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Euthanasia by CO_2 Inhalation Affects Potassium Levels in Mice

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We and others frequently have noted serum potassium levels of 8.0 ± 0.85 mEq/L or greater in laboratory mice; this concentration has even been published as the upper limit of a 'normal' reference range. However, if bone fide, this potassium concentration would be incompatible with life in all species. We investigated conditions frequently encountered in the research setting to distinguish artifactual from true hyperkalemia. Variables evaluated included site of collection, time allowed for clot formation before serum separation, time elapsed between collection and analysis of samples collected in a serum separator tube, precollection method of anesthesia, and euthanasia technique. Serum potassium was measured from 75 C57BL/6NTac 10-wk-old female mice and divided into at least 5 mice per variable. Animals were euthanized by exsanguination immediately after terminal CO_2 or ketamine–xylazine (KX) administration. Mice euthanized with CO_2 had higher mean serum potassium (7.0 ± 0.5 mEq/L) and range serum potassium (6.0 to 8.1 mEq/L) than did KX-treated mice. CO_2 inhalation resulted in significantly lower blood pH (6.9 ± 0.1), higher pCO_2 (153.3 ± 38.8 mm Hg), and higher lactate levels (3.9 ± 0.9 mmol/L) than did KX anesthesia followed by exsanguination. These results suggest that antemortem respiratory acidosis from CO_2 administration causes artifactual hyperkalemia in mice. Therefore, blood collection under KX anesthesia is preferable over CO_2 inhalation to obtain accurate potassium values from mice.

Abbreviation: KX, ketamine–xylazine.

Laboratory mice are widely used as experimental models for many human diseases. To be useful, these models need to have standard responses to usual procedures or treatments and to function as expected. However, great variation of serum potassium levels has been observed in mice used for research.[7,22,24,32,39,40,51]

High levels of serum potassium (hyperkalemia) can be attributed to many factors. One of these is acidemia, which occurs when intracellular potassium is exchanged for excessive extracellular protons. Hyperkalemia is thought to be uncommon in respiratory acidosis, perhaps due to CO_2's diffusible nature which allows pH equalization across the cell membrane.[15] However, CO_2 used for euthanasia of laboratory animals has been reported to cause acidosis.[11,23] In one study, hypercapnia induced after prolonged CO_2 inhalation resulted in hyperkalemia in dogs.[25]

In addition, sex-associated differences in serum potassium levels may occur. Sexual dimorphism was the explanation for the differences in renal and plasma potassium levels observed in CD1 and C57BL/6 mice.[7,10] This sex-associated difference may be related to the presence of testosterone, because CD1 female mice treated with testosterone exhibited increased potassium, whereas orchiectomized CD1 male mice had a significant decrease in plasma potassium levels.[10] In addition, CD1 male mice showed a higher mortality rate than females when treated with potassium-sparing diuretics.[10]

High serum potassium in animals may be due to artifacts associated with preanalytical sample handling or to blood dyscrasias, such as extremely high leukocytosis and thrombocytosis.[15] Delaying the separation of serum from clotted blood can result in pseudohyperkalemia; increased levels of phosphorous, lactate dehydrogenase, and bilirubin; and decreased glucose.[31,47] In addition, the site or technique used for blood collection can result in increased serum potassium due to hemolysis or tissue damage.[44] Numerous changes in clinical chemistry results, not limited to potassium levels, have been associated with specific collection sites in studies done with mice.[1,21,36,40,44,47] In mice, samples collected through cardiocentesis have increased aspartate aminotransferase, creatine kinase, lactate dehydrogenase, and alanine aminotransferase values due to muscle damage.[47] Blind cardiocentesis may also result in hemolysis and contamination of sample by bodily fluids.[1] One study showed that compared with jugular venipuncture, cardiocentesis resulted in lower creatine kinase and higher glucose values.[36]

The technique used to obtain blood for analysis can affect various parameters. Blood collection by use of the tail vein may produce variation in blood values, and only small volumes can be collected because of clotting or vasoconstriction.[1,40] Sample-to-sample variation and increased PCV and hemoglobin levels have been reported in samples collected by tail venipuncture in mice.[21,40] In addition, collection of blood from the orbital venous sinus can elevate lactate dehydrogenase, aspartate aminotransferase, creatine kinase, alkaline phosphatase, calcium, aldolase, and albumin levels.[1,44,47] Retroorbital collection results in mean serum potassium levels as high as 6.6 mmol/L with only moderate hemolysis in C57BL/6J mice.[28] Retroorbital collection and free peritoneal collection can both lead to significant variation in creatine kinase values when compared with cardiocentesis and caudal vena cava collection. Next to cardiocentesis, blood

