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Isoflurane Provides Neuroprotection in Neonatal Hypoxic Ischemic Brain Injury

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Abstract

Isoflurane is a volatile anesthetic that is widely used clinically as an inhalational anesthetic. In recent years, several studies have indicated that isoflurane has neuroprotective properties. This has led to the beneficial effects of isoflurane being analyzed in both cell culture and animal models, including various models of brain injury. Neonatal hypoxia ischemia may be characterized as injury that occurs in the immature brain, resulting in delayed cell death via excitotoxicity and oxidative stress. These adverse events in the developing brain often lead to detrimental neurological defects in the future. Currently, there are no well-established effective therapies for neonatal hypoxia ischemia. In line with this, isoflurane, which displays neuroprotective properties in several paradigms and has been shown to improve neurological deficits caused by brain injuries, has the capability to be an extremely relevant clinical therapy for the resolution of deficits concomitant with neonatal hypoxic ischemic brain injuries. This review will therefore seek to explore and analyze the current information on isoflurane, looking at general isoflurane anesthetic properties, and the protection it confers in different animal models, focusing particularly on neuroprotection as shown in studies with neonatal hypoxic ischemic brain injury.

Keywords

Isoflurane; Long-term neuroprotection; Pre-conditioning; Post-conditioning; Isoflurane mechanism; Neonatal hypoxia ischemia

Isoflurane and Other Volatile Anesthetics

Isoflurane is a volatile anesthetic that has been in clinical practice for several decades. Volatile anesthetics are also known as inhalational anesthetics and, apart from isoflurane, also include sevoflurane, desflurane, halothane, and enflurane. Of the five major volatile anesthetics, isoflurane, sevoflurane, and desflurane are most commonly used today. Halothane and enflurane are not used widely due to undesirable side effects¹. The volatile anesthetics all differ in potency, adverse effects, and cost, and are used extensively during surgery in human neonates and during neonatal animal research. Moreover, volatile

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anesthetics are employed in about 80% of the surgeries in the United States that are performed under general anesthesia². Volatile anesthetics have been reported to produce their effects by a number of mechanisms, including antagonism of ionic channels, activation of glycine and γ -aminobutyric acid (GABA) receptors, and alteration of the function and activity of other cellular proteins, as well as through utilizing high lipid solubility characteristics^{3,4}.

Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane, $\text{CHF}_2\text{-O-CHCl-CF}_3$), has been shown to be more potent than several other anesthetics. It is much more widely used in comparison to the other inhalational fluorinated inhalational anesthetics⁵. Isoflurane was discovered in 1965 by Dr. Ross Terrell and associates in their quest for a better inhaled anesthetic than was available at the time and was the 469th compound found, following enflurane and halothane⁵. Isoflurane has a minimum alveolar concentration (MAC) of 1.15%, and blood/gas partition coefficient of 1.4, lower than that of most other inhaled anesthetics⁶. Also, isoflurane is very stable and is resistant to biodegradation³. These properties of isoflurane allow it to rapidly induce unconsciousness and also to be quickly eliminated from circulation.

Isoflurane is Protective in Various Animal Models

Isoflurane has been studied in animal models of various diseased states, such as Lipopolysaccharide (LPS)-induced Acute Inflammation of the Lung⁷, Acute Lung Injury⁸, glucose-induced oxidative stress⁹, renal ischemia/reperfusion injury^{10,11}, cardiac injury¹², etc. In these models, it is shown to provide protection from injury and improve various negative functional outcomes. The protective effects of isoflurane in these models are reported to occur through the activation of several pathways, and are briefly outlined in Table 1.

In particular, isoflurane shows neuroprotection in several stroke models including subarachnoid hemorrhage (SAH)¹³, middle cerebral artery occlusion (MCAO)^{14,15}, intracerebral hemorrhage¹⁶, traumatic brain injury¹⁷, and Neonatal Hypoxic Ischemic Brain Injury (HI)¹⁸ (Table 1). For this review, the main focus will be on the neuroprotection of isoflurane as studied in neonatal HI, its possible mechanisms of action, and what direction this could be leading us in terms of clinical applications of isoflurane treatment.

