Rats Sniff off Toxic Air

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TOC

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(*RSTair*)

36 Abstract

37 Breathing air is a fundamental human need, yet its safety, when challenged by
 38 various harmful or lethal substances, is often not properly guarded. For example, air
 39 toxicity is currently monitored only for single or limited number of known toxicants,
 40 thus failing to fully warn against possible hazardous air. Here, we discovered that
 41 within minutes living rats emitted distinctive profiles of volatile organic compounds
 42 (VOCs) via breath when exposed to various airborne toxicants such as endotoxin, O₃,
 43 ricin, and CO₂. Compared to background indoor air, when exposed to ricin or
 44 endotoxin aerosols breath-borne VOC levels, especially that of carbon disulfide, were
 45 shown to decrease; while their elevated levels were observed for O₃ and CO₂
 46 exposures. A clear contrast in breath-borne VOCs profiles of rats between different
 47 toxicant exposures was observed with a statistical significance. Differences in
 48 MicroRNA regulations such as miR-33, miR-146a and miR-155 from rats' blood
 49 samples revealed different mechanisms used by the rats in combating different air
 50 toxicant challenges. Similar to dogs, rats were found here to be able to sniff against
 51 toxic air by releasing a specific breath-borne VOC profile. The discovered science
 52 opens a new arena for online monitoring air toxicity and health effects of pollutants.

53

54 **Keywords:** Online Monitoring Toxic Air, Rat, Volatile Organic Compound,
 55 microRNA,

56

1. Introduction

Polluted air is a complex mixture of various pollutants, including particulate matter (PM), biologicals, and also gaseous substances such as O₃ and NO_x. Inhaling these pollutants can cause a variety of serious health problems: respiratory and cardiovascular diseases, and even death.¹⁻² For example, PM alone was shown to have resulted in 4.2 million deaths in 2015.²⁻³ Exhibiting a positive correlation with daily mean mortality, ground ozone exposure resulted in decreased lung function and airway inflammation.⁴⁻⁵ In the meantime, exposure to pathogenic bioaerosols including bacteria, fungi, virus, et al in the air can cause respiratory infections with grave human and economic costs.⁶⁻⁹ Apart from these, there is a growing risk of terrorist attacks by intentionally releasing biological and chemical agents into the air to cause substantial civilian casualties.¹⁰⁻¹² Apparently, inhaling unsafe air has become an increasing health concern. Yet, in many high profile events, in addition to the public sectors, the air being inhaled is not readily protected or properly guarded. Real-time monitoring of air toxicity is of great importance, which however is a long-standing significant challenge in the field.

For monitoring hazardous substances in the air, a variety of real-time online monitoring methods have been previously developed or tested for individual pollutants such as the PM and other chemicals.¹³⁻¹⁶ While for bioaerosols, the adenosine triphosphate (ATP) bioluminescence technology, surface-enhanced Raman spectroscopy (SERS), bioaerosol mass spectrometry (BAMS), ultraviolet aerodynamic particle sizer (UVAPS) as well as silicon nanowire (SiNW) biosensor were investigated and attempted over the years.¹⁷⁻¹⁹ It is well known that these existing or developed technologies are mostly restricted to either single agent or

82 overall microbial concentration levels without identifying species. In addition,
 83 airborne pollutants and toxicity could vary greatly from one location to another ²⁰⁻²¹,
 84 thus presenting location-specific air toxicity and health effects. Current
 85 epidemiological or toxicological methods involving data analysis or animal and cells
 86 experiments cannot provide in situ air toxicity information, accordingly failing to
 87 represent the response at the time of exposure since biomarker levels evolve over time.
 88 ²² In addition, under certain scenarios (high profile meetings or locations) a rapid
 89 response to air toxicity needs to be in place in order to protect the interests. However,
 90 the response time is very demanding for an immediate effective counter-measure, for
 91 example, usually several minutes can be tolerated. ^{10, 23} In many air environments,
 92 multiple hazardous pollutants could also co-exist even with unknown ones at a
 93 particular time, which makes protecting the air rather difficult, if not impossible,
 94 using current technologies of species level detection and warning.

95
 96 Previously, olfactory receptors of mouse cells for odors ²⁴, immune B cells ²⁵ for
 97 pathogen detections, and silicon nanowire sensor arrays for explosives²⁶ were studied.
 98 Recently, a breath-borne biomarker detection system (dLABer) integrating rat's breath
 99 sampling, microfluidics and a silicon nanowire field effect transistor (SiNW FET)
 100 device has been developed for real-time tracking biological molecules in the breath of
 101 rats exposed to particulate matter (PM).²⁷ The dLABer system was shown to be able
 102 to online detect interleukins-6 (IL-6) level in rat's breath, and capable of
 103 differentiating between different PM toxicities from different cities using the
 104 biomarker level. However, as observed in the study the production of protein
 105 biomarkers could significantly lag behind the pollutant exposure, thus falling short of
 106 providing a timely warning against toxic air. Nonetheless, exhaled breath is