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collection from the caudal vena cava is considered to be the method of choice by some authors. Both methods yield the most consistent clinical chemistry values when compared with those of free peritoneal blood and retroorbital phlebotomy.[1,44] When using the caudal vena cava, the operator must collect blood slowly because of the thin-walled vessel's tendency to collapse. This demand may help prevent hemolysis.[44]

Despite all these findings, little has been reported on the effects of collection techniques on serum potassium levels. Preanalytical handling procedures may be responsible for the consistently high serum potassium levels observed for many mouse strains, but no study has been performed to differentiate elevated serum potassium levels due to artifactual from true hyperkalemia.

Potassium levels are essential in preclinical toxicity studies and are needed for accurate evaluation of chemistry parameters as an integral part of phenotyping procedures. Furthermore the ability to identify true hyperkalemia is crucial when working with transgenic mouse models with the expected phenotype of hyperkalemia. An example of this situation is the *ENaC* knockout mouse, in which the deleted subunit of the highly amiloride-sensitive epithelial sodium channel predisposes the animal to hyperkalemia.[9] Mice with homozygous deletions in the *ENaC* gene suffer from a salt-wasting syndrome with hyperkalemia in the face of increased plasma aldosterone concentrations.[5,29] This condition mirrors human pseudohypoaldosteronism type 1.[9,14] Therefore, the hyperkalemia in this and similar genetically modified mouse models must be differentiated from artifactual hyperkalemia induced by preanalytical handling procedures.

In the current study, we evaluated blood serum potassium in samples collected under conditions frequently found in the research laboratory setting. We investigated various procedures that may affect serum potassium levels and assorted factors that can be used to differentiate true hyperkalemia. To that end, we analyzed site of blood collection, serum separator use, method of delivery, time allowed for clot formation before serum separation, time elapsed between collection and analysis of samples, precollection method of anesthesia, and euthanasia technique for effects on potassium concentrations. By identifying a technique to minimize variation of potassium levels in laboratory mice, we aim to help the research community to obtain standardized values for potassium levels in mice used in preclinical toxicity studies and as disease models. In addition, our observations may be useful in studies using other laboratory animals that are commonly euthanized by carbon dioxide, including rats, gerbils, and hamsters.

Materials and Methods

Animals. A total of 75 adult C57BL/6NTac mice (age, 10 wk; Taconic, Germantown, NY) were used in this study. Female mice were selected to avoid variations in potassium levels caused by testosterone.[7,10] The C57BL/6 strain was chosen because it is the strain used most often for creating transgenics.[49] Mice were housed in the AAALAC-accredited animal facility of Memorial Sloan–Kettering Cancer Center in accordance with current regulations and standards of the US Department of Agriculture, Department of Health and Human Services, and National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee of Memorial Sloan–Kettering Cancer Center. All animals were housed in individually ventilated polysulfone shoebox cages (Thoren Systems, Hazelton, PA) on aspen-chip bedding (PWI Industries Canada, Quebec, Canada) and had ad libitum access to feed (PicoLab 5058, PMI, Richmond, IN) and acidified water (pH 2.5 to 2.8). Animal were free of mouse hepatitis virus, Sendai virus, mouse parvovirus, minute virus of mice, pneumonia virus of mice, mouse rotavirus, lymphocytic choriomeningitis virus, ectromelia virus, murine norovirus, reovirus type 3, Theiler mouse encephalomyelitis virus, mouse adenovirus, K virus, polyoma virus, mouse cytomegalovirus, mouse thymic virus, Haanta virus, lactic dehydrogenase elevating virus, cilia-associated respiratory bacillus, *Mycoplasma pulmonis*, *Helicobacter* spp., *Salmonella* spp., *Clostridium piliforme*, *Corynebacterium kutscheri*, and *Citrobacter rodentium* as well as endoparasites and ectoparasites. Animal rooms were maintained at 22 ± 1 ºC (72 ± 2 °F) and a relative humidity of 30% to 70% with 15 air changes hourly and a 12:12-h light:dark cycle.

