Rats Sniff Off Toxic Air

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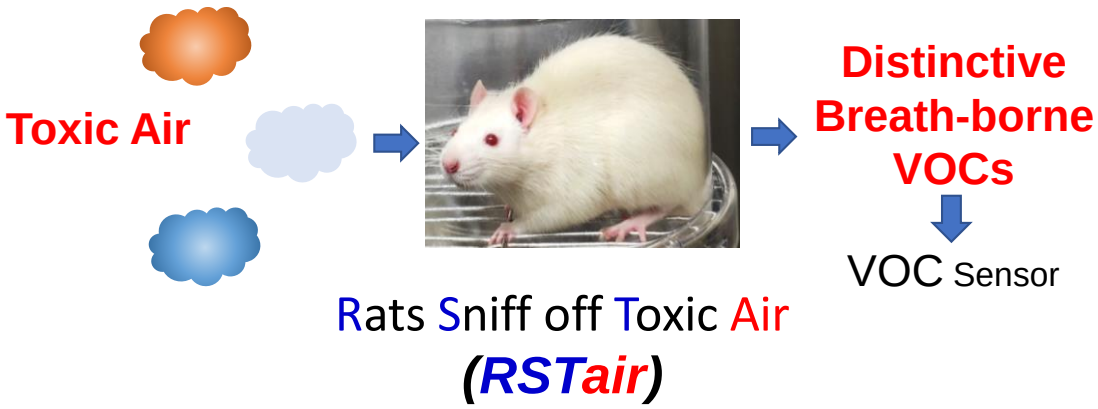
Abstract Graphic



Abstract

Breathing air is a fundamental human need, yet its safety, e.g., when challenged by various harmful or lethal substances, is often not properly guarded. Currently, air toxicity is monitored only for single or limited number of known toxicants, thus failing to fully warn against possible hazardous air. Here, using a photoionization detector (PID) or GC-MS/FID we found that within minutes living rats emitted distinctive profiles of volatile organic compounds (VOCs) via breath when exposed to various airborne toxicants such as endotoxin, O_3, ricin, and CO_2. Compared to background indoor air, when exposed to ricin or endotoxin aerosols breath-borne VOC levels, especially that of carbon disulfide, were shown to decrease, while elevated levels were observed for O_3 and CO_2 exposures. Principal component analysis (PCA) revealed a clear contrast in breath-borne VOCs profiles of rats among different toxicant exposures. MicroRNA regulations such as miR-33, miR-146a and miR-155 from rats' blood samples also suggested varying mechanisms used by the rats in combating different air toxicant challenges. By integrating living rats, breath sampling, and VOC online detection, we pioneered a system that can real-time monitor air toxicity without the need of detecting specific species. Importantly, rats were shown to be able to sniff off toxic air.

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

1. Introduction

Breathing air is a fundamental human need, however its safety is often not properly guarded. Common pollutants in the air include particulate matter (PM), biologicals, and also gaseous substances such as O_3 and NOx. Inhaling these pollutants can cause a variety of health problems such as respiratory, cardiovascular diseases, and even death [1-2]. For example, PM alone was shown to have resulted in 4.2 billion 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]. On the other hand, exposure to pathogenic bioaerosols including bacteria, fungi, virus, et al in the air can cause respiratory infections. Airborne exposure to some infectious agents can also lead to respiratory infection outbreaks: the severe acute respiratory syndrome (SARS) in 2003, H1N1 flu in 2009, H7N9 avian influenza in 2013, and Middle East Respiratory Syndrome (MERS) in 2015. These outbreaks have resulted in grave human and economic costs. Besides, there is a growing concern about the risk of terrorist attack by intentionally releasing biological and chemical agents into the air to cause huge civilian casualties [10]. In the past, the Tokyo Subway Sarin Attack in 1995, for example, have caused 11 deaths and more than 5,000 injuries [11]. In 2001, anthrax spores attacks via postal mails in the United States have resulted in five deaths and 17 infections as a result of inhaling anthrax spores [12]. It is evidenced that 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 overall microbial concentration levels without identifying species. In addition, airborne pollutants and toxicity could vary greatly from one location to another [20-21], thus presenting location-specific air toxicity and health effects. Current epidemiological or toxicological methods involving data analysis or animal and cells experiments cannot provide in situ air toxicity information, accordingly failing to represent the response at the time of exposure because biomarker levels evolve over time [22]. In addition, under certain scenarios (high profile meetings or locations) a rapid response to air toxicity needs to be in place in order to protect the interest. However, the response time is very demanding for an immediate effective countermeasure, for example, usually several minutes can be tolerated [10, 23]. In many air environments, multiple hazardous pollutants could also co-exist even with unknown ones at a particular time, which makes protecting the air rather difficult, if not impossible, using current technologies of species level detection and warning.

Previously, olfactory receptors of mouse cells for odors [24], immune B cells [25]for pathogen detections, and silicon nanowire sensor arrays for explosives were studied. Recently, a breath-borne biomarker detection system (dLABer) integrating rat's breath sampling, microfluidics and a silicon nanowire field effect transistor (SiNW FET)

device has been developed for real-time tracking biological molecules in the breath of rats exposed to particulate matter (PM). The dLABer system was shown to be able to online detect interleukins-6 (IL-6) level in rat's breath, and capable of differentiating between different PM toxicities from different cities using the biomarker level. However, as observed in the study the production of protein biomarkers could significantly lag behind the pollutant exposure, thus falling short of providing a timely warning against toxic air. Nonetheless, exhaled breath is increasingly being used in biomarker analysis in both medical and environmental health studies [28-30]. In addition to protein biomarkers, a large number of volatile compounds (e.g., nitric oxide, carbon monoxide, hydrocarbons) have been also studied to assess health status and even developed for clinical diagnosis. For example, ethane and n-pentane detected in the breath were linked to the *in vivo* level of lipid peroxidation and oxidative stress ; breath-borne acetone was shown to correlate with the metabolic state of diabetic patients. In addition, exhaled VOCs have been used for the diagnosis of asthma, lung cancer and other diseases. Undoubtedly, breath-borne VOC has emerged as a promising biomarker for health or environmental exposure monitoring.

Inspired by the dog sniff for explosive, the work here was conducted to investigate if we can use breath-borne VOCs from living rats to real-time monitor toxic air. Particularly, we wanted to study: 1) When rats are exposed to air toxicants, whether the VOC species and concentration in the exhaled breath change? If yes, how long does the change need to occur? 2) Are there any specific exhaled VOCs in response to different toxicants exposure including both chemical and biological agents? 3) To develop an online air toxicity analyzing system based on the real-time monitoring of exhaled VOCs of rats. The work here has demonstrated a great promise of online air toxicity

131 monitoring, and expected to revolutionize the field.