107 increasingly being used in biomarker analysis in both medical and environmental
108 health studies.²⁸⁻³⁰ In addition to protein biomarkers, a large number of volatile
109 compounds (e.g., nitric oxide, carbon monoxide, hydrocarbons) have been also
110 studied to assess health status and even developed for clinical diagnosis.³¹⁻³² For
111 example, ethane and n-pentane detected in the breath were linked to the *in vivo* level
112 of lipid peroxidation and oxidative stress³³; breath-borne acetone was shown to
113 correlate with the metabolic state of diabetic patients.³⁴ In addition, exhaled VOCs
114 have been used for the diagnosis of asthma, lung cancer and other diseases.³⁵⁻³⁷
115 Undoubtedly, breath-borne VOC has emerged as a promising biomarker for health or
116 environmental exposure monitoring.

117

118 Inspired by the dog sniff for explosive, the work here was conducted to
119 investigate if we can use breath-borne VOCs from living rats to real-time monitor
120 toxic air. Particularly, we wanted to investigate: 1) When rats are exposed to air
121 toxicants, whether the VOC species and concentration in the exhaled breath change?
122 If yes, how long does the change need to occur? 2) Are there any specific exhaled
123 VOCs in response to different toxicants exposure including both chemical and
124 biological agents? 3) To develop an online air toxicity analyzing system via real-time
125 monitoring of exhaled VOCs of rats. The work here has demonstrated a great promise
126 for online air toxicity monitoring using the method developed, and opened a new
127 arena for studying health effects of air pollution.

128

129 **2. Materials and methods**

130 **2.1 Rat breeding**

131 The Jugular Vascular Catheterizations (JVC) rat model described in our previous

study was used in this work.³⁸ Weighing 200–240 g at an age of 10 weeks, a total of 20 male Wistar rats with jugular vein catheterization operation and a 100 mL/min inhalation rate were purchased from a local provider (Beijing Vital River Laboratory Animal Technology Co., Ltd.). With about 1 centimeter out of the skin, a flexible sterile catheter was embedded into the jugular vein and fixed on the back with staples. Under a 12 h light/12 h dark cycle, all the rats were raised in an animal care facility naturally with a normal chow diet. After 1 week of acclimation, the rats were randomly divided into 5 groups (4 rats in each group) for the exposure of different air toxicants. All animal experiments were approved by the Institutional Review Board of Peking University and relevant experiments were performed in accordance with the ethical standards (approval # LA2019294).

2.2 Preparation of toxic air

In this work, four different exposure toxicants (ricin, endotoxin, ozone and carbon dioxide) and indoor air (as a background control) (5 groups) for rats were prepared for the exposure experiments. Ricin was extracted from the seeds of castor produced in Xinjiang, China, and prepared by Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences in Beijing. The endotoxin was purchased from Associates of Cape Cod, Inc., USA. The ricin and endotoxin suspensions were prepared by vigorously vortexing 40 µg of ricin or 50 ng endotoxin per ml deionized (DI) water for 20 min at a vortex rate of 3200 rpm (Vortex Genie-2, Scientific Industries Co., Ltd., USA). Detailed information about ricin preparation and exposure can be also found in another work.³⁹ Here, ozone was generated by an ozone generator (Guangzhou Environmental Protection Electric Co., Ltd., China) using corona discharge method. The ozone was further diluted with indoor air for rat

157 exposure experiments, and the ozone concentration in the exposed chamber was
158 approximately 5 ppm. Carbon dioxide was purchased from Beijing Haike Yuanchang
159 Utility Gas Co., Ltd., and diluted 20 times with indoor air to a concentration of about
160 5% (5×10^4 ppm). The major objective of this work was to study whether there will be
161 specific VOC release by rats when challenged with different toxicants, not specific
162 dose-response for each toxicant. Nonetheless, the selection of specific exposure levels
163 for different toxicants was provided and discussed in Supporting Information.

164

165 ***2.3 Rats sniff off toxic air (RST_{air}) system and experimental procedure***

166 To investigate whether we can use breath-borne VOCs from living systems to
167 real-time monitor toxic air, we have developed the system named as *RSTair* (Rats
168 Sniff Off Toxic Air). As shown in Figure 1 and Figure S1 (Supporting Information),
169 the system is composed of four major parts: toxicant generator, exposure chamber,
170 exhaled breath sampling and online VOC analysis. Indoor air was used as carrier gas
171 for generating toxicant aerosols (ricin and endotoxin) using a Collison Nebulizer (BGI,
172 Inc., USA) or diluting toxicants gas (ozone and carbon dioxide). The toxicant aerosol
173 or toxicant gas was introduced together with indoor air into the exposure chamber at a
174 flow rate of 1 L/min. As shown in Figure S1, a cage was used as the exposure
175 chamber which can allow rat's feces and urine to fall from below quickly so as to
176 reduce their influences on VOC analysis. In addition, teflon tubes and vials were also
177 applied to reducing adsorption loss of VOCs. For ricin and endotoxin, they are small
178 molecules and relevant particle loss on the aerosolization tubing could be negligible.
179 When performing the experiments, the rats were first placed in the exposure chamber.
180 Indoor air was continuously introduced into the chamber at a flow rate of 1 L/min for
181 10 minutes, then followed by each of tested toxicants together with indoor air at the

182 same flow rate for about 10 minutes to conduct exposure tests.

