Developing a standardized system of exposure and intervention endpoints for isoflurane in preclinical stroke models

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Abstract

Isoflurane is a regularly used anesthetic in translational research. Isoflurane facilitates invasive surgery and a rapid recovery. Specifically, in the pathology of stroke, controversy has surrounded isoflurane's intrinsic neuroprotective abilities, affecting apoptosis, excitotoxicity, and blood brain barrier disruption. Due to the intrinsic neuroprotective nature and lack of standardized guidelines for the use of isoflurane, research has shifted away from this gas in most animal models. Antagonistically, studies have also reported that no neuroprotective effects are observed when a surgery is accompanied with isoflurane exposure under 20 minutes. Isoflurane affects the pathophysiology in stroke patients by altering critical pathways in endothelial, neuronal, and microglial cells. Current studies have elucidated isoflurane neuroprotection to be time dependent and may be minimized in experimental designs if the exposure time is limited to a specific window. Therefore, with detailed and extensive literature on anesthetics, we can hypothesize that isoflurane exposure under the 20-minute benchmark, behavior and molecular pathways can be evaluated at any time-point following ischemic insult without confounding artifacts from isoflurane; however, If the exposure to isoflurane exceeds 20 minutes, the acute neuroprotective effects are evident for 2 weeks in the model, which should be accounted for in molecular and behavioral assessments, with either isoflurane inhibitors or a control group at 2 weeks post middle cerebral artery occlusion. The purpose of this review is to suggest a detailed and standardized outline for interventions and behavioral assessments after the use of isoflurane in experimental designs.

Key words: isoflurane; stroke; surgery; exposure; neuroprotection; pathophysiology; anesthetics; preclinical; animal model

doi: 10.4103/2045-9912.254640
How to cite this article: Hillman TC, Matei N, Tang J, Zhang JH. Developing a standardized system of exposure and intervention endpoints for isoflurane in preclinical stroke models. Med Gas Res. 2019;9(1):46-51.
Funding: This work was funded by an NIH grant, No. NS081740 (to JHZ).

Introduction

Previous research

Although isoflurane is a commonly used anesthetic in preclinical trials as well as in vertebrae models,[1-6] controversy has developed due to its acute neuroprotective effects.[7-12] Isoflurane has been shown to decrease infarct volume, neuronal damage[3,6,13] and improve neurobehavioral outcomes in vertebrae stroke models.[14] In cerebral ischemic models, exposure of isoflurane between 30–90 minutes resulted in neuroprotection, reduced infarct volume, and improved neurobehavioral outcomes.[3,6] Neurobehavioral outcomes were evaluated by open field activity, prepulse inhibition, novel object recognition, acoustic startle, rotarod, and water maze.[6] Antagonistically, with the same exposure, Lay et al.[2] showed no significant effect in infarct volume or neurobehavioral outcomes with both sodium pentobarbital, another type of anesthetic, and isoflurane compared to vehicle during cerebral ischemia. Significant research suggests that, when exposure to isoflurane is over 30 minutes, molecular and behavioral tests should be evaluated at 2 weeks to ensure the acute neuroprotection of isoflurane dissipates and the effects observed are solely due to treatment.[15,16]

Concerns with the use of isoflurane

To date, research has shifted away from isoflurane to non-gas anesthetics, avoiding any confounding artifacts from isoflurane that may interfere with treatment in ischemic models.[15] However, in this review we suggest that an ample amount of experiments have shown no conflict in molecular and behavioral analysis at 24–48 hours in the evaluation of novel treatments when isoflurane exposure was kept below 20 minutes.[1,16] Failure to account for isoflurane's neuroprotective characteristics and pursue either molecular or neurobehavioral tests may lead to artifacts in data and report an exacerbated drug efficacy.[1,16] Of interest, no neuroprotective effects were observed and normal experimental evaluations may be evaluated at any time point following ischemia when exposure to isoflurane was less than 20 minutes.[1,15,16] Without established standards for anesthesia use, concerns with isoflurane in pre-clinical studies have risen from the acute neuroprotective effects and interference in the evaluation of treatments in brain injury models.[1,15,16] In order to avoid errors in data, Gaidhani et al.[1] suggested a standard system be created for the use of isoflurane. This system should establish a timeframe of isoflurane exposure during surgery to minimize interference in pre-clinical work. Supporting this standardized system, Kawaguchi et al.[15,16] suggested that the neuroprotective effects of isoflurane are due to longer exposure times during surgery and is not advantageous at shorter exposure times. Therefore, with a thorough review of current literature, a standardized exposure time for isoflurane during surgery may be suggested to reduce inconsistent results

and misleading treatment effects.[15,16]

