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Original Article

Surgery Induces Activation of Indoleamine 2, 3-Dioxygenase, Increases in Pro-Inflammatory Cytokines and Cognitive Impairment in Old Rats

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ABSTRACT

Background: Postoperative cognitive dysfunction (POCD) is one of the most common complications in senior patients after surgery. However, the cause is largely unknown. Activation of indoleamine 2, 3-dioxygenase (IDO), a rate-limiting enzyme of kynurenine pathway, which converts tryptophan into kynurenine in extrahepatic tissues, may contribute to cognitive impairment. We therefore set out to assess whether surgery under anesthesia can induce activation of IDO, neuroinflammation and cognitive impairment in old rats.

Methods: Male Sprague-Dawley rats (24-month-old) were randomly assigned into three groups: 40% oxygen inhalation, 1.8% isoflurane anesthesia and left nephrectomy under 1.8% isoflurane anesthesia. Cognitive function was assessed daily in Y maze. Levels of interferon gamma (IFN-γ) and interleukin-6 (IL-6), IDO, tryptophan and kynurenine in serum and hippocampus were determined at 6 hours, 1, 3 and 7 days after anesthesia or surgery by using MILLIPLEX MAP Rat Cytokine Panel, enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC), respectively.

Results: Anesthesia with 1.8% isoflurane for three hours increased IL-6 level in serum at six hours which returned to normal level at 24 hours after the anesthesia. The isoflurane anesthesia alone did not induce cognitive impairment. Nephrectomy plus isoflurane elevated IFN-γ level in serum, increased levels of IL-6 and IDO, and IDO activity, determined by the ratio of tryptophan and kynurenine, in both serum and hippocampus, and finally induced cognitive impairment. These data showed that surgery under isoflurane anesthesia may induce activation of IDO, neuroinflammation and cognitive impairment in old rats.

Conclusions: These results suggested the potential role of IDO pathway in POCD pathogenesis, pending on further studies.

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Postoperative cognitive dysfunction (POCD) is a postoperative complication characterized by impairment of memory or attention, which has become a significant and increasing problem associated with increased mortality, risk of leaving the labor market prematurely, and dependency on social transfer payments (1, 2). The precise neuropathogenesis of POCD is mainly unknown despite of enormous research efforts (3). Recently, many studies showed that inflammatory response, especially neuroinflammatory response, might have an important role in neuropathogenesis of POCD (4-6). However, the

underlying mechanism by which inflammatory response leads to cognitive impairment and contributes to POCD remains largely unknown and needs to be further studied.

Some studies implicated that an activated indoleamine 2, 3- dioxygenase (IDO) induced by inflammation was likely the initial factor of the development and progression of depression (7, 8), schizophrenia (9), Huntington disease (10), and Alzheimer disease (AD) (11) among others, some of which, e.g. AD, might share common neuropathogenesis with POCD (12). IDO is a rate-limiting enzyme of the kynurenine pathway, which converts tryptophan into kynurenine in extrahepatic tissues. Under normal conditions, IDO is modestly expressed in many tissues but will be highly up-regulated locally or systemically by inflammation with consequently generated metabolites, which has been demonstrated to induce neurotoxicity (13, 14).

Therefore, we hypothesize that an up-regulation of IDO induced by inflammatory response (generated some neurotoxic intermediates) plays an important role in the development and progression of POCD. The current study was carried out to test this hypothesis in a clinically relevant surgical model of elderly rats.

METHODS

Animal Treatment

After obtaining approval from the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, Sichuan, China), 108 healthy male Sprague- Dawley rats aged 24- month- old and weight 400-600 g, were randomly assigned to three groups, receiving 40% O_2 for three hours only, 1.8% isoflurane in 40% O_2 for three hours or left nephrectomy under 3- hour 1.8% isoflurane anesthesia, respectively. One week before treatment, all rats were housed in standard cages, in a temperature (23℃) and humidity (45-55%) controlled environment with a 12/12-hour modified dark- light cycle (light on 11:00 PM- 11:00 AM). Food and water were available ad libitum.