183

184 For each toxicant experiment, the TVOC was first measured for the control (rat
185 with indoor air at a flow rate of 1 L/min), then followed by the toxicant aerosol/gas
186 exposure (rat + toxic air) at the same flow rate for 10 min. Here, the photoionization
187 detector (PID) (MOCON, Inc., USA) coupled with an air pump was used to real-time
188 monitor TVOC at a flow rate of 0.6 L/min. After the exposure, the TVOC was
189 continuously measured again using the PID sensor, reaching a relatively stable level
190 approximately within 7-8 min. In the meantime, VOC samples were also collected
191 both before (control) and after the toxicant exposure. A 3.2 L Silonite canister (Entech
192 Instruments, Inc., USA) was used to collect VOC samples at the flow rate of 0.8
193 L/min at least 10 min after the exposure test, and the VOCs species were further
194 analyzed using a gas chromatograph-mass spectrometry/flame ionization detection
195 (GC-MS/FID) system (Agilent Technologies, Inc., USA). A total of 117 VOC species
196 were screened and quantified for all exposure tests using the GC-MS system. During
197 the VOC sample collection, the PID sensor was switched to measure the indoor air
198 TVOC levels. The detailed descriptions of the working principles of PID and VOC
199 species analysis by GC-MS/FID are provided in Supporting Information. In this work,
200 both TVOC and VOC species in the exposure chamber were analyzed for all the
201 experiments: 1) when the rats were not in the chamber (background air); 2) rats in the
202 chamber (before toxicant exposure) and rats in the chamber (after toxicant exposure).
203 The air flows in and out of the exposure chamber were the same both for TVOC
204 measurements and VOC collection. Based on the dimensions of the exposure chamber,
205 the residence time of pollutants in the chamber was about 5 min under the
206 experimental conditions, e.g., the air flow rate was about 1 L/min. We collected VOC

samples at least 10 min after the toxicant exposure, therefore remaining secondary pollutants produced, if any, during the exposure tests such as O₃ will be flushed out by the indoor air. For each toxicant, the above experiments were repeated four times with a different rat each time. Each rat (total four) either for toxicant or control exposure group experienced the exposure only once, not repeated exposures. For each of four tested toxicants, the same experiments were repeated. All tests were performed inside a Class 2 Type A2 Biological Safety Cabinet (NuAire, Plymouth, MN), and all exposure toxicants were ventilated out via the built-in and lab ventilation system.

Figure 1. Rats sniff off toxic air (*RSTair*) system: different toxicants were introduced or aerosolized into the chamber for exposure. The VOCs from the rat placed in the chamber before and after the toxicant exposure for 10 min were analyzed by the PID directly and also by GC–MS/FID method coupled with a VOC sampler silonite canister. During the VOC collection and measurements by the PID, the toxicant exposure was terminated. Each time only one rat was placed into the chamber. For each toxicant, the experiment was independently repeated four times with a different

227 rat each time.

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229

230 **2.4 Blood microRNA detection**

231 Right before and 20 min after each 10-min toxicant exposure, 0.75 mL blood
232 samples from rats were taken through the catheter using sterile syringes with 23G
233 flat-end needles and kept at -20 °C for microRNA analysis. The blood microRNAs in
234 the blood samples such as miR-125b, miR-155, miR-146a, miR-21, miR-20b,
235 miR-210, miR-122, and miR-33 were analyzed using a RT-qPCR array (Wcgene
236 Biotech, Inc., China). Total RNAs in the blood samples, including microRNAs, were
237 extracted using a Trizol reagent (Sigma Aldrich, Inc., USA) according to the
238 manufacturer's instructions. Subsequently, the purified RNAs were polyadenylated
239 through a poly(A) polymerase reaction and was then reversed-transcribed into
240 complementary DNA (cDNA). TIANGEN® miRcute Plus miRNA First-Strand cDNA
241 Kit (Code No. KR211) was used in the reverse transcriptional reaction system of total
242 10 µL, including 5 µL 2x miRNA RT Reaction Buffer, 1 µL miRNA RT Enzyme
243 Mix and 4 µL RNA sample. The reaction conditions are 40 °C for 60 mins and 95 °C
244 for 3 mins. The cDNA was quantified in real-time SYBR Green RT-qPCR reactions
245 with the specific microRNA qPCR Assay Primers. TIANGEN® miRcute Plus miRNA
246 qPCR Kit (SYBR Green) (Code No. FP411) was used in the qPCR reaction system of
247 total 10 µL, including 5 µL 2x miRcute Plus miRNA PreMix (SYBR&ROX),
248 0.2 µL Forward Primer, 0.2 µL Reverse Primer, 1 µL 50X ROX Reference Dye,
249 1 µL DNA Sample and 2.6 µL ddH₂O. The cycling conditions were 95 °C for 15
250 min, followed by 40 cycles at 94 °C for 20s, 60 °C for 15s and 72°C for 30s. The
251 primers used for qPCR are presented in Table S1 (Supporting Information).

