on an intubation platform suspended
104 by their upper incisors with the tongue gently pulled out of the mouth (5, 10). The fluid dose was
105 then pipetted directly onto the distal oropharynx at 50 µl volume with both nares covered to
106 obligate breathing through the mouth. After ~30 seconds, mice were removed from the platform
107 and placed supine until sample collection.

108 *Non-surgical intratracheal dosing*

109 Mice were positioned as with o.a. dosing, with transillumination and adjustment of body position
110 used to visually identify the larynx (**Figure 4B**). Mice were orotracheally intubated with a 22G 1”
111 Safelet IV Catheter (Nipro, #CI+22225-2C) customized into an endotracheal tube and stylet with
112 addition of cushions (see **Figure 4A**). Tracheal positioning was confirmed by attaching a
113 manometer to the endotracheal tube and checking for oscillation of water column with breathing
114 movements (**Figure 4C**) (11). Dose at 50 µl volume was then injected through the endotracheal
115 tube using a customized 28G ½” insulin syringe (BD, #329461) with added PE-10 tubing and
116 cushion for placement within catheter (see **Figure 4A**), followed by 120 µl air.

117 *Experimental design and statistical analysis*

118 Mice were randomly assigned to groups with blocking by cage, and samples were collected and
119 quantified with investigators blinded to groups. For the influenza infection study, the handler
120 dosing mice was also blinded during dosing, with a second unblinded handler in control of
121 anesthesia. Group *n* was set prior to study initiation and analysis. Where necessary, data were
122 transformed prior to statistical testing according to distribution. Statistical analyses used
123 InVivoStat 4.4 (body weight and power analysis) or GraphPad Prism 9 (other comparisons). The

124 tests used for each analysis are stated in figure legends with P=0.05 as α threshold. Data are
125 reported as means ± standard deviation.

126 **Results**

127 In previous experiments we noticed that B6 mice anesthetized with ketamine/xylazine smoothly
128 aspirated i.n. doses, whereas i.n. doses sometimes bubbled back out of the nares of isoflurane-
129 anesthetized mice (7, 8). As previous studies assessing effects of anesthesia on i.n. delivery to
130 lungs used anesthetics or mouse strains not used in our protocols (1, 12, 13), we aimed to
131 determine whether use of isoflurane or ketamine/xylazine anesthesia during i.n. dosing affected
132 delivery of dose to lungs of B6 mice.

133 We found that relative to isoflurane anesthesia, use of ketamine/xylazine anesthesia during i.n.
134 dosing resulted in delivery of dose to more distal regions of lung (**Figure 1A**) and increased
135 pulmonary dosing efficiency (**Figure 1B**).

136 The i.n. dosing route is in widespread use in respiratory virus infection models. Current
137 protocols suggest that handlers can use either isoflurane or ketamine/xylazine anesthesia
138 during i.n. infection with influenza A virus (14). We therefore formally tested whether the effect of
139 anesthetic approach on intranasal dosing to lungs could be a factor altering outcomes and
140 reproducibility of studies of respiratory viral infection.

141 With one handler delivering anesthesia, and a second handler blinded to anesthetic approach
142 dosing and assessing mice, we gave B6 mice randomized to isoflurane or ketamine/xylazine
143 anesthesia prior to i.n. doses containing 50 p.f.u. of PR8 influenza A virus.

144 We observed bubbling of dose back out of nares and down the philtrum in mice in our
145 biodistribution study. During infection with PR8, the handler blinded to anesthesia approach
146 therefore recorded whether dose reflux was observed. We found that isoflurane-anesthetized
147 mice consistently refluxed some dose back out of their nares, whereas mice anesthetized with
148 ketamine/xylazine smoothly aspirated doses without visible reflux (**Figure 2A,B**).

149 We also monitored body weight daily as an index of general health status. All mice anesthetized
150 with ketamine/xylazine at time of infection had lost weight at day 9, but weight loss was
151 significantly lower in the isoflurane-anesthetized group from 5 to 9 days post infection, with
152 some mice in the isoflurane group gaining weight after inoculation (**Figure 2C**).

