The effect of repeated isoflurane exposure on serine synthesis pathway during the developmental period in *Caenorhabditis elegans*

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A B S T R A C T

Background: Serine synthetic pathway plays an essential role in the development and function of the nervous system. This study investigated whether the serine synthetic pathway was affected by repeated volatile anesthetic exposure using *C. elegans* and its relationship with anesthesia-induced neurotoxicity.
Methods: Synchronized worms were divided into two groups: the control and isoflurane groups. Worms in the isoflurane group were exposed to isoflurane for 1 h at each larval stage. The chemotaxis index was evaluated when they reached the young adult-stage in both groups. Also, RNA was extracted from the young adult-worms, and the expressions of *C31C9.2*, *F26H9.5*, and *Y62E10 A.13* were evaluated using real-time polymerase chain reaction in both groups. At the same time, the L-serine level was measured. After phosphoserine phosphatase inhibitor – glycerophosphorylcholine (GPC) – and L-serine were treated, the change of chemotaxis index was determined.
Results: In young adult worms exposed to isoflurane, the genetic expressions of *C31C9.2*, *F26H9.5*, and *Y62E10 A.13* were decreased, and a significant decrease was shown in *Y62E10 A.13*. The serine level in worms was also lower in the isoflurane group than in the control group (5.13 ± 1.44 vs. 7.65 ± 0.81 pM, n = 5 in each group, p = 0.009). Exposure to GPC reduced the chemotaxis index to a similar degree as repeated isoflurane exposure (52.9% in GPC group vs 58.7% in the isoflurane group). The chemotaxis index (61.1%) was not decreased by repeated isoflurane anesthesia in GPC-treated worms. In this condition, the L-serine level was low similarly in both groups (5.22 ± 1.19 vs. 4.90 ± 1.36 pM, n = 5 in each group, p = 0.702). When L-serine was supplied to *C. elegans*, the deteriorated chemotaxis index by isoflurane exposure recovered (78.1% in the control group vs. 75.5% in the isoflurane group, p = 0.465).
Conclusion: Serine synthetic pathway was negatively affected in *C. elegans* by repeated isoflurane exposure. *Y62E10 A.13*, which corresponds to phosphoserine phosphatase, was mostly influenced, followed by low L-serine level. Supplementation with L-serine could restore the chemotaxis index.

1. Introduction

Volatile anesthetic agents have widely been used in patients undergoing various surgeries under general anesthesia. However, given its prevalence, concerns about neurotoxicity have been raised especially for those at a young developmental age or repeatedly exposure throughout their life time.

Many preclinical and clinical studies have investigated the effects of volatile anesthetics on neurocognitive function. Although most preclinical studies confirmed volatile anesthesia-induced neurotoxicity (Jevtovic-Todorovic et al., 2003; Coleman et al., 2017; Liang et al., 2017), various clinical studies have shown conflicting results (Davidson et al., 2016; Sun et al., 2016). Unlike animal studies, it is difficult to evaluate the isolated effect of volatile anesthetic exposure in humans due to the complex nature of surgery, underlying disease, and surrounding environment. However, it was found that extended and repeated exposure to volatile anesthetics resulted in lower language scores in children (Ing et al., 2017), and impacted neurodevelopment (Amrock et al., 2015; Warner et al., 2018). Thus, the potential effect of volatile anesthetics on neurodevelopment should not be ignored.

In astrocytes, serine is synthesized from 3-phosphoglycerate via the sequential action of phosphoglycerate dehydrogenase, phosphoserine aminotransferase, and phosphoserine phosphatase (Rowsell et al., 1969; Tabatabaie et al., 2010). It has become clear that the serine

synthetic pathway plays an essential role in the development and function of the central nervous system (Tabatabaie et al., 2010). Notably, serine is an essential amino acid for the development of neurons (Savoca et al., 1995).

