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Effects of General Anesthesia on 2 Urinary Biomarkers of Kidney Injury—Hepatitis A Virus Cellular Receptor 1 and Lipocalin 2—in Male C57BL/6J Mice

Krista M Gibbs,[1,*] Jenelle M Izer,[1] W Brian Reeves,[2] Ronald P Wilson,[1] and Timothy K Cooper[1]

Urinary biomarkers are used increasingly for sensitive prediction of kidney injury in preclinical and clinical studies. Given the frequent requirement of anesthesia in various animal models of disease, it is important to define the effects of anesthesia on kidney injury biomarkers to guide the appropriate selection of anesthetic agents and to avoid potential confounders in the interpretation of data. Therefore, we performed a prospective study using male C57BL/6J mice (*n* = 45) exposed to a single anesthetic episode to determine the effects several common anesthesia regimens on the urinary excretion of 2 commonly used kidney injury biomarkers: hepatitis A virus cellular receptor 1 (HAVCR1, also known as KIM1) and lipocalin 2 (LCN2, also known as NGAL). We evaluated 3 injectable regimens (ketamine–xylazine, tiletamine–zolazepam, and pentobarbital) and 2 inhalational agents (isoflurane and sevoflurane). Concentrations of HAVCR1 and LCN2 in urine collected at various time points after anesthesia were measured by using ELISA. Administration of ketamine–xylazine resulted in a significant increase in HAVCR1 levels at 6 h after anesthesia but a decrease in LCN2 levels compared with baseline. LCN2 levels steadily increased over the first 24 h after inhalant anesthesia, with a significant increase at 24 h after sevoflurane. These results suggest that injectable anesthesia had early effects on HAVCR1 and LCN2 levels, whereas inhalational agents increased these biomarkers over prolonged time.

Abbreviations: AKI, acute kidney injury; HAVCR1, hepatitis A virus cellular receptor 1; LCN2, lipocalin 2

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Anesthesia is commonly used in biomedical research and testing both to prevent pain during surgical procedures and for restraint when animals might otherwise experience prolonged distress. This use is due, in part, to ethical standards of humane care of animals shared among researchers, veterinarians, and the public but also is required by federal regulations and other guidelines.[20,40]

Most general anesthetic agents produce dose-dependent effects on the animal.[20] In addition to the desired outcomes of loss of consciousness and insensitivity to pain, there are several systemic side effects that can result from administration of these agents, including changes in heart rate, cardiac output, and systemic blood pressure and even organ toxicity.[1,34] Therefore, selection of the appropriate anesthetic agent should include consideration of the overall health of the animal, the type and degree of pain or distress related to the procedure, the safety and systemic effects of the agent(s), and the overall goal of the research.

Renal function is often an essential consideration regarding the selection of anesthetic agents. Despite their small size relative to total body weight, the kidneys receive 25% of the resting cardiac output and are a primary site of drug metabolism and potential toxicity.[1] Traditional biomarkers used for assessing kidney function include BUN, serum creatinine, and urine output. Although these indicators remain the 'gold standard' in clinical practice, there are several well-described limitations regarding the sensitivity and specificity of their use.[37] For instance, BUN is a product of protein metabolism and therefore can be elevated after the breakdown of protein within the body—making it particularly nonspecific.[37] In addition, even though serum creatinine is traditionally considered a more specific biomarker of kidney injury than BUN, serum creatinine has been found to be insensitive, unreliable, and slow in response to early changes of acute kidney injury (AKI).[36,38] Because creatinine is a breakdown product of skeletal muscle, there is inter- and intraindividual variation in its accumulation, which is dependent on muscle mass, age, sex, diet, drug exposure, and vigorous exercise.[37,54] Moreover, elevations in serum creatinine are seen only after greater than 50% of the glomerular filtration rate is lost, and these increases may take more than 24 h to be measurable.[37,54] Despite these shortcomings, a more reliable alternative to BUN and serum creatinine has yet to be established.

To overcome these limitations, researchers and clinicians have embraced novel technologies including functional genomics and proteomics to identify several earlier and more sensitive biomarkers of kidney injury. Ongoing research has attempted to validate several of these genes and proteins in both laboratory and clinical settings, with variable results. Instead of relying on a single biomarker as an indicator of renal injury, it has been suggested that a panel of urinary biomarkers be used not only in the diagnosis of kidney injury and evaluating the

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[1]Department of Comparative Medicine, Penn State University College of Medicine, Milton S Hershey Medical Center, Hershey, Pennsylvania, and [2]Department of Medicine, School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, Texas
[*]Corresponding author. Email: Krista.gibbs@ahn.org

response to treatment but also to provide safety markers for the identification of subclinical nephrotoxicity (that is, prior to abnormalities in serum chemistries or overt signs of illness) to previously approved pharmaceuticals and during preclinical drug development.[9,10,16,19,27,37,50]

One such biomarker is hepatitis A virus cellular receptor 1 (HAVCR1), also known as kidney injury molecule 1 (KIM1). HAVCR1 is arguably the best-studied urinary biomarker relating to kidney injury. HAVCR1 is a type I transmembrane glycoprotein that is not detectable in normal urine, but its expression is upregulated in the proximal tubular epithelial cells in the face of injury. This expression is thought to promote apoptotic and necrotic cell clearance required for the remodeling of injured epithelium. As a consequence of injury, the extracellular domain of HAVCR1 is shed into the urine, making it an easily accessible marker that is stable in the urine for prolonged periods of time.[19,42] HAVCR1is considered a reliable marker of tubular cell damage, although there are conflicting reports regarding whether it is an early or late marker of injury.[16,35,49]