Physiological Effects

The use of vaporized anesthetics, specifically isoflurane, has been connected to several physiological changes. In mice the use of vaporized anesthetics was shown to cause hypoglycemia and acidosis.[6] These conditions are tied to the inhibition of the electron transport chain and triphosphate production.[6] The exposure of aerosolized anesthetics greater than 30 minutes has been shown to play a role in the delayed development of cerebral infarction,[6] inhibition of apoptotic pathways, and suppression of excitotoxicity.[1,15] Thus, investigators have tried to precondition ischemic models with isoflurane, desflurane, and sevoflurane, which advantageously preserved the dopaminergic circuit – damage in this circuit leads to injury in the ipsilateral hippocampus.[6,16] Lastly, isoflurane showed significant improvement in neurobehavioral outcomes and decreased infarct volume.[9,14]

Effects on Stroke Physiology

The use of isoflurane in stroke has been used extensively to keep subjects under deep anesthesia, facilitated with a short recovery period.[2,3] Isoflurane has been used in the following research studies: focal cerebral ischemia,[13,16] transient focal ischemia,[17] middle cerebral artery occlusion (MCAO),[1,5,18,19] neonatal hypoxia-ischemia,[6,10,12] intracerebral hemorrhage,[20] germinal matrix hemorrhage,[8,10] and subarachnoid hemorrhage.[21,22] These studies have shown isoflurane pre-treatment delayed the onset of apoptosis, reduced cerebral infarction, and decreased neuronal damage.[13] Specifically, in MCAO and transient focal ischemic models, isoflurane displayed a dose-dependent relationship with its neuroprotective characteristics.[1,19] When models used long isoflurane exposure times, between 20 and 90 minutes, a significant increase in neuroprotection was observed.[1,19] However, at 90 minutes, the protective effects were reversed, and isoflurane became deleterious, increasing infarct size and neurological deficits. In contrast, neonatal hypoxia-ischemia and germinal matrix hemorrhage were the only models not to show significant neuroprotection after isoflurane exposure.[6,8] The hippocampus in neonates did not significantly improve from isoflurane,[6] but negatively increased spatial learning deficits[6] long-term.[10] Similarly, in germinal matrix hemorrhage, pretreatment of isoflurane was not antiapoptotic, but resulted in delayed motor deficits.[8,10]

Some models, *e.g.* MCAO, favor the use of intravenous agents instead of inhalation agents in vertebrae experimental studies to avoid complications from isoflurane. A common intravenous agent used in animal models is ketamine[23] and is thought to not possess the neuroprotective effects of isoflurane or other volatile anesthetics.[24] Two reviews have compared the use and effects of both volatile and intravenous agents, but further discussion is merited for the development of a standardized system of anesthetics, in both intravenous agents and volatile anesthetics.[23,24] Confounding the hypothesis of only using intravenous agents as an anesthetic, ketamine has been reported to show mild neuroprotection in vertebrae studies.[23,24] Therefore, in the stroke model, researchers should consider and account for the intrinsic effects of anesthetics on vertebrae animals to best allow for a translationally strong experimental design with accurate results.[24]

Stroke Pathophysiology

Ischemia induces an array of metabolic events that result in deleterious damage and cellular death. The ischemic insult results in an influx of reactive oxygen species in the brain to initiate inflammation and apoptosis.[25,26] Additionally, the creation of reactive oxygen species, increase in cytokines, and decrease in adhesion molecules results in endothelial damage and blood-brain barrier (BBB) deterioration.[25] With low concentrations of molecular oxygen and glucose in the blood, increased free radicals, and an abundant amount of lactate and hydrogen ions, Caspases are activated after stroke and neuronal cells begin undergoing apoptosis.[25,26] Further, brain damage is caused by a depletion of oxygen or glucose resulting in adenosine triphosphate depletion.[27] Lack of nutrients and energy leads to cell injury and death, resulting in extensive damage to the brain and organism.[27] Additionally, the extent of the damage proportional to the duration, location and severity of the ischemic event.[27] Lack of energy for as little as 5 minutes can lead to irreversible brain damage due to by-products of anaerobic metabolism.[27] Glial and neuronal cells also rapidly swell from an influx of extracellular fluids and undergo apoptosis.[27] Of note, hypoxia in the brain leads deterioration of blood vessels with the release of proteases, which causes damage and breakdown of the blood brain barrier, observed as cerebral edema in ischemia.[27]