Neonatal Hypoxia-Ischemia

Neonatal Hypoxia Ischemia is a major cause of mortality and neurological deficits such as cerebral palsy, mental retardation, and epilepsy in the perinatal period^{19,20}. Several factors have been implicated in the pathophysiology of HI, including inflammatory mediators, excitotoxicity, and oxidative stress²¹. While extensive research has been done on this form of brain injury, there is a gap between the basic and clinical sciences as successful treatments are still lacking. There is still a significant number of neonates who suffer from perinatal HI and its repercussions, approximately 1 in 4000, a figure two to three times higher than the incidence of childhood stroke²². The methods of interventions are often limited for neonatal HI, since neuronal cell death is frequently only delayed, although it usually appears to be prevented shortly after an assault. Thus, it is essential that more preclinical studies be carried out to elucidate the possibility of an effective therapy that will provide long-term neuroprotection against HI in the perinatal period, reducing the effects seen up to adulthood.

To study neonatal hypoxic ischemic brain injury, the Rice-Vannucci model is the one that is most widely used. Induction is most commonly with isoflurane at 3% and occurs for 4 minutes, followed by ligation of the right common carotid artery in rat pups. Isoflurane is

continuously infused for the duration of the surgery (typically not longer than 5 minutes) at 2.5%. The inspired concentration of isoflurane is lowered (from 3% to 2.5%) as this compensates for the decreased uptake due to the equilibration of isoflurane in the body following the induction period⁵. The pups are then subjected to a hypoxic chamber with 8% oxygen at 37°C for 2.5 hours. Infarction volumes are calculated and other neurological assessments are done at different time points after surgery. This method has been modified from that described by Rice, Vannucci, and Brierly, and is most commonly done in postnatal day 7 or 10 rats²³. P7 rat pups represent a human fetus at gestational age 32 to 34 weeks or a newborn infant at this stage²⁴, while P10 rats are similar to the older neonate approaching adolescent stages of development or a full-term neonate²⁵.

Neuroprotection of Isoflurane in Neonatal HI: Possible Mechanisms

The use of isoflurane as an anesthetic in the surgery for modeling neonatal HI, along with the great variations observed in infarct sizes within and among litters, particularly as surgery times varied has led to speculations on the effects that isoflurane may be having on these neonates. This, among other reasons, has therefore sparked the interest in studying whether or not isoflurane may be providing some form of neuroprotection against ischemic injury in neonatal rat pups.

As a result, several studies have found that isoflurane, when it is administered before or after an experimental hypoxic-ischemic insult (pre- and post-conditioning) in neonates, has the ability to reduce negative outcomes. Studies indicate that isoflurane acts through a number of mechanisms that are involved with neuroprotection and an increase in neuronal viability (Figure 1). It has been suggested that isoflurane inhibits excitotoxicity that is initiated by an accumulation of glutamate during ischemia, resulting in a reduction in necrosis. The antagonism of glutamate receptors by isoflurane has been shown in *in vitro* studies, as well as in *in vivo* models where isoflurane reduced extracellular glutamate during ischemia²⁶. In addition, isoflurane has been shown to be a GABA receptor agonist, providing an inhibitory effect against excitotoxicity³.

Under hypoxic conditions isoflurane was shown to be protective via its interaction with the pathways that involve phospholipase C and the release of Ca^{2+} from intracellular stores. Phospholipase C triggers the Phosphatidylinositol-3-kinase/Protein Kinase B and Mitogen-activated protein kinase (PI3K/Akt/MAPK) pathways, which are anti-apoptotic²⁷. Isoflurane also induced neuroprotection by altering the phosphorylation states of players in both the calcium-dependent and calcium-independent MAP kinase pathways, increasing the phosphorylation of some of the kinases (Pyk-2, ERK, MKK-6, JNK), while decreasing the phosphorylation of the downstream MAP kinase dependent transcription factors (p38, pElk-1 p90 RSK, ATF-2). Phosphorylation of the MAP kinases was prevented by an inositol triphosphate (IP3) receptor antagonist, suggesting that this phenomenon after isoflurane preconditioning depends on the small increases in intracellular Ca^{2+} . The study also showed that isoflurane enabled neurons to avoid potentially toxic levels of Ca^{2+} increase. Moreover, there was an observed increase in the levels of Akt, an anti-apoptotic protein, due to isoflurane preconditioning^{27,28}. The activation of the Akt pathway was also observed in a model of oxidative and inflammatory stress on cardiac myocytes. Isoflurane was shown to prevent apoptosis by activating Akt and enhancing B-cell lymphoma-2 (Bcl-2) expression^{29,30}.

