Journal of the American Association for Laboratory Animal Science
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Vol 47, No 2
March 2008
Pages 57–62

Ammonia and Carbon Dioxide Concentrations in Disposable and Reusable Ventilated Mouse Cages

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This study compares resuable and disposable individually ventilated mouse cages in terms of the formation of intracage CO_2 and NH_3. Crl:CD-1(ICR) female mice were placed in either disposable or reusable ventilated cages in a positive pressure animal rack. Intracage CO_2 and NH_3 were measured once daily for 9 d; temperature and relative humidity were monitored for the first 7 d. Results indicated higher CO_2 levels in the rear of the disposable cages and in the front of the reusable cages. This pattern corresponded to where the mice tended to congregate. However, CO_2 concentrations did not differ significantly between the 2 cage types. Average CO_2 levels in both cage types never exceeded approximately 3000 ppm. Intracage NH_3 began to rise in the reusable cages on day 4, reached approximately 50 ppm by day 5 and by day 9 was greater than 150 ppm at the cages' rear sampling port while remaining at approximately 70 ppm at the front sampling port. Intracage NH_3 levels in the disposable cages remained less than or equal to 3.2 ppm. Intracage temperature and relative humidity were approximately the same in both cage types. We concluded that the disposable ventilated cage performed satisfactorily under the conditions of the study.

Abbreviations: ACH, air changes per hour; IVC, individually ventilated cage

A growing body of literature documents the health effects on laboratory animals of intracage gases such as NH_3 and CO_2.[2,4,13,21,22,29] In general, static containment cages (that is, cages with filtered tops that are not connected to, or in the path of, a directional air flow) are more likely to create conditions conducive to the production of high concentrations of intracage NH_3 and CO_2 than are individually ventilated cages (IVCs).[15] Nevertheless, few published studies address the comparative efficacy of different styles of IVCs in evacuating these gases. Recently, disposable rodent cages have been developed for both routine and nonroutine animal housing (for example, biocontainment) and offer animal facilities potential cost savings in labor and equipment. We therefore studied the levels of CO_2 and NH_3 produced by mice housed in disposable IVCs placed in a positive-pressure animal rack and compared them with the NH_3 and CO_2 concentrations developed in a reusable IVC. We hypothesized that both cage types would be similarly efficacious in controlling CO_2 and NH_3 levels.

Materials and Methods

Disposable caging and rack. Polyethylene terephthalate cages and cage tops were used. Cage bottoms measured 27.3 × 18.0 cm at the level of the top of the bedding. The cage top, which had a small filter area, snap-fitted tightly to the rim of the cage bottom. The top also had 2 preformed plastic ports; one for air to enter and another to exhaust cage air. These ports were located approximately 14 cm above the cage bottom and directly articulated with air supply and exhaust ports on the rack. The disposable water bottle was largely outside of the cage, fitting into an indentation in the cage top. The total interior volume of the bottom and top (including preformed indentations in the cage top) was 5876 ml.

The disposable IVCs were placed in a positive-air–pressure double-sided rack that was capable of holding 112 mouse cages when full, although not all cage slots had to be occupied for proper operation. The rack was equipped with exhaust and supply blowers. The rack provided HEPA-filtered air, was set at 60 air changes/h (ACH), and exhausted into the room through a HEPA filter built into the exhaust blower.

Reusable caging and rack. Polysulfone cages were used. Cage bottoms measured 28.0 × 17.3 cm at the level of the top of the bedding. The cage top overhung the cage bottom and had a relatively large filter area. The total interior volume (excluding the portion of the cage top that overhung the bottom of the cage) was 6320 ml. The space occupied by the water bottle and the wire-bar top was excluded from the volume determination. There was 1 air entry port at the rear of the bottom section, approximately 3 cm above the cage floor. Most exhaust air escaped around the interface between the cage top and bottom, was collected by an air-exhaust plenum on the cage rack (with exhaust vents located immediately adjacent and horizontally parallel to the rear of the cage top), and subsequently was evacuated from the rack through a direct connection to the building's exhaust system. The reusable IVC's water bottle was entirely enclosed within the cage.

