

Peripheral surgical wounding may induce cognitive impairment through interleukin-6-dependent mechanisms in aged mice

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Abstract

Post-operative cognitive dysfunction (POCD) is associated with morbidity, mortality and increased cost of medical care. However, the neuropathogenesis and targeted interventions of POCD remain largely to be determined. We have found that the peripheral surgical wounding induces an age-dependent A β accumulation, neuroinflammation and cognitive impairment in aged mice. Pro-inflammatory cytokine interleukin-6 (IL-6) has been reported to be associated with cognitive impairment in rodents and humans. However, the role of IL-6 in the neuropathogenesis of POCD is unknown. We therefore employed pharmacological (IL-6 antibody) and genetic (knock-out of IL-6) approach to investigate whether IL-6 contributed to the peripheral surgical wounding-induced cognitive impairment in aged mice. Abdominal surgery under local anesthesia (peripheral surgical wounding) was established in 18-month-old wild-type and IL-6 knockout mice ($n = 6$ to 10 in each group). Brain level of IL-6 and cognitive function in the mice were determined by western blot, ELISA at the end of procedure, and Fear Conditioning System at 7 days after the procedure. The peripheral surgical wounding increased the level of IL-6 in the hippocampus of aged wild-type, but not IL-6 knockout mice. IL-6 antibody ameliorated the peripheral surgical wounding-induced cognitive impairment in the aged wild-type mice. Finally, the peripheral surgical wounding did not induce cognitive impairment in the aged IL-6 knockout mice. These data suggested that IL-6 would be a required pro-inflammatory cytokine for the peripheral surgical wounding-induced cognitive impairment. Given this, further studies are warranted to investigate the role of IL-6 in the neuropathogenesis and targeted interventions of POCD.

Key words: peripheral surgical wounding; interleukin-6; cognition; mice; fear conditioning system; neuroinflammation

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INTRODUCTION

Post-operative cognitive dysfunction (POCD), one of the most common post-operative complications in senior patients (Liu and Leung, 2000), is associated with increased cost of medical care, morbidity, and mortality (Monk et al., 2008; Deiner and Silverstein, 2009; Steinmetz et al., 2009). However, the causes and neuropathogenesis of POCD re-

main largely to be determined.

Many studies aim to determine the role of general anesthesia alone (Culley et al., 2004; Xie et al., 2006, 2007, 2008; Bianchi et al., 2008; Dong et al., 2009; Zhang et al., 2012; Hu et al., 2014; reviewed by Vutskits and Xie, 2016) or general anesthesia plus surgery (Wan et al., 2007, 2010; Cibelli et al., 2010; Terrando et al.,



2010b) in POCD neuropathogenesis. However, increasing evidence suggests that there is no significant difference in the incidence of POCD between surgery with general anesthesia and surgery without it (with epidural, spinal or local anesthesia) (Karhunen and Jonn, 1982; Ghoneim et al., 1988; Berant et al., 1995; Williams-Russo et al., 1995; Rasmussen et al., 2003; Steinmetz et al., 2009; reviewed by Newman et al., 2007 and Mason et al. 2010). Thus, it is important to determine the potential neurotoxicity of surgery without general anesthesia and its role in POCD. Since it is technically difficult to perform an epidural or spinal anesthesia in mice, we have established an animal model to assess the effects of open abdominal surgery under local anesthesia on the neurotoxicity in mice.

We have shown that open abdominal surgery under local anesthesia (peripheral surgical wounding) in mice can induce A β accumulation, neuroinflammation and cognitive impairment in aged mice (18 months old) (Xu et al., 2014a, b). Other studies have also shown that neuroinflammation contributes to the cognitive impairment induced by surgery under general anesthesia (Wan et al., 2007, 2010; Cibelli et al., 2010; Terrando et al., 2010a; reviewed by Terrando et al., 2011 and Xu et al., 2014b).

