Coadministration of Hydrogen Gas as Part of the Carrier Gas Mixture Suppresses Neuronal Apoptosis and Subsequent Behavioral Deficits Caused by Neonatal Exposure to Sevoflurane in Mice

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ABSTRACT

Background: In animal models, several anesthetics induce widespread increases in neuronal apoptosis in the developing brain with subsequent neurologic deficits. Although the mechanisms are largely unknown, the neurotoxicity may at least in part, be due to elevated oxidative stress caused by mitochondrial dysfunction. In an investigation of potential therapies that could protect against this type of damage, we studied the effects of molecular hydrogen on anesthetic-induced neurotoxicity in the developing mouse brain.

Methods: Six-day-old C57BL/6 mice were exposed to 3% sevoflurane for 6 h with or without hydrogen (<1.3%) as part of the carrier gas mixture. Apoptosis was evaluated by immunohistochemical staining for cleaved caspase-3 (n = 8–10/group). Western blot analysis for cleaved poly-(adenosine diphosphate-ribose) polymerase was also performed to examine apoptosis (n = 3–6/group). Oxidative stress was assessed by immunohistochemical staining for 4-hydroxy-2-nonenal (n = 8/group). Long-term memory and social behavior were examined using the fear conditioning test and the sociability test, respectively (n = 18–20/group).

Results: Western blot analysis showed that coadministration of 1.3% hydrogen gas significantly (P < 0.001) reduced the level of neuronal apoptosis to approximately 40% compared with sevoflurane exposure alone. Immunohistochemical analysis showed that hydrogen reduced oxidative stress induced by neonatal sevoflurane exposure. Although neonatal sevoflurane exposure caused impairment in long-term memory and abnormal social behaviors in adulthood, mice coadministered hydrogen gas with sevoflurane did not exhibit these deficits.

Conclusions: Inhalation of hydrogen gas robustly decreased neuronal apoptosis and subsequent cognitive impairments caused by neonatal exposure to sevoflurane.

What We Already Know about This Topic

• General anesthetics produce widespread neurodegeneration in the developing nervous system of animal models that are associated with subsequent neurocognitive deficits
• Antioxidants can reduce reactive oxygen species generation and neurotoxicity when administered with volatile anesthetics

What This Article Tells Us That Is New

• Hydrogen gas coadministered with sevoflurane in neonatal mice reduced oxidative stress, neuronal apoptosis, and subsequent neurobehavioral deficits
• Coadministration of hydrogen gas provides a promising approach to reduce developmental anesthetic neurotoxicity

SEVERAL anesthetics at clinically effective concentrations induce widespread neurodegeneration in the developing brain of a variety of animals ranging from rodents1-8 to rhesus monkeys.9,10 Furthermore, a number of preclinical studies have shown that, in neonatal animals, anesthetic exposure induces neurologic deficits, including cognitive decline1-5,8 and abnormal social behaviors9 in adulthood, although the underlying mechanisms are largely unknown. Consequently, there is growing concern about the use of general anesthetics in obstetric and pediatric medicine, although we should be cautious to extrapolate animal findings to humans.

It was reported that mitochondria are affected by general anesthetics in the developing rat brain.11 Because dysfunctional mitochondria could be a source of excess reactive oxygen species (ROS), oxidative stress may contribute to anesthetic-induced neurotoxicity in the developing brain. Thus, it is tempting to speculate that antioxidants may be able to protect against neurotoxicity caused by anesthetic...
exposure to neonate. It has also been reported that a synthetic ROS scavenger, EUK-134 effectively inhibits anesthesia-induced ROS production in animal model.\textsuperscript{12} This antioxidant decreased neuronal apoptosis and also alleviated the learning impairment induced by neonatal anesthetic exposure.\textsuperscript{12} Here, we show that molecular hydrogen gas, which can be easily supplied as part of the carrier gas mixture under anesthesia, is an effective antioxidant and has the potential to be used in the management of neurotoxicity caused by neonatal exposure to anesthetics in the developing brain.