Reusable IVCs were placed in a positive-air–pressure mouse rack that was capable of holding 90 cages when full, although not all cage slots had to be occupied for proper operation. The rack was equipped with a supply blower providing HEPA-filtered air. The rack was set for 60 ACH and connected to the building exhaust, which was set at 50 ft^3/min.

Animals. Retired breeder female Crl:CD-1(ICR) mice (Charles River Laboratories, Wilmington, MA) were used. Serologic

Received: 17 Sep 2007. Revision requested: 1 Nov 2007. Accepted: 28 Dec 2007.
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Editor's note: Vendor donation of the ventilated rack and disposable caging represents a potential conflict of interest regarding the data presented in this article.

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monitoring while they were in our animal facility (Charles River Laboratories Diagnostic Services, Wilmington, MA) confirmed freedom from murine norovirus, mouse parvoviruses, mouse hepatitis virus, reovirus (types 1 and 3), lymphocytic choriomeningitis virus, lactate dehydrogenase-elevating virus, mouse rotavirus, Theiler murine encephalomyelitis virus, *Ectromelia*, hantavirus, mouse adenovirus, Sendai virus, and *Mycoplasma* spp. Animals also were free of common pathogenic mouse ectoparasites and endoparasites.

Husbandry. Mice were housed at 5 animals/cage in a room free of other animals. They were fed a commercial irradiated laboratory mouse diet (Purina LabDiet 5P76, Ralston Purina, St Louis, MO). A reversed 12:12-h light:dark cycle was used so that the dark cycle occurred during working hours. This adjustment was done to help ensure maximal animal activity during the sampling periods.[12,29] Animals were acclimated to this cycle for 2 wk prior to initiating testing. Lights were turned on during sampling. Each cage had 270 g of food placed on its feed tray. Animals were given 275 ml of acidified reverse-osmosis water in a water bottle for reusable IVCs and disposable water bottles for the disposable IVCs. Bottles were placed carefully to minimize spillage. Paper chip bedding (160 g; Paperchip Soft Texture, Shepherd Specialty Papers, Kalamazoo, MI), approximately 1 cm deep, was placed in each cage. Paper chip bedding was used because studies suggested that it would be somewhat less effective in maintaining cage homeostasis than corncob bedding.[3,19]

Control cages (without mice) had the same amount of food, water, and bedding as did cages with mice. The room was ventilated at approximately 22 ACH. Room supply airflow was approximately 1163 ft^3/min, and exhaust was approximately 1064 ft^3/min.

Gas, temperature, and humidity measurements. The stainless steel gas sampling ports were commercial bulkhead fittings with barbed tubing connections (MBHA-1332-316, Beswick Engineering, Greenland, NH). They were placed in the front and left-rear of each cage through holes drilled in the plastic. The bottom of the port was 1.9 cm above the top of the bedding, which was the approximate height above the bedding of a mouse's nose. A small piece of plastic intravenous tubing (about 4 cm in length) was attached to the exterior of the sampling port, and a standard Luer adapter was placed on the other end of the tubing (Figure 1). Air flow in or out of the port was effectively stopped by a standard pinch clamp on the tubing and a Luer lock plug placed over the end of the Luer adapter. The plug provided an additional barrier against air escape or entry and was removed during sampling. A stainless steel mesh sink strainer (manufacturer unknown) was secured over the inside of each sampling port to prevent the mice from breathing directly on the port during sampling. The strainer was 4.0 cm in diameter and extended 2.5 cm into the cage. It kept mice at least 2 cm from the ports. All screws used had rubber washers on the outside of the cage and were secured by standard steel nuts.