Interleukin 6 (IL-6) has been reported to be associated with cognitive impairment in animals (Braidia et al., 2004; Huang et al., 2008; Cao et al., 2010), cognitive dysfunction (Hudetz et al., 2010; Patanella et al., 2010), mild cognitive impairment (MCI) (Schuitemaker et al., 2009), and delirium in medical (Katsumata et al., 2007) and surgical patients (Bjornsson et al., 2007). However, the role of IL-6 in POCD neuropathogenesis has not been investigated.

The objective of the studies was to determine the specific role of IL-6 in the neuropathogenesis of POCD. The hypothesis of the study was that the peripheral surgical wounding might induce cognitive impairment through IL-6-dependent mechanisms in aged mice. Therefore, we assessed the effects of the peripheral surgical wounding, in the absence of general anesthesia, on the cognitive function in the aged mice with pretreatment of IL-6 antibody, and in the aged IL-6 knockout (KO) mice.

MATERIALS AND METHODS

Mouse surgery and treatment

All experiments followed the National Institutes of Health guidelines and the animal protocol was approved by the Massachusetts General Hospital (Boston, MA, USA) Standing Committee on the Use of Animals in Research and Teaching. Efforts were made to minimize the number of animals used. Aged wild-type (WT) C57BL/6J female mice (18 months old; National Institute of Aging, Bethesda, MD, USA) and IL-6 KO female and male mice (18 months old, B6.129S2-

Il6tm1Kopf/J, stock number 002650; Jackson Lab, Bar Harbor, ME, USA) were used in the study.

The surgery under local anesthesia was performed as described in our previous studies (Xu et al., 2014a, b). Specifically, the mice were randomly assigned to a surgery or sham group and sub-group (e.g., saline and IL-6 antibody group) by weight and sex with randomization ($n = 10$ in behavior studies, and $n = 6$ in biochemistry studies). The mice were gently restrained to a heating pad (37°C) using paper tape. A local anesthetic bupivacaine (0.5% and 0.1 mL) was injected into the skin and subcutaneous tissue of the abdominal area. A 2.5 cm incision was made in the middle of the abdomen to open and then close the abdominal cavity in the mouse. The procedure lasted about 5 minutes. We did not use sedative medicine in an effort to reveal the effects of surgery without sedation or general anesthesia and to minimize all other variables. EMLA cream (2.5% lidocaine and 2.5% prilocaine) was used every 8 hours for the 1st and 2nd post-operative days to treat the surgery-associated pain. We did not use antibiotics because the procedure was aseptic. The non-surgery (sham) mice underwent the same procedure, only without the incision. For the interaction studies, IL-6 antibody (10 μ g/mouse, IP, eBioscience Inc., San Diego, CA, USA, Cat. Number: 16-7061) or the same volume of saline was administered to the mice 18 hours before the surgery (Pieraut et al., 2011). The dosage of 10 μ g per mouse was determined from our pilot studies. We used the pre-operative treatment of IL-6 antibody because a previous study had shown that pre-operative, but not post-operative, treatment of tumor necrosis factor (TNF)- α antibody attenuated cognitive impairment induced by surgery plus general anesthesia (Terrando et al., 2010a). There was no significant difference in blood pressure, blood gas, levels of blood glucose and epinephrine, locomotor activity, or pain threshold between the surgery and sham mice, as demonstrated in our previous studies (Xu et al., 2014a). Local anesthetic induced neither neuroinflammation nor cognitive impairment in the aged mice (18 months old) (Xu et al., 2014a).

Brain tissue harvest, lysis, and protein amount quantification

The mouse hippocampus was harvested at 12 hours after the procedure. The harvested hippocampus tissues were homogenized on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40) plus protease inhibitors (1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin A). The lysates were collected, centrifuged at 12,000 r/min for 15 minutes, and quantified for total proteins by bicinchoninic acid protein assay kit (Pierce, Iselin, NJ, USA).