Standard method of sample collection and handling. Blood samples were collected at the same time of day (1100) for all animals to avoid circadian cycle variation in blood chemistry values. The standard protocol entailed euthanasia of mice by CO_2 inhalation followed by maximal terminal collection (approximately 1 mL) by cardiocentesis with a 1-mL syringe and 25-gauge, 5/8-in. tuberculin-type needle. In accordance with published guidelines,[2,48] euthanasia was achieved by delivering 100% CO_2 for a minimum of 3 min from a pressurized system at 5 lb/in. into an enclosed non-precharged cage containing the animal, followed by exsanguination.

Samples were collected aerobically in microcollection tubes (BD Microtainers, Becton Dickinson, Franklin Lakes, NJ) with silica clot activators and gel serum separators. Needle tips were removed when blood was delivered to the microcollection tubes. All samples were allowed to clot at room temperature for 15 min; under these conditions normal clotting time for mice is 2 to 10 min.[30] Samples were centrifuged for 10 min at 20 °C and 1000 × *g* and analyzed (model AU400 chemistry analyzer, Irving, TX). This instrument uses an ion-selective electrode to measure potassium.

Blood delivery technique. The method of blood delivery was evaluated to test the hypothesis that leaving the needle attached to the syringe while depositing blood into a collection tube would result in excessive pressure, causing red cell lysis and, consequently, elevated potassium levels in the samples. To this end, half of the blood collected from each of 5 mice (approximately 500 µL per mouse) was transferred through the needle into microcollection tubes with serum separators. The remaining half of each sample (approximately 500 µL per mouse) was transferred similarly but with the needle detached.

Clotting time before centrifugation. The effect of clotting time before centrifugation of whole blood was evaluated to test the hypothesis that spinning blood immediately after collection without sufficient clot formation would result in hemolysis and elevated serum potassium levels. Ten mice were exposed to CO_2 for 3 min, followed by immediate blood collection. Half of the blood collected from each mouse (approximately 500 µL) was allowed to clot for 15 min, centrifuged, and used as the standard control. The animals then were divided in 2 groups. In one group (no clot), blood was centrifuged immediately without clot formation, whereas in the other group (clot), blood was allowed to clot for 30 min. All samples were submitted for analysis 30 min after centrifugation.

Method of serum separation. Potassium levels of samples that had the serum separated manually were compared with samples in which a gel serum separator was used. Half of the blood collected from each of 5 mice (approximately 500 µL per mouse) was separated into 150-µL aliquots and added to microcollection tubes (BD Microtainer Tubes, Becton Dickinson) with a serum separator. The remaining half (approximately 500 µL)

was aliquoted into microcollection tubes without gel serum separator; serum from these samples was separated manually immediately after clotting for 15 min and centrifugation. Samples were analyzed 30 min after centrifugation. This variable was evaluated to determine whether potassium was able to leak from red blood cells and platelets that formed the clot and diffuse through the gel disc into the serum.

Latency between collection and analysis. The time elapsed between collection and analysis was evaluated to determine whether potassium could diffuse from the clot into the serum through the gel separator when samples are stored for prolonged periods.

Blood from 3 groups of 5 mice (groups 4, 8, and 24) was assessed at 4, 8, and 24 h after collection. Half of the blood (approximately 500 µL) from mice in each group was analyzed immediately after collection (time 0) according to our standard protocol. The remaining blood was centrifuged, refrigerated, and analyzed 4 h (Group 4), 8 h (Group 8), and 24 h (Group 24) after collection. These intervals were chosen to correspond to the most likely delay periods from the time of blood collection to the time of analysis in our laboratory.

Collection method. The effects of intracardiac and caudal vena cava collection on serum potassium levels were compared. Blood was collected from 5 mice by cardiocentesis in accordance with our standard protocol. In a second group of 5 mice, the peritoneum was opened and abdominal viscera moved to the side to expose the spinal column. Blood then was collected from the caudal vena cava by aspirating slowly, as described elsewhere.[44] Samples were analyzed 30 min after collection. We postulated that cardiocentesis would result in higher potassium levels, due to greater tissue trauma and hemolysis, than would collection from the caudal vena cava.

Euthanasia method. Serum potassium levels were compared in mice euthanized by terminal ketamine–xylazine (KX) anesthesia or by CO_2 inhalation. A common dose for KX anesthesia in the mouse is 100 mg/kg ketamine and 10 mg/kg xylazine, although cocktails consisting of 120 mg/kg ketamine and 16 mg/kg xylazine have been described for use for embryo transfers in mice.[20] In the current study, we used 110 mg/kg ketamine and 16 mg/kg xylazine to anesthetize 5 mice prior to terminal exsanguination. Samples were handled according to the standard protocol. The resulting data were compared with those obtained from mice euthanized with CO_2 during previous phases of the study.

