Ischemia-reperfusion (I/R) injury, myocardial infarction, creatine kinase release, lactate dehydrogenase (LDH) release, mitochondrial DNA depletion, and apoptosis can occur as consequences of organ dysfunction, due to impaired circulation and oxygen deprivation.1–3 Because I/R injury studies are cumbersome in a clinical setting, cell culture presents some advantages that include predictive responses and better control and selectivity of the study factors.4 Hepatocyte cultures can be utilized for drug-induced organ injury in small animals.5 In search of better treatments and preventive approaches for I/R injury, many anesthetic drugs are investigated as cellular protectors and inflammatory modulators.6–9 Sevoflurane and isoflurane-loaded lipid emulsions have been investigated in patients going under organ transplantation10–12 or procedures that impair organ circulation by prolonged periods of oxygen deprivation.13,14 Published literature14–16 supports that when applied to core organs in animals, such as the heart, brain, and kidneys before, during, or after a predictive ischemic event, emulsified anesthetics will produce a pre- or postconditioning effect, promoting cell viability and diminishing the signaling of the apoptosis cascade initiation.

Target organs designated for cultured cell study subjected to hypoxic environments have 

OBJECTIVE
Emulsified isoflurane and sevoflurane have immunomodulating and anti-inflammatory effects in vital organs such as the brain, myocardium, and kidneys subjected to ischemia-reperfusion injury. This study aims to investigate the cellular protective effects of both emulsified anesthetics in cultured canine hepatocytes.

PROCEDURES
We analyzed the apoptosis and viability responses of cultured primary canine hepatocytes exposed to 1% O2 for 30 versus 120 minutes after being treated with emulsified isoflurane or sevoflurane in 10% lipid, or 10% lipid alone or no-treatment control at 24 hours of reoxygenation (21% O2).

RESULTS
After 120 minutes of hypoxia, the hepatocytes that received either emulsified isoflurane or sevoflurane treatments had significantly decreased apoptosis at 24 hours of reoxygenation in comparison to the 10% lipid treatment. Also, the no-treatment control group had significantly higher apoptosis at 24 hours of reoxygenation when exposed to 120 minutes of hypoxia compared to 30 minutes of hypoxia. Neither 30 nor 120 minutes of hypoxia or exposure to 10% lipid, emulsified isoflurane, or emulsified sevoflurane altered overall cellular viability at 24 hours of reoxygenation.

CLINICAL RELEVANCE
This study demonstrated that both isoflurane and sevoflurane, in the emulsified form, have the potential to reduce the apoptotic response of cells under oxygen deprivation. Therefore, this attribute of both halogenated anesthetics suggests an alternative treatment to be applied in live patients submitted to surgical stabilization of organs and tissues under the risk of ischemia and reperfusion injury.

Keywords: apoptosis, cellular protection, hypoxia, lipid emulsion, volatile anesthetics

Received September 7, 2023
Accepted December 9, 2023
doi.org/10.2460/ajvr.23.08.0192
demonstrated beneficial responses after the administration of halogenated anesthetic emulsions. There is a decrease in cellular apoptosis\textsuperscript{17-19} and LDH leakage,\textsuperscript{2,20,21} lower reactive oxygen species production,\textsuperscript{22} lower malondialdehyde concentrations,\textsuperscript{17,23} and diminishment of the release of proinflammatory mediators such as IL-1, IL-6, and tumor necrosis factor-α.\textsuperscript{24,25} Also, emulsified isoflurane promotes the synthesis of nitric oxide and superoxide dismutase, 2 important inflammatory modulators and cellular protectors.\textsuperscript{2,17}

These emulsified anesthetics are originally available in liquid form containing 99% of the anesthetic agent to be vaporized and delivered to the patient’s pulmonary system. In search of a more versatile alternative administration to inhalation, halogenated anesthetics can be successfully emulsified as shown in this study with lipids or perfluorocarbon compounds by their high oil/gas partitioning coefficient and fluoride ligands.\textsuperscript{26-28} When a stable emulsion is obtained, the solution yields an anesthetic that does not need to be vaporized but can be administered IV\textsuperscript{29-31} or through other routes.\textsuperscript{32-37} Even though there is no approval for clinical use yet, emulsified anesthetics could be an interesting option for use as therapeutic drugs by this means, a stable halogenated anesthetic emulsion would be useful as an anesthetic agent and a potential therapeutic drug due to its potential protective effects.\textsuperscript{12,15,38}

The primary objective of our study was to characterize the effects of both emulsified isoflurane and sevoflurane produced by ultrasonic sonication on cultured primary canine hepatocytes when applied before a hypoxic event followed by reoxygenation (21% O\textsubscript{2}). We hypothesized that these hepatocytes would have reduced apoptosis while maintaining cell viability when subjected to hypoxia of 1% O\textsubscript{2} if pretreated with emulsified isoflurane or sevoflurane in comparison to the controls (lipid 10% vol/vol; or no treatment).