NH_3 and CO_2 measurements were made with a chip measurement analyzer (model 6405300, Dräger Safety, Pittsburgh, PA). A small piece of rubber tubing (approximately 5 cm long) was attached to the sampling port of the analyzer. During testing, the other end of this tubing was attached to the Luer adapter on the sampling port of the cage. The gas analysis system was self-calibrating in that all calibration information was prestored on the bar code of the NH_3 or CO_2 analysis chip that was inserted into the analyzer. An electronic system self-test was performed every time the analyzer was switched on. When the chip was inserted into the analyzer, the calibration information from the chip was automatically downloaded to the analyzer. A sampling system test was automatically performed before each gas concentration analysis, and the analyzer was flushed with room air after testing each cage.

For NH_3 measurements, chips with a detection range of 2.0 to 50 ppm or 10 to 150 ppm were used (the higher range chip was used only if a value greater than 50 ppm was detected initially by using the lower range chip). Chips were accurate to 7% to 8% of the measured value and reproducible at 10% to 12% (SD).[7,8]

For CO_2 measurements, chips with a detection range of 200 to 3000 ppm or 1000 to 25,000 ppm were used (the higher range chip was used only if initial findings with the lower range chip indicated a value greater than 3000 ppm). Chips were accurate to 5% to 7% of the measured value and reproducible at 7% to 10% (SD).[7,8] For either NH_3 or CO_2 gas analysis, the analyzer withdrew 15 ml air/min. Maximum sampling time was 2.75 min (which occurred when very low levels of NH_3 or CO_2 were detected). More typically, sampling times were 45 to 60 s.

Intracage temperature and humidity were measured and recorded hourly for the first 7 d by using a remote data logger (HO8-004-02, Anset Computer, Bourne, MA) preprogrammed for 7 d of recording and attached to the inside of the cage above the food hopper. The study was initially designed to terminate in 7 d, but because we allowed it to progress for a total of 9 d and because the cage could not be opened during the study, temperature and humidity recordings are for the first 7 d only. Ambient room temperature and humidity were recorded once daily at the beginning of the testing session by using a sling psychrometer (Bacharach, Pittsburgh, PA).

Animal randomization. Each mouse was marked for identification and weighed; those with weights outside of 1.5 SDs were excluded. All included animals were then placed in rank order, by weight, from lowest to highest. This weight ranking was divided into 5 groups with 6 animals in each group. By use of a random number generator, 1 animal from each of the 5 weight groups was placed in either a disposable or reusable IVC. This process was repeated until each of 6 cages had 5 animals. When the weights of all the mice in any 1 cage were averaged, the 6-cage average ranged from 38.6 to 38.9 g.

General study design. The study was designed to be performed in triplicate (3 animal-containing cages and 3 nonanimal control cages of each cage type), but due to a technical problem, we only used duplicate data for the disposable IVCs. One cage (n = 5 mice) was placed in each of the 3 middle rows of their respective ventilated rack. Cages with mice were on the outer edge of the rack, and each cage had an unoccupied control cage next to it. The 2 racks faced each other, being separate by approximately 1.3 m. At the same time each day, air samples were taken from each sampling port of each cage and analyzed for either CO_2 or NH_3 concentration. All cages were sampled first for NH_3. After all NH_3 samples were taken from all cages, the same cages then were sampled for CO_2. The pattern was to first take samples from a disposable IVC with animals (front then rear port), then from the associated control cage (front then rear ports). After all disposable IVCs were sampled, the reusable IVCs were sampled. Intracage temperature and humidity were recorded continually remotely once every hour. Cages were otherwise left undisturbed during the course of the study. The study was ended after 9 d. All work was approved by the University of Massachusetts Medical School's institutional animal care and use committee.

Preliminary testing. Preliminary testing suggested that NH_3 and CO_2 concentrations might differ between the front and

back of the cages, therefore sampling ports were placed at those 2 locations. We did not find significant gas concentration differences between higher and lower levels of the cages. To test for the air tightness of the sampling ports, smoke sticks were placed within cages (Tel-Tru Smoke Stick, Liberty Industries, East Berlin, CT). We found no overt escape of smoke through closed ports or screw holes.