Hydrogen has recently received attention because of its small size and electrically neutral properties, enabling it to reach target organs easily, to diffuse across membrane rapidly, and to penetrate the blood–brain barrier for the protection of neurons. Furthermore, molecular hydrogen can selectively neutralize the most toxic ROS, the hydroxyl radical, while having a minimal effect on beneficial ROS.\textsuperscript{13} Thus, we sought to investigate whether hydrogen inhalation could effectively protect neurons against toxicity caused by neonatal anesthetic exposure. Our results demonstrate that concomitant hydrogen inhalation as part of the carrier gas mixture significantly suppressed neurodegeneration caused by neonatal sevoflurane exposure in animal model. Furthermore, it mitigated the impairment of long-term memory and the deficit in social behaviors in adulthood caused by neonatal sevoflurane exposure. This simple method of hydrogen coadministration may have potential for the management of neurotoxicity caused by neonatal exposure to anesthetics.

**Materials and Methods**

**Animals**

All experiments were conducted according to institutional ethical guidelines for animal experiments of the National Defense Medical College and were approved by the Committee for Animal Research at National Defense Medical College (Tokorozawa, Saitama, Japan). Inbred strain C57BL/6 mice were used in this study and were maintained as described previously.\textsuperscript{4}

**Anesthesia and Hydrogen Treatment**

On postnatal day 6, immediately after removal of mice from the maternal cage, mice were placed in a humid chamber that has manipulating gloves. Hydrogen gas was supplied as part of the premixed carrier gas, which was produced by a manufacturer (Saisan Co., Saitama, Japan). Sevoflurane was administered in either 30% O$_2$/air or (30% O$_2$ + H$_2$)/air. The concentrations of hydrogen in this study (0.3, 0.6, and 1.3%) were low enough to avoid explosion\textsuperscript{14}; hydrogen gas poses no risk of explosion when present at concentrations <4% (vol/vol).\textsuperscript{13} Total gas flow was 2 l/min.

We measured the oxygen and anesthetic fractions using a gas analysis system (Capnomac Ultima; GE Healthcare, Tokyo, Japan). Hydrogen gas concentration was determined by a company (Breath Lab Co., Nara, Japan) using gas chromatography. During exposure to the anesthetic, mice were kept warm on a mat heated to 38 ± 1°C.

**Arterial Blood Gas Analysis**

Arterial blood sampling from the left cardiac ventricle was performed as described previously,\textsuperscript{4} with some modifications. Before blood sampling, all pups (including those not already exposed to sevoflurane) were treated with 3% sevoflurane in 30% oxygen for 5 min to prevent pain and hyperpnea.

**Histopathologic Studies**

Immunohistochemical staining was performed as described previously.\textsuperscript{4,16} Primary antibodies used in this study were anti-active caspase-3 (rabbit polyclonal; Cell Signaling Technology, Beverly, MA) and anti-4-hydroxy-2-nonenal (mouse monoclonal; Japan Institute for the Control of Aging, Shizuoka, Japan) antibodies. For fluorescence staining, Alexa-Fluor 546–conjugated anti-mouse IgG antibody (Life Technologies, Eugene, OR) was used as a secondary antibody. Terminal deoxynucleotidyl transferase dUTP nick end labeling was performed as described previously.\textsuperscript{4} Samples from eight to ten mice per group were examined in each experiment.

**Western Blot Analysis**

Western blot analysis was performed as described previously.\textsuperscript{4}

**Behavioral Studies**

Behavioral studies were performed as described previously.\textsuperscript{3,4,16} All mice used in behavioral studies were age-matched male littermates. The olfactory test was conducted as described previously,\textsuperscript{3} with some modifications. Briefly, mice were habituated to the flavor of a novel food (blueberry cheese) on the first day. After 48-h food deprivation, the time required to find buried treat was measured: a piece of blueberry cheese was buried under 2 cm of bedding in a clean cage. Open-field test, Y-maze test, sociability test in the open-field chamber, olfactory test, and novelty test were performed at 12 weeks of age. The fear conditioning test was performed at 13 weeks of age. Mice subjected to the fear conditioning test were not used for any further testing. The same set of mice was used for all behavioral tests.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA). Comparisons of the means of each group were performed using a one-way ANOVA followed by Newman–Keuls post hoc test or two-way ANOVA followed by Bonferroni post hoc test. In the Y-maze test, comparisons of group performance relative to random performance were carried out using a two-tailed one-sample t test. P values less than 0.05 were considered statistically significant. Values are presented as mean ± SEM.
Results