153 At 9 days post infection we collected bronchoalveolar lavage (BAL) fluid from infected mice to
154 measure vascular leak and leukocyte recruitment into lung airspaces as indices of lung injury

155 and inflammation. Both supernatant protein concentration and leukocyte counts were higher in
156 BAL fluid from ketamine/xylazine-anesthetized mice compared to isoflurane-anesthetized mice
157 (**Figure 2D,E**).

158 Using the BAL protein data in **Figure 2D** we ran a power analysis to determine the group size
159 needed for future experiments aimed at detection of a 30% change in BAL fluid protein
160 concentration using unpaired two-tailed t-tests. We found that 9 mice per group would be
161 needed to run such an experiment with 95% power with ketamine/xylazine anesthesia (**Figure**
162 **2F**). In comparison, the isoflurane anesthesia approach would likely not be feasible for
163 experimental use as an experiment with 25 mice per group would still have less than 50%
164 power (**Figure 2F**).

165 Together, these results indicate that isoflurane anesthesia spares a glottic reflex involving
166 sensing of fluid in the upper airways and limitation of aspiration into lungs. In contrast,
167 ketamine/xylazine anesthesia blocks this reflex, facilitating aspiration of a greater proportion of
168 i.n. dose. This effect means that the two anesthesia approaches yield different efficiency and
169 distribution of i.n. dosing efficiency to the lungs, affecting key outcomes in a respiratory virus
170 infection model.

171 Compared with i.n. dosing, the o.a. route involving aspiration from the distal oropharynx can
172 result in less exposure of nasal sinuses to inoculum and increased dosing efficiency to the
173 lungs. Since anesthetic type affected i.n. dosing, we sought to also determine whether different
174 anesthesia approaches altered delivery of o.a. doses to the lungs.

175 Tracking dose delivery visually and quantitatively, we did not detect any effect of anesthesia
176 approach on o.a. dose delivery to the lungs (**Figure 3A,B**). Breath-holding responses were
177 observed in some isoflurane-anesthetized mice after the dose was dropped onto the
178 oropharynx, but doses were eventually aspirated with reflux prevented by retraction of the
179 tongue and covering the nares.

180 We conclude from this study that anesthetic approach is therefore unlikely to have a major
181 impact on dosing to the lungs via the o.a. route.

182 Non-surgical i.t. dosing approaches have potential for more precise lung dosing relative to i.n.
183 and o.a. dosing. Previous studies suggest that o.a. dosing can yield similar dosing efficiency
184 compared to i.t. dosing, but these reports have not directly measured lung delivery using the
185 latest non-surgical i.t. approaches (15–18). We have optimized an approach for i.t. dosing

186 involving direct visualization of the larynx, orotracheal intubation with customized catheter,
187 confirmation of airway placement using a manometer, and then injection using a customized
188 syringe (5, 6, 10, 11) (**Figure 4A-C**), We therefore sought to measure pulmonary dosing
189 efficiency using our non-surgical approach for i.t. dosing, comparing to a control group dosed
190 with the o.a. approach, using ketamine/xylazine anesthesia.

191 We found that i.t. dosing yielded increased pulmonary dose deposition relative to o.a. dosing
192 (**Figure 4D,E**). This result is indicative that although o.a. and i.t. routes deliver the majority of
193 injected dose to the lungs, i.t. dosing might be desirable in situations where precise dosing to
194 lungs is needed.

195 **Discussion**

196 In this study, we found that anesthetic approach and administration route can affect the
197 efficiency of fluid dosing to lungs of mice.

198 We conclude from our results that ketamine/xylazine anesthesia is preferable where consistent
199 i.n. dosing to lungs of B6 mice is needed. Our results in the influenza infection model
200 demonstrate that optimal sedation for i.n. dosing has potential to reduce the number of mice
201 needed in experiments, and that the anesthesia approach used during i.n. dosing should be
202 reported for experimental reproducibility. The effects we found also suggest that tracking
203 biodistribution of i.n.-dosed radiotracers might be useful for quantitative in vivo studies of the
204 interaction of anesthetics with airway-protective glottic reflexes.