132

133 **2. Materials and methods**

134 **2.1 Rat breeding**

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

146

147 **2.2 Preparation of toxic air**

148 In this work, four different exposure toxicants (ricin, endotoxin, ozone and carbon
149 dioxide) and indoor air (as a background control) for rats were prepared for the
150 exposure experiments. Ricin was extracted from the seeds of castor produced in
151 Xinjiang, China, and prepared by Institute of Microbiology and Epidemiology,
152 Academy of Military Medical Sciences in Beijing. The endotoxin was purchased from
153 Associates of Cape Cod, Inc., USA. The ricin and endotoxin suspensions were prepared
154 by vigorously vortexing 40 μg of ricin or 50 ng endotoxin per ml deionized (DI) water
155 for 20 min at a vortex rate of 3200 rpm (Vortex Genie-2, Scientific Industries Co., Ltd.,

156 USA). Detailed information about ricin preparation and exposure can be also found in
157 our previous work. Here, ozone was generated by an ozone generator (Guangzhou
158 Environmental Protection Electric Co., Ltd., China) using corona discharge method.
159 The ozone was further diluted with indoor air for rat exposure experiments, and the
160 ozone concentration in the exposed chamber was approximately 5 ppm. Carbon dioxide
161 was purchased from Beijing Haike Yuanchang Utility Gas Co., Ltd., and diluted 20
162 times with indoor air to a concentration of about 5%.
163
164 **2.3 Rat sniffs off toxic air (*RST*$_{air}$) system and experimental procedure**
165 To investigate whether we can use breath-borne VOCs from living systems to real-
166 time monitor toxic air, we have developed the system named as *RSTair* (Rat Sniffs off
167 Toxic Air). As shown in Figure 1 and Figure S1 (Supporting Information), the system
168 is composed of four major parts: toxicant generator, exposure chamber, exhaled breath
169 sampling and online VOC analysis. Indoor air was used as carrier gas for generating
170 toxicant aerosols (ricin and endotoxin) using a Collison Nebulizer (BGI, Inc., USA) or
171 diluting toxicants gas (ozone and carbon dioxide). The toxicant aerosol or toxicant gas
172 was introduced into the exposure chamber at a flow rate of 1 L/min. As shown in Figure
173 S1, a metabolic cage was used as the exposure chamber which can allow rat's feces and
174 urine to fall from below quickly so as to reduce their influences on VOC analysis. In
175 addition, teflon tubes and vales were also applied to reducing adsorption loss of VOCs.
176 When performing the experiments, the rats were placed in the exposure chamber.
177 Indoor air was first introduced into the chamber for 10 minutes, then followed by each
178 of tested toxicants for about 10 minutes to conduct exposure tests. Before and after the
179 exposure, bloods of the rats were taken. As for the exhaled breath sampling and VOC
180 analysis, the photoionization detector (PID) (MOCON, Inc., USA) coupled with an air

pump was used to real-time monitor TVOC at a flow rate of 0.6 L/min. Besides, the breath samples in the chamber were also collected by using a Silonite canister (Entech Instruments, Inc., USA), and VOCs species were analyzed by a gas chromatograph-mass spectrometry/flame ionization detection (GC–MS/FID) system (Agilent Technologies, Inc., USA). Each of the experiments were conducted with three rats and repeated three times independently.

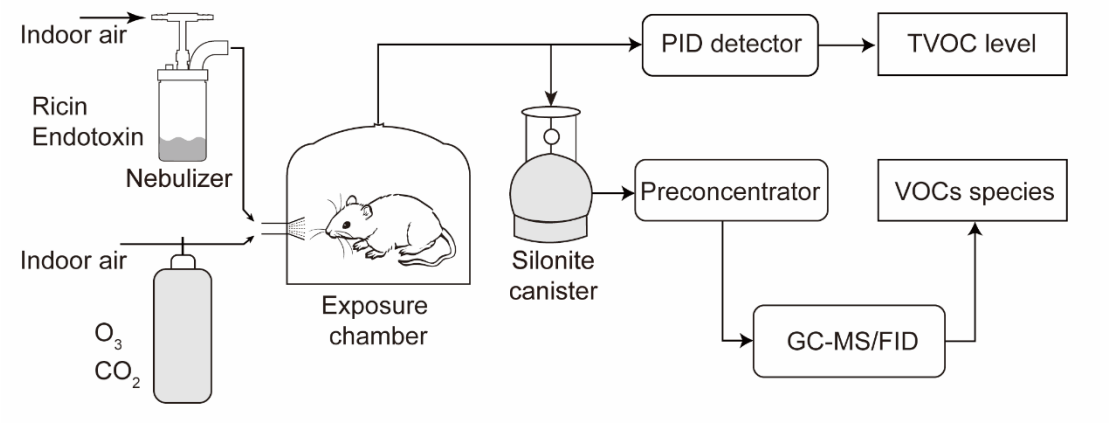


Figure 1. Rat sniffs off toxic air (RST_{air}) system: ricin and endotoxin were aerosolized by a Collison nebulizer (BGI, Inc., USA) using indoor air; ozone and carbon dioxide were diluted with indoor air before introduced into the chamber. The metabolic cage was used as the chamber for exposure and air sampling. After placing rats into the chamber, the VOCs in the chamber before and after the toxicant exposure were analyzed by the PID directly and also by GC–MS/FID system after being collected by using the Silonite canister. During the VOC measurements, the toxicant exposure was terminated. Each time only one rat was placed into the chamber.

2.4 Measurements of exhaled VOCs by PID and GC-MS/FID

In this work, total VOCs (TVOC) level in the exposure chamber was real-time monitored by a PID sensor during all the experiments: 1) when the rats were not in the chamber (background air); 2) rats in the chamber (before toxicant exposure) and rats in

the chamber (after toxicant exposure). The working procedure of the PID sensor is to first ionize the VOCs gas with a high-energy UV lamp, and then the ionized fragments are collected by the ion chamber to generate a current signal. The signal in general is proportional to the concentration of the target VOCs. In this study, the PID sensor is assembled with the oil-free pump and pre-filter and a signal encoder, and the signal is transmitted and displayed in real time. The sensor was calibrated using 1 ppm isobutylene prior to use.