Western blot analysis

IL-6 antibody (1:1,000 dilution; Abcam, Cambridge, MA,



USA, Cat. Number: ab6672) was used to recognize IL-6 (24 kDa). Western blot quantification of the hippocampal tissues was performed as described by Xie et al. (2008). Briefly, signal intensity was analyzed using a Bio-Rad (Hercules, CA, USA) image program (Quantity One). We quantified Western blots in two steps. First, we used β -actin levels to normalize (*e.g.*, determining ratio of IL-6 to β -actin) protein levels and control for loading differences in total protein amount. Second, we presented protein level changes in the mice receiving the surgery as a percentage of those in the sham group mice. 100% of protein level changes refer to control levels for the purpose of comparison to experimental conditions.

ELISA determination of IL-6

The mouse IL-6 immunoassay kit (R&D Systems, Rochester, MN, USA, Catalog number: M6000B) was used to determine the level of IL-6 in the hippocampus tissues of the mice. Briefly, a monoclonal antibody specific for mouse IL-6 had been coated onto microplates. We added 50 μ L of standard or samples, and then added 50 μ L of assay diluent RD1-14 to the center of each well. Wells were incubated for 2 hours at room temperature, and washed five times. Then 100 μ L of mouse IL-6 conjugate was added to each well and incubated for another 2 hours and repeated the washing. At last, wells were incubated in 100 μ L of substrate solution for 30 minutes and stopped with stop solution. Determination of the optical density of each well was set at 450 nm, and corrected at 570 nm.

Fear conditioning system (FCS)

We employed FCS, but not Morris Water Maze, in the current experiments because our previous studies showed that the surgery under local anesthesia was able to induce cognitive impairment in the FCS, but not Morris Water Maze, in the 18-month-old mice (Xu et al., 2014a). The FCS studies were performed as described in our previous studies with modifications (Xu et al., 2014a). Specifically, the pairing in the FCS (Stoelting Co., Wood Dale, IL, USA) was performed at 24 hours post-surgery, mimicking the condition that patients may have difficulty in learning new things after surgery. The pairing was performed twice with 2 minutes in between. For pairing, each mouse was allowed to explore the FCS chamber for 180 seconds before presentation of a 2-Hz pulsating tone (80 dB, 3,600 Hz) that persisted for 60 seconds. The tone was immediately followed by a mild foot shock (0.8 mA for 0.5 second). The mice were tested in FCS at 7 days after the procedure. Each mouse was allowed to stay in the same chamber for a total of 390 seconds. Cognitive functions (*e.g.*, learning and memory) in the context and

tone test were assessed by measuring the amount of time (“freezing time”) the mouse demonstrated “freezing behavior”, which is defined as a completely immobile posture except for respiratory efforts during the second 180 seconds. “Freezing behavior” was analyzed by Any-Maze (freezing on threshold: 10; freezing off threshold: 20; minimum freezing duration: 1 second) (Stoelting Co.). The percentage of freezing time was calculated by dividing the actual freezing time with the observed time (180 seconds).

Statistical analysis

The nature of the hypothesis testing was two-tailed. Data were expressed as the mean \pm SD. There were 10 mice in each group in the behavior studies, and 6 mice in each group in the biochemistry studies. Two-way analysis of variance (ANOVA) was used to determine the interaction between treatment (saline or IL-6 antibody) and group (sham and peripheral surgical wounding) in FCS studies. Student *t*-test was used to determine the difference in IL-6 levels between the peripheral surgical wounding and sham. *P* values less than 0.05 and 0.01 were considered statistically significant. Prism 6 software (La Jolla, CA, USA) was used for all statistical analyses.

RESULTS

Peripheral surgical wounding increased IL-6 levels in the hippocampus of aged mice at 12 hours after the procedure

We first determined the effects of the peripheral surgical wounding on IL-6 levels in hippocampus of aged WT mice at 12 hours after the procedure by employing quantitative Western blot analysis. The immunoblotting of IL-6 showed a visible increase in the density of the bands associated with the surgery as compared to the bands associated with sham condition. There was no significantly visible difference in the amount of β -actin between sham group and surgery group (**Figure 1A**). The quantification of the western blot showed that the peripheral surgical wounding increased the levels of IL-6 as compared to sham condition ($248 \pm 14\%$ vs. $100 \pm 21\%$, $P = 0.0004 < 0.01$, student *t*-test; **Figure 1B**). These data suggested that the peripheral surgical wounding without influence of general anesthesia was able to increase the levels of IL-6 in the hippocampus of aged WT mice.