To confirm the consistency of the remote temperature and humidity monitors (described earlier), we tested the monitors against the sling psychrometer and then against each other. All readings were nearly identical.

Although the original intent of this study was to use the mouse racks as supplied by the manufacturer (that is, without further validation of air flows), we nevertheless performed an initial verification of rack air flows by using a digital manometer for the disposable IVC rack and a specialty manometer for the reusable IVC rack. Both racks performed at or near 60 ACH.

Statistical methods. For the evaluation of CO_2 data, we used actual measured values. For NH_3 data evaluation, we used actual measured values, but when measured concentrations were less than 2.0 ppm, we assigned a value of 1.0 ppm (the midpoint in the range of 0 to 2 ppm). For values greater than 150.0 ppm, we assigned a value of 274.5 ppm (an estimate of the median value in this range based on the normal distribution of log-transformed values). As indicated earlier, all disposable IVC data came from 2 animal-containing or control cages, whereas data from reusable IVCs came from 3 animal-containing or control cages. The effects of cage type, sampling port location, and time were evaluated by using general linear mixed models[16] to fit repeated measures growth curve models for NH_3 and CO_2. In the presence of significant differences among means, pairwise comparisons were made by using the Tukey Honestly Significant Difference test (using the estimated covariance matrix to account for correlated observations).[11] The distributional characteristics of outcome measures were evaluated both graphically and by the Kolmogorov–Smirnov Goodness of Fit Test for Normality.[6] Natural logarithms of outcomes were applied to better approximate normally distributed residuals. All computations were performed by using the SAS Proc Mixed procedure[24] and SAS version 9.1.3[25] statistical software package. Statistical significance was defined as present when associated *P* values were less than 0.050. For this study design, power analyses showed that the sample sizes provided 85% power for detecting a true difference of 20 ppm NH_3 between cage types and greater than 90% power for detecting a true difference of 75 ppm CO_2.

Results

CO_2. All unoccupied cages maintained CO_2 concentrations near ambient room levels (440 to 530 ppm) throughout the study. Unoccupied cages showed no significant differences in CO_2 concentrations between the front and rear sampling ports or between the 2 types of cages.

At the first sampling time point, which occurred approximately 60 min after placing animals in the cages, CO_2 concentrations were higher in both disposable and reusable IVCs as compared with the ambient level (Figure 1). Over the 9 d of the study, CO_2 concentration differed significantly between samples taken from the front sampling ports compared with the rear sampling ports of reusable IVCs ($P = 0.0064$, with 1.126 times more CO_2 at the front port). For the disposable IVCs, differences approached but did not reach statistical significance ($P = 0.0511$, with 1.103 times more CO_2 at the rear port). For the disposable IVCs, mice tended to cluster in the back half of their cages, where CO_2 was detected at higher levels. The opposite was found for reusable IVCs, where higher CO_2 levels usually were detected from the front ports, and the animals tended to congregate in the front of those cages. When the CO_2 concentrations from either of the sampling ports were compared between disposable IVCs and reusable IVCs, there were no significant differences between the 2 cage types. The lowest CO_2 concentration recorded in an individual animal-containing cage was 1040 ppm, and the highest was 4000 ppm.

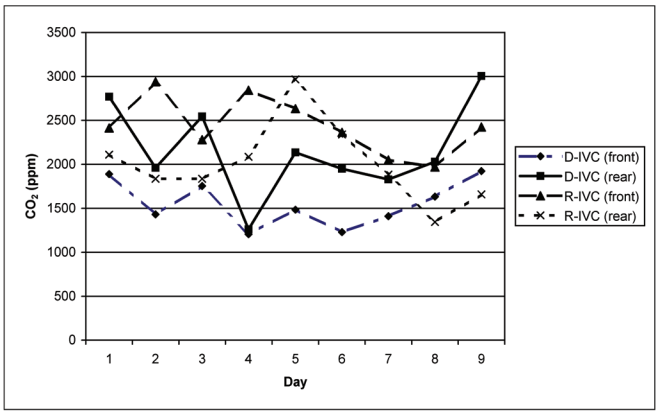

Figure 1. Average daily CO_2 concentrations from front or rear sampling ports of 2 disposable (D-IVC) and 3 reusable (R-IVC) ventilated cages.