\section*{Methods}

\subsection*{Emulsion preparation}

The emulsion preparation and some of the methods used were based on previous publications.\textsuperscript{39,40} For the aqueous phase, 2% vol/vol of polysorbate 80 (Tween80, Sigma-Aldrich Products) and 2.5% wt/vol of sorbitol F (Sigma-Aldrich Products) were added to purified water (7.16 mL; Millipore Milli-Q Advantage A10 Water Purification System) in an Erlenmeyer flask with a stir bar and placed on a heated magnetic stirrer (Cimarec Stirring Hot Plates; Barnstead International) at 30 °C with a speed range of 60 to 125 rpm (1,000 to 3,000 X g) for 1 to 3 minutes until the solutes were completely solved. The lipid stock solution was prepared with 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol (Lipoid S75 [Lip]; Lipoid, GmbH) and 90% medium-chain triglyceride (Ultrafiltered Pharmaceutical MCT Oil 60/40; Medical Lab Supply) placed on a heated magnetic stirrer (Cimarec Stirring Hot Plates) at 30 °C with a speed range of 60 to 125 rpm (1,000 to 3,000 X g) for 10 to 15 minutes until the lipid starch was completely solved in the MCT.

To make the 10% lipid/15% anesthetic emulsions, 1 mL of the lipid stock solution was added to 1.5 mL of either isoflurane (EI; Isothelia; Covetrus, Inc) or sevoflurane (ES; Sevoflo, Covetrus, Inc). The oily phase was poured into the conic tube containing the aqueous phase once both were at 18 °C. The tube was immediately covered and sealed. To perform the emulsification, the tube was completely immersed in ice and the sonicator (Q55; QSonica Sonicators) was turned on at 80% amplitude (~16,000 X g) for 1 minute. Both isoflurane and sevoflurane emulsions were visually inspected for phase separation within the first 30 minutes after the sonication. If no clear layer was visible in the bottom of the tube, the emulsion was considered a stable homogeneous solution (100% stability). The flask was then stored in a cooler at 18 to 22 °C for 24 hours before the experiment.

\subsection*{Cell culture}

The cellular media were prepared with William’s E medium, no phenol red (ThermoFisher Scientific), 4% of primary hepatocyte maintenance supplement + 1 µL of DMSO (GIBCO, ThermoFisher Scientific), and 5% of qualified heat-inactivated FBS (GIBCO, ThermoFisher Scientific). Approximately 13.5 X 10\textsuperscript{3} dog primary hepatocytes (Nunc, ThermoScientific) were added to each well of the 96-well plates (Nunc, ThermoScientific) distributed according to the study design. After the cells were added, 100 µL of the prepared warmed media was added. The plates were then placed in the incubator (Isotemp CO\textsubscript{2} Incubator; FisherScientific) at 37 °C 5%CO\textsubscript{2}/95% air for 12 hours.

\subsection*{Apoptosis and viability before and following hypoxia}

The groups were divided into 30 (H30) and 120 minutes (H120) of 1% O\textsubscript{2}, where each plate was separated for the viability and apoptosis assays (Supplementary Figure S1). The concentrations of the emulsified anesthetics to be added to each well were 67.5 µg/well (0.675 µg/µL), 135 µg/well (1.35 µg/µL), and 240 µg/well (2.4 µg/µL) of either the 15% vol/vol emulsified isoflurane or sevoflurane with 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol. The control treatments containing 10% lipid alone were separated into 192.9 µg/well, 385.8 µg/well, and 771.6 µg/well.

After the treatments were added for a period of 30 minutes, the CellEvent (5 µM/10 µL) apoptosis reagent or the PrestoBlue HS cell viability reagent was added to each well containing the treatments and to control A (no treatment). The plates were protected from direct light and incubated for 30 minutes (37 °C 5% CO\textsubscript{2}/95% air). After that, they were placed in a cell imaging multimode plate reader (Cytation5 BioTek Instruments, Inc) for an endpoint study as baseline data for the CellEvent

\section*{Unauthenticated | Downloaded 01/17/24 09:09 AM UTC}
Effects of 30 or 120 minutes of hypoxia after pretreatment with Lip, EI, or ES on induction of apoptosis

The effects of pretreatment with Lip, EI, or ES before hypoxic insult for 30 or 120 minutes followed by reoxygenation for 24 hours were explored to determine the potential protective effects of these emulsified anesthetics. Pretreatment with EI (135 µg) before 30 minutes of hypoxia (Figure 1) produced a significant reduction in apoptosis compared to Lip (385.8 µg). Thus, EI (135 µg) appeared to have a protective effect compared to its corresponding vehicle control. Pretreatment with ES (270 µg) or ES (270 µg) before 120 minutes of hypoxia (Figure 2) significantly decreased induction of apoptosis compared to Lip (771.6 µg). Thus, pretreatment with both EI and ES at 270 µg before the more prolonged hypoxic insult of 120 minutes appeared to have a protective effect compared to their corresponding vehicle control.