252

253 3. Statistical analysis

254 In this study, the TVOC levels for all samples detected by the PID sensor were
 255 not normally distributed, while their TVOC change rates were. The TVOC change
 256 rate was calculated by dividing the TVOC level after exposure using the TVOC level
 257 before exposure. Therefore, the Mann-Whitney rank sum test was used to analyze the
 258 differences in TVOC levels before and after each toxicant exposure, and t-test was
 259 used to analyze the differences in TVOC change rates between each toxicant exposure
 260 group and the control group (indoor air). For individual VOC concentrations by
 261 GC-MS/FID, the paired t-test was used to analyze differences for each VOC species
 262 before and after the exposure. The software Canoco 4.5 was used to visualize the
 263 VOC profile distance and relatedness between the samples of different groups using
 264 the principal component analysis (PCA). Besides, the concentrations of micro-RNAs
 265 in blood samples from different rat groups were determined by RT-qPCR. For the
 266 group exposed to carbon dioxide, blood samples were only taken from two rats
 267 (before and after the 10-min exposure) because of the catheter blockage (unable to
 268 draw bloods) for the other two. For the other three groups, blood samples were
 269 obtained for all four rats. The differences between micro-RNA levels in blood samples
 270 before and after the exposure in one group were analyzed using a paired t-test (data
 271 exhibited a normal distribution) or Wilcoxon signed rank test (data did not follow a
 272 normal distribution). The outliers for normally distributed data were examined and
 273 eliminated by a Grubbs test. All the statistical tests were performed via the statistical
 274 component of SigmaPlot 12.5 (Systat Software, Inc., USA), and a *p-value* of less than
 275 0.05 indicated a statistically significant difference at a confidence level of 95%.

276

277

278 **4. Results and discussion**

279 ***4.1 TVOC monitoring for the toxicants exposure***

280 As described in the experimental section, four toxicants (ricin, endotoxin, ozone
281 and carbon dioxide) and indoor air (as a background control) were used for inhalation
282 exposure in rats. For each group, the TVOC levels of only 3 rats (PID
283 instrument/software failure for one out of 4 rats) were shown in Figure 2. The indoor
284 air background TVOC was found to be less than 0.04 ± 0.02 ppm. After one rat was
285 placed in the exposure chamber, the TVOC level in the cage was shown to first
286 gradually increase, then reach a relatively stable level after about 500 seconds. The
287 TVOC level before the exposure (rat + indoor air) when one rat was in the chamber
288 was about 2 ppm, except for the CO₂ group it was about 0.5-0.8 ppm (These
289 differences, if any, applied to both before and after exposure in one test, thus
290 presenting no influences on the same experiments). The differences in TVOC levels
291 for indoor air exposures (different times: “before” and “after”, but the same indoor air)
292 were small (the average change rate was about $-4\% \pm 1.4\%$ (95% confidence interval)),
293 although the Mann-Whitney Rank Sum Test showed that for each of the rats, the
294 difference (over some time for the indoor air) was significant ($p\text{-value} < 0.001$). The
295 background indoor air TVOC levels were 1.22-5.1% (detected) of the chamber TVOC
296 with rats together with indoor air or other toxicants). The fluctuations were taken into
297 account for each toxicant exposure test. Nonetheless, the fluctuations, if any, from
298 background indoor air had minor impacts on the TVOC levels measured for rats’
299 exposure tests. The change rates (n=3) of the control group (indoor air) then served as
300 the reference for other toxicant exposures in the statistical analysis. During the indoor
301 air experiment, the rats were seen to carry out normal life activities in the exposure

chamber, and correspondingly the TVOCs in the chamber were shown to remain relatively stable.

304

305

306

Figure 2. Real-time continuous measurements of exhaled TVOC levels in the chamber when rats were exposed to different toxicants via inhalation for 10 mins: Indoor air, ricin, endotoxin, O₃ and CO₂. During the exposure processes, the PID sensor was turned off. Data lines (measurement time was 1000 s) represent results from three individual rats (#1, #2, #3) before or after exposure to each of the air toxicants (aerosolized amounts described in the experimental section) tested. Each

313 exposure test was independently repeated with four rats from the same group (PID
314 instrument/software failure for one rat).