Lipocalin 2 (LCN2) is another well-studied urinary biomarker of kidney injury. LCN2 is a small secreted polypeptide more commonly known as neutrophil gelatinase-associated lipocalin (NGAL). Under normal conditions, LCN2 is readily filtered by the glomerulus and efficiently resorbed by the proximal tubules, resulting in only 0.1% to 0.2% excretion in the urine in a healthy subject.[4,39] LCN2 has 4 normal physiologic functions. First, LCN2 exerts a bacteriostatic effect by binding to siderophores synthesized by various bacteria for gathering iron, thus preventing the bacteria from obtaining a necessary metabolite for growth. Second, LCN2 produces an antioxidant effect by assisting in the transport of iron into target cells to prevent free iron from producing oxygen free radicals. Third, increasing evidence supports LCN2's function as a growth factor through its regulation of cell proliferation, apoptosis, and differentiation. Finally, LCN2 is hypothesized to scavenge intracellular iron for export.[37] LCN2 protein and gene expression are rapidly increased after injury to epithelial cells, including renal tubular epithelia.[35,36] LCN2 has been evaluated across a vast array of nephropathies, including glomerular disorders, diabetic nephropathy, obstructive nephropathy, interstitial nephritis, nephrotoxicities, and ischemia-reperfusion injury.[27,35,37] In addition, LCN2 is protease-resistant, making it a robust molecule for evaluation in preclinical testing.

Given that the perioperative development of AKI is a common challenge in patients undergoing major surgical procedures, there is frequent overlap in the use of urinary biomarkers and general anesthesia in the field of AKI research.[5,7] In addition to its frequent use in human clinical patients, general anesthesia is used in the development of many animal models of AKI, such as ischemia–reperfusion injury and obstructive uropathy.[6,48]

Because of this overlap, it is important to describe the effects of general anesthesia on urinary biomarkers of kidney injury, because they may have significant confounding effects in the interpretation of study results. Furthermore, the *Guide for the Care and Use of Laboratory Animals*, 9th edition, states that "Guidelines for the selection and proper use of analgesic and anesthetic drugs should be developed and periodically reviewed and updated as standards and techniques are refined."[20] Therefore, as urinary biomarkers become validated as sensitive and specific markers of subclinical nephrotoxicity, current anesthetic protocols need to be reevaluated with regard to safety, efficacy, and potential effects on research outcomes.

Controlled studies outlining the effects of general anesthesia alone on these sensitive biomarkers of renal injury are sparse. To begin the investigation of these effects, this study evaluated changes in 2 of the most widely used urinary biomarkers of kidney injury (HAVCR1 and LCN2) in response to 5 general anesthetic agents commonly used in laboratory animal medicine. We hypothesized that significant increases in measured urinary biomarkers would be identified in mice anesthetized with various anesthetics or anesthetic combinations. We further postulated that the greatest elevations would occur in mice undergoing injectable anesthesia compared with inhalational anesthesia, given that several studies have revealed a renal protective effect of inhalational anesthesia in both human and animal patients.[11,12,17,30]

Materials and Methods

Animals. A total of 45 male C57BL/6J mice (age, 8 to 12 wk) were purchased from The Jackson Laboratory (stock no. 000664, Bar Harbor, ME) and housed in an AAALAC-accredited facility in compliance with the *Guide for the Care and Use of Laboratory Animals*, 9th edition.[44] All procedures were approved by the IACUC of the Pennsylvania State University College of Medicine, Milton S Hershey Medical Center (Hershey, PA). According to serology (Opti-Spot Mouse Comprehensive Panel, IDEXX BioResearch, Columbia, MO) of dirty-bedding testing of female sentinel mice (Crl:CD1(ICR), Charles River Labs, Wilmington, MA; age, 4 to 6 wk) housed on the same rack, all mice were free of the following pathogens: mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse norovirus, Theiler murine encephalomyelitis virus, mouse rotavirus, Sendai virus, pneumonia virus of mice, reovirus 3, lymphocytic choriomeningitis virus, ectromelia virus, mouse adenovirus types 1 and 2, mouse polyoma virus, mouse cytomegalovirus, *Encephalitozoon cuniculi*, cilia-associated respiratory bacillus, *Clostridium piliforme*, and *Mycoplasma pulmonis*. In addition, sentinel mice were negative for *Helicobacter* spp. (real-time PCR analysis of feces; IDEXX BioResearch) and free of endo- and ectoparasites.

Husbandry, housing, and experimental groups. On arrival, mice were housed in stable groups of 5 per cage in open-top, solid-bottom polycarbonate cages (Max75, Alternative Design, Siloam Springs, AR) with wire-bar lids, and on corncob bedding (Tekland 7097 Corncob Bedding, Harlan, Frederick, MD). Each cage included a red igloo (K3327, BioServ, Flemington, NJ) for environmental enrichment. Mice had unrestricted access to standard commercial rodent chow (Tekland 2018 Global 18% Protein Rodent Diet, Harlan) and municipal tap water (provided in bottles). The animal room was maintained on a 12:12-h light:dark cycle with no twilight, at a temperature of 70 to 73 °F (21.1 to 22.8 °C), and at a relative humidity of 30% to 70%. Cage changing was performed once per week. Mice were acclimated to their environment for a minimum of 7 d prior to experimentation.