205 A likely consequence of poor delivery to lungs using i.n. dosing under isoflurane anesthesia is
206 that inoculating doses containing greater quantities of virus will be used to produce infections
207 that consistently result in robust lung inflammation, exposing extrapulmonary tissues to higher
208 quantities of virus. This is not desirable as exposure of the nasal sinuses to high quantities of
209 viral particles could cause serious adverse effects, as recently demonstrated in a study showing
210 lethal SARS-CoV-2 neuroinvasion when K18-hACE2 mice were infected using intranasal dosing
211 but not when mice were infected using aerosolized virus (19).

212 Injectable ketamine/xylazine anesthesia may not always be preferable as recovery time can be
213 longer than with isoflurane, and use of needles is discouraged where possible due to safety
214 risks. In our study, we found that it was feasible to give mice i.p. ketamine/xylazine injections in
215 a biosafety cabinet separate from that used for handling virus to minimize infection risk to
216 handlers. Limiting dose reflux using ketamine/xylazine anesthesia might also reduce risk of

aerosolization and surface contamination by inoculum. In studies where severe viral pneumonia is not of interest, the limited pneumonia and patchy pathology we observed using isoflurane anesthesia for i.n. infection with PR8 may be useful for studying infected lung lesions with minimal animal suffering.

Our study provides quantification of pulmonary dosing efficiency comparing i.n., o.a. and i.t. methods using anesthetics and mouse strains in current widespread usage. The percentage lung delivery values that we measured are largely consistent with those from previous studies which used a range of mouse strains, anesthetic approaches and methods for measuring dose distribution (1, 4, 12, 13). Our conclusion that anesthetic type can alter lung deposition of i.n. doses differs from that of a previous study which found no effect of different anesthetics (isoflurane, halothane, Avertin) during i.n. dosing on pulmonary dosing efficiency in BALB/c mice, although ketamine/xylazine was not examined in this previous report (1). Curiously, another study using BALB/c mice found increased bacterial content of lungs after intranasal dosing with *Francisella tularensis* under isoflurane compared to ketamine/xylazine anesthesia (20). Effects of anesthetics may therefore vary depending on mouse strain and pathogen biology.

In summary, we recommend the use of ketamine/xylazine anesthesia over isoflurane anesthesia for i.n. dosing into lungs of B6-background mice. Where needed, pulmonary dosing efficiency can be increased using the o.a. route, and further still using i.t. dosing. Anesthetic approach and administration method are factors that can alter outcomes of studies involving dosing to lungs, affecting the number of mice required for experiments. To increase reproducibility and decrease animal usage, these factors should therefore be considered during experimental design and clearly reported in publications.

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Disclosures

The authors report no conflicts of interest.

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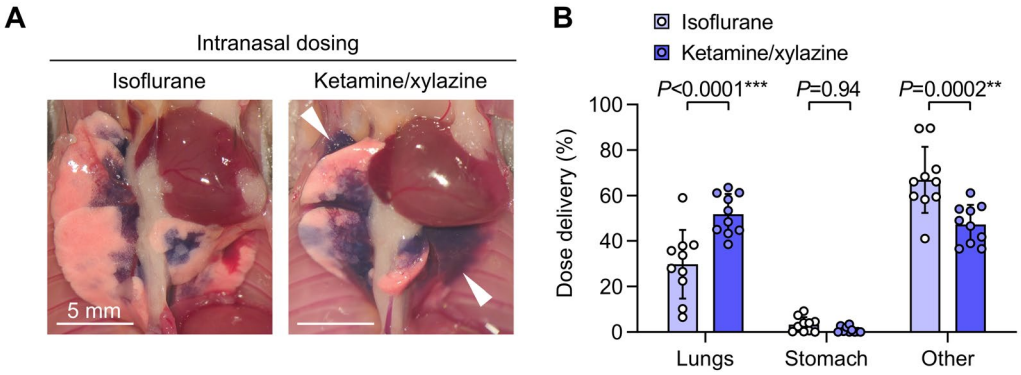

Figure 1. Increased pulmonary dosing efficiency with ketamine/xylazine versus isoflurane anesthesia during intranasal dosing.