Blood gas analysis. Blood gases were analyzed from samples from 12 CO_2 mice and 16 KX mice. Animals were exsanguinated by cardiocentesis anaerobically with lithium heparinized 1-mL arterial blood gas samplers (MarQuest Aspirator, MarQuest, Englewood, CO). Samples then were transported on crushed ice and analyzed within 10 min of collection by using a RAPIDlab 1265 blood-gas analyzer (Siemens, Deerfield, IL). In addition to blood gas values, plasma electrolyte values were obtained. Blood smears were prepared from 7 CO_2 and 7 KX mice from this cohort to manually estimate platelet counts. The slides were stained with a modified Wright–Giemsa solution and examined microscopically (magnification, ×500).

Reference intervals. The ALX laboratory (New York, New York) generated reference clinical chemistry values for each assay. The parameters were based on values obtained from serum analysis of 60 to 100 normal healthy mice from a variety of background strains. The parametric reference interval was calculated by using a commercial software package (Analyze-it for Microsoft Excel [Microsoft, Redmond, WA], 2008, version 2.11, Analyze-it Software, Leeds, UK). For serum potassium, the lower confidence interval of 95% was 3.154 to 3.612 mEq/L, and the upper confidence interval was 5.228 to 5.686 mEq/L. The 95% upper and lower limits were set at 3.383 and 5.457 mEq/L, respectively.

Statistical analysis. All analyses were completed by using SAS 9.1 (SAS Institute, Cary, NC); P values less than 0.05 were considered statistically significant. The Wilcoxon rank sum test was used for comparing groups by serum potassium, plasma potassium, collection method, and blood gas analyzer values. The average value across the aliquots was used when multiple aliquots from the same mouse were tested. The Wilcoxon signed ranked test compared paired data from the same subject and method of serum separation, delivery method, time to clot, time for analysis, and plasma potassium and serum potassium.

Results

Among the mice and potential factors we tested, the method of deep anesthesia or euthanasia had the greatest effect on serum potassium levels and other blood parameters. Blood gases and serum potassium levels were compared between mice euthanized by CO_2 inhalation and exsanguination (CO_2 group) or deeply anesthetized with KX and exsanguinated (KX group). The CO_2 group had significantly ($P < 0.05$) higher mean pCO_2, sodium, calcium, serum potassium, and plasma potassium and significantly ($P < 0.05$) lower mean blood pH and glucose than did the KX group (Table 1). The potassium levels of CO_2 mice were well above our normal reference range of 3.4 to 5.5 mEq/L and other published reference ranges,[7,32] regardless of the analyzer used. The mean lactate level of CO_2 mice generally was higher than that of KX mice. The blood smears from the 2 groups revealed a range of 500 to 1000×10^3 platelets per microliter. The anion gap was significantly ($P < 0.05$) higher in the CO_2 than the KX group. Significant differences between the 2 groups were not observed for chloride, bicarbonate, and pO_2. In addition, the CO_2 group showed a trend ($P = 0.05$) toward a base excess lower than that in the KX group.

Both paired and unpaired comparisons were made for serum and plasma potassium. The mean serum potassium for the CO_2 group was not significantly different from the mean plasma potassium for the same group. Similarly, a significant difference between mean plasma and serum potassium values was not observed for the KX group. The mean of the differences between serum and plasma potassium levels within individual mice was not significantly different from 0 in the 6 subjects in CO_2 group (-0.3 ± 0.8 mEq/L, $P = 0.44$) nor in the 4 subjects in the KX group (-0.4 ± 0.3 mEq/L, $P = 0.13$).

In addition other variables tested, including site of collection, delivery method, use of serum separators, time allowed for clot formation before serum separation, and time elapsed between collection and analysis of samples collected in serum separator tubes, lacked significant effects on serum potassium concentration.

Discussion

Despite the well-known physiologic effects of hyperkalemia, disagreement exists regarding the normal reference interval for serum potassium in mice. Serum potassium levels of 6.0 to 6.5 mEq/L have been reported to cause an increase in atrioventricular conduction and tachycardia both experimentally and clinically.[18] Serum potassium levels higher than 7.5 mEq/L have been associated with decreased atrioventricular conduction and cardiac standstill.[18] Nonetheless, in several reference texts, potassium values as high as 8.0 mEq/L are considered normal

Table 1. Comparison of blood parameters between mice euthanized by using CO_2 or ketamine–xylazine