Figure 1—Primary canine hepatocytes were treated with either control (no-treatment), Lipoid S75 (Lip; 10% vol/vol fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol), 15% vol/vol emulsified isoflurane (EI), or 15% vol/vol emulsified sevoflurane (ES) at concentrations that mimic 1, 2, or 4 hours (192.9 µg, 385.8 µg, 771.6 µg for Lip, respectively, and 67.5 µg, 135 µg, 270 µg respectively for both EI and ES) of constant rate infusion in live dogs to maintain general anesthesia. This bar graph illustrates the significant difference between the moderate concentration of Lip (385.8 µg) and the corresponding concentration of EI (135 µg) at 24 h following 30 minutes of hypoxia (1%). The apoptosis response of the hepatocytes measured in relative fluorescence units (RFU) by the assay CellEvent (502-nm excitation [ex] and 530-nm emission [em]) is demonstrated for the comparisons between treatments at 24 hours of reoxygenation (21% O₂) subtracted from the baseline disregarding the outliers. All results are expressed as the mean (bars) ± SEM (bar whisker) of n = 7 to 8 replicates/group. *P < .05 significant difference.

Results

Emulsions stability and volume quantification

By visual inspection, the emulsions of 15% vol/vol isoflurane and sevoflurane with 10% Lipoid S75 prepared by ultrasonic sonication were stable for 24 hours before the experimental study. No phase separation was visualized, indicating 100% stability. No decrease in volume was observed during this period. The prepared emulsions were kept at 18 to 20°C for the primary 24 hours and then were refrigerated at 4°C. The volume of emulsions after the experiment execution remained the same for 290 days. This analysis indicates that evaporation did not occur and the initial concentration of 15% vol/vol of each anesthetic was maintained.

Statistical analysis

The data were analyzed using GraphPad Prism (GraphPad Prism version 10.0.0 for Windows, GraphPad Software). All results are expressed as the mean ± SEM of n = 7 to 8 technical replicates per group. To determine induction of apoptosis, baseline values prior to hypoxia were subtracted from the values obtained following 24 hours of reoxygenation for each replicate. A Grubb test was utilized to detect outliers within each group of experimental data. A one-way ANOVA with Tukey post hoc test for pairwise comparisons was utilized to examine treatment effects on apoptosis and viability at 24 hours of reoxygenation for comparisons with 3 or more groups. An unpaired Student t test was utilized to determine statistically significant effects in comparisons with 2 groups. A P value ≤ .05 was considered a statistically significant difference between groups.
Effects of duration of hypoxia in untreated cells or after pretreatment with Lip, EI, or ES on induction of apoptosis

The effects of 30 or 120 minutes of hypoxia in untreated cells and pretreated cells were examined to determine if duration of hypoxia affected induction of apoptosis. In untreated hepatocytes, exposure to hypoxia for 120 minutes significantly increased induction of apoptosis compared to exposure to hypoxia for 30 minutes (Figure 3). Therefore, the duration of hypoxic insult did affect induction of apoptosis in untreated hepatocytes. In hepatocytes pretreated with either Lip, EI, or ES (Figure 4), the moderate concentration of Lip (385.8 µg) and the high concentration of ES (270 µg) displayed significantly lower levels of apoptosis after long-term hypoxia (120 minutes) compared to short-term hypoxia (30 minutes). Thus, the differences between the hypoxia period in these groups influenced the apoptosis response of 10% Lip and ES but not EI.

Effects of 30 and 120 minutes of hypoxia after pretreatment with Lip, EI, or ES on cellular viability

The effects of pretreatment and hypoxic duration on overall cellular viability were examined to see if induction of apoptosis corresponded with alterations in cellular viability. Pretreatment with Lip, EI, or ES before 30 minutes or 120 minutes of hypoxia did not significantly alter cellular viability (Figure 5). Thus, pretreatments in combination with either 30 or 120 minutes of hypoxia did not have a detrimental
effect on overall cellular viability at the 24-hour reoxygenation time point.