Meanwhile, as mentioned above air samples were also collected by a 3.2 L Silonite canister (Entech Instruments, Inc., USA) at the flow rate of 0.8 L/min for the qualitative and quantitative VOC species detection. The air samples were then transferred into the pre-concentrator (7200, Entech Instruments, Inc., USA) and analyzed by gas chromatography (GC) together with Mass Spectrometry (MS) and Flame Ionization Detector (FID) (5975C/7890A, Agilent Technologies, Inc., USA) within 12 hours. The GC-MS/FID analysis work was provided by Tianjin Zhongfei Huazheng Testing Technology Co., Ltd, and is briefly described as follows: the Entech 7200 system was used to pre-concentrate the sample, including enrichment of the sample, and removal of water and carbon dioxide from the sample. Then, an Agilent 5975C/7890A gas chromatography mass spectrometer was used to qualitatively analyze sample according to the standard mass spectrometry library and the mixed gas standard compound. The multi-point calibration working curve was developed by the external and internal standard method for quantitative analysis. The external standard gases used are: mixed standard gas of 57 Photochemical Assessment Monitoring Stations (PAMS) gas, 12 kinds of aldehyde and ketone gases and 47 TO-15&17 gases (including aromatic hydrocarbons, halogenated hydrocarbons and oxygenated organic compounds). The

internal standard gases are mixtures of bromochloromethane, 1,4-difluorobenzene, deuterated chlorobenzene, and 1-bromo-3-fluorobenzene. The above standard gases were purchased from Scott Specialty Gases, USA.

2.5 Blood microRNA detection

Before and after each toxicant exposure, 0.75 mL blood samples from rats were taken through the catheter using sterile syringes with 23G flat-end needles and kept in -20 ºC for microRNA analysis. The blood microRNAs in the blood samples such as miR-125b, miR-155, miR-146a, miR-21, miR-20b, miR-210, miR-122, and miR-33 were analyzed using a RT-qPCR array (Wcgene Biotech, Inc., China). Total RNAs in the blood samples, including microRNAs, were extracted using a Trizol reagent (Sigma Aldrich, Inc., USA) according to the manufacturer's instructions. Subsequently, the purified RNAs were polyadenylated through a poly(A) polymerase reaction and was then reversed-transcribed into complementary DNA (cDNA). TIANGEN® miRcute Plus miRNA First-Strand cDNA Kit (Code No. KR211) was used in the reverse transcriptional reaction system of total 10 µL, including 5 µL 2x miRNA RT Reaction Buffer, 1 µL miRNA RT Enzyme Mix and 4 µL RNA sample. The reaction conditions are 40 ºC for 60 mins and 95 ºC for 3 mins. The cDNA was quantified in real-time SYBR Green RT-qPCR reactions with the specific microRNA qPCR Assay Primers. TIANGEN® miRcute Plus miRNA qPCR Kit (SYBR Green) (Code No. FP411) was used in the qPCR reaction system of total 10 µL, including 5 µL 2x miRcute Plus miRNA PreMix (SYBR&ROX), 0.2 µL Forward Primer, 0.2 µL Reverse Primer, 1 µL 50X ROX Reference Dye, 1 µL DNA Sample and 2.6 µL ddH_2O. The cycling conditions are 95 ºC for 15 min, followed by 40 cycles at 94 ºC for 20s, 60 ºC for 15s and 72ºC for 30s. The primers used for qPCR are presented in Table S1 (Supporting

Information).

3. Statistical analysis

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

4. Results and discussion

4.1 Results

TVOC monitoring for the toxicants exposure

As described in the experimental section, four toxicants (ricin, endotoxin, ozone and carbon dioxide) and indoor air (as a background control) were used for inhalation exposure in rats. Before and after the exposure, the TVOC level in the exposure chamber was monitored by the PID sensor. For each group, the TVOC levels of only 3 rats (PID instrument failure for one rat) were shown in Figure 2. The indoor air background TVOC was found to be less than 0.02 ppm. After one rat was placed in the exposure chamber, the TVOC level in the cage was shown to first gradually increase as shown in Figure 2, then reach a relatively stable level after about 500 seconds. The TVOC level before the exposure (indoor air) when one rat was in the chamber was about 2 ppm as shown in Figure 2, except for the CO_2 group of which was about 0.5-0.8 ppm (These background differences, if any, applied to both control and exposure tests, thus presenting no influences on the same experiments). The air pump of the PID sensor was then turned off, and air in the exposure chamber was sampled using a Silonite canister. After the sampling (about four minutes) was completed, each of the tested toxicants was then introduced into the exposure chamber. After the exposure (ten minutes) was completed, the original indoor air supply was provided again to each rat, and the TVOC monitoring by the PID sensor was resumed. As shown in Figure 2, the differences in TVOC levels for indoor air exposures (different times: "before" and "after", but the same indoor air) were small (the average change rate was about -4%±1.4% (95% confidence interval)), although the Mann-Whitney Rank Sum Test showed that for each of the rats, the difference (over some time for the indoor air) was significant (p-value<0.001). The difference may be caused by the error of PID itself or variations of indoor air compositions over the time, and this change rates (n=3) of the control

group (indoor air) was then served as the reference for other toxicant exposures in the statistical analysis. During the indoor air experiment, the rats were seen to carry out normal life activities when in the exposure chamber, and correspondingly the TVOCs in the chamber were shown to remain relatively stable.