Caenorhabditis elegans is an ideal animal model for investigating molecular mechanisms and biological processes. Human homologs to most *C. elegans* genes have been discovered, and the pharmacological mechanisms of *C. elegans* are similar to those of human (Shaye and Greenwald, 2011). Thus, we chose *C. elegans* as our model system to evaluate the gene expression following repeated volatile anesthetic exposure. The aim of this study was to investigate whether there was involvement of the serine synthetic pathway in anesthesia-induced neurotoxicity by repeated volatile anesthetic exposure.

2. Materials and methods

2.1. C. elegans strains and culture condition

Wild type N2 worms were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). All worms were grown and maintained on *Escherichia coli* strain OP50–seeded nutrient growth medium (NGM) Petri plates (OP50 plates) at 20 °C. NGM was made of 3 g of sodium chloride, 2.5 g of peptone, and 17 g of agar in distilled water. After autoclaving for 50 min, the medium was cooled down to 55 °C, then 1 ml of 1 M magnesium sulfate, 1 ml of cholesterol (5 mg/ml), 1 ml of 1 M calcium chloride, and 25 ml of 1 M potassium phosphate buffer were added to a final volume of 1 L. The NGM agar was poured into each Petri plate aseptically, which was left at room temperature for 2 days before use. OP50 was added and spread evenly and aseptically; these OP50 plates were incubated at 37 °C for 8 h and then cooled to room temperature.

2.2. Synchronization and anesthesia exposure

Thirty reproductively active adult worms were placed on 35-mm OP50 plates and removed after 1 h. Age-synchronized eggs were then incubated at 20 °C for 12 h, until all eggs hatched and became first-stage (L1) larvae. To anesthetize the L1 worms, the uncovered plates were put into a sealed glass vacuum desiccator. Liquid isoflurane was injected rapidly through the stopcock. The injected volume of isoflurane was considered the 99.9% effective immobilizing dose, which was determined by our previous pilot experiment. After 1 h of anesthesia, the plates were removed from the desiccator, and the L1 worms gradually recovered from anesthesia over a period of 3 to 4 h L1 worms were grown at 20 °C, and when they reached the second (L2), third (L3), and fourth larval stages (L4), they were anesthetized again as per the original protocol. These worms were designated to the isoflurane group. The worms of the control group were grown at 20 °C without exposure to isoflurane, and the following experimental procedures were for both groups.

2.3. C. elegans chemotaxis assay

Chemotaxis plates were prepared in 9-cm Petri plates containing NGM, as shown in Fig. 1. For chemotaxis, synchronized young adult worms from both groups were used. Once they reached the young adult stage, they were washed from the Petri plate using the S-basal buffer and collected into a microcentrifuge tube. They were briefly centrifuged and the supernatant was removed. This washing process was repeated two more times. The remaining worm pellet was transferred in the center of the chemotaxis plate and the number of worms was counted using a microscope. Each chemotaxis assay involved approximately 50–100 worms. The worms were allowed to move on the chemotaxis plate for 60 min. The immobilized worms within a 1.5-cm diameter of the attractant or control site as well as those outside these borders were manually counted. The chemotaxis index (%) was calculated in the
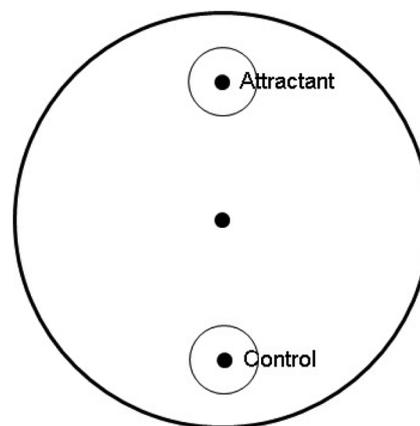

Fig. 1. A plate for the chemotaxis assay.

following manner: (number of worms at attractant site - number of worms at control site)/total number of worms × 100. The chemotaxis assay was repeated using 5 different batches.

A point in the center of the plate was the starting point for the worms. Both opposite sites, which were equidistant from the center and from each side, were blank control and OP50 attractant sites, respectively. Immediately before the chemotaxis assay, 1 μl of 1 M sodium azide was added in both control and attractant sites from immobilization of worm when they were reached either site.