Left nephrectomy was conducted under general anesthesia with 1.8% isoflurane under aseptic conditions by a professional urologist. Simply, after the surgical site was shaved and sterilized, a local anesthetic bupivacaine (0.375% and 2 ml) was injected into the skin and the subcutaneous tissue of abdominal area, and then a 1-cm incision was made in the upper left quadrant through the skin and muscles. The kidney was mobilized, isolated, and removed, and then the muscle and skin were closed. Finally, EMLA cream (2.5% lidocaine and 2.5% prilocaine) was used every eight hours for the first and second postoperative days.

During surgery and/or anesthesia, the continuous electrocardiogram monitoring, pulse oximetry and noninvasive blood pressure were measured by using a 150B3 monitor (Philips, Suzhou, China). The rectal temperature was measured and maintained at 37-39℃ by using a heating board as needed. After surgery and/or anesthesia, rats were allowed to awaken in an oxygen-enriched environment, and then were transferred to their own cage after completely awakened with free access to food and water.

Behavioral Tests

A well- trained observer who was blinded to group assignment performed behavioral tests of rats daily. 12 rats in each group were used for the behavioral tests.

Y Maze Test

After treatment, the learning and memory ability of rats was assessed daily by using a modified Y-maze apparatus in a dark and quiet room (15). The Y shaped apparatus (50 cm × 6 cm × 22 cm) consists of three black plastic branches with an illuminant at each end and a joint area. The "floor" of Y- maze is installed with copper rods through which an electric foot shock (30 ± 5 V for 10 seconds) can be applied. Firstly, rats were placed in the joint area of the Y maze, and then one of the arms was randomly illuminated, followed by electric shock applied in 5 seconds in the other two dark arms and the joint area. Normally, rats would run to the other two dark arms for they preferred to avoid an illuminated area. After receiving electric foot shocks, rats would run out, explore and finally enter the illuminated arm. Secondly, rats were manually returned to the joint area of the Y maze and the next episode began. The same episode was repeated for 20 times for each rat every day. If the

rat run directly into the illuminated arm before electric shocks were applied, which was defined as active response, this suggested a good learning and memory ability. Learning and memory impairment was presented by a decrease of the number of active response.

Locomotor Activity Test

Locomotor activity of animals was assessed daily in an open field apparatus (160 cm × 160 cm × 20 cm) which was divided into 36 grids by black lines. Rats were individually placed in the center of the apparatus, and then the number of grid crossings (motor activity) and rearings (exploratory activity) was counted in a five-minute period (16).

Samples Preparation

In the present study, rats in the three groups were harvested on 6 hours, 1, 3, and 7 days post-treatment for biochemistry studies, and six rats in each group were used for the biochemistry studies at every time point. Blood was collected from retinal vein under 4% isoflurane anesthesia, put in pre-heparinized Eppendorf tubes, and then centrifuged at 5000 rpm for 20 minutes at 4℃, finally the serum was stored at -80℃ until assay. For hippocampal samples, animals were rapidly decapitated with their brain quickly removed. All dissections were performed on an ice-cold frosted glass plate. Hippocampus was disrupted in an immunoprecipitation buffer (10 mmol Tris-HCl, pH 7.4, 150 mmol NaCl, 2 mmol EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) (Roche, Indianapolis, IN, USA), and then was homogenized. The homogenate was then centrifuged at 4℃ and 13,000 rpm for 15 minutes. The supernatant was removed and stored at -80℃ until assay. The protein content of each sample was determined by using the bicinchoninic acid protein assay kit (Pierce, Iselin, NJ, USA) according to the manufacturer's protocol.

Determination of Interferon Gamma (IFN-γ) and Interleukin-6 (IL-6) Level in Hippocampus and Serum by Employing A MILLIPLEX MAP Rat Cytokine Panel

Luminex Corporation has created an open source hardware platform combining flow cytometry- and bead-based antibody capture that is able to detect multiple analytes from a single sample and have a greater detection capacity (17). In the present study, IFN-γ and IL-6 levels in hippocampus and serum were quantified on 6 hours, 1, 3, and 7 days post-treatment by using MILLIPLEXTM MAP Rat Cytokine Kits (cat # RCYTO-80K, Millipore, Billerica, MA, USA) in a MILLIPLEX MAP Chemokine Panel which employed Luminex xMAP® technology, according to manufactures' recommended procedures (18). The multiplex assay was sensitive to 4.88 pg/ml IFN-γ and 9.80 pg/ml IL-6, and the inter-assay and intra-assay coefficients of variation were 8%.