In cerebral infarction, the inflammatory process is critical in determining the extent of damage. Inflammation occurs at multiple levels, including the molecular and cellular level, of blood-microvascular endothelial cell interface.[27] Ischemic brain injuries results in the release of cytokines such as interleukin-1β and tumor necrosis factor alpha (TNF-α).[27] During ischemic injury there is an increased expression of proinflammatory genes including genes like heat shock proteins, adhesion molecules, and cytokines.[27] The aforementioned processes that normally occur in the stroke pathophysiology may be affected by certain exposure times of anesthetically used isoflurane in translational models.

Effects on apoptosis

Isoflurane has been shown to ameliorate apoptosis in cerebral ischemic models.[15] Isoflurane regulates apoptosis through the sphingosine-1-phosphate (S1P)/phosphoinositide 3-kinases (PI3K)/kinase B (Akt) signaling pathway (**Figure 1**).[10] By phosphorylating Akt, cleaved caspase-3 and activated caspase-9 are reduced to promote cell-survival post-infarction.[10,15] Supporting the premise of isoflurane activation of S1P, Zhou et al.[10] used an inhibitor to block the S1P receptor followed by the administration of 2% isoflurane as a treatment to cerebral ischemia and reversed the neuroprotection of the anesthetic drug. Additionally, isoflurane neuroprotection was reversed with VPC23019, an inhibitor of the S1P receptor, and wortmannin, an inhibitor of PI3K, confirming the dependence of isoflurane to the Isoflurane regulates apoptosis through the S1P/phosphoinositide pathway.[10] Literature has reported that isoflurane activates sphingosine kinase, leading to phosphorylated sphingosine, which produces S1P in neurons.[8,10] After S1P activation, Akt is phosphorylated, leading to Bad phosphory-

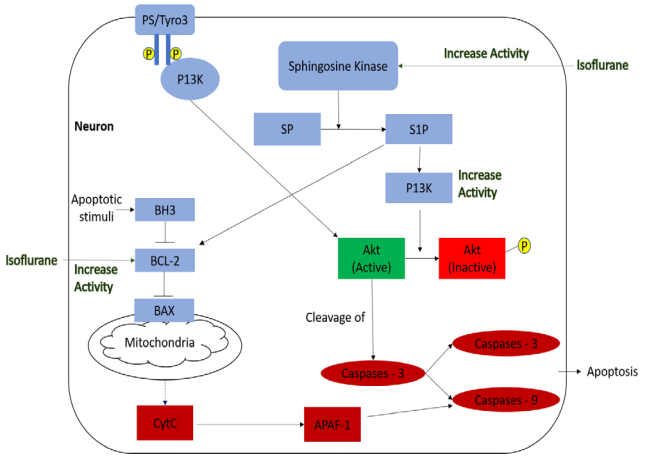

Figure 1: Isoflurane's anti-apoptotic pathway.
Note: Isoflurane increases sphingosine kinase activity, decreasing active Akt *via* phosphorylation of the Akt protein. Akt, when activated, cleaves caspase-3 and induces apoptosis. So, by decreasing the activity of Akt, less Caspase-3 is cleaved and promotes neuron survival. Isoflurane also increases the activity of BCL-2, which attenuates cleavage of caspase-9 and inhibits BAX. BAX is an apoptotic protein responsible for the expression of CytC/APAF-1 activation that cleaves of caspase-9, resulting in cell-death.[8,10,11,15,17,41] CytC: Cytochrome c protein; APAF-1: apoptotic protease activating factor 1; Akt: protein kinase B; BCL-2: B-cell lymphoma 2, apoptosis regulator; BAX: BCL2 associated X, apoptosis regulator; S1P: sphingosine-1-phosphate;PI3K: phosphoinositide 3-kinases.