Hydrogen Administration Reduces Neuronal Apoptosis Caused by Sevoflurane Exposure in the Neonate

To investigate the potential protective effects of hydrogen gas against neurotoxicity caused by neonatal sevoflurane exposure, we performed Western blot analysis of forebrain extracts using an antibody to cleaved poly-adenosine diphosphate-ribose polymerase (PARP; a biomarker of apoptotic cell death). Although cleaved PARP immunoreactivities in mice brains exposed to oxygen/air or (oxygen + H₂)/air were below the detection level, mice exposed to 3% sevoflurane in oxygen/air for 6h induced PARP reactivity (fig. 1A). In contrast, mice exposed to 3% sevoflurane in (oxygen + H₂)/air exhibited significantly reduced immunoreactivity to cleaved PARP compared with mice exposed to sevoflurane in oxygen/air (fig. 1A, 1B), indicating that 1.3% hydrogen gas protects against neuronal apoptosis caused by neonatal sevoflurane exposure. Two-way ANOVA confirmed these differences, indicating a main effect of hydrogen administration (F = 12.17, P < 0.01), a main effect of sevoflurane administration (F = 45.66, P < 0.0001), and interaction (hydrogen administration × sevoflurane administration; F = 15.28, P < 0.01). The effect of hydrogen on the attenuation of PARP level was concentration dependent from 0 to 1.3% (fig. 1C).

Next, we performed histologic analysis using an antibody against activated caspase-3, another biomarker of apoptotic cell death. Because Western blot analysis showed that mice exposed to (oxygen + H₂)/air exhibited a level of apoptosis similar to those exposed to oxygen/air as described above, we performed histologic evaluation for only three groups: oxygen/air (hereafter termed control), 3% sevoflurane in oxygen/air (hereafter termed sevoflurane), and 3% sevoflurane in (oxygen + H₂)/air (hereafter termed sevoflurane + hydrogen). Exposure to sevoflurane for 6h induced dramatic increases in the number of activated caspase-3+ cells compared with sham controls in some regions of the brain immediately after the 6-h anesthesia period (figs. 2 and 3), consistent with previous reports. In contrast, the numbers of activated caspase-3+ cells in mice exposed to sevoflurane + hydrogen were significantly smaller than in mice treated with sevoflurane alone (figs. 2 and 3). We also performed terminal deoxynucleotidyl transferase dUTP nick end labeling as an independent measure of apoptotic cell death. The pattern of terminal deoxynucleotidyl transferase dUTP nick end labeling staining 6h after anesthesia was similar to activated caspase-3 staining (fig. 4). These results indicate that 1.3% hydrogen significantly reduces neuronal apoptosis caused by sevoflurane exposure in the neonate.

Hydrogen Reduces Oxidative Stress Induced by Sevoflurane Exposure in the Neonate

Hydroxyl radicals react with lipids, resulting in lipid peroxidation and production of 4-hydroxy-2-nonenal. Thus, 4-hydroxy-2-nonenal is widely used as a marker of lipid peroxidation and oxidative stress. Figure 5 shows that much greater lipid peroxidation was induced in neurons 6h after sevoflurane exposure (fig. 5B) compared with sham controls (fig. 5A). In contrast, staining for 4-hydroxy-2-nonenal was significantly reduced in the brains of mice exposed to sevoflurane + hydrogen (fig. 5C) compared with sevoflurane alone (fig. 5B). These results indicate that neonatal exposure...
to 3% sevoflurane induces oxidative stress in the brains and that 1.3% hydrogen reduces it.

**Arterial Blood Gas Analysis**
Analysis of arterial blood before and after the 6-h anesthesia period revealed no significant differences in pH or in PaO₂ between the various groups (table 1). PaCO₂ was increased both in sevoflurane and in sevoflurane + hydrogen groups compared with 0-h control group (table 1). However, no significant difference was detected between the sevoflurane and sevoflurane + hydrogen groups. These results indicate that 1.3% hydrogen administration concomitant with 3% sevoflurane did not have significant adverse effects on pH, PaO₂, or PaCO₂ compared with sevoflurane treatment alone.