	CO_2 group			Ketamine–xylazine group			
	n	Mean (1 SD)	Median (range)	*n*	Mean (1 SD)	Median (range)	*P*[a]
RAPIDlab 1265 analyzer							
pH	12	6.9 (0.1)	6.9 (6.8–7.0)	16	7.2 (0.1)	7.2 (7.1–7.3)	0.0001
pCO_2 (mm Hg)	11	153.3 (38.8)	169.5 (76.9–200.9)	15	61.6 (14.8)	61.8 (36.9–86.3)	0.0003
Na^+ (mmol/L)	12	152.5 (2.7)	151.9 (150.2–159.5)	16	141.2 (3.9)	141.7 (134.9–146.2)	0.0001
Ca^+ (mg/dL)	12	5.4 (0.3)	5.3 (4.8–5.9)	16	4.0 (1.0)	4.4 (2.1–5.1)	0.0002
Glucose (mg/dL)	11	167.9 (51.0)	174.0 (58.0–257.0)	15	265.1 (43.0)	260.0 (223.0–387.0)	0.0007
Lactate (mmol/L)	12	3.9 (0.9)	4.0 (1.8–5.3)	14	2.1 (0.8)	2.0 (1.1–3.6)	0.0020
Cl^- (mmol/L)	12	111.9 (4.1)	111.0 (107.0–121.0)	15	113.3 (3.3)	113.0 (105.0–118.0)	0.1700
HCO_3^- (mmol/L)	12	25.0 (10.2)	28.7 (3.6–34.9)	15	23.1 (3.2)	24.1 (17.0–27.1)	0.1400
Anion gap (mEq/L)	12	22.5 (7.5)	18.2 (15.5–37.3)	15	8.8 (5.8)	8.6 (–2.2–23.4)	0.0004
Plasma K^+ (mmol/L)	12	6.9 (0.7)	6.9 (6.0–8.3)	16	4.2 (0.6)	4.2 (3.5–5.3)	0.0001
Olympus AU400 analyzer							
Serum K^+ (mmol/L)	38	7.0 (0.5)	7.0 (6.0–8.1)	8	3.8 (0.3)	3.8 (3.5–4.6)	<0.0001

[a]Mean values of the same parameter were compared.

for healthy adult mice.[3,22,39] The findings of our study reject the dominant paradigm of potassium reference ranges in mice in favor of a more physiologically appropriate reference range and provide evidence that this historic discrepancy may be due to the commonly used method of CO_2 euthanasia.

The normal reference range for mouse serum potassium (3.4 to 5.5 mEq/L) used by our institution (ALX Laboratory, New York, NY) is more consistent with normal physiologic states than are historic norms and parallels that for other small mammals. By use of unspecified sampling techniques, other investigators[32] found the reference range of serum potassium in mice to be 5.2 to 5.5 mEq/L. Still others,[7] using either aortic puncture or left ventricular cardiocentesis and 100 mg/kg thiopental for terminal anesthesia of female C57BL/6 mice, reported serum potassium levels (mean, 4.3 mEq/L; reference range, 3.5 to 5.1 mEq/L) similar to those levels we describe in the current study. Conversely, authors who used CO_2 euthanasia and blind cardiocentesis have reported mean potassium levels of 7 to 8 mEq/L.[51] The absence of hyperkalemia without CO_2 administration and its presence when CO_2 euthanasia is used supports our conclusion that CO_2 euthanasia causes the increased serum potassium levels in premortem mice.

Mice that underwent CO_2 inhalation in our study developed hypercapnia with pCO_2 concentrations as high as 200 mmHg, acidemia with pH as low as 6.8, and hyperkalemia with plasma potassium levels as high as 8.3 mmol/L. In contrast, animals managed with the same experimental controls but given KX anesthesia instead of CO_2 revealed physiologically acceptable blood potassium levels that were significantly different from those of the CO_2 group. Compared with those of the CO_2 group, the slight elevation in pCO_2 and correspondingly modest decrease in blood pH of the KX group indicated milder changes in these mice's acid–base balance and may indicate both respiratory and cardiovascular depression due to the anesthetic cocktail. In another study, a mixture comprising 100 mg/kg ketamine and 5 mg/kg xylazine led to moderate respiratory depression and severe cardiovascular depression in mice.[16] Our current study used slightly increased doses of 110 mg/kg ketamine and 16 mg/kg xylazine; this regimen potentially had the same respiratory and cardiovascular depressive effects as in the cited study.[16] Anesthesia due to another xylazine-like α2 agonist, medetomidine, and ketamine resulted in a 70% decrease in respiratory rate in cats.[13] Similarly, sheep under acepromazine–ketamine anesthesia have demonstrated increased pCO_2.[4]