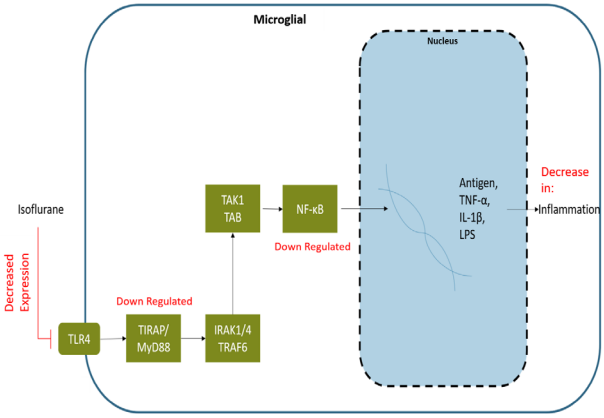

Figure 2: Isoflurane's anti-inflammatory pathway in microglia.
Note: Isoflurane downregulates the TLR4 receptor on microglial cell surface. By inhibiting TLR4, NF-κB is attenuated and a decreased expression of: Antigen, TNF-α, IL-1β and LPS inflammatory proteins. Isoflurane inhibits the MyD88/TRAF6/ TAK1/NF-κB cascade and leads to a decrease in overall inflammation.[28-31] TLR4: Toll like receptor 4; NF-κB: nuclear factor kappa B; TNF-α: tumor necrosis factor alpha; IL: interleukin; LPS: lipopolysaccharide.

lation to increase Bcl-2 expression, preventing cytochrome C release.[8,11] By blocking cytochrome C release, a decrease in neuronal cell death in the penumbra region was observed after cerebral ischemia.[11] Therefore, the S1P/PI3K/Akt is the hypothesized pathway to be responsible for the anti-apoptotic effects of isoflurane.

Effects on inflammation

Research has shown that isoflurane inhibits microglia and astrocyte activation by attenuating toll like receptor 4 (TLR4).[28] The TLR4-MyD88 pathway is responsible for activating microglia and damaginghealthy neurons by releasing interleukin-1β and TNF-α.[29] With 60 minutes of isoflurane pretreatment, the expression of TLR4, MyD88 and nuclear factor-κB (NF-κB) are downregulated, protecting the brain from ischemic damage.[28] TLR4-mediated-MyD88[28,29] pathway activates NF-κB,[30,31] which initiates the release of inflammatory cytokines (*e.g.* TNF-α, interleukin-6 and interleukin-1β)[31]; NF-κB expression peaks at 48 hours after MCAO.[28] In stroke, inflammation peaks at 24 hours and gradually decreases by 72 hours after MCAO.[28] Xiao et al.[28] reported that TLR4, MyD88 and NF-κB were downregulated after treatment with isoflurane. Other researchers have confirmed the ability of isoflurane pretreatment to attenuate TLR4 levels.[28] Overall, it can be concluded that isoflurane treatment reduces infarction size and inflammation (**Figure 2**).[15,28]

Inflammation in cerebral ischemia can be activated by the release of chemokines, cytokines, endotoxins and some adhesion molecules.[28,32] In the brain, glutamate is released during the anoxic conditions of stroke.[33,34] Specifically, isoflurane has been shown to inhibit excitotoxicity, and reduce the release of glutamate.[28] Also, isoflurane has been shown to inhibit post-synaptic glutamate levels in the hippocampus after cerebral ischemia.[35] In addition, the reduction of NMDA and AMPA was observed in cortical injuries after isoflurane treatment.[15,33,36,37] In contrast, with prolonged exposure to isoflurane for over 1 hour, NMDA and GABA receptors are activated and lead to increased neurodegeneration in stroke animal models.[10] A standardized system is needed for isoflurane to elucidate the implications in stroke.