Quantification of IDO Level by Using A Sandwich Enzyme-Linked Immunosorbent Assay (ELISA)

IDO levels in hippocampus and serum were determined with a Sandwich ELISA assay by using a Rat IDO ELISA Kit (Cat. No. E1547Ra, Uscn Life Science Inc., Wuhan, China) according to the instructions supplied by the manufacturers. Specifically, 96-well plates were coated with rat monoclonal antibodies specific to IDO. Following blocking with Block Ace, wells were incubated overnight at 4℃ with test samples, and then an anti-IDO antibody conjugated to horseradish peroxidase was added. Plates were then developed with TMB reagent and well absorbance was measured at 450 nm. IDO levels in test samples were determined by comparison with the signal from unconditioned media spiked with known quantities of IDO.

Determination of IDO Activity by Using High Performance Liquid Chromatography (HPLC)

Serum and hippocampal IDO activity were presented as the ratio of level of tryptophan and kynurenine, and kynurenine aminotransferase (KAT) activity was presented as the ratio of level of kynurenic acid and kynurenine. Serum and hippocampal levels of tryptophan, kynurenine and kynurenic acid were analyzed on 6 hours, 1, 3, and 7 days post-treatment by using an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC was carried out on a Venusil MP C18 analytical column (5 μm, 250 mm × 4.6 mm) operated at 25℃ and a fluores-

cent detector with a 225 nm wavelength according to classic methods (19). The linear ranges were from 0.3366 to 420.8 μmol/L for tryptophan, from 0.1302 to 1627 μmol/L for kynurenine and from 0.246 to 98.4 μmol/L for kynurenic acid.

Statistics

An observer who was blinded to the experimental protocol analyzed all data. MLwiN2.25 statistical software and a two-level logistic regression model were used in the comparison of learning curves (based on the number of active responses) among the three groups (control group was defined as reference group). Active responses were divided into two groups, with 1 denoting active response while 0 denoting no active response. Other analyses were performed using the SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Data are expressed as means ± standard errors of the means (SEM), with (N) indicating the number of subjects. The data were tested for normal distribution or not by the Kolmogorov-Smirnov test. Logarithmic transformation was done if the data did not meet the normal distribution (data not shown). A repeated-measures analysis of variance (ANOVA) was applied to analyze the difference of vital signs, and locomotor activity (based on the number of grid crossings and rearings) among rats in the control group, rats treated with anesthesia and rats treated with nephrectomy under anesthesia, and Bonferroni tests were used for the post-hoc comparisons. Covariates were treatment and time. Finally, basal body weight, the levels of IFN-γ, IL-6 and IDO, and IDO and KAT activity among the three groups were compared using a one-way ANOVA, and Bonferroni tests was used for the post-hoc comparisons. P values less than 0.05 (* or #) and 0.01 (** or ##) were considered statistically significant.

RESULTS

We set out to determine the effects of nephrectomy under isoflurane anesthesia on IDO levels and activation, neuroinflammation and cognitive function in old rats (24-month-old). There were no significant differences in basal body weight and vital signs among the three groups.

Table 1. A Two-Level Logistic Regression Model of Active Response.

Parameter	Effect	SE	OR	95% CI	P
Intercept	-2.336	0.568	0.097	0.032-0.294	<0.001
Treatment (Control)					
Isoflurane	0.105	0.458	1.111	0.453-2.726	0.819
Surgery+isoflurane	-2.483	0.804	0.083	0.017-0.404	0.002
Time	0.343	0.092	1.409	1.177-1.688	<0.001

Nephrectomy under 1.8% isoflurane anesthesia in elderly rat induced learning and memory impairment. There were significant differences in the active responses between rats following control condition and rats following nephrectomy in the Y Maze test. Nephrectomy reduced the number of active response as compared to the control condition. The probability of active responses in rats following nephrectomy was 0.083 times to rats following control condition. Isoflurane did not change number of active responses in the Y Maze test as compared to the control condition.
SE, standard error; OR, odds ratio; CI, confidence interval.