315

316

317 In contrast, the TVOC levels were shown to vary greatly with different toxicant
318 exposures as shown in Figure 2. For example, when rats were exposed to the
319 aerosolized ricin, the TVOC level was observed to exhibit an average change rate of
320 $-3\% \pm 1.6$. Compared to the control group (indoor air + rat before the exposure) shown
321 in Figure 2, the difference of the TVOC change rate was not significant for the ricin
322 exposure (t-test, $p\text{-value}=0.426$). For ricin exposure, its concentration (40 $\mu\text{g/mL}$
323 suspension aerosolized) might be too low in aerosol state after the aerosolization from
324 the liquid to produce a detectable response from the rats. It was previously reported
325 that 0.1 mg/mL ricin solution was used for aerosolization and subsequent exposure to
326 mice (Ref # 5, Supporting Information). After 30 minutes ricin aerosol exposure, the
327 exposed mice became poisoned. The concentration of ricin used in this experiment
328 was lower and the exposure time was shorter, therefore the toxicity reaction may be
329 mild. In contrast to the ricin exposure, however we observed a different phenomenon
330 for the endotoxin (50 ng/mL aerosolized) tests as shown in Figure 2. Upon the
331 endotoxin exposure, the TVOC level was observed to first increase, and then
332 decreased to a level that was about 21-46% below the pre-exposure level after four
333 minutes (Mann-Whitney Rank Sum Test, all $p\text{-values}<0.001$). Compared to the
334 control group (indoor air + rat), the difference of the TVOC change rate was
335 statistically significant for endotoxin (t-test, $p\text{-value}=0.0147$). The observed
336 differences from the ricin and endotoxin exposures could be due to different
337 mechanisms initiated by different substances involved. Ricin is derived from plant,

338 while endotoxin is from Gram-negative bacterial membrane. They could interact
339 differently with relevant human respiratory or other body cells. Nonetheless, for both
340 ricin and endotoxin, they were probably causing health effects by immuno-toxicity,
341 while O₃ and CO₂ both induce harm first by chemical manners.

342

343 After exposure to gaseous toxicants such as ozone and carbon dioxide, the levels
344 of TVOCs in the exposure chamber with rats were observed to have increased
345 significantly, as observed in Figure 2. As can be seen from the figure, the TVOC
346 levels has increased for about 44-110% for ozone and about 109-265% for carbon
347 dioxide exposure (Mann-Whitney Rank Sum Test, all *p-values*<0.001). The t-test
348 showed that differences of the TVOC change rates of both ozone and CO₂ exposures
349 compared to the control group (indoor air) were statistically significant
350 (*p-value*=0.0219 and 0.0296, respectively). These data indicated that rat exposure to
351 both ozone and CO₂ has resulted in significant elevations of TVOCs, suggesting rats
352 were actively responding to the exposure challenges. The behavior observation from a
353 video also indicated that rats after the exposure to O₃ seemed to be suffering from the
354 challenge (Video S1, Supporting Information).

355

356 ***4.2 Changes in exhaled VOCs after exposure to different toxicants***

357 Using GC-MS/FID method, a total of 31 different VOCs out of 117 screened
358 were detected and shown in Figure S2 (Supporting Information) for background
359 indoor air with and without rats. Among detected VOCs as shown in Figure S2
360 (Supporting Information), the VOCs with the highest concentrations in indoor air
361 were n-hexane, ethyl acetate and acetone. When rats were placed in the exposure
362 chamber (one rat at each time), the most abundant VOC species was detected to be

363 acetone, which was about 4 times more than that of the indoor air background.
 364 Statistical tests found that the concentrations of ethylene and ethane in the chamber
 365 containing one rat were significantly lower than those of the background (paired t-test,
 366 p -value<0.05), which in part could be due to the air dilution by the rat's breath.
 367 Namely, when rats' breath with specific higher or lower VOC species replaced
 368 equivalent indoor air inside the chamber, diluting effects for higher indoor VOC
 369 species and enhancing effects for lower indoor VOC species could take place.

370
 371 **Figure 3.** Differentiations of VOC species from indoor air and those from the rats'
 372 exhaled breath under different air toxicity with exposure to ricin, O₃, endotoxin and

CO₂. The red lines show that the average change ratios of every toxicant calculated by the level after the exposure divided by the level before exposure (right axis). The dotted line is the baseline with a change ratio of 1. Percentages refer to specific VOC percentage change before and after the exposure. Values represent averages and standard deviations from three different rats. “*” indicates a significant difference at $p\text{-value}<0.05$.