Another component of our study was hyperlactatemia, although it likely played a limited role in the development of hyperkalemia. Lactate is a common byproduct of cellular anaerobic metabolism. The blood lactate levels for both our CO_2 and KX groups exceeded published reference ranges.[7] Mean blood lactate was significantly higher in the CO_2 group, although the ranges for the 2 groups overlapped. In a study on CO_2-euthanized swine, hypoxia-induced anaerobic metabolism led to increased lactic acid production.[17]

The hyperlactatemia in both our experimental groups may be explained by hypoxemia; however, the difference in lactate levels between the 2 groups did not correlate with their respective pO_2 values. This discrepancy may reflect that circulating lactate concentrations are more dependent on delivery, uptake, and utilization of oxygen in tissues than on the presence of decreased systemic pO_2 levels.[27] It follows that some subjects with normal pO_2 may have increased serum lactate, principally due to perfusion deficits.[27] Indeed, hyperlactatemia can occur whenever tissue energy demands exceed the capacity of aerobic metabolism.[27] In addition, at least some of the increase in blood lactate levels we noted might be attributed to struggling during CO_2 inhalation. Similar changes in blood lactate have been reported in veterinary patients stressed during venapuncture.[41]

The relatively increased levels of serum lactate noted in the CO_2-treated mice were likely the predominant factor in the elevated anion gap noted in members of this group, as compared with KX mice. The presence of this organic acid is unlikely to have contributed to the hyperkalemia, however. Rather, hyperlactatemia serves to indicate underlying tissue hypoxia, a process that may well have contributed to the increase in serum potassium levels.

CO_2 is a potent vasodilator in many tissues and, in high concentrations, has been documented to disrupt normal autonomic vascular tone.[34,35] The CO_2-treated mice in this study likely became profoundly hypotensive, which in turn contributed to a significant decrease in tissue perfusion with tissue hypoxia. We hypothesize that compared with the KX mice, the CO_2 group sustained more tissue hypoxia because of severe CO_2-induced vasodilation, leading to poor tissue perfusion. The significant

circulatory compromise and tissue hypoxia, as further evidenced by hyperlactatemia, could have caused failure of the ATP-driven Na^+/K^+ pump. Hypoxia-induced suppression of the Na^+/K^+ pump has been documented to occur in mammals and has been shown to increase passive Na^+ uptake by cells.[8] This uptake leads to an increased Na^+ cellular load, cell swelling, and necrotic cell death.[8] In the CO_2-treated mice, inhibition of the pump secondary to systemic arterial vasodilatation and tissue hypoxia may have resulted in disruption of cellular membranes with leakage of intracellular potassium stores into the extracellular spaces, leading to hyperkalemia. We did not measure the mice's blood pressure during CO_2 administration, but doing so might help delineate the mechanisms behind the observations we have described.

A second possibility is that the hyperkalemia seen in the CO_2-treated mice may be secondary to the severe acute respiratory acidosis observed in this group. Respiratory acidosis is defined as an increase in pCO_2 generally greater than 45 mm Hg and, in the clinical setting, typically is due to alveolar hypoventilation.[12] Marked increases in pCO_2 levels produce acidemia by virtue of the carbonic anhydrase equation:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

The presence of excess CO_2 drives this equation toward the right, increasing the H^+ concentration and, in turn, decreasing pH:$pH = -\log [H^+]$.Although the effects of hypercapnia on the carbonic anhydrase equation also produces increased levels of HCO_3^-, this buffer cannot effectively titrate the increased acid—to do so, the equation must be driven toward the left, which the excess CO_2 levels prevent.In veterinary and human patients, respiratory acidosis has been associated with inhibition or dysfunction of medullary respiratory centers or respiratory muscles, upper airway obstruction, and impaired gas exchange.[46,50] Being fat-soluble, CO_2 generally freely diffuses across cellular membranes, between tissues and plasma, and into the alveoli, where it is exhaled; therefore, most clinical diseases associated with respiratory acidosis chiefly result in the impairment of CO_2 excretion.[46] The mice that received CO_2 in our study exhibited acute respiratory acidosis characterized by profoundly increased pCO_2, and the observed values (77 to 200 mm Hg) far exceed those generally noted in clinical respiratory disorders. Although our mice had no predisposing diseases that would have impaired the normal excretion of CO_2, the rapid increase in pCO_2 (hypercapnia) was principally due to CO_2 administration. Moreover, acute exposure to extraordinarily high levels of CO_2 could plausibly overwhelm the gas' tendency to equilibrate across cell membranes, causing net accumulation within the extracellular spaces and inhibiting its expedient excretion through exhalation.