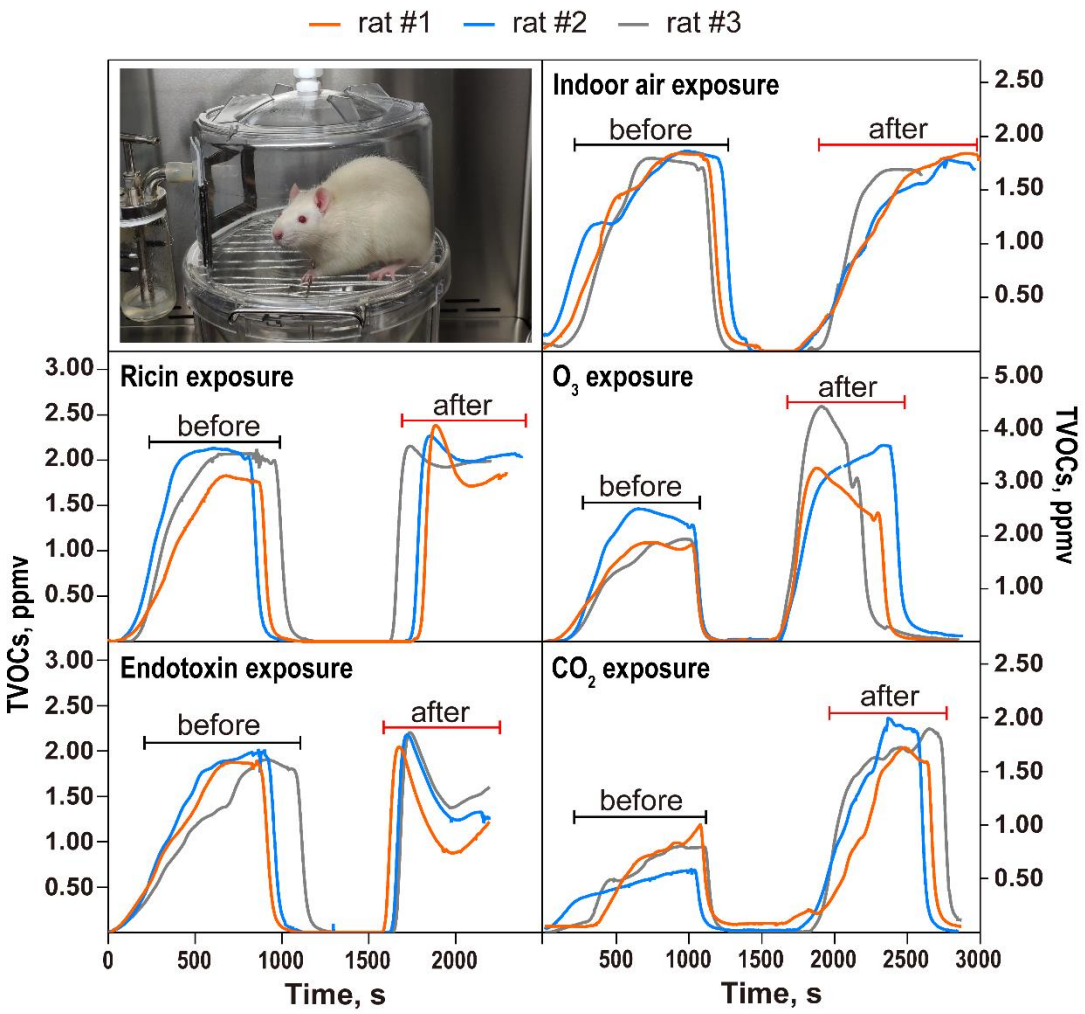


Figure 2. Real-time continuous measurements of exhaled TVOC levels in the chamber when rats were exposed to different toxicants via inhalation for 10 mins: Indoor air, ricin, endotoxin, O_3 and CO_2. During the exposure processes, the PID sensor was turned off. Data lines (measurement time was 1000 s) 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 exposure test was

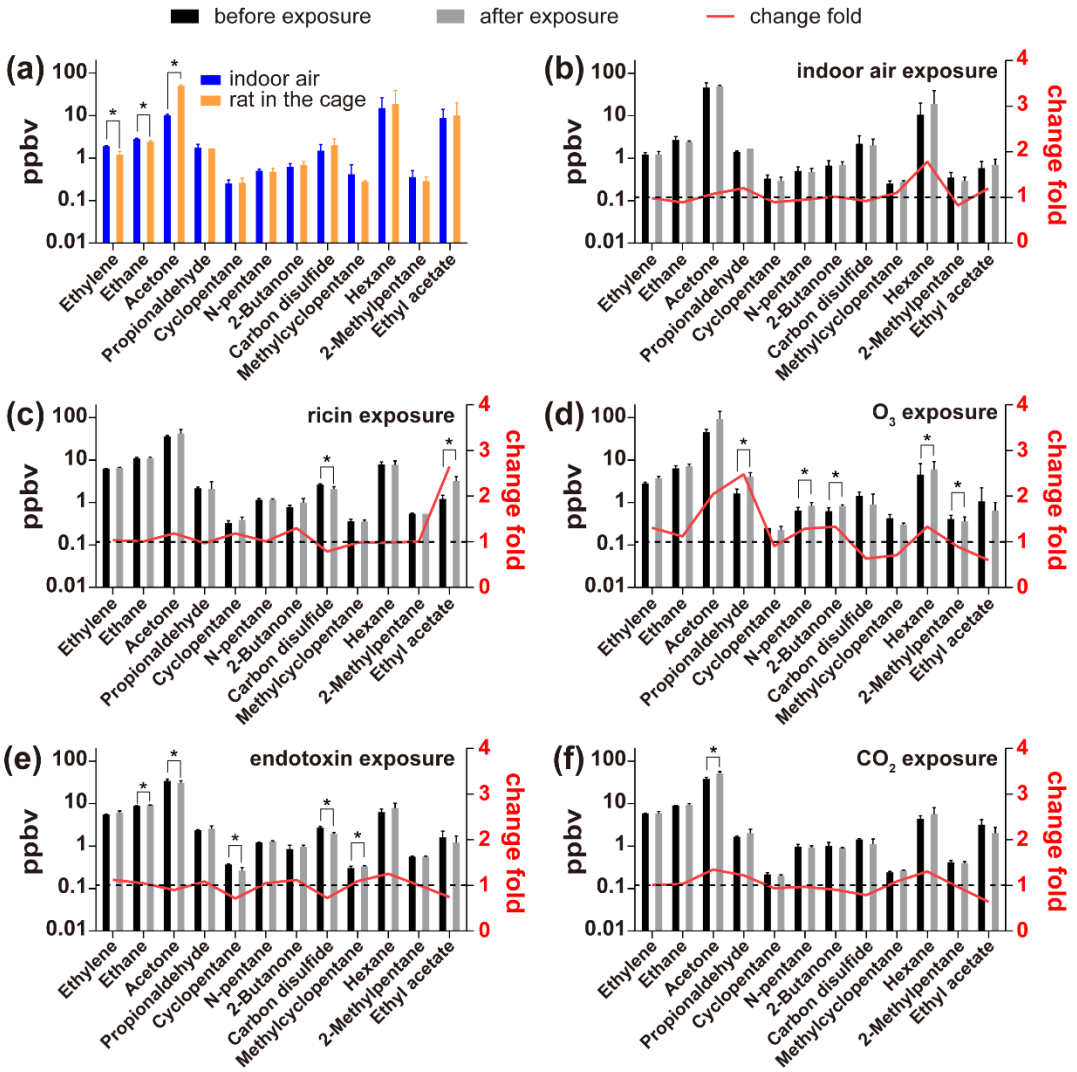
312 independently repeated with four rats from the same group (PID instrument failure for
313 one rat).
314
315 In contrast, TVOC levels were shown to vary greatly with different toxicant
316 exposures as shown in Figure 2. For example, as shown in Figure 2, when rats were
317 exposed to the ricin, the TVOC level was observed first to increase slightly, then
318 decreased to a level comparable to that of before exposure with an average change rate
319 of -3%±1.6 (Mann-Whitney Rank Sum Test, all p-values<0.001). Compared to the
320 control group (indoor air) shown in Figure 2, the difference of the TVOC change rate
321 was not significant for the ricin exposure (t-test, p-value=0.426). For ricin exposure, its
322 concentration (40 μg/mL aerosolized) might be too low in aerosol state after the
323 aerosolization from the liquid to produce a detectable response from the rats. This
324 suggests that ricin, given the amount aerosolized here, presented no additional health
325 challenge compared to the indoor air at the time of the experiment. Compared to the
326 ricin exposure, however we observed a different phenomenon for the endotoxin (50
327 ng/mL aerosolized) tests as shown in Figure 2. Upon the endotoxin exposure, the TVOC
328 level was observed to first increase slightly, and then surprisingly decreased to a level
329 that was about 21-46% below the pre-exposure level after four minutes (Mann-Whitney
330 Rank Sum Test, all p-values<0.001). Compared to the control group (indoor air), the
331 difference of the TVOC change rate was statistically significant for endotoxin (t-test,
332 p-value=0.0147). The observed differences from the ricin and endotoxin exposures
333 could be due to different mechanisms initiated by different substances involved. Ricin
334 is derived from plant, while endotoxin is from Gram-negative bacterial membrane.
335 They could interact differently with relevant human respiratory or other body cells.
336