Differentiations of VOC species from the rat’s exhaled breath under different toxicants exposures were also shown in Figure 3. There were no significant differences in the concentrations of any VOCs before and after the exposure for the control group, i.e., indoor air (t-test, $p\text{-value}=0.05$). This suggests that indoor air is relatively less toxic at a level that is unable to detect a VOC change. In contrast, specific VOC species had experienced significant changes when rats were exposed to ricin, endotoxin, O₃ and CO₂ as observed from Figure 3. For example, exposure to ricin caused significant higher concentration of ethyl acetate (183% higher), while lower concentration of carbon disulfide (22% lower). As shown in Figure 3, after the endotoxin exposure process, concentrations of five VOC species: ethane, acetone, cyclopentane, carbon disulfide and methylcyclopentane were shown to be significantly different with those of before the exposure (t-test, all $p\text{-values}<0.05$). As can be seen from the results of the ozone exposure group in Figure 3, the concentrations of propionaldehyde, pentane, 2-butanone, hexane and 2-methylpentane exhibited significant differences before and after exposure (t-test, all $p\text{-values}<0.05$), in which all the VOCs except 2-methylpentane were elevated. In comparison, rat exposure to CO₂ resulted in acetone level increase by 34% (t-test, $p\text{-value}=0.0016$). These data suggest that exposure to different toxicants had led to production of

398 different profiles of VOC species in addition to their level changes.

399

400 ***4.3 Detection of micro RNAs in Blood Samples***

401 To further explore the VOC response mechanism of rats to toxicants exposure,
402 microRNAs (miRNA) in the blood samples were examined by an RT-qPCR assay.
403 Fold-changes in microRNA levels after toxicants exposure were shown in Table S2
404 (Supporting Information). The level of miR-33 in the blood of rats was shown to be
405 significantly lower than that before ricin exposure ($p\text{-value}<0.05$); after exposure to
406 ozone, miR-146a level in the blood samples of rats was significantly higher than those
407 before the exposure ($p\text{-value}<0.05$), while miR-155 was significantly lower than that
408 before the exposure ($p\text{-value}<0.05$). For other microRNAs as listed in Table S2, the
409 changes seemed to be insignificant (t-test, $p\text{-values}>0.05$).

410

411 As observed from Figure 4, PCA results revealed a clear contrast in breath-borne
412 VOC profiles of rats between different toxicants exposures. The VOC profiles of the
413 ozone exposure group was very different from that of the control group (indoor air)
414 and the VOCs profile of the ricin exposure group was the closest to that of the control
415 group, which agreed with TVOC level and VOC species profiles obtained above.
416 Overall, the experiments showed that rats responded differently to different toxicants
417 by releasing different VOC species owing to different mechanisms of toxicity: ozone
418 caused significant increases in various breath-borne VOCs; while endotoxin exposure
419 generally decreased the releasing of VOCs; and ricin and carbon dioxide exposure
420 resulted in one or two significant VOC species changes. In general, the results of
421 qualitative and quantitative analysis by the GC-MS/FID method agreed with the
422 TVOC level monitored by the PID sensor.

Figure 4. PCA ordinations of exhaled breath-borne VOCs profiles under exposures to different toxicants: ricin, endotoxin, O₃, CO₂ and control (indoor air). PC1 and PC2 are the first and second principal components. The VOCs species involved in the PCA analysis were the 12 species which were shown to have undergone changes after each toxicant exposure. Data presented in the figure were from three independent rats exposed to each toxicant.

As observed from Figure 3, exposure to ricin caused 183±143 % higher concentration of ethyl acetate, while 22±8 % lower concentration of carbon disulfide. It was previously reported the concentration of ethyl acetate was significantly higher in exhaled breath from people with cancer compared to the healthy group.^{40,41} In addition, *in vitro* experiments have shown the human umbilical vein endothelial cells (HUVEC) can produce ethyl acetate, which is presumably generated by a reaction of ethanol with acetic acid.⁴² It was demonstrated that ricin is not only responsible for the ricin intoxication through ribosomal inactivation and subsequent inhibition of protein synthesis and cell death, but also shows endothelial toxicity by acting as a natural disintegrin binding to and damaging human endothelial cells.⁴³ Therefore, the

toxicity of ricin on the endothelial cells might be the source of the higher concentration of ethyl acetate observed in this work. As a disease biomarker, carbon disulfide was observed in the exhaled breath.⁴⁴⁻⁴⁵ Recently, it was suggested that the carbon disulfide may be generated endogenously and play a role as a bioregulator.⁴⁶ Here, we observed that exposure to both ricin and endotoxin resulted in lower levels of breath-borne carbon disulfide compared to the control.

For CO₂ and endotoxin exposure, the observation for acetone was the opposite as shown in Figure 3. Acetone in exhaled breath was widely investigated in many studies as an important biomarker related to blood glucose and diabetes.^{34,47} Acetone is produced in the fatty acids metabolism by hepatocytes via decarboxylation of excess acetyl coenzyme A (Acetyl-CoA), and then oxidized via the Krebs cycle in peripheral tissue.⁴⁸ As shown in Figure 3, the acetone level increased by 34±9% as a result of CO₂ exposure, suggesting CO₂ caused hypoxia in rats, and led to increased respiration from rats. These increases in acetone level corresponded to TVOC level increase as determined by the PID sensor after the exposure to CO₂. However, when exposed to endotoxin, the acetone level in the exposure chamber decreased by about 10±6%, indicating that the respiration of the rats may be attenuated by the exposure of endotoxin. Clearly, the involved mechanisms by which endotoxin and CO₂ cause health effects to rats could be very different.