2.4. RNA preparation

To determine the differentially expressed genes between the control and isoflurane groups, we performed a microarray using RNA extracted from the worms in both groups. Synchronized young adult worms were washed from the plates with M9 buffer, then transferred to 1.5 ml Eppendorf tubes, and pelleted at 0.1 g for 5 min. The supernatant was removed and the worm pellet was washed three times more with the M9 buffer. The *E. coli*-free worm pellet was transferred to a mortar including liquid nitrogen and was ground into a fine powder with a pestle. Liquid nitrogen was added intermittently to keep the worm powder cold. After grinding, the frozen powdered worms were transferred to a 1.5 ml Eppendorf tube and 600 μl of buffer RLT was added. The mixture was centrifuged at 12,000× g for 3 min. The supernatant was discarded and the pellet was mixed with an equal volume of 70% ethanol. An RNeasy mini spin column was placed into a 2-ml collection tube, and 700 μl of the ethanol-worm mixture was added, followed by centrifugation at 8000 ×g for 15 s. After removing the flow-through, 700 μl of RW1 was added and centrifuged at 8000× g centrifuging for 15 s. The same procedure was repeated twice – once for 15 s and another for 2 min – using 500 μl of RPE at 8000× g. After removing the flow-through, the tubes were centrifuged at the maximum speed for 1 min. The column was transferred to a new 1.5 ml collection tube and 30–50 μl of RNase-free water was added directly on the column membrane. The eluted solution was pipetted again on the column membrane two or three more times. The collected RNA was stored at −80 °C until use.

For quality control, RNA purity and integrity were evaluated by the ratio of absorbance at 260 nm–280 nm (OD 260/280 ratio), and the ratio of absorbance at 260 nm to 230 nm (OD 260/230 ratio) using a NanoDrop (ND-2000, Thermo Science), and analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). The samples that met a certain quality (OD 260/280 > 1.5 and OD 260/230 > 1.0) were used for microarray and real time polymerase chain reaction (RT-PCR).

Table 1
Forward and Reverse Primer Sequences for Real-Time PCR.

C31C9.2	Forward	GAATCCCTGAACGCTGGACA
	Reverse	GCGTCAATCGTTGATGCTCC
F26H9.5	Forward	TTCTTTTCATGCAAGGCGGC
	Reverse	TCACAATGTAGTCCGCGTGT
Y62E10 A.13	Forward	ATCCATATCCACATCACC
	Reverse	GGCATACTGTAGAATCAAC
act-1	Forward	TTACTCTTTCACCACCACCGCTGA
	Reverse	TCGTTTCCGACGGTGATGACTTGT

2.5. Real time polymerase chain reaction

Once the quality and quantity of total RNA were determined, cDNA was synthesized using the Thermo Scientific Maxima H minus First strand cDNA Synthesis Kit (Thermo Scientific). RT-PCR was set up with 125 ng of cDNA, 1 μl of each gene-specific forward and reverse primers (Table 1), and 10 μl of Power SYBR Green PCR Master mix (Applied biosystems). The thermocycler was run under the following conditions: 1) 50 °C for 2 min and 95 °C for 10 min; 2) 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; 3) 65–95 °C melt curve with a gradient of 0.5 °C; and 4) 40 °C cooling for 5 min. The ΔCT values were calculated using a housekeeping gene, act-1, as the control.

2.6. Measurement of serine contents

Serine content was measured using the DL-Seine Assay Kit (BioVision, Inc., CA, USA). Synchronized young adult worms (~ 10,000) were rapidly homogenized on ice with 100 μl ice cold Serine Assay Buffer. After centrifuging at 15,000× g for 10 min at 4 °C, the supernatant was transferred to a new microfuge tube. To eliminate the potential metabolites interfering with assay reaction, a Sample Cleanup Mix was added at a 1:25 ratio (4 μl for every aliquot 100 μl of the supernatant). The mixture was incubated at 37 °C for 15 min, and then transferred to 10 kDa MWCO Spin Columns. After centrifuging at 10,000× g for 10 min, the filtrated solution was collected. Once deproteinized, the filtered lysate can be stored at −20 °C for at least 2 months.