**General Behavior Is Not Affected by Neonatal Sevoflurane Exposure, Regardless of Hydrogen Coadministration**
To evaluate behavioral activity in a novel environment, mice exposed to 3% sevoflurane with or without hydrogen for 6 h as neonates were examined in the open-field test as adults. Compared with controls, mice exposed to sevoflurane at the neonatal stage did not show abnormal activity, regardless of hydrogen coadministration, as assessed by the total distance traveled (fig 6A; one-way ANOVA, F = 0.49, P = 0.62).

**Short-term Spatial Working Memory Is Normal in Mice Exposed to Sevoflurane as Neonates, Regardless of Hydrogen Coadministration**
Working memory is the ability to hold information temporarily to perform complex cognitive tasks. To evaluate spatial working memory, mice exposed to sevoflurane with or without hydrogen were tested in the Y-maze task. This task examines whether mice remember the preceding arm position selected. By nature, rodents seek a new arm, different from that of the preceding choice, but if working memory is impaired, the number of correct choices should be reduced. Mice in the control, sevoflurane, and sevoflurane + hydrogen mice performed this task with 61.9 ± 3.5%, 60.3 ± 1.7%, and 60.0 ± 2.0% correct choices, respectively (fig 6B). One-way ANOVA did not indicate significant differences between them (F = 0.16, P = 0.85). The values for control, sevoflurane, and sevoflurane + hydrogen groups were well above those expected if mice chose the arms by random (random choice = 50%; one-sample t test, P < 0.0001 in the above cases). Previously, we reported that neonatal sevoflurane exposure did not affect performance in the Y-maze task, consistent with the results here.

**Hydrogen Suppresses the Impairment of Long-term Memory Caused by Neonatal Sevoflurane Exposure**
To assess the effects of hydrogen on long-term memory impairment caused by neonatal sevoflurane exposure, mice exposed to sevoflurane with or without hydrogen were subjected to contextual/cued fear conditioning testing during the adult stage. The freezing response of mice exposed to sevoflurane was significantly reduced in the contextual test compared with control animals after a 24-h retention delay (fig 6C; one-way ANOVA, F = 7.22, P = 0.0017; Newman–Keuls post hoc test, P < 0.01 for control vs. sevoflurane), indicating that neonatal sevoflurane exposure impairs long-term memory in adulthood. In contrast, mice exposed to sevoflurane + hydrogen exhibited enhanced performance compared with animals given sevoflurane alone (Newman–Keuls post hoc test, P < 0.01 for sevoflurane vs. sevoflurane + hydrogen), performing similarly to controls (Newman–Keuls post hoc test, P > 0.05 for control vs. sevoflurane + hydrogen). Freezing responses of mice exposed to sevoflurane and subjected to cued fear conditioning were also significantly reduced compared with controls after a 48-h retention delay (fig 6D; one-way ANOVA, F = 12.08, P < 0.0001; Newman–Keuls post hoc test, P < 0.001 for control vs.
In contrast, mice exposed to sevoflurane + hydrogen displayed better performance compared with mice exposed to sevoflurane alone (Newman–Keuls post hoc test, \( P < 0.001 \) for sevoflurane vs. sevoflurane + hydrogen) performing similar to controls (Newman–Keuls post hoc test, \( P > 0.05 \) for control vs. sevoflurane + hydrogen). These results indicate that hydrogen can suppress the impairment of the long-term memory caused by neonatal sevoflurane exposure.