After exposure to gaseous toxicants such as ozone and carbon dioxide, the levels of TVOCs in the exposure chamber with rats were observed to have increased significantly, as observed in Figure 2. As can be seen from the figure, the TVOC levels has increased for about 44-110% for ozone and about 109-265% for carbon dioxide exposure (Mann-Whitney Rank Sum Test, all p-values<0.001). The t-test showed that differences of the TVOC change rates of both ozone and CO_2 exposures compared to the control group (indoor air) were statistically significant (p-value=0.0219 and 0.0296, respectively). These data indicated that rat exposure to both ozone and CO_2 has resulted in significant elevations of TVOCs, suggesting rats were actively responding to the exposure challenges. The behavior observation from a video also indicated that rats after the exposure to O_3 seemed to be suffering from the challenge (Video S1, Supporting Information).

Changes in exhaled VOCs after exposure to different toxicants

In order to determine the changes of VOC species exhaled out by rats before and after the exposures to four different substances, the GC-MS/FID method was used to qualitatively and quantitatively analyze the VOCs in the exposure chamber. The background indoor air without and with rat in the chamber were first analyzed. A total of 31 different VOCs were detected and shown in Figure S2 (Supporting Information). Among detected VOCs as shown in Figure S2 (Supporting Information), the VOCs with the highest concentrations in indoor air were n-hexane, ethyl acetate and acetone, which all come from the laboratory air. When rats were placed in the exposure chamber (one rat at each time), the most abundant VOC species was detected to be acetone, which was about 4 times more than that of the indoor air background. Statistical tests found that the concentrations of ethylene and ethane in the chamber containing one rat were

362 significantly lower than that of the background (paired t-test, p-value<0.05), which in
363 part could be due to the air dilution by the rat's breath.



364

365 **Figure 3.** Differentiations of VOC species from indoor air and those from the rats'
366 exhaled breath under different air toxicity with exposure to ricin, O_3, endotoxin and
367 CO_2. The red lines show that the average change ratios of every toxicant calculated by
368 the level after the exposure divided by the level before exposure (right axis). The dotted
369 line is the baseline with a change ratio of 1. "*" indicates a significant difference at p-
370 value=0.05.

371

372 Differentiations of VOC species from the rat's exhaled breath under different
373 toxicants exposures were also shown in Figure 3. There were no significant differences
374 in the concentrations of any VOCs before and after the exposure for the control group,
375 i.e., indoor air (t-test, p-value=0.05). This suggests that indoor air is relatively less toxic
376 to a level that is unable to detect a VOC change. In contrast, specific VOC species had
377 experienced significant changes when rats were exposed to ricin, endotoxin, O_3 and
378 CO_2 as observed from Figure 3. For example, exposure to ricin caused significant
379 higher concentration of ethyl acetate (183% higher), while lower concentration of
380 carbon disulfide (22% lower). As shown in Figure 3, after the endotoxin exposure
381 process, concentrations of five VOC species: ethane, acetone, cyclopentane, carbon
382 disulfide and methylcyclopentane were shown to be significantly different with those
383 of before the exposure (t-test, all p-values<0.05). As can be seen from the results of the
384 ozone exposure group in Figure 3, the concentrations of propionaldehyde, pentane, 2-
385 butanone, hexane and 2-methylpentane exhibited significant differences before and
386 after exposure (t-test, all p-values<0.05), in which all the VOCs except 2-
387 methylpentane were elevated. In comparison, rat exposure to CO_2 resulted in acetone
388 level increase by 34% (t-test, p-value=0.0016). These data suggest that exposure to
389 different toxicants had led to production of different VOC species in addition to their
390 level changes.
391
392 ***Detection of micro RNAs in Blood Samples***
393 To further explore the VOC response mechanism of rats to toxicants exposure,
394 microRNAs (miRNA) in the blood samples were examined by an RT-qPCR assay. Fold-
395 changes in microRNA levels after toxicants exposure were shown in Table S2
396 (Supporting Information). The level of miR-33 in the blood of rats was shown to be

397 significantly lower than that before ricin exposure (p-value<0.05); after exposure to
398 ozone, miR-146a in the blood samples of rats were significantly higher than those
399 before the exposure (p-value <0.05), while miR-155 was significantly lower than that
400 before the exposure (p-value <0.05). For other microRNAs as listed in Table S4, the
401 changes seemed to be insignificant (t-test, p-values>0.05).
402
403 **4.2 Discussion**
404 As observed from Figure 4, PCA results revealed a clear contrast in breath-borne
405 VOC profiles of rats between different toxicants exposures. The VOC profiles of the
406 ozone exposure group was very different from that of the control group (indoor air) and
407 the VOCs profile of the ricin exposure group was the closest to that of the control group,
408 which agreed with TVOC level and VOC species profiles obtained above. Overall, the
409 experiments showed that rats responded differently to different toxicants by releasing
410 different VOC species owing to different mechanisms of toxicity: ozone caused
411 significant increases in various breath-borne VOCs; while endotoxin exposure
412 generally decreased the releasing of VOCs; and ricin and carbon dioxide exposure
413 resulted in one or two significant VOC species changes. In general, the results of
414 qualitative and quantitative analysis by the GC-MS/FID method agreed with the TVOC
415 level monitored by the PID sensor.