An aliquot 20 μl of the prepared filtered lysate was added to the desired wells in a black and flat-bottom 96-well plate, and 40 μl of Serine Assay Buffer was added to make the volume of all wells 60 μl. At least three parallel wells were prepared for each final lysate to determine the D-serine only, total serine (both the D- and L-serine), and sample background control. While preincubating the plate at 37 °C for 10 min with the light blocked, reaction mixes were prepared for D-serine only, total serine, and sample background control according to the manufacturer's recommendation. Prepared reaction mixes were added to the corresponding wells, which were incubated at 37 °C for 60 min in the dark. The fluorescence was measured at Ex/Em 535/587 nm.

The standard curve was drawn by using the D-serine standard stock and serine assay buffer. The zero standard reading (0 pmol/well) was subtracted from the standard readings, and the values were plotted to calculate the slope of the standard curve. For worm lysate, the corrected fluorescence was calculated by subtracting the relative fluorescence unit (RFU) of the sample background from the corresponding RFU of D-Serine Only or Total Serine, respectively. For the unspiked sample, either the D-serine or total serine concentration was calculated using the standard curve.

2.7. Phosphoserine phosphatase inhibitor and L-serine

To evaluate the dose-response effect of a phosphoserine phosphatase inhibitor on the behavior of worms, the worms were incubated with glycerophosphorylcholine (GPC; Sigma, St. Louis, Mo. USA). GPC was diluted in 1 l of NGM; the final concentrations were 1 μM, 10 μM,

30 μM, 100 μM, and 300 μM. The worms were also treated with L-serine at the following concentrations: 1 mM, 3 mM, 10 mM, 30 mM, 100 mM, and 300 mM. A chemotaxis assay was performed on 30 μM of GPC- or 30 mM of L-serine-exposed worms in the same manner as described above after repeated exposure to isoflurane.

2.8. Statistics

The values were presented as the mean ± SD, unless specified otherwise. Student's *t*-test was used to determine the significance of the differences between the two groups. One-way ANOVA was performed to compare the two or more groups, and Bonferroni *t*-test was used for post-hoc analysis. Statistical analyses were performed using SPSS software (ver. 21; IBM Co., Armonk, NY, USA). A P value of less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. Decreased chemotaxis index by repeated isoflurane exposure

Anesthesia-induced neurotoxicity by repeated isoflurane exposure was confirmed via chemotaxis assay. In the control group, the chemotaxis index was 90.1 ± 8.5%; however, that in the isoflurane group decreased by 61.5 ± 11.8% (P = 0.002), as reported by others (Gentry et al., 2013; Na et al., 2017).

3.2. Influence of isoflurane on serine synthetic pathway

Herein, we determined whether serine synthetic pathway was affected when *C. elegans* was repeatedly exposed to isoflurane during the developmental period. Representative enzymes of the serine synthetic pathway were phosphoglycerate dehydrogenase, phosphoserine aminotransferase, and phosphoserine phosphatase, which corresponds to *C31C9.2*, *F26H9.5*, and *Y62E10 A.13* in *C. elegans*, respectively. RT-PCR was performed to validate the expression level of these 3 genes in both groups, and the serine level was measured in *C. elegans* at the same time.