Fig. 3. Numbers of black dots labeled by immunohistochemical staining for cleaved caspase-3 are significantly decreased by hydrogen gas treatment. A comparison of the means between control, sevoflurane, and sevoflurane + hydrogen groups was performed using a one-way ANOVA followed by Newman–Keuls post hoc test (control: \( n = 8 \) mice; sevoflurane: \( n = 10 \) mice; sevoflurane + hydrogen: \( n = 10 \) mice). \( F \) and \( P \) values are presented below each panel. *\( P < 0.05 \), **\( P < 0.01 \) or ***\( P < 0.001 \) compared with controls.
Hydrogen Suppresses the Impairment of Social Behavior Caused by Neonatal Sevoflurane Exposure

Mice are a social species and exhibit social interaction behaviors. We previously reported that mice exposed to sevoflurane as neonate exhibit abnormal social behaviors as adults. Here, we investigated whether hydrogen could suppress the impairment of social behavior caused by neonatal sevoflurane exposure. In a test for social versus inanimate preference (sociability test), all groups spent significantly more time interacting with the social target than with the inanimate target (fig. 7A; t test, all P values < 0.001). However, mice exposed to sevoflurane as neonates exhibited decreased interaction with a social target compared with controls (fig. 7A), consistent with previous result. Coadministration of hydrogen prevented the impairment of the social behavior caused by sevoflurane, performing similar to the control. One-way ANOVA confirmed these differences (F = 6.12, P = 0.004; Newman–Keuls post hoc test, P < 0.01 for control vs. sevoflurane, and P > 0.05 for control vs. sevoflurane + hydrogen).

We did not attribute the differences in social interaction to impaired olfactory sensation or loss of general interest in novelty, because we did not detect significant differences between groups in tests for olfaction (fig. 7B; one-way ANOVA, F = 0.50, P = 0.71) or for novel inanimate object interaction (fig. 7C; one-way ANOVA, F = 0.04, P = 0.96). Therefore, it can be concluded that hydrogen can suppress abnormal social behaviors caused by neonatal sevoflurane exposure.

Discussion

In this study, we observed that hydrogen gas administration markedly reduced neuronal apoptosis caused by sevoflurane exposure in the developing brain. Furthermore, hydrogen could suppress cognitive deficits and impairment of social behaviors.

Recently, it was reported that developing mitochondria are extremely vulnerable to anesthetics. Complex IV, which controls the final step of the mitochondrial electron transport chain, is acutely sensitive to neonatal anesthesia. Dysfunction of complex IV is thought to cause electron leakage from the mitochondrial electron transport chain, thereby increasing ROS production. It is well known that oxygen radicals easily react with macromolecules, including DNA, lipids, and protein, causing cellular injury. Our results demonstrate that

![Fig. 4. Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay showing a labeling pattern similar to that of cleaved caspase-3. (A–C) Light microscopic views of the mouse brain after 6-h exposure to carrier gas (A), 3% sevoflurane (B), or 3% sevoflurane with 1.3% hydrogen (C). Black dots represent TUNEL-positive cells, which indicate apoptosis. Representative images from one of eight mice per group. Scale bar: 100 μm.](image)

![Fig. 5. Hydrogen gas alleviates oxidative stress caused by sevoflurane exposure in the developing brain. (A–C) Fluorescence microscopic views of the mouse brain after 6-h exposure to carrier gas (A), 3% sevoflurane (B), or 3% sevoflurane with 1.3% hydrogen (C). Red staining represents 4-hydroxy-2-nonenal (4-HNE)–positive cells, which indicate oxidative stress. Representative images from one of eight mice per group. Scale bar: 100 μm.](image)
hydrogen gas inhibits oxidative stress induced by sevoflurane exposure in the neonate. Collectively, it is likely that hydrogen suppresses neuronal apoptosis caused by neonatal sevoflurane exposure by exerting an antioxidant effect. Because excessive ROS might induce mitochondrial damage, it is also possible that hydrogen may protect mitochondria by scavenging ROS. Furthermore, the beneficial effects of hydrogen might be, at least in part, mediated by direct mitochondrial protection, although the underlying mechanism is unknown. In this regard, it is worth noting that hydrogen can effectively improve mitochondrial dysfunction in mitochondrial myopathies and other mitochondrial diseases (see review by Ohta). Interestingly, there is a relationship between hydrogen and mitochondria; hydrogen was closely involved with the ancestors of mitochondria (see review by Ohta). Future studies might reveal the role of mitochondria in the protective effect of hydrogen.