Nephrectomy plus Isoflurane Induced Cognitive Impairment

Whether neuroinflammation following anesthesia (20) or surgery (4) can contribute to cognitive impairment in animals and POCD in humans remains largely to be determined. Therefore, the present study was designed to confirm the role of neuroinflammation in cognitive impairment and illustrate the underlying mechanisms. In the study, rats were treated with either isoflurane anesthesia alone or nephrectomy under isoflurane anesthesia. After the treatments, rats were monitored daily for learning and memory function by using a Y maze and for locomotor activity by using an open filed apparatus.

We found that the rats which only had isoflurane anesthesia, did not show any learning and memory impairments. However, nephrectomy under 1.8% isoflurane anesthesia induced a significant less number of active responses in rats (Table 1, P=0.002), which suggested learning and memory impairment. In the locomotor activity test, we were able to show that isoflurane inhalation or nephrectomy under isoflurane anesthesia did not change motility and exploratory activity (presented as the number of grid crossings and rearings), which further suggested that the learning and memory impairment induced by surgery under the isoflurane anesthesia cannot be owed to the alterations of motility ability.

Nephrectomy plus Isoflurane-Induced Inflammation

To explore the role of inflammation in the

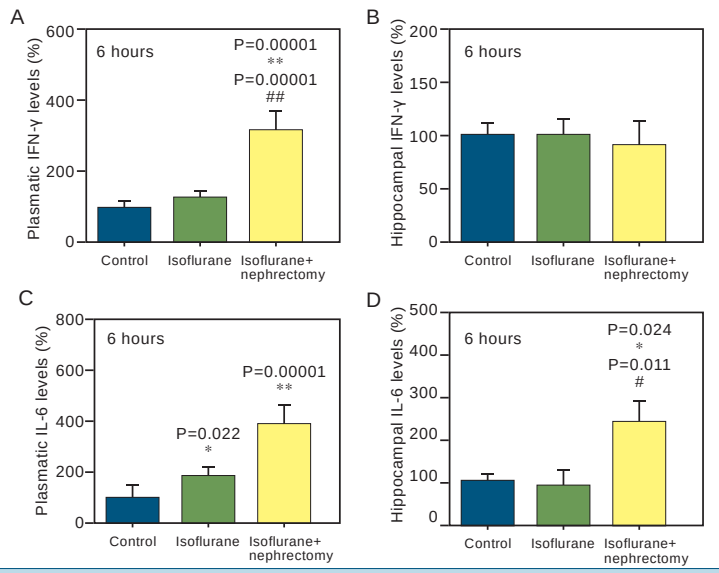

Figure 1. Nephrectomy under 1.8% Isoflurane Anesthesia in Elderly Rat Increased IFN-γ Level and IL-6 Level in Serum and Hippocampus at Six Hours Post-Nephrectomy.
A. Nephrectomy increased serum IFN-γ level as compared to the control condition (** P=0.00001) and the anesthetic condition (## P=0.00001); B. Nephrectomy did not change hippocampal IFN-γ level; C. Nephrectomy increased serum IL-6 level as compared to the control condition (** P=0.00001) and the anesthetic condition (# P=0.023). Isoflurane increased serum IL-6 level as compared to the control condition (* P=0.022); D. Nephrectomy increased hippocampal IL-6 level as compared to the control condition (* P=0.024) and the anesthetic condition (# P=0.011). Data represent means±SEM (N=6).

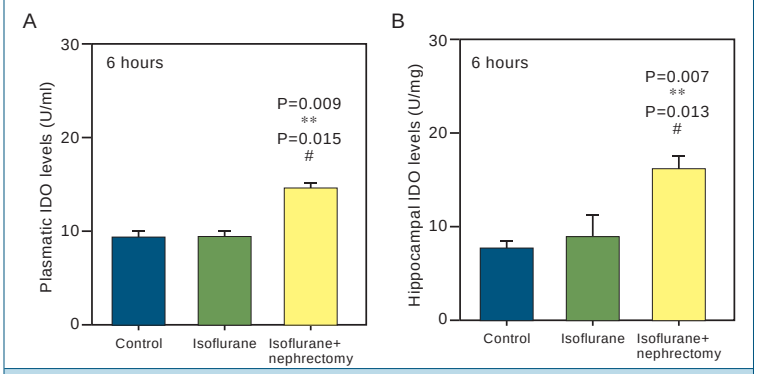

Figure 2. Nephrectomy under 1.8% Isoflurane Anesthesia in Elderly Rat Increased Indoleamine 2, 3-Dioxygenase (IDO) Level in Both Serum and Hippocampus at Six Hours Post-Nephrectomy.
A. Nephrectomy increased serum IDO level as compared to the control condition (** P=0.009) and the anesthetic condition (# P=0.015); B. Nephrectomy increased hippocampal IDO level as compared to the control condition (** P=0.007) and the anesthetic condition (# P=0.013). Data represent means±SEM (N=6).