Effects on blood-brain barrier

The amount of BBB damage can be a strong indicator for assessing neuronal injury.[38] The BBB normally protects against cytokine induced inflammation by preventing passage of inflammatory cells or plasma proteins into the brain.[38] In ischemic stroke, deterioration of the BBB occurs with the attenuation of tight-junction proteins.[38,39] With isoflurane treatment, a reduction of the early BBB disruption was reported in cerebral ischemia.[39] Therefore, the maintained BBB after isoflurane exposure correlates with increased neuroprotection after cerebral ischemia.[39] BBB is composed of vascular endothelial cells that are degraded by matrix metalloproteinase-9.[14] In intracranial hemorrhage, vascular endothelial growth factor activates matrix metalloproteinase-9, degrading the BBB.[14] In cerebral ischemic models, the intracranial hemorrhage and BBB disruption were significantly reduced, lowering the expression of matrix metalloproteinase-9, when exposed to isoflurane for 1 hour.[14]

The anti-apoptotic S1P1/3 pathway, targeted by isoflurane, preserves the BBB.[21] Specifically, impermeability of the BBB is connected to an increased SphK1 expression, which leads to an up-regulation of S1P.[21] S1P activates S1P1, upregulating tight junction proteins.[21] Studies have reported 1-hour exposure of isoflurane preserves the expression and function of tight junctions[21,40] and adherens.[21] Altay et al.[21] suggested that a 2% isoflurane anesthesia may prevent BBB distribution *via* the sphingosine pathway. Activation of the S1P1/3 pathway increased the number of gap junctions (**Figure 3**) and increased the expression of SphK1, which leads to the synthesis of S1P

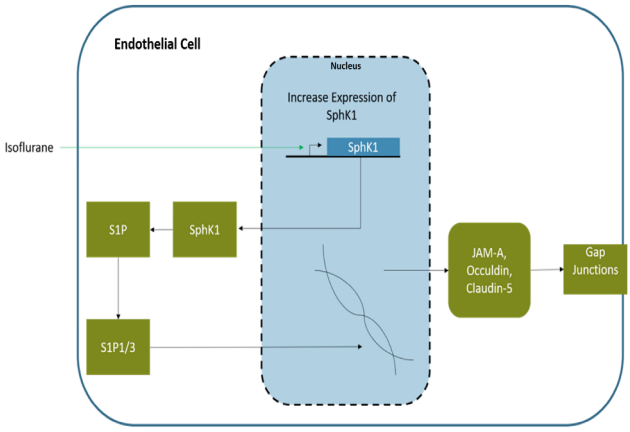

Figure 3: Isoflurane regulation of Gap junction proteins maintains blood-brain barrier integrity.
Note: Isoflurane is going to cause an increase in expression of SpkK1. SphK1 will activate S1P and S1P1/3. These factors will lead to an increase in the expression of JAM-A, Occludin, and Claudin-5.[21,40] S1P: Sphingosine-1-phosphate.

and activation of S1P1/3.[21] Lastly, non-specific tight junctions to the S1P1/3 pathway, such as JAM-A, occludin, claudin-5[21] and claudin-5,[40] suggests an alternative pathway may be targeted by isoflurane to preserve the BBB.[40]

Exposure Times to Isoflurane

Use of isoflurane or other volatile anesthetics are very common, but there are some concerns about the length of exposure to them. Yet, they are still used extensively in models because of their ability to facilitate invasive surgeries and a quick recovery.[2,3] It is well established that isoflurane provides neuroprotection,[3,6,13] but without a standardized system for the use of isoflurane, studies that use volatile anesthetics may be introducing errors into their studies when testing novel drugs or techniques.[2,16] Gaidhani et al.[1] suggested that a window from 20 to 90 minutes of anesthesia leads to neuroprotection. Therefore, according to the studies outline in this review, we have suggested a timeline to prevent artifacts associated with the use of excessive isoflurane in **Figure 4**. Use of isoflurane in experiments can be categorized into two different groups. First, exposure below 20 minutes to isoflurane did not significantly induce neuroprotection and no additional control experiments were required. Second, exposure over 20 minutes to isoflurane induced neuroprotection and two interventive measures should be used to control for isoflurane's artifact: first, isoflurane inhibitors for apoptosis, VPC23019 and wortmannin,[10] can be used to block neuroprotection; second, molecular and neurological evaluations should be conducted 2 weeks after isoflurane exposure, ensuring isoflurane's neuroprotective effects have dissipated.[15,16] In summary, vertebral anesthesia should not exceed 20 minutes, and a molecular and behavioral analysis should be evaluated at 6, 12, 24, 48 and 72 hours following surgery.[1,10,16] If isoflurane exposure exceeds 20 minutes, a control group using specific isoflurane inhibitors and a group at 2 weeks should be used for therapeutic comparision.