IL-6 antibody ameliorated the peripheral surgical wounding-induced cognitive impairment in aged mice

Given that the peripheral surgical wounding without the influence of general anesthesia could increase IL-6 levels (**Figure 1**), and cognitive impairment in aged WT mice (Xu et al., 2014a); next, we determined the cause-effect relationship by

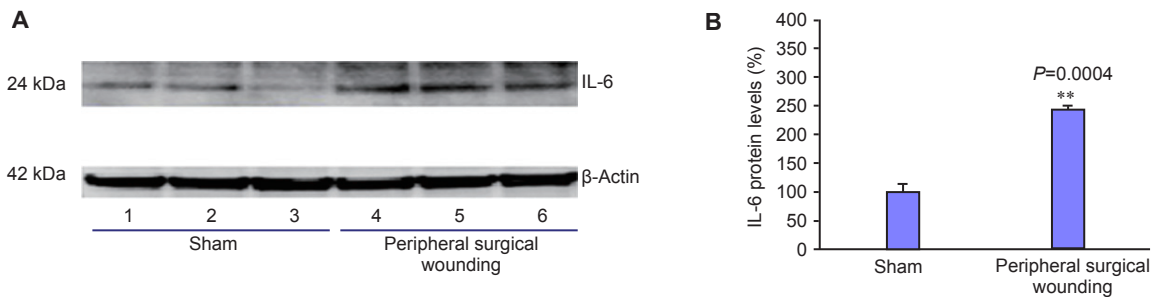


Figure 1: Peripheral surgical wounding increased IL-6 levels in the hippocampus of aged mice.

Note: (A) Western blot analysis showed that the peripheral surgical wounding (lanes 4 to 6) increased the levels of IL-6 in the hippocampus of 18-month-old mice at 12 hours after the peripheral surgical wounding as compared to sham condition (lanes 1 to 3). There was not significant difference in the β -actin levels between the mice in sham group and in surgery group. (B) Quantification of the western blot showed that the peripheral surgical wounding increased the levels of IL-6 in the hippocampus of 18-month-old mice at 12 hours after the peripheral surgical wounding as compared to sham condition. Data are expressed as the mean \pm SD, $n = 6$ in sham or surgery group. ** $P < 0.01$, vs. sham group (student t -test). IL: Interleukin.

assessing whether IL-6 antibody could attenuate the peripheral surgical wounding-induced cognitive impairment, tested by FCS, in aged mice. We found that a treatment with IL-6 antibody before the peripheral surgical wounding ameliorated peripheral surgical wounding-induced cognitive impairments

in aged mice ($P = 0.034 < 0.05$, two-way ANOVA; **Figure 2A**) and ($P = 0.015 < 0.05$, two-way ANOVA; **Figure 2B**). These findings suggested that the peripheral surgical wounding could induce cognitive impairment in aged mice *via* elevating IL-6 levels in brain tissue of the mice.