pathogenesis of POCD in elderly rat, we employed MILLIPLEX MAP Chemokine Panel to determine IL-6 and IFN-γ levels in serum and hippocampus. The results showed that isoflurane anesthesia slightly increased IL-6 level in serum at six hours post-anesthesia (Figure 1C, * P=0.022). Nephrectomy under isoflurane anesthesia induced a robust increase in serum IFN-γ and IL-6 levels (almost 2 and 6 times of control) at six hours post-nephrectomy (Figure 1A and 1C, ** P=0.00001, respectively). Nephrectomy does not change hippocampal IFN-γ level (Figure 1B). A transient elevated IL-6 level in hippocampus was only found at six hours post-nephrectomy (Figure 1D, # P=0.011). On 1, 3, and 7 days post-treatment, no differences were found in IL-6 levels in serum and hippocampus among the three groups (data not shown).

Nephrectomy under Isoflurane Anesthesia Induced Activated IDO and Inactivated KAT

IDO plays a pivotal role in mediating the development of many neuroinflammation diseases, especially AD (11), which may share similar pathogenesis with POCD. To determine if IDO has an important role in the development of POCD, we assessed IDO levels in hippocampus and serum with Sandwich ELISA assay by using a Rat IDO ELISA Kit. We found that serum and hippocampal IDO levels were markedly increased at six hours post-nephrectomy (Figure 2, ** P=0.009 and ** P=0.007), and then returned to baseline levels on one day post-treatment (data not shown). Hippocampal activities of IDO and KAT could not be determined because hippocampal levels of kynurenine were below the threshold of detection, and serum IDO activation observed was usually correlated with hippocampal IDO activation (21). Therefore, hippocampal activities of IDO and KAT were presented by serum activities of IDO and KAT in current studies. We found that there was a significant increase in serum IDO activity on one day post-nephrectomy, and it continued to be higher than that in control group and isoflurane anesthesia group on three and seven days post-nephrectomy (Figure 3). On the contrary, there was a significant decrease in KAT activity one day post-nephrectomy, and it continued to be lower than that in control group and isoflurane anesthesia group on three and seven days post-nephrectomy (Figure 4).

DISCUSSION

In the present study, the results showed that six hours after surgery (nephrectomy) under general anesthesia (isoflurane), rats exhibited a remarkable increase of proinflammatory cytokine: IL-6 (increasing to about 6 times in serum and 2 times in hippocampus) and IFN-γ (increasing to about 2 times in serum). These changes were associated with learning and memory impairment on four days post-treatment (a decrease of active response number). While rats that were subjected to general anesthesia was noted for only slight increase in serum IL-6 level (about 2 times increase compared to control), without following decrease of active response number. The results were consistent with previous experiments, which found that POCD might be resulted from surgery, such as splenectomy (15), partial hepatectomy (4) or orthopaedic surgery (22, 23). Moreover, the results also demonstrated that the increase of proinflammatory cytokine was closely associated with the learning and memory impairment in aged rats.

Furthermore, our results found that nephrectomy under general anesthesia induced a significant increase in IDO level and activity, which was associated with elevated cytokine levels (IL-6 and IFN-γ) and the following learning and memory impairment, suggesting a close relationship among inflammation induced by surgery, IDO activation and the cognitive impairment in aged rats. The results were consistent with the findings from the studies by O'Connor and coworkers that elevation of IFN-γ in response to Bacillus Calmette-Guerin infection induced an elevated IDO level and activity and the following depressive-like behaviors (16). Moreover, neither increase in IDO level and activity nor learning and memory impairment was found after isoflurane anesthesia, further demonstrating the role of surgery during this pathway.