At 24 h prior to anesthesia, mice were singly housed in metabolic cages (Techniplast, West Chester, PA) to facilitate urine collection. After 24 h, baseline urine was retrieved from the urine collection tube, and animals were transported to a nearby procedure room. Mice were weighed and randomly assigned, according to weight, to 1 of 5 experimental groups as follows: 100 mg/kg IP ketamine plus 10 mg/kg IP xylazine (n = 5); 50 mg/kg IP pentobarbital (n = 6); 40 mg/kg IP tiletamine–zolazepam (n = 6); isoflurane (n = 7) delivered in 100% oxygen through a vaporizer set at 3% for induction and at 1% to 2% for maintenance of anesthesia for a total duration of 30 min; and sevoflurane (n = 6) delivered in 100% oxygen through a vaporizer set at 5% to 6% for induction and at 2% to 3% for maintenance of anesthesia for a total duration of 30 min. Three control groups were used for this study: a positive surgical

control group (renal ischemia–reperfusion injury, $n = 5$); a positive pharmaceutical control group resulting from 16 mg/kg IP cisplatin ($n = 5$); and a negative control group (sterile water at 0.1 mL/10 g body weight IP, $n = 5$).

Anesthesia preparation, administration, and monitoring. Drugs used in this study included ketamine hydrochloride (Ketathesia, Butler Animal Health Supply, Dublin, OH) combined with xylazine (AnaSed, Lloyd Labs, Bulacan, Philippines), pentobarbital (Nembutal, Akorn, Buffalo Grove, IL), tiletamine–zolazepam (Telazol, Zoetis, Florham Park, NJ), isoflurane (Isothesia, Henry Schein Animal Health, Dublin, OH), sevoflurane (Sevothesia, Henry Schein Animal Health), and cisplatin (Cisplatin, APP Fresenius Kabi USA, Lake Zurich, IL). Buprenorphine (Buprenex, Reckitt Benckiser Pharmaceuticals, Richmond, VA) was provided at a dose of 0.1 mg/kg SC as analgesia for all mice undergoing surgery to induce renal ischemia–reperfusion injury. Ketamine–xylazine combination, pentobarbital, tiletamine–zolazepam, and buprenorphine were diluted in sterile water prior to injection to allow for more accurate drug administration.

After anesthetic agents had been administer, each mouse was placed on top of a circulating warm-water blanket (Blanketrol II, Cincinnati Sub-Zero, Cincinnati, OH) and a timer was started. All mice were closely monitored for loss of the righting reflex, indicating the end of the induction phase. Once the mouse stopped ambulating, it was placed in lateral recumbency; the mouse was considered to be anesthetized when it was unable to regain a sternal posture within 10 s of placement in lateral recumbency. Once under anesthesia, the mouse was placed in alternating right- or left- lateral recumbency every 5 min to establish a time for the return of the righting reflex. Given these definitions and for the purpose of this study, the period between induction of anesthesia and the return of the righting reflex was recorded as the duration of anesthesia. Subjective observations including spontaneous movement (scratching, twitching, paddling), intermittent response to toe-pinch reflex (withdrawal of the paw after application of moderate manual pressure to the foot) during assessment of the righting reflex, and transition in and out of anesthesia were recorded by a single observer (KMG), who was unblinded to the anesthetic agents administered.

All injected mice, including those in the negative control and cisplatin positive control groups, received 1 mL of 0.9% normal saline subcutaneously immediately after their intraperitoneal injection. Animals under inhalant anesthesia received subcutaneous fluids prior to anesthetic induction, whereas positive surgery control animals received subcutaneous fluids after closure of the surgical incision but before recovery from anesthesia. Once animals recovered from anesthesia, they were returned to their metabolic cages for the duration of the experiment.

Surgery to induce ischemia–reperfusion injury. Surgery was performed as previously described.[3,13] In brief, all surgical instruments were sterilized by autoclaving prior to surgery. Mice ($n = 5$) were anesthetized by using 50 mg/kg IP pentobarbital and received 0.1 mg/kg SC buprenorphine for analgesia. After induction, mice were aseptically prepared and placed on a circulating warm-water blanket with a nose cone delivering 100% oxygen, with the option to supply isoflurane as needed to provide a negative response to noxious stimuli (toe pinch, tail pinch). Once a surgical plane of anesthesia was achieved, the mouse was covered with a sterile drape, and a 1-cm vertical midline skin incision was made over the thoracolumbar region by using sharp scissors. The subcutaneous space lateral to the incision (left and right) was undermined by using blunt dissection. An approximately 1-cm paramedian incision was made into the retroperitoneum overlying the left kidney. The renal pedicle was isolated and then clamped by applying a microaneurysm clip (catalog no. 160-863, George Tiemann and Company, Hauppauge, NY) by using forceps (catalog no. 160-870, George Tiemann and Company). Ischemia was confirmed by a color change from red to dark red or purple; the same procedure then was performed on the contralateral kidney. The clips remained in place for 30 min each, after which they were atraumatically removed by using forceps. Reperfusion was confirmed by a color change from purple to red. The retroperitoneum was closed by using 5-0 polydiaxanone suture in a cruciate pattern, and the skin was closed by using wound clips. After wound closure, all mice received 1 mL of 0.9% normal saline subcutaneously. Once recovery from anesthesia was complete, mice were returned to their individual metabolic cages and monitored hourly for signs of pain during the first 6 h after surgery and then once every 8 h. All mice in this group, regardless of clinical signs, received a second dose of 0.1 mg/kg SC buprenorphine at 8 h after the initial dose. At 24 after surgery, urine was collected and mice were euthanized.

Urine collection and storage. Urine was collected passively from individual mice by using the collection cup on the metabolic cages. Baseline urine was collected during 24 h while mice were housed in the metabolic cage, prior to any other experimental manipulation. Urine was then collected as available at 6, 12, and 24 h after anesthesia, with the exception of the cisplatin group, from which continued to be collected at 48 and 72 h after injection. After collection, urine was centrifuged for 5 min at 19,480 × *g*, separated from debris, and stored at –80 °C for later testing.