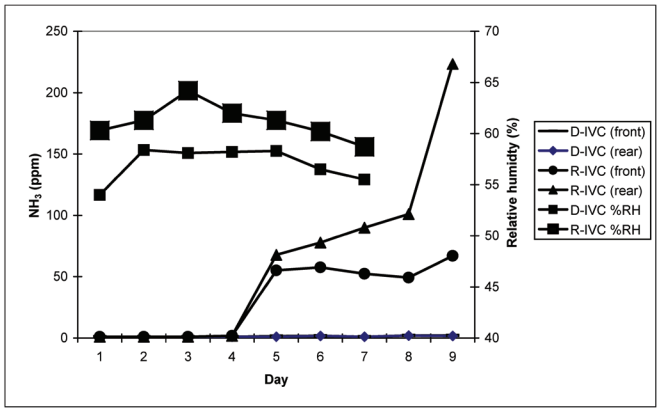

Figure 2. Average daily NH_3 concentrations from front and rear sampling ports of 2 disposable (D-IVC) and 3 reusable (R-IVC) ventilated cages and associated average relative humidity (%RH) at the time of sampling.

The disposable IVC cages showed no clear pattern of temporally changing CO_2 concentrations over the 9 d of the study, however, the reusable IVC cages showed a significant reduction in CO_2 over the same 9 d ($P = 0.0305$). The reason for this is not known.

NH_3. Ambient (room) NH_3 levels remained below 2.0 ppm. There were no statistically significant differences between front and rear sampling ports of either cage type. Mean intracage NH_3 levels in reusable IVCs were occasionally greater than 25 ppm and, in some instances, were greater than 150 ppm (Figure 2). Animals in the cages with high NH_3 concentrations were closely monitored, but no overt problems (that is, sneezing, rubbing of eyes or nose, erythema, changes in behavior patterns) were noticed, and with approval from the institutional animal care and use committee, studies were continued for an additional 2 d. Unoccupied cages maintained negligible NH_3 concentrations throughout the study. Infrequent low concentrations of NH_3 were found in 4 of 90 unoccuppied cage measurements (maximum, 3.8 ppm, data not shown) and most likely occurred

from NH_3 carryover from the previously sampled cage (which contained animals) due to incomplete flushing of the analyzer between cages. In unoccupied cages, NH_3 concentrations did not differ significantly between front and rear ports or between the 2 types of cages.

In cages with mice, NH_3 levels did not rise above the minimal detectable concentration (2.0 ppm) until day 4. At that time, concentrations of approximately 2 to 3 ppm were detected from both sampling ports of 1 reusable IVC and the rear port of another reusable IVC. By day 5, the average NH_3 concentration in the reusable IVCs was near 70 ppm (Figure 2) with the highest concentrations found in 2 of the 3 cages. In the 1 remaining reusable IVC, NH_3 concentrations remained at 5.1 ppm or less until study day 8, at which time concentrations rose to 33 and 48 ppm at the front and rear ports, respectively. By day 9, all reusable IVCs had NH_3 concentrations between 59 and 77 ppm at the front sampling ports and greater than 120 ppm at the rear ports.

Throughout the course of the study, the disposable IVCs with animals maintained NH_3 concentrations that were no greater than 3.2 ppm in any cage. Reusable IVCs with animals had higher NH_3 than did unoccupied cages ($P = 0.0046$). In disposable IVCs, NH_3 did not differ significantly between occupied and unoccupied cages. When occupied, disposable IVCs had lower NH_3 concentrations than reusable IVCs (P=.0176).