Furthermore, isoflurane was shown to protect against oxygen glucose deprivation-induced ischemia in neuronal cultures by activating the cell-surviving protein, hypoxia inducible factor -1 (HIF-1) and the subsequent increase in expression of inducible nitric oxide synthase (iNOS) mRNA. Notably, the protection observed with isoflurane preconditioning

was also dependent on the extracellular signal-related kinase (ERK) pathway; an ERK1/2 inhibitor partially reversed the protective effects that resulted from isoflurane administration³¹. The aforementioned studies were mainly carried out in *in vitro* models of hypoxia, and though they may provide clearer mechanistic pathway descriptions, they differ from the physiology seen in intact animals. However, similar neuroprotection has been observed *in vivo*.

In one study done in an animal model of neonatal HI, Chen et.al, hypothesized that longer isoflurane exposure times may be one of the underlying factors leading to the inconsistent results in infarction volumes observed in the model. The results showed that longer surgery times, and hence longer isoflurane exposure, resulted in decreased infarction severity in the rat pups. Pups that had exposure times to isoflurane of 13 or 21 minutes, had significantly less volume of infarction than those with only 5 minutes of exposure: 33.2% in the 5 minute group, versus 20.3% in the 13 minute group and 11.3% in the 21 minute group in the 7-day old pups. This trend was also observed in the 10-day old rat pups (24.8% in the 5-minute group versus 11.9% in the 13-minute group and 9.3% in the 21-minute group)²⁵.

By extension, this group also did experiments to elucidate the mechanism by which isoflurane was participating in this neuroprotective role. They found that the sphingosine-1-kinase/PI3K/Akt (S1P/PI3K/Akt) pathway is active in this function as administration of selective inhibitors of S1P (VPC23019) and PI3K (Wortmannin) completely reversed the effects of isoflurane-induced neuroprotection. Importantly, isoflurane provided both short and long-term neuroprotection as effects were observed up to 4 weeks after the HI injury, when neurobehavioral tests were carried out. The effect of the S1P and PI3K inhibitors had a long-term effect as well, reversing the improvement in neurobehavior induced by longer exposures to isoflurane. Also, Akt phosphorylation was preserved in the isoflurane pre-treated group, and a significant reduction in cleaved caspase-3 levels was observed. These findings confirmed the speculation of the involvement of the S1P/PI3K/Akt pathway¹⁸. In addition, in a SAH model, isoflurane was also shown to be neuroprotective by activating the sphingosine kinase/S1P (SK/S1P) pathway. Isoflurane treatment attenuated the disruption of the blood-brain-barrier (BBB) that usually occurs after SAH¹³.

The involvement of the S1P/PI3K/Akt pathway in protection has also been implicated by other groups in different models of ischemia. Isoflurane was found to protect against renal dysfunction, significantly lowering plasma creatinine values and tubular necrosis, an effect abrogated by SK and S1P receptor antagonists, providing further evidence that their activity is essential to isoflurane-induced protection against ischemia. Additionally, mice lacking the SK1 enzyme were not protected against injury from ischemia¹⁰. The S1P pathway involvement in protection was seen in cardiac ischemia reperfusion injury also as the administration of S1P, which activated the S1P receptor, resulted in reduced infarct sizes, leukocyte recruitment and adhesion³². In models of liver ischemia, the SK/SIP pathway was also shown to be activated. There was a significant improvement in hepatic function in both normal and cirrhotic livers, increased anti-inflammatory cytokine production, and downregulation of anti-apoptotic genes following ischemia-reperfusion injury due to the activation of S1P³³.