Euthanasia, tissue collection, and processing. All mice were euthanized (in accordance with the *AVMA Guidelines for the Euthanasia of Animals*)[29] after administration of inhalational anesthesia (isoflurane), blood collection through cardiocentesis, and transcardial perfusion with PBS followed by 4% formaldehyde. After perfusion, the kidneys and urinary bladder were removed and placed in 10% neutral buffered formalin for complete fixation prior to trimming and paraffin embedding. Tissues were then sectioned at a thickness of 3 µm and stained with hematoxylin and eosin and periodic acid–Schiff for review. The collected blood was allowed to clot for a minimum of 30 min; serum was then separated by 2 rounds of centrifugation, and analyzed for BUN and serum creatinine levels (Cobas Mira Plus Chemistry System, Roche Diagnostics USA, Indianapolis, IN).

Biomarker quantification. For analysis, HAVCR1 and LCN2 levels were standardized to urinary creatinine concentration. To measure urine creatinine, urine samples were thawed to room temperature, diluted 1:25 in PBS, and measured in duplicate by using a colorimetric assay kit (catalog no. ab65340, Abcam, Cambridge, MA) according to manufacturer instructions. Commercially available mouse ELISA kits were used according to manufacturer instructions to measure urinary HAVCR1 (catalog no. ab119596, Abcam) and LCN2 (catalog no. ab119601, Abcam) levels in duplicate. Optical absorbance was measured on a microplate reader (EL808, BioTek, Winooski, VT). To minimize freeze–thaw cycles, urinary HAVCR1 and LCN2 levels were measured in parallel. Analysis of all assays was performed by using Gen5 data analysis software (BioTek). The mean of the duplicate values for each mouse was then used for further statistical analysis.

Renal scoring. Renal histology was scored by a board-certified diplomate veterinary pathologist (TKC) experienced in renal pathology and blinded to experimental group. A grading system describing the severity of nephropathy was developed on the basis of previous publications (Figure 1).[45,46] Scores for

	Grade	Description
Tubular epithelium		
Minimal	1	Epithelial degeneration or necrosis spectrum changes (cell swelling, cytoplasmic hyalinization and hypereosinophilia, nuclear pyknosis, karyolysis, karyorrhexis) present in 1% to 10% of tubular epithelial cells
Mild	2	Epithelial degeneration or necrosis spectrum changes present in 11% to 30% of tubular epithelial cells
Moderate	3	Epithelial degeneration or necrosis spectrum changes present in 31% to 50% of tubular epithelial cells
Severe	4	Epithelial degeneration or necrosis spectrum changes present in more than 51% of tubular epithelial cells
Interstitial nephritis		
Minimal	1	1 to 3 discrete microscopic foci of inflammation (diameter, 150 μm or less)
Mild	2	4 to 10 discrete microscopic foci or a single large focus of inflammation (diameter, greater than 150 μm)
Moderate	3	10% to 40% of the interstitium is infiltrated with inflammatory cells
Severe	4	More than 40% of the interstitium is infiltrated with inflammatory cells

Figure 1. Criteria for histopathologic grading of the severity of nephropathy.

tubular epithelial degeneration or necrosis and the degree of interstitial inflammation were combined, yielding a total score of 0 (normal) to 8 (severe) for analysis. In addition, urinary bladder mucosa was screened for the presence or absence of inflammatory cell infiltrates.

Statistical analysis. Data were analyzed by using Prism 6 (GraphPad Software, San Diego, CA) and SAS9.4 (SAS Institute, Cary, NC) statistical software. Serum BUN and creatinine levels were analyzed by using one-way ANOVA followed by posthoc Tukey multiple-comparison testing. Urinary HAVCR1 and LCN2 levels were analyzed by using a PROC MIXED repeated-measures model; this method achieves multiple-comparisons testing, which accounts for missing data points that arose due to insufficient urine volume collected from a specific mouse at a given time point. To control for the type 1 error rate, the *P* value was adjusted by using the false discovery rate to minimize false significance. The ordinal values of the kidney scores were analyzed by using the Kruskal–Wallis nonparametric test. In all cases, a probability statistic of $P < 0.05$ defined significance. All data presented throughout the text are expressed as mean ± SEM.

Results

One mouse in the surgery positive-control group died after induction of pentobarbital anesthesia, and all data from this animal were excluded from analysis; no other mice died before euthanasia. At euthanasia and tissue collection, one mouse from the tiletamine–zolazepam group had a large cyst at the cranial pole of the right kidney. Histology revealed that the cyst was lined by urothelium, and there was atrophy, fibrosis, and chronic inflammation of the surrounding parenchyma; all data collected from this mouse were excluded from analysis. In addition, sufficient volumes of urine for analysis were not available from all animals at all time points, resulting in unequal sample sizes as reflected within tables.

At the time of euthanasia, all mice except for the positive controls were clinically normal, with no evidence of dehydration as evaluated through skin tent assessment. According to clinical observation, surgery and cisplatin animals were hunched, lethargic, and 5% to 15% dehydrated at the time of euthanasia; the cisplatin mice were the group most severely affected at the 72-h time point.

Induction and duration of anesthesia. Times for induction and duration of anesthesia due to the injectable agents evaluated are presented in Table 1. Ketamine–xylazine reliably achieved smooth induction and prolonged duration of anesthesia. The toe-pinch reflex was inconsistent in 3 of the 5 animals throughout testing. Pentobarbital anesthesia often resulted in hyperactivity during induction, with stiffening and paddling as the mouse neared the loss of the righting reflex. In addition, the 3 pentobarbital-anesthetized animals often twitched a hindfoot, with occasional efforts to scratch at the neck, but were unable to return to sternal recumbency. The toe-pinch reflex was present at all time points assessed in all animals injected with pentobarbital.