Discussion

The overall purpose of this study was to investigate the preconditioning effects of 15% emulsified isoflurane and sevoflurane with the lipid vehicle (10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol) in comparison to the lipid vehicle alone by reducing apoptosis while maintaining cell viability of cultured canine hepatocytes under severe hypoxia (1% O2). Our study demonstrates that both emulsified anesthetics were able to diminish the apoptosis of these hepatocytes after a prolonged period of 120 minutes of exposure to 1% oxygen but not after a short period of 30 minutes and that 120 minutes of hypoxia was more effective in inducing apoptosis compared to 30 minutes in untreated cells.

Our initial goal was to emulsify the solution components to obtain a nanoemulsion (particle size of 100 to 500 nm, with a mean particle radius < 100 nm). However, to produce a nanoemulsion, established methods require a high-pressure homogenizer, or microfluidization and phase-inversion temperature to overcome the energy barrier. Considering that the specific equipment was not available to us, we only utilized ultrasonic sonication and did not analyze the droplet size or particle dispersion. Another way to evaluate if the particles fall within the nano-range is through visual access to the color or transparency of the solution. The more transparent, or translucent, the smaller the particle size is. Our emulsion had a turbid appearance; therefore, we considered it likely to be a macroemulsion (> 500 nm). The emulsions prepared in our study with 15% vol/vol sevoflurane or isoflurane with 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol had 100% stability because no phase separation was visualized for 30 minutes after they were produced and up to 24 hours before they were added to the cultured primary canine hepatocytes. To the authors’ knowledge, no previous study with halogenated anesthetics had produced an emulsion by ultrasonic sonication alone.

The concentration of each anesthetic was based on approximately 1.5 g wt/vol per mL of liquid isoflurane or sevoflurane (Table 1). The concentrations of the emulsified anesthetics to be added to each well containing the cultured hepatocytes were based on a previous publication, where a constant rate infusion of 0.3 mL/kg/min was delivered IV in healthy dogs. One hour of infusion is represented by 67.5 µg/well (0.675 µg/µL), 2 hours by 135 µg/well (1.35 µg/µL), and 4 hours by 240 µg/well (2.4 µg/µL) of either the 15% vol/vol emulsified isoflurane or sevoflurane with 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol. To maintain the same concentration of 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol, the Lip treatments were separated into 192.9 µg/well, 385.8 µg/well, and 771.6 µg/well to represent 1 hour, 2 hours, and 4 hours of infusion, respectively. In a preliminary study (data not shown), our results indicated that 15% vol/vol sevoflurane or isoflurane with 10% fat-free soybean phospholipids with 70% phosphatidylcholine (Lipoid S75) wt/vol emulsions can maintain a concentration-dependent viability response in cultured canine primary hepatocytes and that no significant increase in apoptosis occurred with either emulsion at normoxia. Therefore, the concentration of the anesthetic agent in the emulsified solutions was adequate and replicable to the present study.

As opposed to exposure to 30 minutes of hypoxia followed by reoxygenation, the cells in the no-treatment control group displayed significant induction of apoptosis following hypoxia for 120 minutes and reoxygenation (Figure 3). These
membranes, the main promoter of cellular protection is likely the volatile anesthetic or a combinatorial effect of time of hypoxia, lipid vehicle, and anesthetic concentrations.

The effects of compound and concentration on apoptosis at 24 hours were examined to determine if there were compound or concentration-dependent effects on apoptosis at the end of reoxygenation at 24 hours. These data indicate exposure to the highest concentrations of E1 and ES appears to have a protective effect against apoptosis induced by prolonged hypoxia (Figure 2). Additionally, the more prolonged 120-minute hypoxia followed by 24 hours of reoxygenation produced a significant increase in apoptosis in control and Lip-treated hepatocytes compared to the brief 30 minutes of hypoxia (Figure 3). The present data indicate that the time of hypoxia can be considered an important factor for the effects on cellular protection of both emulsified anesthetics on cultured canine hepatocytes given pretreatment with ES (270 μg) before 30 minutes of hypoxia significantly decreased apoptosis compared to pretreatment with ES (270 μg) before 30 minutes of hypoxia (Figure 4). In previous studies comparing isoflurane, sevoflurane, and control, both anesthetics demonstrated better protection than the control, and no differences between them were noted. In the same studies, the authors compared the IV infusion for 30 minutes of 6.9% emulsified isoflurane lipid vehicle and 7.5% emulsified sevoflurane or lipid vehicle and emulsified sevoflurane before a coronary artery occlusion in rabbits to evaluate myocardial infarct size. In the emulsified sevoflurane and isoflurane groups, the animals demonstrated smaller infarct size compared to lipid alone and control (saline solution), emphasizing that in comparison to lipid alone, both anesthetics promoted better organ protection and that no difference was observed between them. This suggests that the lipid vehicle, while ideal for creating a stable emulsion with lipophilic substances such as volatile anesthetics, is not essential for cellular protection at higher concentrations or for prolonged exposures.