Volatile anesthetics, including isoflurane, have been proposed to exhibit potency as a result of their high lipid solubility³. This theory was proposed by Meyer and Overton in 1901 and has been used to explain the involvement of the lysophospholipid, Sphingosine-1-phosphate, which results from the activation of sphingomyelin hydrolysis³⁴. Sphingomyelin is a lipid component of the membrane which is hydrolyzed to ceramide, a precursor of sphingosine. Sphingosine is then converted to S1P by the enzyme sphingosine kinase¹⁰. Sphingosine kinase is involved in many different biological processes including cell growth

and cell survival³⁵. In addition, sphingosine kinase and S1P have been shown to upregulate the ERK/MAPK pathway, resulting in increased cell survival^{36,37}. A significant finding was that animals with a knockout of the SK gene could not be protected by isoflurane treatment^{10,38}. These studies all correlate with the Lipid Theory which describes the potency of volatile anesthetics in relation to lipid solubility. However, in recent years, the Protein Theory, which postulates that anesthetics act directly on proteins rather than accumulating in membranes, has stimulated a lot of interest^{3,39}.

Consequently, other mechanisms have been proposed on how isoflurane confers neuroprotection. One such pathway is through the activation of inducible nitric oxide synthases (iNOS). Zhao, et.al found that isoflurane induced an increase in nitric oxide and suggested that this may be the mediator for the neuroprotection induced by isoflurane. They confirmed this by the use of a nitric oxide inhibitor, aminoguanidine, which abolished the neuroprotection conferred by isoflurane²². Isoflurane neuroprotection was shown using a preconditioning model where isoflurane was given at 1.5% for 30 minutes 24 hours before the hypoxic insult. Neuroprotection was measured up to 7 days post injury.

In another study by the same group, it was found that the brain loss induced by HI was significantly attenuated up to one month following injury, when animals were preconditioned with 1.5% isoflurane for 30 minutes 24 hours before injury. This conclusion was made as there were no significant differences between control and isoflurane treated groups in long-term reference memory, as well as short-term working memory. Isoflurane also significantly improved motor coordination one month after injury. Additionally, preconditioning with isoflurane resulted in increased Bcl-2 expression in the hippocampus⁴⁰. Bcl-2 can decrease the release of cytochrome C and mitochondrial membrane permeability caused by ischemic episodes⁴⁰. Again, it was found that the protection by isoflurane could be reversed by administration of an inducible nitric oxide synthase inhibitor, suggesting a role for iNOS in the isoflurane-induced neuroprotection. Small increases in iNOS protein expression have been associated with cardioprotection and neuroprotection. iNOS is believed to be an important factor for protection since protein kinase C(PKC) and MAP kinase are both located downstream of iNOS and are believed to provide protection⁴¹. Notably, isoflurane was found to increase the p38 MAPK phosphorylation status after an ischemic injury and the reduction induced by preconditioning with isoflurane was abolished by the administration of a p38 MAPK inhibitor⁴².

Kapinya et.al. also found that isoflurane preconditioning resulted in an increase in iNOS in an MCAO model and suggested that the increase in iNOS may be the means by which isoflurane reduces infarction volume and other neurological deficits. However, they also stated that further study is needed to identify the underlying anesthetic mechanism as the increase in iNOS was not observed until 6 hours after the preconditioning event, while protection was seen immediately after preconditioning with isoflurane⁴³. Nitric oxide is known to interact with two survival pathways: the Ras/Raf/MEK/ERK pathway and the PI3K/Akt pathway⁴⁴. Both of these pathways are essential to neuronal survival and could therefore be the underlying mechanism by which the isoflurane-induced increase in iNOS is protective in brain injury models. However, there is some contradiction in this area as NO has been shown to be both protective and toxic⁴⁴. Therefore, there are still further studies that need to be done to clarify the neuroprotection of isoflurane via an increase in iNOS.