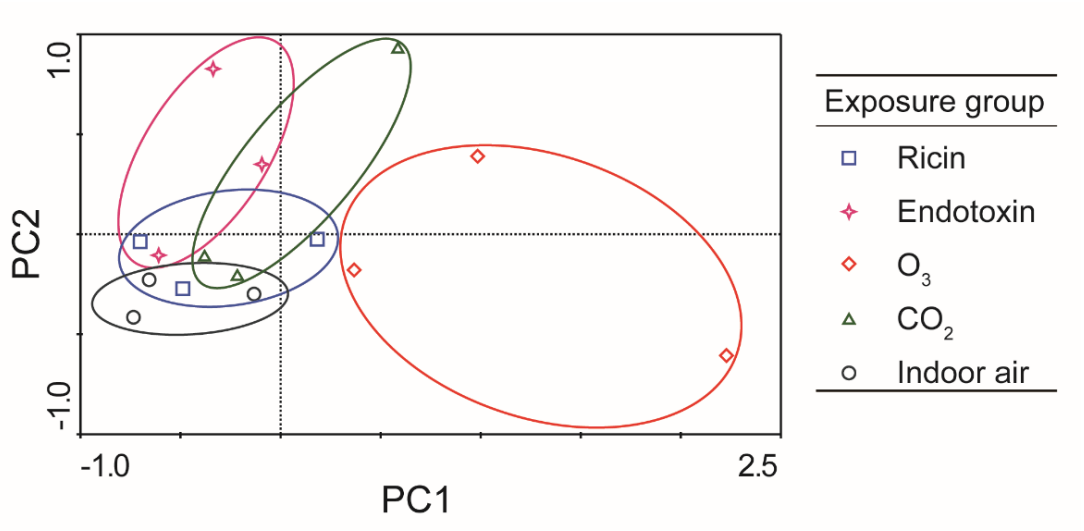


Figure 4. PCA ordinations of exhaled breath-borne VOCs profiles under exposures to different toxicants: ricin, endotoxin, O_3, CO_2 and control (indoor air). PC1 and PC2 are the first and second principal components. The VOCs species involved in the PCA analysis were the 12 species which were shown to 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% 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 . 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 . 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 presents endothelial toxicity by acting as a natural disintegrin binding to and damaging human endothelial cells . Therefore, the toxicity

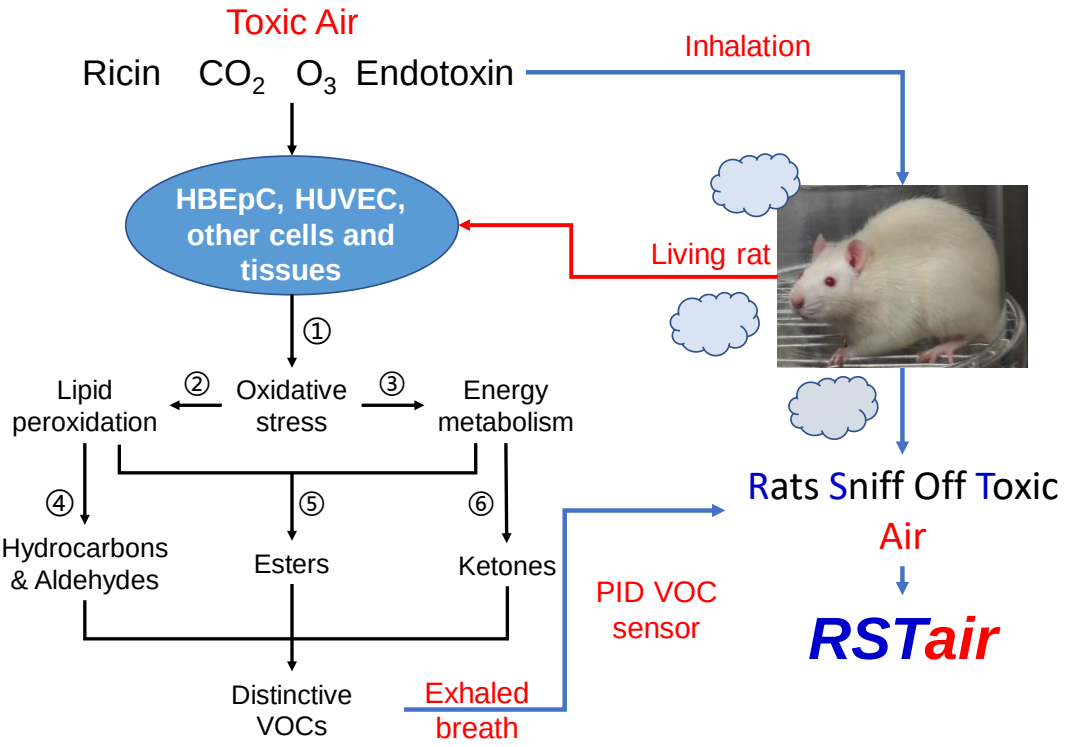
434 of ricin on the endothelial cells might be the source of the higher concentration of ethyl
435 acetate observed in this work. As a disease biomarker, carbon disulfide was observed
436 in the exhaled breath [44-45]. Recently, it was suggested that the carbon disulfide may be
437 generated endogenously and play a role as a bioregulator [46]. Here, we observed that
438 exposure to both ricin and endotoxin resulted in lower levels of breath-borne carbon
439 disulfide compared to the control.
440
441 For CO_2 and endotoxin exposure, the observation for acetone was the opposite as
442 shown in Figure 3. Acetone in exhaled breath was widely investigated in many studies
443 as an important biomarker related to blood glucose and diabetes . Acetone is produced
444 in the fatty acids metabolism by hepatocytes via decarboxylation of excess acetyl
445 coenzyme A (Acetyl–CoA), and then oxidized via the Krebs cycle in peripheral tissue .
446 As shown in Figure 3, the acetone level increased by 34±9% as a result of CO_2 exposure,
447 suggesting CO_2 caused hypoxia in rats, and led to increased respiration from rats. These
448 increases in acetone level corresponded to TVOC level increase as determined by the
449 PID sensor after the exposure to CO_2. However, when exposed to endotoxin, the
450 acetone level in the exposure chamber decreased by about 10±6%, indicating that the
451 respiration of the rats may be attenuated by the exposure of endotoxin. Clearly, the
452 involved mechanisms by which endotoxin and CO_2 cause health effects to rats could be
453 very different.
454
455 As observed in Figure 3, the increase of ethane level by endotoxin exposure
456 suggested that lipid damage was induced by oxidative stress in the rat's body since
457 ethane is acknowledged as a marker of lipid peroxidation and described to be generated
458 by peroxidation of ω-3 polyunsaturated fatty acids . In addition to ethane, the increase

459 of methylcyclopentane level as shown in Figure 3 might also be the result of the
460 endotoxin exposure. Endotoxin has been shown to trigger inflammation through its
461 interaction with the TLR4/CD14/MD2 receptor and then initiates a signal cascade. This
462 reaction correspondingly results in the activation of transcription factor such as NF-κB
463 leading to the production of pro-inflammatory cytokines and type 1 interferons (IFNs),
464 and finally results in systemic inflammatory response syndrome . In general, in terms
465 with the average fold changes, the concentration of total VOCs in the exhaled breath
466 was relatively lower after the endotoxin exposure, which agreed with the results of
467 TVOC obtained by the PID sensors.
468
469 In addition to these biologicals, we have also shown that exposure to chemicals
470 such as ozone and CO_2 also resulted in in-vivo changes in VOC levels. From the fold
471 changes of various VOC species such as propionaldehyde, N-pentane, 2-Butanone, and
472 Hexane, the ozone exposure has resulted in an overall increase of VOCs in rats' exhaled
473 breath, which agreed with the TOVC monitoring shown in Figure 2 by the PID sensors.
474 It was previously indicated that increase in propionaldehydes, further products of lipid
475 peroxidation, indicated more severe oxidative damage in rats following exposure to
476 ozone . Ozone was described as a strong oxidizing agent, and can cause intracellular
477 oxidative stress through ozonide and hydroperoxide formation . The mechanism of
478 ozone oxidative damage involves the activation of Nrf2, heat shock protein 70, NF-κB,
479 thus increasing expression of a range of proinflammatory cytokines such as TNFα and
480 interleukin 1β, and chemokines such as interleukin 8 . The results above show that
481 regardless of toxicant types breath-borne VOC from rats experienced *in vivo* changes.
482
483 In this work, miRNAs from rats' blood were also analyzed as shown in Table S4