Moreover, our results also showed that serum KAT activity decreased significantly on one day post-surgery, and continued to decrease till seven days post-surgery. KAT is a downstream enzyme of IDO in the kynurenine pathway, which converts kynurenine into kynurenic acid (KA) in extrahepatic tissues. Kynurenine hydroxylase is another downstream enzyme of IDO in the kynurenine pathway, which converts kynurenine into quinolinic acid (QA) and 3-hydroxykynurenine (3-HAA) (11). These data suggested that the kynurenine pathway of tryptophan degradation was modulated headily to QA and 3-HAA productions, at the same time KA production level decreased after nephrectomy in aged rats.

It is widely accepted that QA is a potent excitotoxic N-methyl-D-aspartate (NMDA) receptor agonist and may produce overstimulation of hippocampal NMDA receptors, leading to neuron apoptosis and hippocampal atrophy (13, 14). Furthermore, 3-HAA is able to produce oxidative stress by increasing the production of reactive oxygen species which have well-established neurotoxic effects (13, 14). On the other hand, KA is identified as the only endogenous NMDA receptor antagonist and could modulate the neurotoxic effects of QA as well as disrupt excitato-

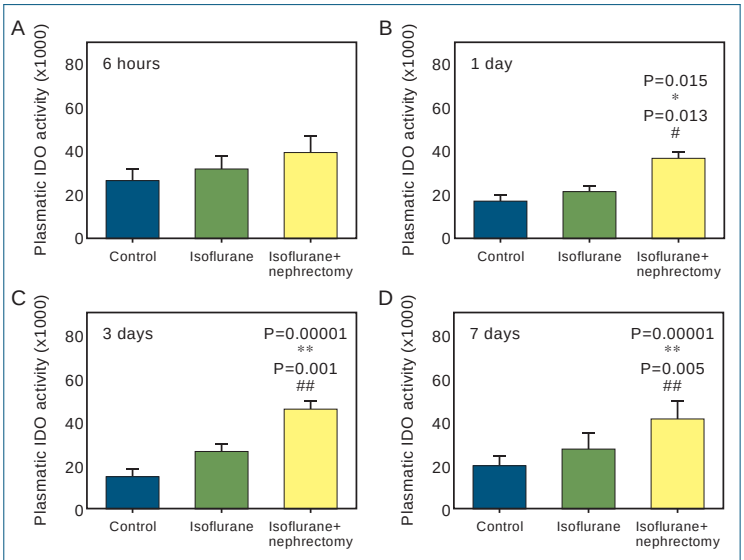

Figure 3. Nephrectomy under 1.8% Isoflurane Anesthesia in Elderly Rat Increased Serum Indoleamine 2, 3-Dioxygenase (IDO) Activity at 1, 3 and 7 Days Post-Nephrectomy.

A. Nephrectomy did not change serum IDO activity at six hours post-nephrectomy; B. Nephrectomy increased serum IDO activity at one day post-nephrectomy as compared to the control condition (* P=0.015) and the anesthetic condition (# P=0.013); C. Nephrectomy increased serum IDO activity at three days post-nephrectomy as compared to the control condition (** P=0.00001) and the anesthetic condition (## P=0.001); D. Nephrectomy increased serum IDO activity at seven days post-nephrectomy as compared to the control condition (** P=0.00001) and the anesthetic condition (## P=0.005). Data represent means±SEM (N=6).