Table 1. Time (mean ± SEM) to induction and duration of anesthesia due to injectable agents tested

	Induction (s)	Duration (min)
Ketamine–xylazine	164 ± 48	56.6 ± 11.2
Pentobarbital	261 ± 103	52.9 ± 26.1
Tiletamine–zolazapam	130 ± 41	12.6 ± 5.3

Injection with tiletamine–zolazepam resulted in a rapid induction and duration of anesthesia. Mice were often hyperactive during induction and had an elevated respiratory rate and effort throughout anesthesia. Two of the 5 mice chattered or vocalized during recovery, and response to toe pinch was present in all animals at all time points.

Administration of either isoflurane or sevoflurane resulted in a brief period of hyperactivity, followed by smooth induction, 30 min of surgical plane anesthesia, and rapid (less than 1.5 min) recovery from anesthesia. Toe pinch was consistently negative for all animals under inhalational anesthesia.

Serum chemistry. Serum BUN and creatinine levels were measured after terminal blood collection. Serum BUN was significantly ($P = 0.0002$) elevated in the surgical control and cisplatin control groups compared with all other groups (Table 2). For a single mouse in the cisplatin group, there was insufficient serum to quantify creatinine, due to collection and processing error. Serum creatinine was significantly ($P = 0.0002$) higher in the surgical control group compared with all other groups tested (Table 2).

Urinary biomarkers. Urine was collected in the urine collection container of the metabolic cage after spontaneous urination by the mice. Therefore, biomarker quantification was limited to those samples of sufficient volume (at least 150 μL) to run urine creatinine and biomarker assays. As a result, sample number varied among testing time points, and the number of samples assayed per group is indicated (Tables 3 and 4).

HAVCR1. Anesthesia variably affected HAVCR1 levels (Table 3). Relative to baseline, HAVCR1 levels were increased 18 fold at 12 h after surgery and 14 fold at 24 h afterward. HAVCR1 was significantly ($P = 0.0352$) increased 13.7 fold at 24 h after surgery and 2.4 to 6.0 fold from 24 through 72 h after cisplatin injection ($P = 0.0171$). HAVCR1 levels were doubled those at baseline at 6 h after ketamine–xylazine anesthesia ($P = 0.0413$) but returned to normal at 12 h. In all other experimental groups, HAVCR1 levels did not change significantly relative to baseline.

LCN2. LCN2 levels decreased in all injectable anesthetic groups but increased in inhalational anesthesia groups (Table 4).

Table 2. Serum BUN and creatinine levels (mg/dL; mean ± SEM) after terminal blood collection at endpoint

	BUN (29.2–36.2)[a]	Creatinine (0.42–0.58)[a]
Ketamine–xylazine	27 ± 2	0.18 ± 0.02
Pentobarbital	27 ± 1	0.20 ± 0.00
Tiletamine–zolazapam	30 ± 2	0.16 ± 0.02
Isoflurane	26 ± 2	0.20 ± 0.00
Sevoflurane	27 ± 1	0.18 ± 0.03
Negative control	30. ± 2	0.20 ± 0.00
Surgery control	150 ± 59[b,c]	1.60 ± 0.72[b,c]
Cisplatin control	87 ± 19[b,c]	0.45 ± 0.05[b]

Data are given as mean ± SEM; $n = 5$ for all experimental groups except sevoflurane ($n = 4$; due to processing error); $n = 4$ for the negative control group; $n = 3$ for the surgery positive-control group; $n = 2$ for the cisplatin positive-control group (due to insufficient quanity).
[a]Normal reference ranges for BUN[52] and creatinine[52] of mice are shown.
[b]$P < 0.05$ compared with values for all other groups.
[c]Value exceeds upper limit of published reference range.[52]

Compared with baseline values, LCN2 was increased 21 fold at 6 h after ischemia-reperfusion surgery and 17 fold at 24 h ($P = 0.0068$). Although LCN2 levels were undetectable 6 h after injection of cisplatin ($P = 0.0095$), significant increases were present at 12 h (2.4 fold), 24 h (3.8 fold), and 72 h (4.1 fold) compared with the 6-h time point ($P < 0.03$). LCN2 levels were significantly ($P = 0.0334$) decreased 0.15 fold from baseline 6 h after ketamine–xylazine anesthesia but were increased ($P = 0.02$) by 1.3 fold at 24 h after sevoflurane anesthesia compared with 6 and 12 h.

Renal histology. Renal histology scores (Figure 2) did not differ significantly among all groups (score [mean ± SEM]: ketamine–xylazine, 0.6 ± 0.2; pentobarbital, 0.2 ± 0.2; tiletamine–zolazapam, 0.4 ± 0.4; isoflurane, 1.0 ± 0.3; sevoflurane, 1.0 ± 0.3; negative control, 0.25 ± 0.25; surgery control, 3.7 ± 1.3; and cisplatin, 1.7 ± 0.3). However, according to histology, renal ischemia–reperfusion injury was incomplete in one mouse (no. Sx2). When renal histology scoring data from this mouse are excluded, the renal histology score of the surgery group is significantly different when compared with all other groups (5.0 ± 0.0, $P = 0.0277$). In addition, no inflammatory cells were present in the evaluated urinary bladder sections.

Discussion

In the present study, we hypothesized that 1 of 5 anesthetic regimens would induce significant increases in measured urinary biomarkers in mice, due to the direct metabolic effect of these drugs on the kidneys and to the secondary effects of these drugs on renal blood flow.[44] However, relative to baseline, both HAVCR1 and LCN2 typically decreased in most experimental groups within the timeframe studied. In addition, we further hypothesized that the greatest elevations would occur in mice undergoing injectable anesthesia compared with inhalational anesthesia, given that several studies have revealed a protective effect of volatile anesthetics on renal function.[12,17,21] However, this effect was not true for LCN2, which increased during inhalational anesthesia.