Temperature and humidity. During the study, the mean daily ambient (room) temperature at the time of sampling was 21.4 °C, and the mean relative humidity was 43.9%. These readings closely correlated with the recordings from the control cages. The ranges of room ambient temperature and relative humidity at the time of sampling were 20.6 to 21.6 °C and 41% to 50%, respectively.

The mean high and mean low intracage temperature and humidity readings for the entire study (Table 1) indicate minimal differences between disposable and reusable IVCs. At the actual time of NH_3 sampling, humidity ranged from 54.0% to 58.4% in the disposable IVCs and 58.7% to 64.2% in the reusable cages. For both cage types, at the time of sampling, mean relative humidity peaked near day 3 and then gradually decreased (Figure 3).

Discussion

The primary intent of this study was to evaluate intracage NH_3 and CO_2 levels that developed in a disposable IVC as compared with a reusable IVC that we had used for many years. Specific effects on animal health or behavior were not evaluated. Based on our observations from routine husbandry and the findings of others who used various types of mouse cages to study NH_3 and CO_2,[17,27] our working hypothesis was that concentrations of these gasses would not significantly differ between disposable and reusable IVCs. Nevertheless, over the course of the study, we found significantly higher NH_3 concentrations in the reusable IVCs. We also found significant differences in CO_2 concentrations between front and rear sampling ports of the cages, although overall there were no CO_2 concentration differences between the disposable and reusable IVCs. Given that animals in the disposable cages congregated near the rear of their cages and mice in reusable cages congregated near the front of their cages at the time of sampling, it was not surprising that CO_2 concentrations were higher where the animals were located.

CO_2. CO_2 concentrations were greater at the end of the cage where animals congregated. At this time, we can only hypothesize why the mice tended to congregate at different ends of the 2 cage types. The reusable IVC used a low-velocity air flow with a perforated metal air inlet located at the back of the cage at the level of the mice. The air is pushed downward and forward toward the front of the cage. For the disposable IVC, the supply air port is on the top of the cage toward the front, and air is exhausted through a port near the top rear of the cage. There is a high-volume, low-pressure air flow (0.2 to 2.5 in. H_2O), which is further interrupted by the presence of the food tray. We speculate that these differences may influence preferred areas of animal congregation within the cage. In a study with BALB/c female mice housed in IVCs with 60 ACH, the animals preferred having the air flow enter from the top of the cage.[1]

Although considerable differences in methodology (for example, different cages, cage racks, gas analysis methodology, animal strains and stocks) do not allow for detailed comparisons with other studies, the CO_2 levels detected in this study were in general agreement with those found by others using IVCs.[17,21,22,27] Room ventilation rates will affect CO_2 levels in cages, but even with static caging in rooms with 20 ACH, CO_2 concentrations similar to those in the present study have been reported.[20] Further, after 6 d, IVCs at 60 cage ACH demonstrated CO_2 levels similar to those in the present study, although NH_3 levels were lower (approximately 1.2 ppm).[22] Intracage temperature was approximately the same as in the current study, whereas intracage relative humidity was slightly lower. In our study CO_2 levels in the reusable IVCs fell over time, but a reason for this was not apparent in a review of hourly intracage temperature and humidity recordings and animal activity observations made during the sampling times.

The present human occupational exposure limit for CO_2 is 5000 ppm for an 8-h time-weighted average exposure duration,[30] although continuous around-the-clock exposures of 2500 to 5000 ppm may cause headaches.[10] Currently, there are no recommended CO_2 limits for mice. Even though mice are continuously exposed to CO_2 in a cage environment, human limits may not be appropriate for laboratory animals due to evolutionary changes resulting from the adaptation of many rodent species to spending a portion of their lives underground.[13] The authors of the previous study[13] suggested that until further evidence is provided, a CO_2 concentration of 1.5% (15,000 ppm) should be considered the experimental limit, requiring a few days of recovery after exposure. That recommendation may have been based on the work of others who found CO_2 levels as high as 1.4% in artificial rat burrows.[28] More recently, levels greater than 50,000 ppm have been deemed inappropriate for animal welfare.[14] In the present study, the mean daily CO_2 levels ranged from approximately 1250 to 3000 ppm.