Conclusion

When isoflurane and other volatile anesthetics are used, close regulation of exposure times need to be reported and controlled. To prevent misleading artifacts in research, this review recommends guidelines for isoflurane use into two groups, above and below the 20-minute mark. If exposure to isoflurane is kept below 20 minutes, no neuroprotection occurs and efficacy of the drug can be evaluated at any time-point.[16] However, with exposure to isoflurane longer than 20 minutes, two measures are recommended to strengthen the scientific rigor of studies. First, specific inhibitors for isoflurane apoptosis pathways should be used: VPC23019 and wortmannin.

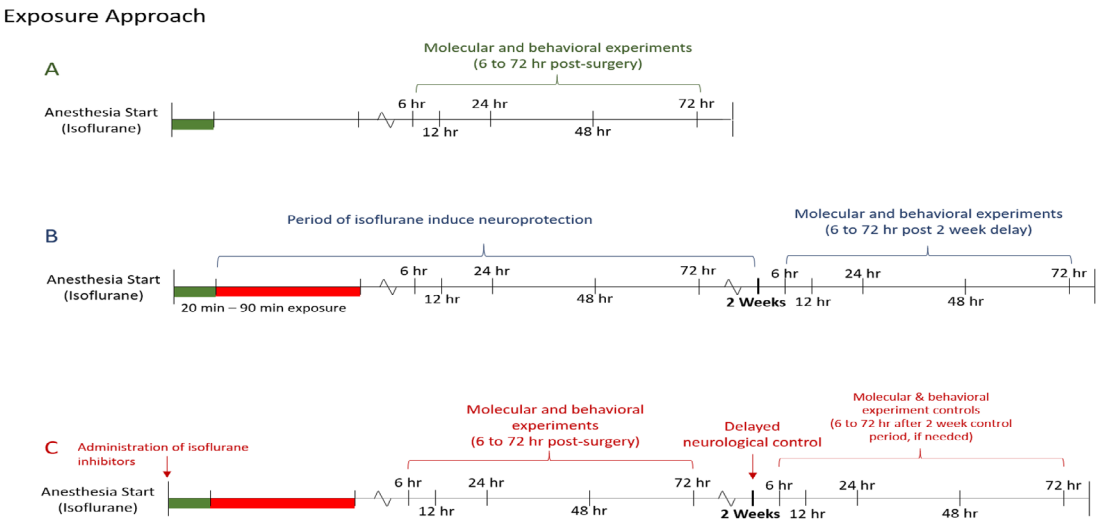

Figure 4: Standardized experimental periods for exposure to isoflurane.
Note: (A) Isoflurane exposure that is limited to under 20 minutes allows for molecular and behavioral experiments to be conducted between 6 and 72 hours post-surgery/post-exposure. (B) With exposure to isoflurane during surgery greater than 20 minutes but less than 90 minutes, experimental data should be evaluated after two weeks to ensure that neuroprotection from isoflurane has dissipated. Molecular and behavioral tests can be conducted 6 to 72 hours post 2-week isoflurane neuroprotective window. (C) Utilization of apoptotic inhibitors, such as VPC23019 and wortmannin,[10] before administration of isoflurane, when the exposure ranges from 20 to 90 minutes. Molecular and behavioral experiments should be conducted from 6 to 72 hours post-surgery/post-exposure. hr: Hours; min: minutes.

Second, a control group can be added at the 2-week mark after isoflurane exposure, giving enough time for all isoflurane effects to wear off. Understanding isoflurane and physiological impact may help accurately investigate drug efficacy and understand their molecular mechanisms.

Author contributions
Conception and literature search: were conducted by TCH and NM; drafting: TCH; revision: TCH, NM, JT, JHZ. All authors read and approved the final version of the paper for publication.
Conflicts of interest
The authors have no conflict of interest.
Financial support
This work was funded by an NIH grant, No. NS081740 (to JHZ).

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Received: February 14, 2019
Accepted: March 12, 2019