A. B6 mice were given intranasal (i.n.) doses containing ^{125}I-albumin radiotracer and Evans blue dye under either isoflurane or ketamine/xylazine anesthesia. Photographs show lungs after euthanasia and thoracotomy, with delivery of dye to more distal regions of the lungs with ketamine/xylazine anesthesia (white arrowheads).

B. Effect of anesthetic type on dose distribution quantified using radiotracer**.**

Means ± standard deviation, n=10. *P*-values are from an ordinary two-way ANOVA with Holm-Šídák tests for effect of dosing route within each location.

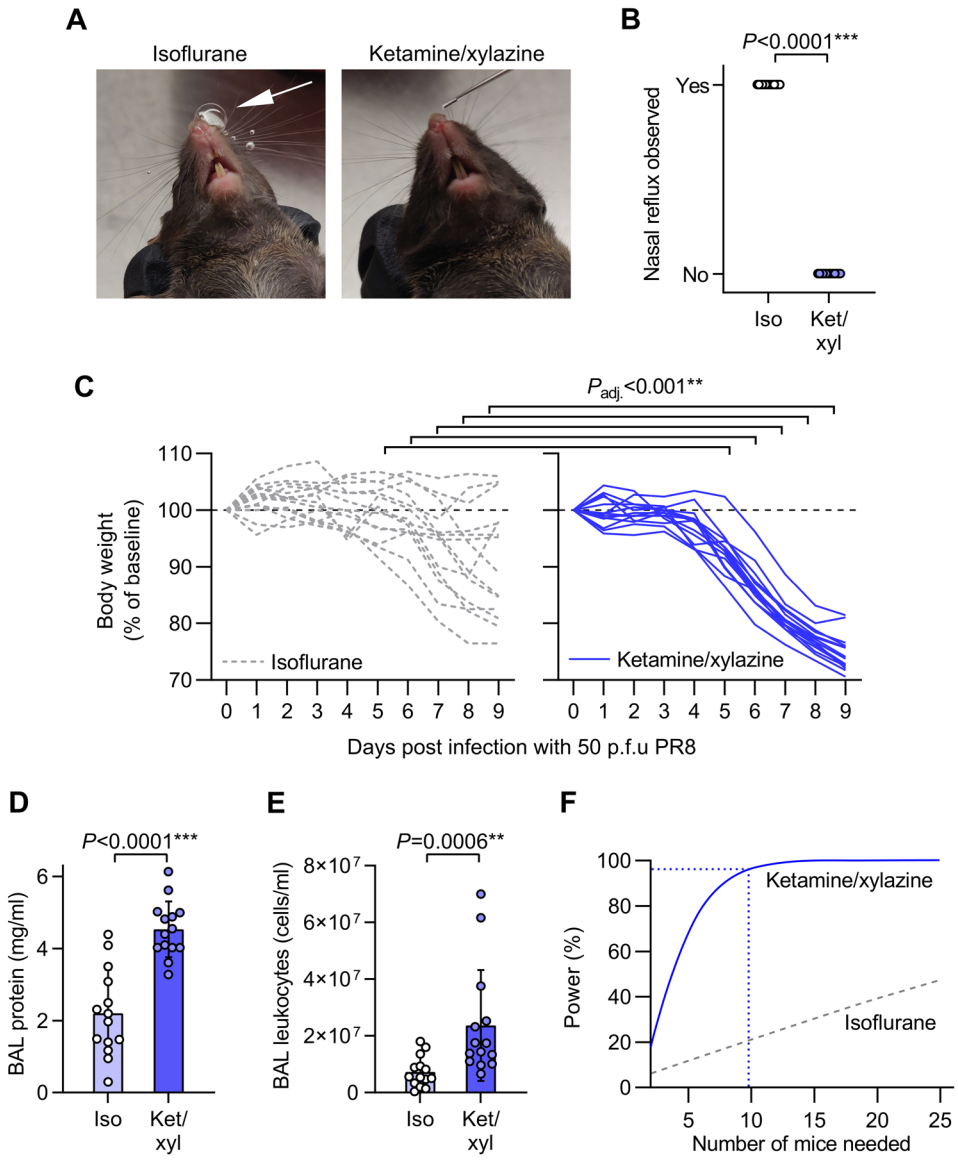

Figure 2. Increased body weight loss and lung inflammation after intranasal infection with influenza A virus under ketamine/xylazine relative to isoflurane anesthesia.