The studies described above show a long-term neuroprotective role of the anesthetic, isoflurane. This is significant as a large percentage of infants who suffer from neonatal HI do survive into adulthood; however, most of them have residual motor and cognitive deficits²¹. However, importantly, there have been contradictions on whether or not the neuroprotection provided by isoflurane is long-term, or only transient. A study done by

McAuliffe, et.al, suggested that isoflurane may be useful in preventing the deficits seen long after the neonatal injury, or at least reducing the severity. This conclusion was made from experiments that showed that preconditioning with isoflurane improved striatal function in adulthood. Neurobehavioral testing was also done by the use of a water maze with adult mice that had undergone neonatal HI, and was done at 70 days old. Mice preconditioned with isoflurane performed as well as sham in this neurobehavioral test and this therefore provides evidence for long-term neuroprotection⁴⁵.

By contrast, Sasaoka, et.al found that preconditioning with isoflurane at 2% for either 60 or 90 minutes before the HI insult induced ischemic tolerance in the hippocampus of P7 rat pups up to 7 days after the injury, but not at 49 days²⁰. These results suggest that the neuroprotective effect of isoflurane is only transient. This may be due to the rapid removal of isoflurane from the system as a result of small amounts of biodegradation, as stated before.

It has also been suggested that isoflurane preconditioning may simply be delaying neuronal injury, not preventing it. This idea was proposed based on results that showed that brain infarction in the isoflurane treated group was significantly reduced in comparison to controls two days following ischemic injury, but this reduction was not seen 14 days after the insult. However, isoflurane did reduce the number of necrotic cells in the penumbral area two weeks after ischemia was induced⁴⁶. This experiment was carried out in an adult model of ischemia. Nevertheless, it still presents a significant consideration to be made with regards to the use of isoflurane for neuroprotection clinically because the delay in neuronal injury provided by isoflurane may provide a longer therapeutic window for other interventions.

Future Directions

It is clear that isoflurane exhibits neuroprotective properties, and harnessing this capability of the volatile anesthetic may be very relevant clinically. However, while isoflurane has been shown to be beneficial in numerous cases, before it can be used as a neuroprotective therapy for neonatal HI, several concerns have to be addressed. One such consideration is the length of time that isoflurane is administered. Studies have shown that isoflurane exposure for periods of 3 to 6 hours resulted in caspase activation and neuronal death^{47,48}, in contrast to the potent neuroprotection observed with shorter exposure times^{25,40}. Therefore, studies specifically aimed at establishing an optimal time period within which there is still effective neuroprotection from isoflurane, without causing neurotoxicity, have to be done.

There are contradictory evidences in support of isoflurane playing a neuroprotective role, while at the same time, potentially being neurotoxic. As discussed above, there are many animal studies that show isoflurane to be convincingly neuroprotective. However, evidence based on clinical studies in humans is not as strong⁴. And while there is not much evidence on the neurotoxicity resulting from isoflurane anesthesia, the fact that there is any, is reason for considerations to be made in this regard before clinical isoflurane therapy can progress substantially.

Isoflurane studies are clinically relevant and translatable because the percentage of isoflurane used experimentally is around 1.15% to 2%, which is comparable to a MAC of 1.15% in human adults and 1.6% in human neonates⁴⁰. However, different researchers have found the most effective dosages to be different in their models. With these discrepancies, much more would need to be done to establish what the optimal dosage of isoflurane is that will be most effective, providing maximal neuroprotection to infants.