We cannot rule out that hyperkalemia in the CO_2-treated group may, in part, have been due to a compensatory cation shift, where intracellular potassium ions are exchanged for extracellular protons, specifically H^+ ions. However, this compensation is not widely accepted to occur in disease-induced respiratory acidosis. We suggest that this process, although more frequently associated with metabolic acidosis due to increased concentrations of mineral acids, may have been accentuated here due to the periacute nature of the CO_2-induced acidosis as well as to the profound hypercapnia that developed. The potassium values in the CO_2 group averaged nearly 3 mmol/L higher than those in the KX group (Table 1). The hyperkalemia seen in the CO_2-treated mice is suggested, at least in part, to be a manifestation of the severe and abrupt respiratory acidosis produced by CO_2 euthanasia.

In humans, hyperkalemia due to respiratory acidosis has been cited in the *Tietz Textbook of Clinical Chemistry* but was noted to be less predictable than in some forms of metabolic acidosis, specifically those attributable to a mineral rather than an organic acid.[50] Further, this reference indicates that in respiratory acidosis, for every 0.1 unit decrease in pH, there is generally an increase of 0.6 mmol/L in serum potassium, and this increase is largely attributed to cation exchange.[50] This phenomenon has been described in other settings involving CO_2 administration. Hypercapnia caused a rapid hyperkalemia in healthy women undergoing peritoneoscopy by CO_2 insufflation.[19] Similarly increased potassium levels developed in pigs after prolonged peritoneal insufflation with CO_2, although normocapnia was maintained by supplemental ventilation, suggesting that CO_2 effects on serum potassium may be independent of pCO_2 levels.[37] Conversely, the mechanism of cation exchange has been associated with hypokalemia after respiratory alkalosis was induced with hyperventilation in anesthetized dogs; [33] the study suggested that each 10 mm Hg decrease in pCO_2 was associated with a 0.4 mEq/L decrease in serum potassium.[33]

In clotted blood samples, potassium is released into the serum from platelets. In animals with normal platelet numbers, this release is taken into account when establishing normal reference ranges.[42] Moreover, this process should be minimized in nonclotted blood, from which plasma is derived.[42] Dogs with thrombocytosis greater than 600×10^3 platelets per microliter had a mean difference of 1.55 ± 0.73 mEq/L between serum and plasma potassium concentrations, compared with a mean difference of 0.63 ± 0.17 mEq/L for dogs with normal platelet counts;[42] in addition, a positive correlation between platelet count and potassium levels was established.[42]

Reference values for mouse platelet counts often range higher than 1000×10^3 per microliter.[38] C57BL/6 mice had a mean platelet count of 985×10^3 per microliter and a range of 620×10^3 to 1200×10^3 per microliter.[6] In our current study, the volume of specimen required for the simultaneous biochemical and acid–base determinations precluded obtaining sufficient blood to evaluate hematologic parameters, specifically automated platelet counts. Therefore, we estimated platelet counts by examining 7 blood smears from each group. Platelet numbers appeared to be similar and within normal range for the CO_2 and KX groups, both of which demonstrated mild platelet clumping at the feathered edges of slides. Estimation of platelet numbers often yields falsely decreased values due to the presence of variable-sized platelet clumps within the sample; therefore we cannot eliminate the possibility of mice with absolute thrombocytosis in either, or both, groups.

If thrombocytosis contributed to increased serum potassium, we would expect to see hyperkalemia in the group showing the highest mean platelet counts. More importantly, we would expect to see a difference between serum and plasma potassium levels in that group, with the plasma-derived potassium concentrations lower than those from serum. However, this was not the case in our mice. In fact, the mean difference in serum and plasma potassium in mice from both CO_2 and KX groups did not exceed 0.4 mmol/L, which fails to meet the criterion for pseudohyperkalemia in humans.[45] Although the evidence we have provided is highly suggestive that platelets had no significant effect on potassium levels, the relationship between hyperkalemia and platelet counts in mice needs to be further investigated, particularly with the use of automated platelet

assays of specimens collected in appropriate anticoagulant-treated tubes.