A. Mice were randomized to receive either isoflurane or ketamine/xylazine anesthesia for intranasal dosing with 50 p.f.u. of H1N1 influenza A virus A/PR/8/1934 (PR8). Photographs of mice show presence of nasal reflux during intranasal dosing under isoflurane anesthesia (white arrow) but not ketamine/xylazine anesthesia.

B. Quantification of incidence of nasal reflux during intranasal dosing under isoflurane (iso) versus ketamine/xylazine (ket/xyl)

C. Body weight changes over 9 days post infection.

D. BAL supernatant protein concentration at day 9 post infection.

E. Leukocyte counts from BAL fluid at day 9 post infection.

F. Output of power analysis using total protein data in Figure 2D to estimate number of mice needed per group to detect a 30% change in BAL protein concentration using an unpaired two-tailed t-test.

Means ± standard deviation, n=14. *P*-values are from: B: Fisher's exact test, C: repeated measures mixed model approach with baseline values as covariates and adjusted (adj.) *P*-values from Holm's tests for effect of anesthesia within each time point; D,E: unpaired two-tailed t-tests.

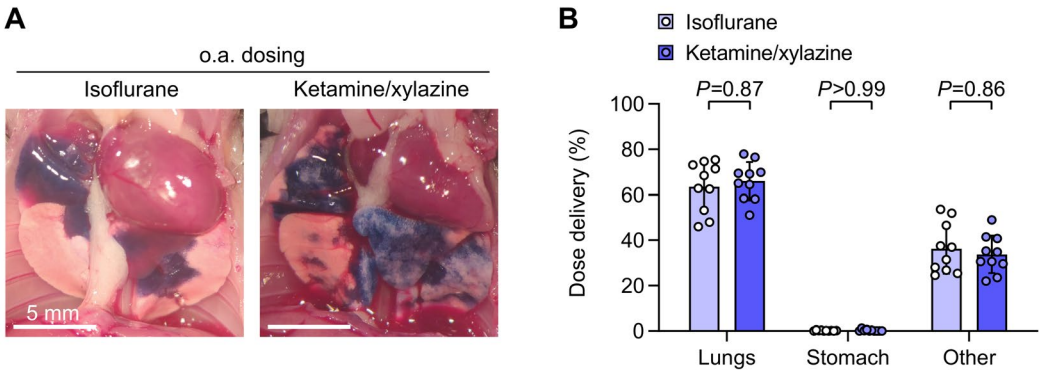

Figure 3. Pulmonary dosing efficiency using oropharyngeal aspiration is not altered by anesthetic type.

A. B6 mice were given intranasal (i.n.) doses containing ^{125}I-albumin radiotracer and Evans blue dye under either isoflurane or ketamine/xylazine anesthesia and euthanized 10 minutes later. Assessment of lungs after thoracotomy showed bilateral delivery of dye to distal regions of lungs with both anesthetic types.

B. No effect of anesthesia approach was found on biodistribution of radiotracer.

Means ± standard deviation, n=10. *P*-values are from an ordinary two-way ANOVA with Holm-Šídák tests for effect of dosing route within each location.