484 to investigate the VOC emission mechanism by rats upon exposure to various types of
485 toxicants. miRNAs are short non-coding RNA sequences that regulate gene expression
486 at the posttranscriptional level; and many miRNAs have already been identified to
487 influence physiological processes such as immune reaction, adaptation to stress, and
488 widely investigated in environmental exposure studies . Among these microRNAs,
489 miR-125b, miR-155, miR-146a, and miR-21 are mainly shown to regulate oxidative
490 stress and inflammatory processes *in vivo*, and widely investigated in air pollution
491 related studies . For example, among them, miR-155 has a positive regulation function,
492 and the other three are negative regulation . However, in this study, the ozone exposure
493 resulted in decreased levels of miR-155, which are contrary to previous reports in the
494 literature . The specific reasons need further investigations. Previous studies have
495 shown that miR-20b and miR-210 are hypoxia regulators in animals, and miR-122 and
496 miR-33 are mainly responsible for regulating lipid metabolism and glucose metabolism
497 in the body . However, these microRNAs were shown to have not undergone significant
498 changes in this study after the exposures to four different toxicants. The possible reason
499 may be that microRNAs act as post-transcriptional regulators by degrading mRNA or
500 inhibiting their translation , thus failing to respond in a timely manner during short-term
501 exposure (10 min used here for taking blood samples). Nonetheless, observed changes
502 in microRNA levels after the toxicant exposure are related to those VOC level and
503 species changes.
504
505 The experimental results here showed that the indoor air supply to the rats in the
506 exposure chamber did not cause obvious changes in the TVOC level. This indicated
507 that the indoor air exhaled by the rats were relatively less toxic, for example, unable to
508 induce a detectable TOC change from exhaled breath. When exposed to aerosolized

509 ricin of the tested amount for 10 mins, the TVOC did not change significantly; while
510 the endotoxin exposure resulted in lower TVOC levels. In contrast, when exposed to
511 ozone or CO_2 the TVOC levels were shown to have increased by more than 200%.
512 Clearly, these results indicate that when rats are exposed to toxic substances their
513 certain metabolic activities are immediately affected, i.e., these exposures promoted or
514 inhibited specific VOC productions. Based on the results we obtained from this work,
515 the following VOC emission mechanisms of rats when exposed to different toxicants
516 are proposed and illustrated in Figure 5. Previously, it was suggested that VOCs are
517 produced during the normal metabolism in the body; while pathological processes, such
518 as metabolic disorders, can also produce new species of VOCs or alter the levels of
519 existing VOCs . Therefore, cell or tissue injuries caused by external toxicants exposure
520 also can alter the exhaled VOCs profile by disturbing the normal process. The exact
521 toxic effect mechanism as observed from this work could vary from one toxicant to
522 another. For some pollutants such as endotoxin and ricin, there are specific receptors to
523 recognize them and then start the chain of responses or reactions . Among these various
524 mechanisms, the ROS (reactive oxygen species) and oxidative stress are recognized to
525 be the central and the common mechanism in various forms of pathophysiology, as well
526 as the health effect of various air pollutants including ambient particulate matter (PM) .
527 Oxidative stress is essentially a compensatory state of the body and can trigger redox-
528 sensitive pathways leading to different biological processes such as inflammation and
529 cell death . For example, the strong oxidants such as ozone might cause oxidative stress
530 through direct effects on lipids and proteins , which mostly caused the generation and
531 release of hydrocarbons and aldehydes, such as ethane, ethylene, and propionaldehyde .
532 While carbon dioxide tends to make the redox balance tilted toward the reduction side
533 by reducing oxygen supply and thus influencing the energy metabolism in cells . For

534 ricin and endotoxin exposure, the underlying mechanisms seem to be different from
535 ozone and CO_2, and they could cause oxidative stress indirectly through the activation
536 of intracellular oxidant pathways. Nonetheless, all toxicants share a common effect of
537 disrupting the redox balance, and thus interfere with normal biochemical reactions or
538 cause material damage in cells, accordingly changing the VOC profile and releasing
539 into the breath. As discussed above, in this work, the VOCs profile of rats changed
540 significantly after exposure to different toxicants. Therefore, regardless of toxicant
541 types, breath-borne VOCs from the rats seem to be capable of serving as a proxy for
542 real-time monitoring air toxicity.



543

544 **Figure 5.** Proposed mechanisms of toxic effects and VOCs releasing in rats when
545 exposed to the environmental toxicants via inhalation. The black arrows represent the
546 toxic effects of different toxicants and the possible pathways of VOCs generation. The
547 blue arrows stand for the principle and working process of the invented RSTair system
548 for real-time air toxicity monitoring. The corresponding references cited are: ① [59]; ②

549 [68]; ③ [62]; ④ [48]; ⑤ [42]; ⑥ [48, 69].

550

551 **5. Conclusions**

552 Recently, exhaled VOCs have increasingly been used as non-invasive samples for
553 exposure studies as well as clinical diagnosis. In this study, we examined the possibility
554 of using living animals' exhaled VOCs in real-time monitoring air toxicity. Our
555 experimental data showed that when the rats were exposed to air containing various
556 toxicants, characteristic VOC profiles were *in vivo* produced in the body within 10
557 minutes or shorter, and further emitted via exhaled breath. In addition, different toxicant
558 exposures were shown to have caused productions of distinctive profiles of VOC
559 species from the living rats. Therefore, the developed *RSTair* system, i.e., by integrating
560 inhalation exposure, living rats, breath sampling and online VOC sensor, was capable
561 of real-time monitoring toxic air. The *RSTair* system can detect a breath-borne VOC
562 change when the air is becoming toxic to rats. In doing so, the system can real-time
563 alter people of possible air toxicity change or hazardous air. Nonetheless, the PID sensor
564 used in this system only reports the TVOC level without classifying individual VOC
565 species. It should be also noted that the PID sensor responds to different VOCs
566 differently. For example, the PID could respond to one VOC with stronger signal, but
567 to another with weaker one even for the same concentration level. However, this can be
568 readily remedied by using portable direct-injection mass spectrum such as Proton
569 Transfer Reaction-Mass Spectrometry (PTR-MS). This study revealed that living
570 systems such as rats will *in vivo* alter specific VOC productions in response to external
571 toxicant exposure challenges from air. By using this discovered fundamental science,
572 the invented *RSTair* system here showed its great promise of revolutionizing the air
573 toxicity monitoring, and providing significant technological advances for air security