As observed in Figure 3, the increase of ethane level by endotoxin exposure suggested that lipid damage was induced by oxidative stress in the rat's body since ethane is acknowledged as a marker of lipid peroxidation and described to be generated by peroxidation of ω-3 polyunsaturated fatty acids.³³ In addition to ethane,

the increase of methylcyclopentane level as shown in Figure 3 might also be the result of the endotoxin exposure. Endotoxin has been shown to trigger inflammation through its interaction with the TLR4/CD14/MD2 receptor and then initiates a signal cascade. This reaction correspondingly results in the activation of transcription factor such as NF- κ B leading to the production of pro-inflammatory cytokines and type 1 interferons (IFNs), and finally results in systemic inflammatory response syndrome.⁴⁹ In general, in terms with the average fold changes, the concentration of total VOCs in the exhaled breath was relatively lower after the endotoxin exposure, which agreed with the results of TVOC obtained by the PID sensors.

475

In addition to these biologicals, we have also shown that exposure to chemicals such as ozone and CO₂ also resulted in in-vivo changes in VOC levels. From the fold changes of various VOC species such as propionaldehyde, N-pentane, 2-Butanone, and Hexane, the ozone exposure has resulted in an overall increase of VOCs in rats' exhaled breath, which agreed with the TOVC monitoring shown in Figure 2 by the PID sensors. It was previously indicated that increase in propionaldehydes, further products of lipid peroxidation, indicated more severe oxidative damage in rats following exposure to ozone.⁵⁰ Here, we also showed that after exposure to ozone changes in specific breath-borne VOCs and also certain miRNA regulations were detected. Ozone was described as a strong oxidizing agent, and can cause intracellular oxidative stress through ozonide and hydroperoxide formation.⁵¹ The mechanism of ozone oxidative damage involves the activation of Nrf2, heat shock protein 70, and NF- κ B, thus increasing expression of a range of proinflammatory cytokines such as TNF α and interleukin 1 β , and chemokines such as interleukin 8.⁵² The results above show that regardless of toxicant types breath-borne VOCs from rats experienced *in*

491 *vivo* changes.

492

493 Results of miRNAs from rats' blood as shown in Table S4 revealed different
 494 mechanisms by rats when exposed to various types of toxicants. miRNAs are short
 495 non-coding RNA sequences that regulate gene expression at the posttranscriptional
 496 level; and many miRNAs have already been identified to influence physiological
 497 processes such as immune reaction, adaptation to stress, and widely investigated in
 498 environmental exposure studies.⁵³ Among these microRNAs, miR-125b, miR-155,
 499 miR-146a, and miR-21 are mainly shown to regulate oxidative stress and
 500 inflammatory processes *in vivo*, and widely investigated in air pollution related
 501 studies.⁵³⁻⁵⁴ For example, among them, miR-155 has a positive regulation function,
 502 and the other three are negative regulation.⁵⁵⁻⁵⁶ However, in this study, the ozone
 503 exposure resulted in decreased levels of miR-155 (t-test, *p-value*<0.05), which are
 504 different from previous reports in the literature.⁵⁵ The difference could be due to
 505 different exposure toxicant, i.e., ozone used here, leading to acute lung damages
 506 compared to mild airway inflammation or asthma problems in other studies. Previous
 507 studies have shown that miR-20b and miR-210 are hypoxia regulators in animals, and
 508 miR-122 and miR-33 are mainly responsible for regulating lipid metabolism and
 509 glucose metabolism in the body.⁵⁷⁻⁵⁸ However, these microRNAs, except for the
 510 decreased level of miR-33 and increased level of miR-146a caused by ricin and ozone
 511 exposure, respectively, were shown to have not undergone significant changes in this
 512 study after the exposures to four different toxicants (*p-values*=0.05). The possible
 513 reason may be that microRNAs act as post-transcriptional regulators by degrading
 514 mRNA or inhibiting their translation, thus failing to respond in a timely manner
 515 during short-term exposure (blood samples taken 20 min after the 10-min exposure).

516 To further understand the problem, a yeast model is currently being used to fully
 517 investigate the mechanisms underlying the miRNA regulation when exposed to the
 518 toxicants used here. Nonetheless, these results here reflected that exposure to the
 519 toxicants led to specific miRNAs either up- or down-regulated. On the other hand, the
 520 results here also suggest, especially for those no changes observed for the miRNAs,
 521 higher toxicant level or specific longer time might be needed to allow miRNA
 522 regulation change to occur.