In young adult worms exposed to isoflurane, the genetic expressions of *C31C9.2*, *F26H9.5*, and *Y62E10 A.13* were decreased; a significant decrease was observed in *Y62E10 A.13* (Fig. 2). The serine level in worms was also lower in isoflurane group than in the control group (5.13 ± 1.44 in the isoflurane group vs. 7.65 ± 0.81 pM in the control
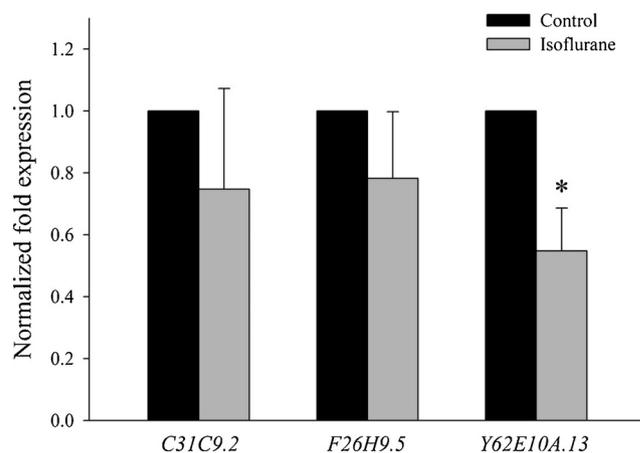
Fig. 2. The expression level of serine synthetic pathway-related genes.
Three enzymes, *C31C9.2*, *F26H9.5*, and *Y62E10 A.13* corresponded to phosphoglycerate dehydrogenase, phosphoserine aminotransferase, phosphoserine phosphatase, respectively. Experiments were conducted in triplicate. Every experiment included 3 plates in each group. Error bar is standard deviation of the mean. *Different expression of *Y62E10 A.13* gene from the control group, P < 0.001.

A

B
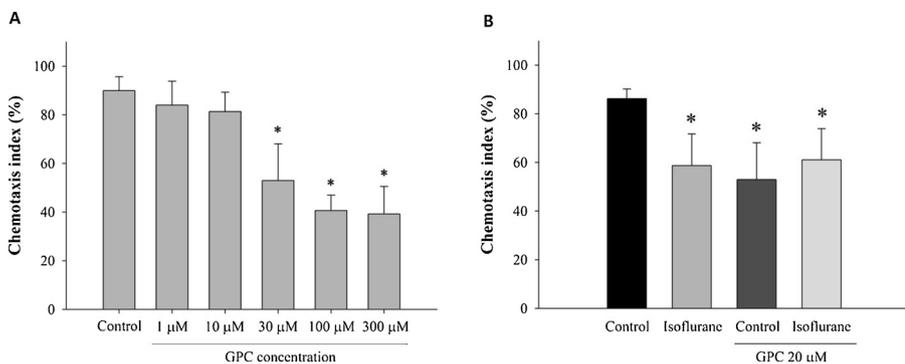
Fig. 3. Chemotaxis indices of young adult C. elegans according to the treatment of phosphoserine phosphatase.
(A) The change of chemotaxis index according to the concentration of GPC. (B) In 20 μM of GPC-treated worms, the change of chemotaxis index after repeated isoflurane exposure. Experiments were conducted in 5 replicates. Every experiment included 3 worm plates in each group. Error bars are standard deviations of the means. *Different from the chemotaxis index of the control group, P < 0.01. GPC, glycerophosphorylcholine.
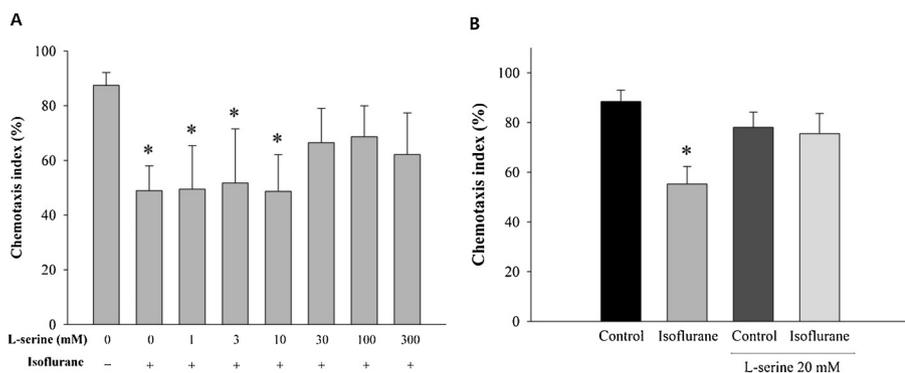
group, n = 5 in each group, p = 0.009).