574 in related fields such as military defense, customs, counter-terrorism and security
575 assurances for important events or special locations.
576
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581
582 **Conflict of interests**
583 The authors declare no competing financial interest.
584

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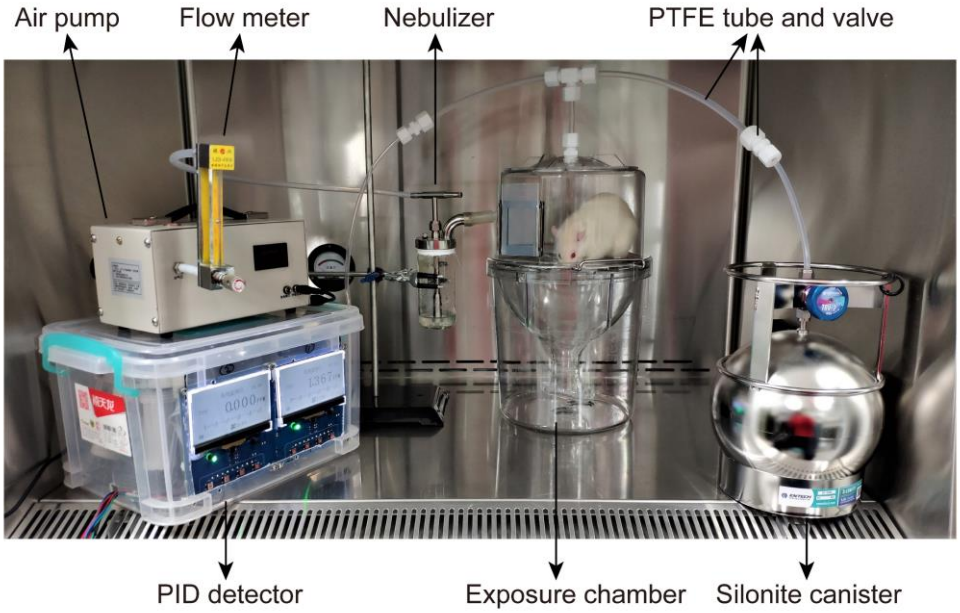
758

759 **Supporting Information**

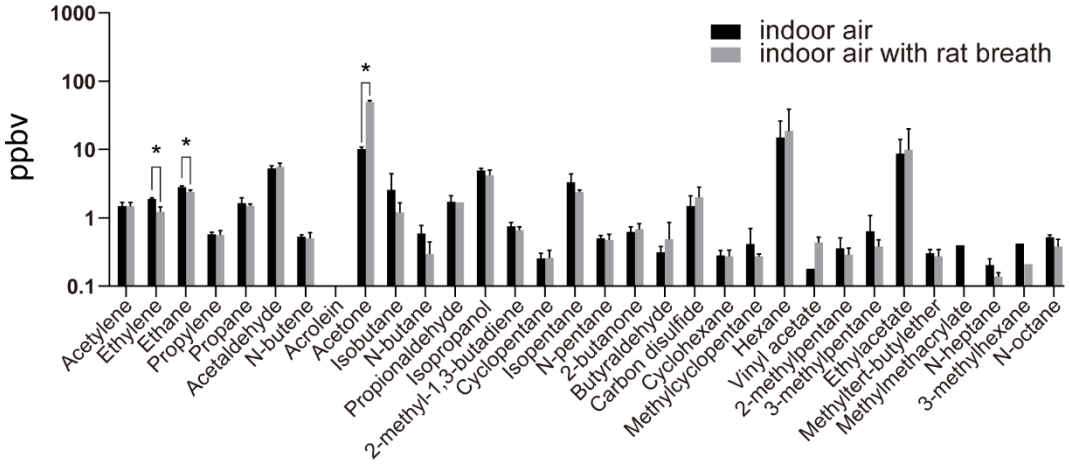


760

761 **Figure S1.** Experimental setup (RSTair) for measuring total VOCs from the rats with

762 and without different toxicant exposures.

763



764

765 **Figure S2.** Differentiations of VOC species from indoor air and those from rats'

766 exhaled breath. The data represent average levels of three independent experiments,

767 and error bars stand for the standard deviations. "*" indicates a statistically significant

768 difference at p-value =0.05.

769

Table S1 Primers used for RT-qPCR analysis of microRNA. The reverse primers are included in the TIANGEN® miRcute Plus miRNA qPCR Kit (SYBR Green) (Code No. FP411).

microRNA	Forward primers
MIR-122-5P	GAGTGTGACAATGGTGTTTG
MIR-125B-5P	CCTGAGACCCTAACTTGTGA
MIR-146A-5P	GAGAACTGAATTCCATGGGTT
MIR-155	TAATGCTAATCGTGATAGGGGTT
MIR-20B	AGTGCTCATAGTGCAGGTAG
MIR-210	CCTGCCCACCGCACACTG
MIR-21-5P	AGCTTATCAGACTGATGTTGA
MIR-33	GCATTGTAGTTGCATTGCA

Table S2 Expression level changes (before and after the exposure) of microRNA levels in blood samples from rats before and after exposure to ricin, O_3, endotoxin and CO_2. The blood samples were taken before exposure and 10 minutes after exposure. SD represents a standard deviation value. "*" indicates significant difference at p-value=0.05.

microRNA	Ricin		Endotoxin		Ozone		Carbon dioxide	
	Expression level change	SD	Expression level change	SD	Expression level change	SD	Expression level change	SD
miR-122	1.03	2.73	-0.70	0.19	5.22	8.50	-0.27	0.41
miR-125b	1.04	2.74	1.85	4.21	0.87	1.66	-0.26	0.17
miR-146a	0.37	1.14	-0.37	0.59	0.52*	0.26	0.48	0.71
miR-155	2.59	3.47	4.24	5.01	-0.47*	0.07	-0.26	0.04
miR-20b	-0.53	0.47	-0.81	0.16	0.48	0.69	0.65	0.64
miR-210	0.24	1.66	-0.68	0.33	2.07	2.69	0.12	0.17
miR-21	0.46	1.77	-0.36	0.55	-0.11	0.59	0.65	0.46
miR-33	-0.43*	0.17	-0.60	0.35	1.64	2.17	0.23	0.30

Video S1. Video clip of rat with the exposure to ozone for 10 minutes.