Given the well-known limitations of the use of serum BUN and creatinine for the evaluation of kidney function, vast amounts of time and funding have been expended toward the identification and validation of other, more sensitive markers of kidney injury. Several proteins found in the urine have been identified as candidates for this purpose, including cystatin C, liver-type fatty acid binding protein, and IL18.[4,33,37] Once validated, urinary proteins will be preferable over serum markers because urinary proteins offer the advantages of reflecting real-time damage and requiring noninvasive sampling methods. Despite these advantages, several limitations remain associated with the evaluation and validation of these proteins, including instability in urine, delayed appearance after injury, inconsistency among various mechanisms of kidney injury, and a lack of high-throughput detection methods.[50] In part due to these limitations, cut-off values indicating clinically or biologically relevant values remain largely unavailable. With these factors in mind, we selected HAVCR1 and LCN2 in light of 2 main factors: stability in urine and the availability of commercial detection and quantification methods that have been validated for mouse urine.[19,35,50]

The most noteworthy changes were seen within 6 h after ketamine–xylazine anesthesia. HAVCR1 levels were significantly increased relative to baseline during this period, whereas LCN2 levels were significantly decreased. In previous studies, both biomarkers have shown elevations within this time frame, so it is unlikely this response was a temporal effect. Therefore, we suspect that the elevation in HAVCR1 is reflective of the mechanism of action or location of injury due to the administered agent. HAVCR1 mRNA and protein are expressed at very low levels in healthy rodent kidneys, and the increases in HAVCR1 expression after ischemic injury are reported to be the greatest among tested biomarkers.[16,18] This pattern is different from LCN2, which is normally present at low levels and reabsorbed by the proximal tubular epithelium.[27,37] Due to these differences, we postulate that ketamine–xylazine anesthesia results in a degree of ischemia, upregulating HAVCR1 within the proximal convoluted tubule, because it is the most ischemia-sensitive portion of the nephron. This degree of ischemia is likely to be subclinical, given that it does not appear to impair the absorptive capacity of the proximal tubular epithelium, thus retaining its ability to resorb LCN2. This explanation is further supported by previous literature describing dramatic reductions in mean arterial pressure in rodents after ketamine–xylazine anesthesia; with some papers describing these effects continuing beyond the recovery period.[2,43] Further mechanistic research is necessary to confirm this speculation.

LCN2 levels were significantly elevated at 24 h but not at 6 or 12 h after sevoflurane anesthesia. Although differences were not statistically significant, renal histology scores in mice appeared to increase with sevoflurane anesthesia, suggesting a gradual effect of sevoflurane on the renal tubules in the hours after recovery. This effect could be due in part to the metabolism and excretion of sevoflurane. Although the mechanism of action of sevoflurane remains largely unknown, approximately 3% of inhaled sevoflurane is metabolized in the liver and then excreted by the kidneys, resulting in inorganic fluoride ions which have been thought to contribute to the anesthetic's nephrotoxic potential.[1] This delayed effect may contribute to the elevation of LCN2 at 24 h after sevoflurane anesthesia. In addition, decreased renal perfusion secondary to hypotension and reduced myocardial function may play a role in these changes.[44]

This elevation in LCN2 after sevoflurane is surprising, given prior studies that have described antiinflammatory and multiorgan protective effects of volatile anesthetics.[8,12,17,21,28,41] Most of these conclusions were based on histology, immunohistochemistry, serum analysis, and in some cases, Western blot or flow cytometry methods. A key difference between those studies and ours is that our current study used urinary

Table 3. Fold change in urinary HAVCR1 relative to baseline

	6 h	12 h	24 h	48 h	72 h
Ketamine–xylazine	2.14 ± 0.02[a] (2)	0.03 ± 0.00 (2)	0.42 ± 0.02 (5)	not done	not done
Pentobarbital	1.09 ± 0.02 (3)	1.00 ± 0.00 (4)	0.20 ± 0.00 (4)	not done	not done
Tiletamine–zolazapam	0.16 ± 0.00 (4)	0.34 ± 0.00 (4)	0.29 ± 0.00 (5)	not done	not done
Isoflurane	0.28 ± 0.00 (3)	0.81 ± 0.01 (3)	0.70 ± 0.00 (5)	not done	not done
Sevoflurane	0 ± 0.00 (3)	0.93 ± 0.00 (4)	0.46 ± 0.00 (5)	not done	not done
Negative control	0 (1)	0.12 ± 0.00 (3)	0.90 ± 0.00 (4)	not done	not done
Surgery control	0 (1)	17.80 (1)	13.70 ± 0.01[a] (3)	not done	not done
Cisplatin control	0 (1)	0.48 (1)	2.41 ± 0.00[a] (3)	5.97 ± 0.01[a] (3)	5.32 ± 0.01[a] (3)

Data presented as mean ± SEM, where appropriate. Numbers in parentheses indicate the number of samples analyzed at that time point.
[a]$P < 0.05$ compared with baseline value.

Table 4. Fold change in urinary LCN2 relative to baseline

	6 h	12 h	24 h	48 h	72 h
Ketamine–xylazine	0.15 ± 0.03[a] (2)	0.04 ± 0.03 (2)	0.55 ± 0.03 (5)	not done	not done
Pentobarbital	0.84 ± 0.07 (3)	0.52 ± 0.19 (4)	0.67 ± 0.02 (4)	not done	not done
Tiletamine–zolazapam	0.99 ± 0.02 (4)	0.86 ± 0.08 (4)	0.51 ± 0.03 (5)	not done	not done
Isoflurane	0.69 ± 0.07 (3)	1.73 ± 0.06 (3)	1.25 ± 0.07 (5)	not done	1.21 ± 0.09 (3)
Sevoflurane	0 ± 0.00 (3)	0.72 ± 0.05 (4)	1.28 ± 0.06[b](5)	not done	0.19 ± 0.02 (3)
Negative control	0.04 (1)	0.55 ± 0.08 (3)	0.37 ± 0.04 (4)	not done	not done
Surgery control	21.48[a] (1)	2.18 (1)	16.55 ± 0.60[a] (3)	not done	not done
Cisplatin control	0 (1)[a]	2.36[b] (1)	3.77 ± 0.09[b] (3)	2.86 ± 0.12 (3)	4.08 ± 0.04[b] (3)

Data presented as mean ± SEM where appropriate. Numbers in parentheses indicate the number of samples analyzed at that time point.
[a]$P < 0.04$ compared with baseline value.
[b]$P < 0.03$ compared with value at 6 h.