3.3. Restoration of chemotaxis index by L-serine treatment

To confirm how phosphoserine phosphatase could affect the behavior of worms, C. elegans was treated with phosphoserine phosphatase inhibitor, GPC. The chemotaxis indices decreased in GPC-treated worm in a dose-dependent manner, and significant difference was observed from the 30 μM of GPC (Fig. 3A). The half maximal inhibitory concentration (IC_{50}) of GPC was 21.2 μM. Thus, worms were incubated with 20 μM of GPC during the developmental period, and they were divided into two groups, either the control or isoflurane group. Irrespective of the repeated isoflurane exposure, the chemotaxis index of GPC-treated worms were as low as that of the isoflurane group (P = 0.002; Fig. 3B). Repeated isoflurane exposure did not worsen the chemotaxis index in GPC-treated worms. In GPC-treated worms, the L-serine level of the control group was also as low as that of the isoflurane group (4.90 ± 1.36 pM in the isoflurane group vs. 5.22 ± 1.19 pM in the control group, n = 5 in each group, p = 0.702).

Because L-serine is produced from phosphoserine by phosphoserine phosphatase, we supplied it during isoflurane exposure. When the worms were treated with L-serine during isoflurane exposure, the chemotaxis index recovered at 30, 100, and 300 mM of L-serine even after repeated isoflurane exposure (Fig. 4A). The half maximal effective concentration (EC_{50}) of L-serine was 18.67 mM. Finally, when 20 mM of L-serine was supplied during the isoflurane exposure period, the deteriorated chemotaxis index by isoflurane was not observed (Fig. 4B), and the L-serine level of the isoflurane group was similar with the control group (7.27 ± 1.00 pM in the isoflurane group vs. 8.64 ± 1.19 pM in the control group, n = 5 in each group, p = 0.084).

4. Discussion

There is increasing concern about the neurotoxicity of volatile anesthetics, which are widely used in general anesthesia. However, the causal relationship between volatile anesthetics and neurotoxicity remains unclear. Moreover, the exact mechanism responsible for anesthetic-induced neurotoxicity also remains uncertain. Although there is a consensus that there may not be long-term risk from brief exposure to anesthetics in clinical situations (Davidson and Sun, 2018), the potential effects of prolonged or repeated exposure to anesthetics should be carefully investigated.

In this study, we attempted to find the association between serine synthetic pathway and neurotoxicity induced by repeated isoflurane exposure during the developmental period of C. elegans. This endeavor was undertaken because serine has been reported to play a critical role in neural development and its importance in preventive strategy of neurological or psychiatric diseased has been increasingly recognized (Savoca et al., 1995; Tabatabaie et al., 2010; Zhai et al., 2015; Dunlop et al., 2018a; Metcalf et al., 2018).

Three main enzymes related to the serine synthetic pathway of C. elegans were under-expressed after repeated isoflurane exposure during development. Of those, Y62E10A.13 was downregulated significantly, which was followed by low L-serine level in C. elegans. The worsened chemotaxis index in the isoflurane group might be attributed to the down-regulated Y62E10A.13 and subsequent low L- serine level. Unfortunately, the exact neuron that is affected by isoflurane exposure in C. elegans remains unknown.

Chemosensation is an essential behavior of C. elegans. A plenty of neurons are involved in chemosensation, and well-controlled regulatory and behavioral mechanisms mediate a complex and versatile chemosensory system. In our study, anesthesia-induced neurotoxicity and preventive effect of L-serine were confirmed by the chemotaxis assay, which have already been used as a simple method to evaluate anesthesia-induced neurotoxicity in previous studies (Gentry et al., 2013; Na et al., 2017). In C. elegans, serine-threonine kinase and phosphatase are a part of chemosensory homeostasis and adaptation (Bargmann, 2006). Serine is an amino acid residue that is most commonly phosphorylated by kinases during cell signaling in eukaryotes (Nestler and Greengard, 1999), and the addition or removal of phosphate residues is the basis for mediating cell cycle, apoptosis, and signal transduction (Hunter, 2012). In the next study, we should investigate how repeated exposure to volatile anesthetic agent during developmental phase may impact cell signaling via post-translational modification.