Another factor that is very important to consider is elucidating the exact mechanism by which isoflurane provides protection from cell death and other negative outcomes. Several

different pathways have been implicated to date. It was first postulated that isoflurane, and volatile anesthetics by extension, were more potent the more lipid-solubility they exhibited³. Since the membrane is composed mainly of lipids, they were thought to act through membrane lipids. Hence, sphingosine and sphingosine kinase were suggested to be involved. From this theory, several studies carried out in various models have shown that isoflurane provides neuroprotection by activating this pathway, which then upregulates the ERK/MAPK/Akt pathway, resulting in increased cell growth and survival. However, newer theories indicate a more protein-based mechanism of action, via several ion channels, as well as GABA and glutamate receptors. The involvement of iNOS in isoflurane-mediated neuroprotection is another popular mechanism proposed and is more in line with the Protein Theory. Notably, many of these pathways, including sphingosine-1-phosphate and iNOS, exhibit protection via the activation of the ERK/MAPK/Akt pathway. This is significant and may be the way to connect the different pathways implicated, thereby establishing how isoflurane is protecting neurons from cell death.

Finally, neonatal HI results from several different pathophysiological manifestations. Hence, it may be necessary to target more than one of these to provide sufficient neuroprotection. Consequently, using isoflurane along with another intervention may be much more effective. Moreover, with the suggestion by some studies that isoflurane is merely delaying repercussions from ischemia, rather than preventing them, this may open a longer therapeutic window for other interventions to be applied, and may also provide an additive protective effect.

The possibility of using isoflurane clinically is very promising, and with the fact that it is already so widely used, addressing the current concerns would prove very beneficial in progressing not only the field of neonatal brain injury, but also many diseases related to hypoxia and ischemia.

Abbreviations

MAC	minimum alveolar concentration
LPS	Lipopolysaccharide
SAH	Subarachnoid hemorrhage
MCAO	Middle Cerebral Artery Occlusion
HI	Hypoxia Ischemia
GABA	-aminobutyric acid
PI3K	Phosphoinositol-3-Kinase
AKT	Protein Kinase B
MAPK	Mitogen-activated Protein Kinase
ERK	Extracellular Signal-Related Kinase
Bcl-2	B-cell lymphoma 2
iNOS	inducible Nitric Oxide Synthase
S1P	Sphingosine-1-phosphate
SK	Sphingosine kinase
LDH	lactate dehydrogenase
CK-MB	Creatine kinase MB

PKC	Protein Kinase C
ALDH2	Aldehyde dehydrogenase 2
BBB	Blood brain barrier
NF- B	Nuclear Factor- B

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Figure 1. Proposed Mechanisms of Isoflurane Neuroprotection. Other potential mechanisms have been explored; however, these are the most extensively addressed in this review^{18,29,40,41}.

Table 1

Isoflurane shows protection in various animal models

Animal Model	Isoflurane Protection	Source
General Models		
LPS-Induced Acute Lung Inflammation (Rat)	Inhibited ROS burst, NF- κ B activation, and pro-inflammatory cytokines	Chung, I.S. et.al. 2013
T/HS-induced ALI	Inhibited platelets through the ADP pathway	Harr, N. et.al. 2012
Renal Ischemia/ Reperfusion Injury	Reduced renal dysfunction and tubular necrosis via the SK/SIP pathway	Kim, M. et.al.2007; Lee, H.T. et.al. 2004
Cardiac Injury	Attenuated the release of LDH and CK-MB, and reduced infarct size via PKC γ /ALDH2 pathway	Lang, X. et.al. 2013
High Glucose-induced Oxidative Stress in Isolated Human Artery	Preserved vascular function by restoring ATP-sensitive K ⁺ channel function	Kinoshita, H. et.al. 2012
Stroke Models		
Subarachnoid Hemorrhage	Prevented post SAH neuronal apoptosis and BBB disruption in ipsilateral hemisphere through SIP pathway	Altay, O. et.al. 2012
Middle Cerebral Artery Occlusion	Improved neurological outcome, and reduced inflammatory cytokine production via attenuation of NF- κ B activation	Li, H. et.al. 2013
Intracerebral Hemorrhage	Reduced brain edema, apoptotic cell death, and neurological deficits	Khatibi, N. H. et.al. 2011
Traumatic Brain Injury	Increased CA3 neuronal survival, and improved histopathology	Statler, K.D. et.al. 2006
Neonatal Hypoxic Ischemic Brain Injury	Reduced infarction volume, improved neurobehavioral outcome, and increased pAkt	Zhou, Y. et.al. 2010