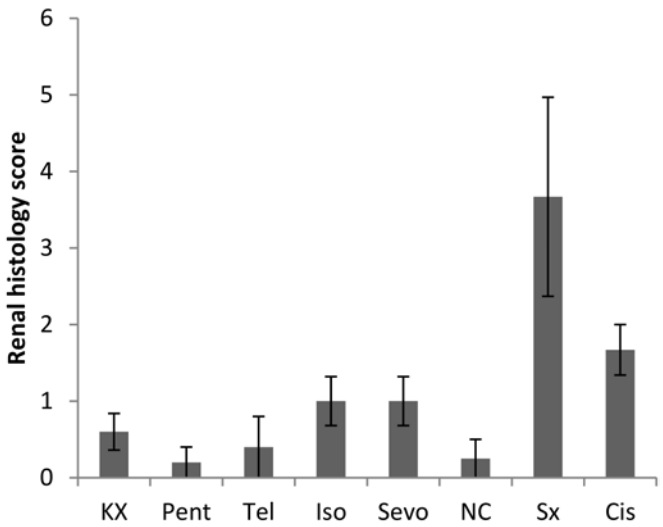

Figure 2. Renal histology scores (mean ± SEM) at study endpoint. Cis, cisplatin positive control; Iso, isoflurane; KX, ketamine–xylazine; NC, negative control; Pent, pentobarbital; Sevo, sevoflurane; Sx, surgery positive control; Tel, tiletamine–zolazepam.

biomarker measurement, whereas others relied on serum markers, including serum LCN2.[26] It could be assumed that the increased LCN2 in our study resulted from damage to the renal tubules, however the purpose of this study was not to correlate urinary biomarker levels and clinical damage.

Interestingly, there was a significant decrease in HAVCR1 and LCN2 levels at 6 h after intraperitoneal injection of sterile water and cisplatin, respectively. Although not statistically significant, HAVCR1 levels appeared to decrease in all experimental groups during the first 6 h of injection. Although unlikely to be biologically relevant—when considering the decrease in experimental, positive, and negative control groups—this change may be due to a common event among all groups: the subcutaneous injection of 0.9% saline (admittedly not a physiologic fluid replacement) for fluid support. Administration of subcutaneous fluids to rodents under anesthesia is both recommended and commonplace, but published literature regarding its effects are sparse.[15,56] Given the aforementioned change in biomarkers within the first 6 h, the most likely explanation is that the subcutaneous fluids promoted diuresis during recovery. This diuresis may have resulted in the dilution of the excreted proteins in the urine and may have further been enhanced in the ketamine–xylazine group, consistent with evidence that xylazine decreases the release of antidiuretic hormone.[44,53] Although this influence could have been controlled for through normalizing to urinary creatinine, this method of calculation has its own limitations.[4,51] Taking this information into account, the administration of subcutaneous fluids could be masking potential effects of anesthesia on urinary HAVCR1 and LCN2 expression and lends anecdotal support to the beneficial effects of fluid administration during or after (or both) anesthesia in rodents. This possibility could be explored in future studies through the inclusion of a negative control group that does not receive subcutaneous fluids.

Consistent with published literature regarding traditional serum biomarkers, all anesthetics used in this study had no significant clinically measurable effect on renal function. BUN and creatinine were significantly elevated only in the ischemia–reperfusion injury group, and BUN was significantly elevated in the cisplatin-treated group. Although not statistically significant, serum creatinine was elevated in the cisplatin-treated group; the reduced sample size in this group (due to insufficient blood sample collection in one animal) likely prevented this value from

reaching significance. The elevation of BUN and creatinine in these 2 groups was expected, as acute kidney injury was induced in both of these animal groups. These biochemical changes validate the use of ischemia–reperfusion injury and cisplatin nephrotoxicity for the study of AKI and subsequent determination of unique biomarkers of disease.[10,36,39,48,49,54]

Unrelated to the main focus of this study were the qualitative effects of the various general anesthetics on the depth and duration of unconsciousness, and although inconsistently controlled with regard to applied pressure and time points assessed, the subjective effects provided some valuable insights. Although well cited in the literature as an effective anesthetic for surgical procedures in male C57BL/6 mice, 50 mg/kg IP pentobarbital did not reliably result in a surgical plane of anesthesia during our study.[25,31,44,53] All mice undergoing ischemia–reperfusion injury surgery required supplementation with isoflurane to maintain an anesthetic depth appropriate for surgical manipulation. In addition, mice receiving only anesthesia with pentobarbital (no surgery) gave positive responses to toe pinch at all times tested and frequently exhibited spontaneous motor function without overt stimulation. Therefore, in addition to being cost prohibitive, we contend that injectable pentobarbital at 50 mg/kg IP should not be used as a sole agent for the purpose of surgical anesthesia in 8- to 12-wk-old male C57BL6/J mice. We also recognize that ketamine-containing combinations are not ideal options, given that unchanged ketamine and its metabolites are eliminated through the kidneys and result in prolonged recovery in kidney-injured animals.[44] Therefore, we encourage the consideration of isoflurane in combination with appropriate analgesia for kidney injury models, with the caveat that the selection of anesthetic agents considers all of the factors we discuss in this article.