A

B

Fig. 4. Chemotaxis indices of young adult C. elegans with supplementation of L-serine.
(A) The change of chemotaxis index according to the concentration of L-serine. (B) In 20 mM of L-serine-treated worms, the change of chemotaxis index after repeated isoflurane exposure.
Experiments were conducted in triplicate. Every experiment included 3 worm plates in each group. Error bars are standard deviations of the means. *Different from the chemotaxis index of the control group, P < 0.01.

Moreover, L-serine plays a pivotal role in the development and function of the central nervous system, and acts as a precursor for nucleotides, phospholipids, glycine, and D-serine (de Koning and Klomp, 2004; Tabatabaie et al., 2010). Clinically, phosphoserine phosphatase deficiency is an extremely rare form of serine deficiency syndrome; however, it was reported that decreased serine levels in plasma and cerebrospinal fluid is accompanied by pre-and postnatal growth retardation, psychomotor retardation, and facial dysmorphism (Jaeken et al., 1997).

When a phosphoserine phosphatase inhibitor was applied to *C. elegans*, the chemotaxis index deteriorated to a similar degree as that of the isoflurane-exposed worms. In addition, co-treatment of the phosphoserine phosphatase inhibitor and isoflurane did not show a synergistic effect on the deterioration of the chemotaxis index. Equally, these animals showed a low L-serine level. Thus, our results suggest that isoflurane inhibits serine synthetic pathway, decreasing L-serine, and ultimately mediating isoflurane-induced behavioral deficiency in *C. elegans* model.

Our result is further supported by the finding that L-serine restored the chemotaxis index to the level equivalent to the control group. In other studies, external supplementation of L-serine improved the development and survival of cultured hippocampal neurons and Purkinje cells (Mitoma et al., 1998; Furuya et al., 2000). L-serine is an important amino acid in neural development, neurological signaling and function, and synaptic plasticity (Metcalf et al., 2018). The neuroprotective effect of L-serine has been reported in animal models of cerebral ischemia (Wang et al., 2010; Ren et al., 2013). Unfortunately, the exact role of serine in the neural development of *C. elegans* is not known; however, in a previous study, serine was reported to be one of the amino acids capable of extending the lifespan of *C. elegans* by activating the stress-response pathways, such as the DAF-16/FOXO or SKN-1/Nrf-2 pathway (Edwards et al., 2015).

Several potential mechanisms, such as neuronal apoptosis, endoplasmic reticulum-associated stress response, oxidative stress and mitochondrial damage, calcium/calmodulin-dependent protein kinase II signaling pathway, and others, have been proposed to play a role in anesthesia-induced neurotoxicity through the preclinical animal studies (Zhang et al., 2011; Li et al., 2015; Fang et al., 2017; Na et al., 2017; Jevtovic-Todorovic, 2018). In particular, endoplasmic reticulum mediated-neuronal apoptosis was observed after an exposure to volatile anesthesia in animal models (Chen et al., 2013; Zhu et al., 2017). L-serine was reported to show a neuroprotective effect by reducing endoplasmic reticulum stress responses (Dunlop et al., 2018a, b). Moreover, serine is converted to glycine, which is further required to produce glutathione. Glutathione acts as an antioxidant through neutralizing the reactive oxygen species (Chaneton et al., 2012; Diaz-Vivancos et al., 2015). Thus, L-serine should be further investigated as a substance that prevents neurotoxicity by repeated anesthesia exposure. Future studies evaluating whether there are any histopathologic changes upon L-serine supplementation are necessary. Furthermore, it is also necessary to identify how enzyme activity, which is related ro the serine synthetic pathway, may be affected by the repeated isoflurane exposure.

5. Conclusion

In conclusion, serine synthetic pathway might be negatively affected by repeated isoflurane exposure. Phosphoserine phosphatase of the three representative enzymes was mostly influenced, which may play an important role in the neurobehavioral deficiency of *C. elegans* induced by repeated isoflurane exposure during the developmental period. Supplementation of L-serine was helpful in restoring the deteriorated chemotaxis index; thus, L-serine should further be investigated as a potential strategy for preventing anesthesia-induced neurotoxicity.

Conflicts of interest

The authors declare no competing interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

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