While a prolonged hypoxia produced increased apoptosis response in a treatment and concentration-dependent manner in the cultured canine hepatocytes evaluated in this study, it was not known if these alterations were significantly altering overall cellular viability at the 24-hour reoxygenation time point. Therefore, overall treatment and concentration-dependent effects on cellular viability were assessed after 24 hours of reoxygenation following 30 minutes or 120 minutes of hypoxia. Surprisingly, no significant differences were observed between the 30 minutes of hypoxia group of treatments or between the 120 minutes of hypoxia group of treatments (Figure 5). This may indicate that either the activation of apoptosis was not sufficient to decrease cellular viability or that the 24-hour period of reoxygenation was not sufficient for a decreased cellular viability to be observed following activation of the apoptotic pathway. In a recent study, no gross morphological changes were observed in human primary hepatocytes subjected to 1% oxygen for 30 minutes compared to 240 minutes. However, it is unclear if the apoptotic cascade was initiated in this study with human hepatocytes.

Isoflurane alone has been studied as a cellular protector of multiple organs such as the kidneys, brain, and myocardium due to its safety and non-toxic effects in cultured cells as well as in vivo studies. Some studies performed with volatile anesthetics vaporized into the cultured cells showed a decrease in cellular signaling, inflammatory response, apoptosis, and death. The inconvenience of this technique, however, would be the anesthetic apparatus, atmospheric contamination, and limited time to apply the vaporized anesthetic into the cellular culture, whereas an emulsified volatile anesthetic permits a higher time of exposure, better control of the applied concentration, and no anesthetic loss into the atmosphere.

In conclusion, our study demonstrated that the 15% vol/vol isoflurane and sevoflurane with 10% fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol emulsions produced cellular protection in cultured primary canine hepatocytes subjected to 1% oxygen for 120 minutes compared to the lipid alone in a concentration-dependent manner. The prolonged 120 minutes of hypoxia was more effective in inducing apoptosis in canine hepatocytes compared to the more abbreviated 30

Table 1—Emulsified anesthetic compositions and volumes applied per well.

<table>
<thead>
<tr>
<th>Anesthetic agent</th>
<th>Anesthetic concentration (% vol/vol)</th>
<th>Lipid component (% vol/vol)</th>
<th>Volume per well (µL)</th>
<th>Concentration per well (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane</td>
<td>15</td>
<td>10</td>
<td>0.3</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>10</td>
<td>0.3</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>10</td>
<td>0.3</td>
<td>270</td>
</tr>
<tr>
<td>Sevoflurane</td>
<td>15</td>
<td>10</td>
<td>0.3</td>
<td>67.5</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>10</td>
<td>0.3</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>10</td>
<td>0.3</td>
<td>270</td>
</tr>
<tr>
<td>None (Lip)</td>
<td>0</td>
<td>10</td>
<td>0.3</td>
<td>192.9</td>
</tr>
<tr>
<td></td>
<td>0.6</td>
<td>10</td>
<td>0.3</td>
<td>385.8</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>10</td>
<td>0.3</td>
<td>771.6</td>
</tr>
</tbody>
</table>

* Lip = Lipid (10% vol/vol fat-free soybean phospholipids with 70% phosphatidylcholine wt/vol).
minutes of hypoxia. Future studies are warranted to elucidate the relationships between the time of hypoxia and concentrations of emulsified anesthetics apoposis/viability in canine hepatocytes, clarify a potential clinical use of emulsified anesthetics agents to reduce ischemia-reperfusion injury in patients with compromised hepatic blood supply, and determine if these emulsions can protect other vital organs.

Acknowledgments

This manuscript is dedicated to the late Dr. Claudio C. Natalini for his mentorship and dedication. The authors acknowledge Dr. Lakshmi Narayanan, Dr. Sandeep Kundakala, Ben Ardahl (technical), and Dr. Henrique Lupiano for their technical support and laboratory operation. The authors also thank Dr. Robert Wills for helping with the initial statistical design and analysis and Lipoid, GmbH for donating Lipoid S75.

Disclosure

The authors have nothing to disclose.

Funding

This work was supported by a preliminary data grant from the Mississippi State University College of Veterinary Medicine Office of Research and Graduate Studies.

References


Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org