Unexpectedly, contrary to previously reported limitations to the use of tiletamine–zolazepam in mice, there were no anesthesia-related deaths associated with its administration at the dose used in this study. In addition, tiletamine–zolazepam provided the shortest induction and duration of anesthesia, thus directly conflicting with reports of long duration and recovery in mice.[53] This difference could be due in part to the age, sex, or strain of the mice used in this study compared with those previously reported.[14,47] One group found that when used in combination with xylazine, tiletamine–zolazepam provided safe and adequate anesthesia in CD1(ICR) outbred mice, as determined through the loss of response to toe pinch.[14]

There were several limitations present in the design and execution of the current study, including variability in the duration of anesthesia between injectable and inhalational agents, data collection by an unblinded observer, volume of urine collected, the numbers of animals and samples, and lack of advanced anesthetic monitoring (that is, blood pressure). It is important to note that the duration of anesthesia for the inhalant group was roughly half those for 2 of the injectable groups. One of the benefits of using an inhalant agent over an injectable (or combination) is the reduction in duration of anesthesia due to improved recovery times. So although the shortened timeframe that we used is realistic for the completion of a rodent surgical procedure, it does present a variable when comparing with the ketamine–xylazine and pentobarbital groups, in which the pharmacologic effects of the drug(s) lasted nearly double that time. Although subjective data were collected by an unblinded observer, all attempts were made to minimize any potential effect of unconscious bias, by using objective measurements including time to the return of the righting reflex, quantitative measurements of urinary biomarkers and serum chemistry, and blinding of renal histologic scoring.

An additional potential complication was the administration of subcutaneous fluids at the completion of the surgical manipulation in the surgery positive-control group, whereas all others animals received fluids at the time of anesthesia induction. We recommend providing subcutaneous fluids to anesthetized rodents at the time of induction or soon before or afterward, because doing so seems to improve recovery time at the completion of the procedure. This provision would have been challenging to accomplish for the surgery positive–control group, because the surgical incision and dissection of tissue could have disrupted the fluid pocket due to the location of the incision and approach to the kidneys, resulting in variable loss of the fluid or entry of the fluid into the retroperitoneal space. Because all experimental groups received fluids during the same phase, we do not anticipate that this discrepancy negatively affected the quality of the study or results presented.

Perhaps the most significant limitation of this study was the volume of urine that was readily accessible for analysis. Healthy mice, such as those used in this study, have a very high concentrating ability. Therefore, the volume of excreted urine in the normal state is low. Combined with a passive collection method that relied on spontaneous urination, we rarely collected a measurable volume of urine from the same mouse at all time points. Alternatives to the passive collection method include induced collection through frequent manual manipulation of the bladder, urinary catheterization, and serial euthanasia at given time points. These alternatives were not used in the present study because we felt that increased invasiveness of collection methods was not justified for the purposes of this study, but we may consider these alternatives for future studies to validate and explore the current results. These other collection methods would provide the added benefit of eliminating the use of the metabolic cage, which has been well documented to affect normal mouse physiology.[22,55] Although this variable was controlled in this study through inclusion of the negative control group, which also used the metabolic cage, further explorations of the effect of the stressful environment on urinary biomarkers of kidney injury may be worthwhile.

Also contributing to the low sample number per time point was the small number of animals used to execute this study. A small sample size was used in an effort to use the fewest number of animals possible to achieve statistical significance for this study, in accordance with the 3 Rs. In addition, a decreased number of animals were dedicated for use in the positive control groups, because these methods of inducing AKI are well established.[30,36,48,49,54] Given these limitations, future studies should include a larger sample size, either through serial euthanasia methods or increased numbers per groups, to improve the overall sample analysis.

To that end, the inclusion of female mice in future studies is necessary also, because sex-associated differences in responses to general anesthesia and susceptibility to ischemic injury have been described.[15,23,24] We strongly considered including female mice, but the inclusion of 2 sexes in a multifaceted research design would have further complicated the statistical evaluation to determine whether general anesthesia alone affected urinary biomarkers of kidney injury and warranted further evaluation. We selected male mice for this study given the larger proportion of male compared with female mice used in kidney injury models, because males are considered more susceptible to ischemic injury.[23,24] Given the results herein, more informed decisions can be made for future investigations and to align

studies with the NIH's expectation that the consideration of sex as a biologic variable is addressed in their funded research. Similarly, we hypothesize that the results we described are dependent on the mouse strain or stock used, and this and other aspects of experimental design should be considered with these differences in mind.[15,52,53]

An additional limitation to our study is that blood pressure was not monitored while the mice were under anesthesia. One group monitored blood pressure in their studies of the influences of isoflurane, sevoflurane, and pentobarbital on kidney injury in mice. However, they reported no significant influences on blood pressure, even though general anesthesia with these agents is known to have systemic effects, including hypotension.[30-32] Measuring blood pressure in the present investigation would have permitted correlation of elevations in biomarkers with systemic blood pressure, which would be of particular interest in the ketamine–xylazine group, but was beyond the scope of our study.

Despite these limitations, the goal of establishing the effects of general anesthesia alone on commonly used urinary biomarkers of kidney injury was met. The described changes are unlikely to be biologically relevant, and well-controlled general anesthesia using these agents itself is unlikely to cause AKI. However, toxic effects on the kidney act in synergy. Therefore, in patients or studies using animals with compromised renal function and in those receiving other nephrotoxic agents (such as β-lactam antibiotics and NSAID), the selection and use of general anesthesia become more important, as does the effect of those agents on experimental results.[34] It is in this context that the results of the current study provide the most insight.

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