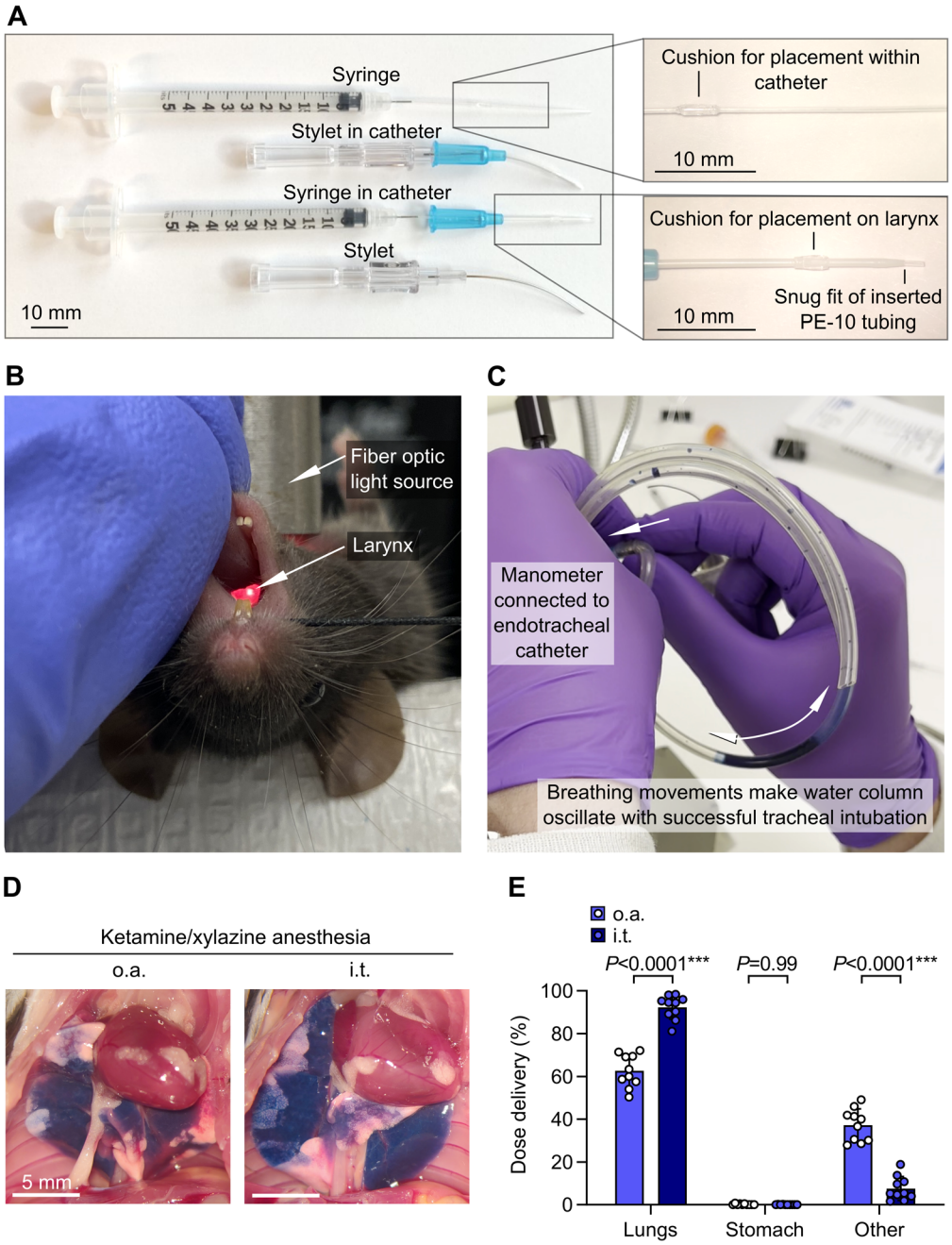

Figure 4. Increased pulmonary dosing efficiency with non-surgical intratracheal dosing relative to oropharyngeal aspiration.

A. Customized catheters and syringes for orotracheal intubation and precise intratracheal injections through endotracheal tube.

B. Mouse on intubation platform showing direct visualization of laryngeal inlet through transillumination the trachea.

C. Manometer used for confirmation of correct airway placement after orotracheal intubation.

D. B6 mice were anesthetized with ketamine/xylazine and then given ^{125}I-albumin and Evans blue by either oropharyngeal aspiration (o.a.) or intratracheal (i.t.) routes. Photographs show representative dose distribution.

E. Effect of administration approach on dose distribution quantified using radiotracer.

Means ± standard deviation, n=10. P-values are from an ordinary two-way ANOVA with Holm-Šídák tests for effect of dosing route within each location.