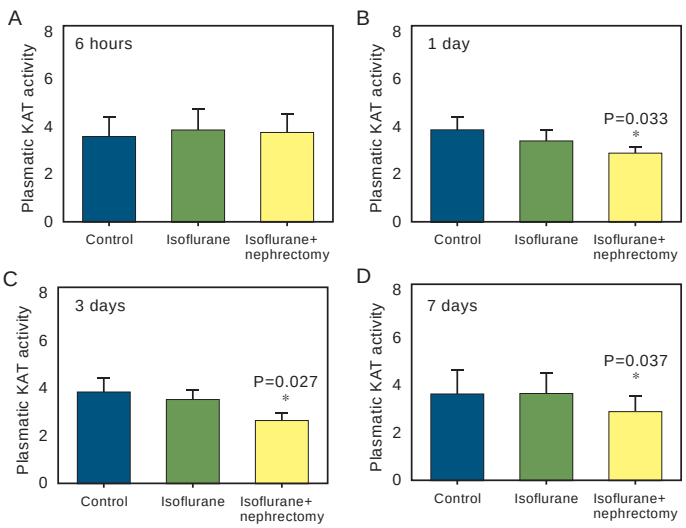

Figure 4. Nephrectomy under 1.8% Isoflurane Anesthesia Decreased Serum Kynurenine Aminotransferase (KAT) Activity at 1, 3 and 7 Days Post-Nephrectomy in Elderly Rat.
A. Nephrectomy did not change serum KAT activity at six hours post-nephrectomy; B. Nephrectomy decreases serum KAT activity at one day post-nephrectomy as compared to the control condition (* P=0.033); C. Nephrectomy decreased serum KAT activity at three days post-nephrectomy as compared to the control condition (* P=0.027); D. Nephrectomy decreased serum KAT activity at seven days post-nephrectomy as compared to the control condition (* P=0.037). Data represent means±SEM (N=6).

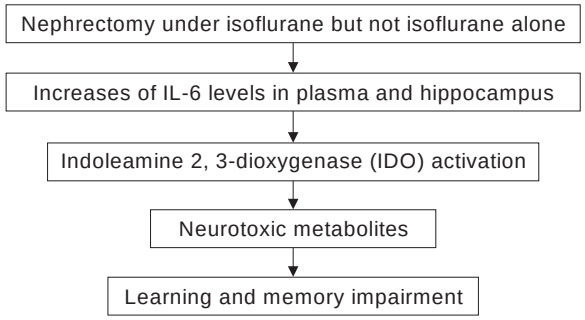

Figure 5. Hypothetical Pathway by Which Surgery Induced Learning and Memory Impairment.
Nephrectomy under isoflurane anesthesia, but not isoflurane alone, induced increases in IL-6 levels in both plasma and hippocampus, which then facilitated indoleamine 2, 3-dioxygenase (IDO) activation, demonstrated by increases in IDO levels and activity. IDO activation would cause increases in levels of neurotoxic metabolites, including quinolinic acid (QA) and 3-hydroxykynurenine (3-HAA), leading to neurotoxicity and finally impairment of learning and memory.

ry amino acid neurotransmission. In the present study, although the levels of QA and 3-HAA were not determined, according to increases in IDO level and activity and decrease in KAT activity, it was proposed that up-regulation of the kynurenine pathway of tryptophan degradation, led mainly to the excessive local production of QA due to neuroinflammatory processes, and also to a decrease in KA level, which made it unable to alleviate the deleterious neurotoxic effects of QA. Finally, all of these changes had an important role in cognitive decline. Our results suggested the potential role of IDO pathway in POCD pathogenesis after nephrectomy in aged rats, pending on further studies.

In this study, we only employed a clinically relevant isoflurane inhalation (1.8% isoflurane, 1.3 minimum alveolar concentration [MAC]) (24) and a nephrectomy associated cognitive impairment model on spatial learning and memory in rat by using the Y-maze. It is possible that isoflurane of different concentrations and other kinds of surgery may have different effects on the learning and memory function. In addition, although cognition in rats is quite different from that in human, our results in rats would promote more studies aiming at exploring cognition impairment in human. In the next study, the role of IDO in surgery-associated learning and memory impairment needs to be further determined. Some methods aiming to inhibit activation of IDO, including specific IDO inhibitor (1-MT), would be used to explore if inactivation of IDO could abolish surgery-associated learning and memory impairment. Nevertheless, the findings that nephrectomy under 1.8% isoflurane anesthesia induced increase of cytokine and IDO activation, and impaired learning and memory suggested for the first time that surgery-induced IDO activation may be associated with surgery-induced neurotoxicity and impairment of learning and memory, and more studies are needed to further test this hypothesis (Figure 5).

In conclusion, we established an animal model of nephrectomy under isoflurane anesthesia in rats and found that nephrectomy increased pro-inflammatory cytokines IFN-γ level in serum, and IL-6 levels in serum and hippocampus of old rats. IDO, a rate-limiting enzyme of kynurenine pathway, was also up-regulated by nephrectomy under isoflurane anesthesia. Finally, nephrectomy impaired learning and memory. Results of the present experiments demonstrated that an over-activated IDO induced by surgery-associat-

ed inflammatory response might play an important role in the development and progression of POCD in old rats. These findings would promote more studies to further determine the role of IDO in cognitive function decline in patients.

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The authors deny any other conflict of interests.

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