NH_3.

Intracage NH_3 levels are potentially subject to multiple variables, such as the bedding used, cage design, cage ventilation (including air changes, filter cleanliness, and methods used to evacuate cage air), cage and room temperature and humidity, number and size of animals in the cage, cage cleaning frequency, animal health, and so forth. In the present study, we controlled all of these variables except the cage and rack design and function (which were specific to each manufacturer) and found that NH_3 levels in 2 of the 3 reusable IVCs began to increase on day 4 and were greater than 50 ppm by day 5. This rapid rise differs from findings in other studies using different methodology [21,22]and highlights the difficulty in making direct comparisons. Similarly, a study involving rats indicated that bedding type did not have an effect on NH_3 concentrations,[4] whereas other experiments[21,23] suggested the opposite conclusion for mice. Intracage NH_3 concentrations for the 2 disposable IVCs with mice never rose above 3.2 ppm.

Table 1. Mean intracage temperature and percentage relative humidity over 7 d in 2 disposable and 3 reusable IVCs.

	Mean low temperature (°C)	Mean high temperature (°C)	Temperature range (°C)	Mean low relative humidity (%)	Mean high relative humidity (%)	Relative humidity range (%)
Disposable, with mice	22.4	25.0	22.0–25.0	50.9	63.0	50.7–64.0
Reusable, with mice	22.1	23.6	22.0–24.4	52.8	70.4	51.0–72.5
Disposable, no mice	20.9	21.7	20.9–21.7	37.5	48.0	36.0–48.0
Reusable, no mice	20.6	21.3	20.6–21.7	38.3	46.4	31.6–50.0

In both the CO_2 and NH_3 experiments in the current study, intracage temperature remained within the recommended secondary enclosure (that is, room) range of the *Guide for the Care and Use of Laboratory Animals*.[18] Intracage humidity was almost always within the *Guide*'s recommended range (30% to 70%), although a single reading from a reusable cage was just outside of that range (72.5%, Table 1) and returned to an acceptable level in approximately 2 h (data not shown). Each day, at the time of NH_3 sampling, the intracage humidity was 3 to 4 percentage points higher in the reusable cages than the disposable ones. Relative humidity is well known to affect NH_3 production,[15,17,23] but whether the somewhat small humidity differences we recorded significantly affected NH_3 production is unclear. Intracage humidity in 4 different types of ventilated cages showed statistically nonsignificant differences, and the associated NH_3 levels were essentially the same.[17]

In the current study, the mice did not demonstrate clinical abnormalities, although other authors have reported health problems due to high or prolonged NH_3 exposure.[2,4,9,26,29,30] In contrast, no atypical clinical findings were associated with NH_3 levels as high as 140 ppm,[27] and another study reported that NH_3 concentrations as high as 241 ppm had no effect on nasal passage histology.[22] These previous findings are in line with results from another study,[5] which demonstrated no significant clinical problems in rats exposed to levels of NH_3 greater than the maximum in our study. Currently, there are no upper level NH_3 exposure guidelines for mice; for humans, the 8-h time-weighted average exposure limit is 50 ppm.[30]

In summary, our findings indicate that the disposable IVC studied performed satisfactorily under the conditions used. NH_3 levels were equal to or less than 3.2 ppm over the course of 9 d, and CO_2 levels never rose above 0.3% (3000 ppm). Temperature and humidity remained within the secondary enclosure boundaries of the *Guide*.[18] Extrapolation of our results should be made with caution, because published reports indicate extensive variations in findings depending on the methodology used.

Acknowledgment

The authors acknowledge that a vendor supplied a ventilated rack and caging for the disposable system.

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