523

524 To address the major objective of this work, we repeated a total of 20 times using
 525 5 different exposure agents such as ozone, CO₂, ricin, endotoxin together with the
 526 background indoor air as a control, and 20 different rats. Clearly, above results
 527 indicate that when rats are exposed to toxic substances their certain metabolic
 528 activities are immediately affected, i.e., these exposures promoted or inhibited
 529 specific VOC productions. Based on the results we obtained from this work, the
 530 following VOC emission mechanisms of rats when exposed to different toxicants are
 531 proposed and illustrated in Figure 5. Previously, it was suggested that VOCs are
 532 produced during the normal metabolism in the body; while pathological processes,
 533 such as metabolic disorders, can also produce new species of VOCs or alter the levels
 534 of existing VOCs.⁵⁹ Therefore, cell or tissue injuries caused by external toxicants
 535 exposure also can alter the exhaled VOCs profile by disturbing the normal process.⁶⁰
 536 The exact toxic effect mechanism as observed from this work could vary from one
 537 toxicant to another. For some pollutants such as endotoxin and ricin, there are specific
 538 receptors to recognize them and then start the chain of responses or reactions.^{49,61}
 539 Among these various mechanisms, the ROS (reactive oxygen species) and oxidative
 540 stress are recognized to be the central and the common mechanism in various forms of

541 pathophysiology, as well as the health effect of various air pollutants including
542 ambient particulate matter (PM).^{1, 62-63} Oxidative stress is essentially a compensatory
543 state of the body and can trigger redox-sensitive pathways leading to different
544 biological processes such as inflammation and cell death.^{51,64} For example, the strong
545 oxidants such as ozone might cause oxidative stress through direct effects on lipids
546 and protein⁶⁵⁻⁶⁶, which mostly caused the generation and release of hydrocarbons and
547 aldehydes, such as ethane, ethylene, and propionaldehyde.^{50,67} While carbon dioxide
548 tends to make the redox balance tilted toward the reduction side by reducing oxygen
549 supply and thus influencing the energy metabolism in cells.⁶⁸⁻⁷⁰ For ricin and
550 endotoxin exposure, the underlying mechanisms seem to be different from ozone and
551 CO₂, and they could cause oxidative stress indirectly through the activation of
552 intracellular oxidant pathways. Nonetheless, all toxicants share a common effect of
553 disrupting the redox balance, and thus interfere with normal biochemical reactions or
554 cause material damages in cells, accordingly changing the VOC profile released into
555 the breath. As discussed above, in this work, the VOCs profile of rats changed
556 significantly after exposure to different toxicants. Therefore, regardless of toxicant
557 types, breath-borne VOCs from the rats seem to be capable of serving as a proxy for
558 real-time monitoring air toxicity. It was reported about 100 years ago that three mice
559 were also carried on all British submarines for sensing small leakage of gasoline as
560 the rats could squeak to notify the crew (Figure S3, Supporting Information). Some
561 previously detected VOCs such as hexane, pentane, acetaldehyde, butanone, and
562 acetone from rat's exhaled breath^{71,72} were also detected in this work. Nonetheless,
563 due to variations in rat breed, experimental conditions, VOC sampling and analysis
564 methods, it is rather difficult to exactly compare the VOCs from rat's exhaled breath
565 across different studies. There might be some breath-borne VOC species not identified

566 yet for the rats used here. Interestingly, rat L6 skeletal muscle cells cultivated with
567 α -MEM containing 10% FCS were shown to release 7 VOC species and uptake 16
568 VOC species.⁷³ Similarly, the increased VOCs detected here from rat's exhaled breath
569 might be released from lung cells or other impacted ones by the exposure. On another
570 front, previous work showed that changes were also detected in breath-borne VOCS
571 from people with upper respiratory infections compared to the health individuals (Ref
572 #7, Supporting Information). All these studies and data support the results and validity
573 of our work from various aspects. For improving *RST*_{air} performance, GC-MS can be
574 also replaced using the Proton Transfer Reaction-Mass Spectrometry (PTR-MS) for
575 fast online VOC species analysis. By using this discovered fundamental science, the
576 invented *RST*_{air} system here showed its great promise of revolutionizing the air
577 toxicity monitoring, and providing significant technological advances for air security
578 in related fields such as military defense, customs, counter-terrorism and security
579 assurances for important events or special locations.

Figure 5. Proposed mechanisms of toxic effects and VOCs releasing in rats when exposed to the environmental toxicants via inhalation. The black arrows represent the toxic effects of different toxicants and the possible pathways of VOCs generation. The blue arrows stand for the principle and working process of the invented *RSTair* system for real-time air toxicity monitoring. The corresponding references cited are: ①⁵⁹; ②⁶⁶; ③⁶³; ④⁴⁸; ⑤⁴²; ⑥^{48, 70}.

Supporting Information

Measurements of exhaled VOCs by PID and GC-MS/FID, Selection of exposure levels for different toxicant, experimental setup for *RSTair* system, indoor air background VOCs, Photo of UK postcard for three mice carried on British submarines, Primers used for RT-qPCR analysis of microRNA and mi-RNA expression level changes after exposure, video of rat after exposed to ozone were

594 provided as Supporting Information.

595

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600

601 **Conflict of interests**

602 The authors declare no competing financial interest.

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