In addition, artifactual hyperkalemia has been associated with excessive in vitro leakage of potassium from erythrocytes into serum or plasma specimens.[15,31] This outcome is generally due to a prolonged period between sample collection and appropriate processing, including centrifugation and separation of the serum or plasma from cellular blood components.[15,31] Furthermore exposing prespun samples to high ambient temperatures or other detrimental handling practices traumatizes erythrocytes, encouraging enhanced release of potassium during specimen transportation.[15]

Lastly, some species, including horses, and particular canine breeds, such as Akitas and other cold-weather breeds and their crosses, manifest artifactual hyperkalemia as a result of increased intraerythrocyte potassium concentrations.[15,31] Initially, we could not exclude the possibility that mice, including the cohort used in this study, may have high intraerythrocyte potassium concentrations compared with other species. Careful sample handling, including expedited centrifugation and plasma and serum separation, should have minimized the contribution of red cell-associated potassium to the hyperkalemia seen herein. Furthermore, given that sample handling was identical for specimens from both groups, this phenomenon would not explain the difference in potassium levels observed between CO_2- and KX-treated mice.

An additional method to confirm increased erythrocyte-associated potassium is repeated daily analysis of serum or plasma that has been in contact with the red cells from a suspect patient. Over time, these specimens show a progressive increase in red cell potassium leakage into the serum, resulting in increasing measured potassium levels.[31] Even though increased due to CO_2 usage, serum potassium values in our mice remained stable regardless to the amount of time that the clot was allowed to remain in contact with the serum or plasma, and no significant changes were noted at 24 or 48 h. Samples from Akita dogs can artifactually produce serum potassium levels of greater than 12 mmol/L if the clot remains in contact with the serum for more than 24 h.[26] In light of this experience, we suspect that serum samples from the mice analyzed in the current study were not saturated.

We also observed a significant difference in blood glucose between the CO_2 and KX groups. Hypertonicity resulting from hyperglycemia can drive water out of cells, resulting in an increase in intracellular potassium.[46] This effect could lead to potassium moving down its concentration gradient into the extracellular fluid.[46] However, even accounting for overlap in the ranges, the mean glucose value of the CO_2 group was lower than that seen in the KX group, suggesting that hyperglycemia did not play a role in hyperkalemia. This conclusion was further supported by the fact that the mean glucose levels in both groups were within normal reference ranges for our lab and within ranges reported by others (76 to 222 mg/dL and 106 to 300 mg/dL, respectively).[7] The slight elevation of the mean glucose values in the KX-treated mice was likely a direct effect of the KX cocktail itself: hyperglycemia has been documented in rats under KX anesthesia.[43]

Many factors could have resulted in the pO_2 variability present in both groups of mice. Error associated with exposure to environmental air during sampling for blood gas analysis can be manifested by increases in pH and pO_2 and decreases in pCO_2 and bicarbonate.[46] Conversely, delayed analysis and failure to chill samples have been associated with decreases in pH and pO_2.[46] Because our experimental design limited these shortcomings, the pO_2 variability in our study was most likely due to the nature of performing blind cardiocentesis, during which venous, arterial, or a mixture of both types of blood may have been obtained. Nonetheless, a mixed arterial and venous sample is unlikely to account for the approximately 3-fold increase in pCO_2 observed in the CO_2 group.[46]

The distinction between premortem and antemortem sampling may be difficult to make. We have observed in other circumstances that after CO_2 inhalation, mice often have residual cardiac function for as long as 3 min, and clinical death is achieved only by exsanguination. Although there is no doubt that these animals would be dead shortly after CO_2 inhalation, with or without sampling, it is imperative that the specimen itself does not represent an early postmortem collection. This point needs to be emphasized because extracellular potassium levels quickly rise in an animal that has died recently.[46] Relevant membrane potentials dissipate in dead and dying cells, and highly concentrated intracellular ions, such as potassium, rapidly equilibrate with the extracellular spaces.[46] In this study, we found that the method of euthanasia had the greatest effect on potassium levels. CO_2 inhalation, used by many animal facilities for euthanasia, directly correlated with respiratory acidosis and hyperkalemia. In addition, all other variables tested in this study, as well as testosterone levels (not addressed herein), may affect serum and plasma potassium levels and should be reevaluated in the absence of CO_2.

We believe that our findings may help investigators avoid obtaining inaccurate serum potassium measurements when working with the mouse as an animal model or in toxicologic studies. Our findings may also apply to other laboratory animal that are commonly euthanized by CO_2, including rats, gerbils, and hamsters, for which the alternative use of the KX cocktail or other forms of anesthesia for blood collection should be considered.

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