Melatonin, a sleep-promoting reagent and antioxidant, reduces the severity of anesthesia-induced apoptotic neurodegeneration in the developing rat brain. L-carnitine, a dietary supplement with antioxidant properties, effectively blocks neuronal apoptosis caused by inhalation anesthesia in the developing rat brain. Furthermore, it has also been reported that a synthetic ROS scavenger, EUK-134 effectively inhibited neuronal apoptosis and also alleviated the learning impairment induced by neonatal anesthetic exposure. These observations support the hypothesis that oxidative stress has important roles in anesthetic-induced neurotoxicity in the neonate.

Table 1. Arterial Blood Gas Analysis

<table>
<thead>
<tr>
<th></th>
<th>Control 0 h</th>
<th>Control 6 h</th>
<th>Sevoflurane 6 h</th>
<th>Sevoflurane + Hydrogen 6 h</th>
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</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.41±0.02</td>
<td>7.33±0.06</td>
<td>7.40±0.06</td>
<td>7.43±0.02</td>
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<tr>
<td>PaO₂, mm Hg</td>
<td>137.5±2.0</td>
<td>137.0±3.8</td>
<td>129.0±3.2</td>
<td>129.3±1.0</td>
</tr>
<tr>
<td>PaCO₂, mm Hg</td>
<td>25.3±1.3</td>
<td>34.2±7.6</td>
<td>43.7±3.2*</td>
<td>43.8±3.7*</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SEM. No significant differences in pH and PaO₂ were observed between the various groups (one-way ANOVA, P > 0.05). PaCO₂ was increased both in sevoflurane and sevoflurane + hydrogen groups compared with 0-h control group (one-way ANOVA, F = 3.80, P = 0.03). * P < 0.05 compared with 0-h control (Newman–Keuls post hoc test). n = 6 mice for each group.

PaCO₂ = partial pressure of arterial carbon dioxide, PaO₂ = partial pressure of arterial oxygen.
developing brain. Thus, it is tempting to speculate that these antioxidants could be potent drugs against neurotoxicity caused by anesthetic exposure in the neonate. However, these antioxidants may react with beneficial ROS as well. Several ROS have important roles in synaptic transmission and cellular homeostasis at low concentrations. For example, \( \text{H}_2\text{O}_2 \) may be important for modulating synaptic transmission. In comparison, the hydroxyl radical is extremely toxic, and no defense mechanism has been found against this ROS in mammalian cells. In this context, it is noteworthy that molecular hydrogen can selectively neutralize hydroxyl radicals with minimal effect on beneficial ROS.

Numerous studies have shown that hydrogen can be used as an effective antioxidant for the treatment of several diseases, including myocardial ischemia–reperfusion injury, atherosclerosis, carcinogenesis, neurodegenerative disorders, and hearing disorders. In these studies, no serious harmful side effect of hydrogen was reported at clinical concentrations. It is possible that hydrogen may promote wound healing after injury, which can be considered another advantage of the clinical use of hydrogen. In addition, hydrogen gas is already used in clinic for the prevention of decompression sickness in divers.

With the obvious interpretative limitation to translate animal model to humans, we previously demonstrated the potential of sevoflurane to induce disturbances in social behaviors that resemble those observed in autistic subjects. Although we do not know the underlying mechanism, it is nonetheless interesting to note that concurrent inhalation of hydrogen gas significantly reduced cognitive deficits and impairment of social behaviors caused by neonatal anesthetic exposure.

Our Western blot analysis revealed slight increase in neuronal apoptosis in the brain of mice given hydrogen under sevoflurane anesthesia compared with control. Although we do not know the reason for this result, it is possible that mechanism other than oxidative stress may contribute to neuronal apoptosis. Another possibility is that the concentration of hydrogen used in this study was not sufficient to fully suppress neuronal apoptosis.

It seems that, in general, continuous gas inhalation therapy is not suitable for preventative use. However, our results indicate that hydrogen inhalation as part of the carrier gas mixture during the anesthesia period can effectively suppress neuronal toxicity caused by sevoflurane in the developing brain. Therefore, coadministration of hydrogen gas should provide an effective therapy to manage neurotoxicity caused by volatile anesthetic exposure in the neonate. Furthermore, hydrogen gas has negligible pungency and minimally affects the airway. We concluded that coadministration of hydrogen gas is a promising therapeutic strategy to mitigate neurotoxicity caused by anesthetic exposure in the developing brain.

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