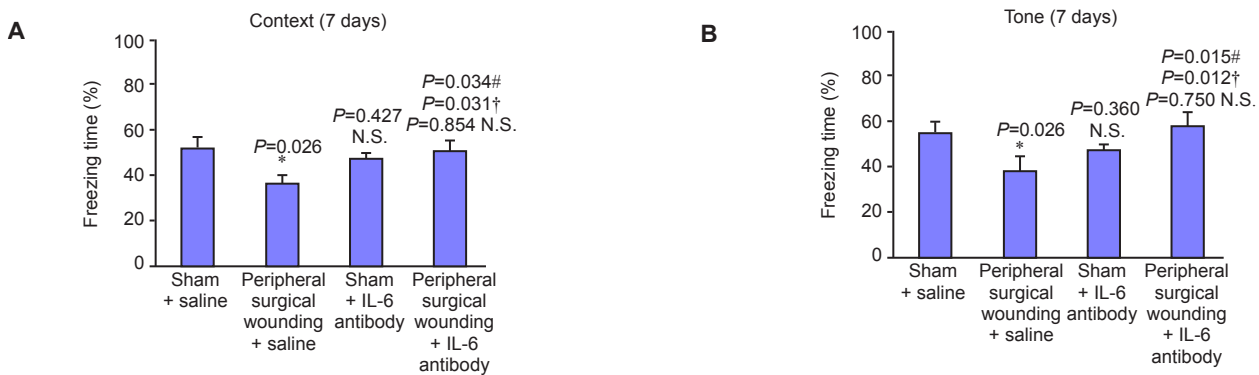


Figure 2: IL-6 antibody attenuated the peripheral surgical wounding-induced cognitive impairment in aged mice.

Note: Peripheral surgical wounding without the influence of general anesthesia induced cognitive impairment in the context test (A) and tone test (B) of the fear conditioning system (FCS) at 7 days post-procedure in 18-month-old mice. IL-6 antibody attenuated the peripheral surgical wounding-induced reduction in freezing time of the context test (A) and the tone test (B) of the FCS at 7 days post-procedure. Data are expressed as the mean \pm SD, $n = 10$ in each group. * $P < 0.05$, vs. sham + saline group; $\#P < 0.05$, vs. saline + surgery group (two-way analysis of variance); $\dagger P < 0.05$: the interaction between the group and the treatment (two-way analysis of variance). IL: Interleukin.

Peripheral surgical wounding did not increase IL-6 levels in the hippocampus of aged IL-6 KO mice at 12 hours after the procedure

Given the findings that the peripheral surgical wounding increased IL-6 levels in the hippocampus of aged mice, and IL-6 antibody was able to attenuate the peripheral surgical wounding-induced cognitive impairment in the aged mice, next, we further investigated the role of IL-6 in the peripheral surgical wounding-induced cognitive impairment by employing aged IL-6 KO mice. ELISA showed that the peripheral surgical wounding did not increase IL-6 levels in the hippocampus of aged IL-6 KO mice as compared to sham condition (94% vs. 100%, $P = 0.427 > 0.05$, student t -test; **Figure 3**).

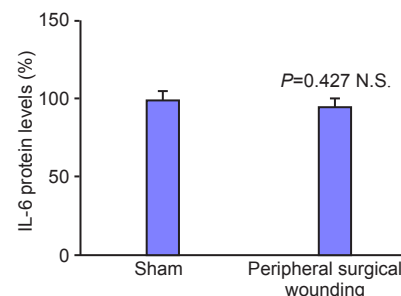


Figure 3: Peripheral surgical wounding did not increase IL-6 levels in the hippocampus of aged IL-6 knockout (KO) mice.

Note: ELISA showed that the peripheral surgical wounding did not increase the levels of IL-6 in the hippocampus of 18-month-old IL-6 KO mice at 12 hours after the peripheral surgical wounding as compared to sham condition. Data are expressed as the mean \pm SD, $n = 6$ in sham or surgery group, and analyzed by student t -test. IL: Interleukin.

Peripheral surgical wounding did not induce cognitive impairment in aged IL-6 KO mice at 7 days after the procedure

Finally, we assessed the effects of the peripheral surgical wounding on cognitive function in aged IL-6 KO mice in FCS at 7 days after the surgery. The peripheral surgical wounding did not reduce the freezing time in the context test ($P = 0.6444 > 0.05$, student *t*-test; **Figure 4A**) nor

tone test ($P = 0.6467 > 0.05$, student *t*-test; **Figure 4B**) of FCS as compared to sham condition). These data showed that the peripheral surgical wounding might not be able to induce cognitive impairment in the aged IL-6 KO mice. Collectively, these results suggested that IL-6 should be one of the inflammatory cytokines required for the peripheral surgical wounding-induced cognitive impairment in aged mice.

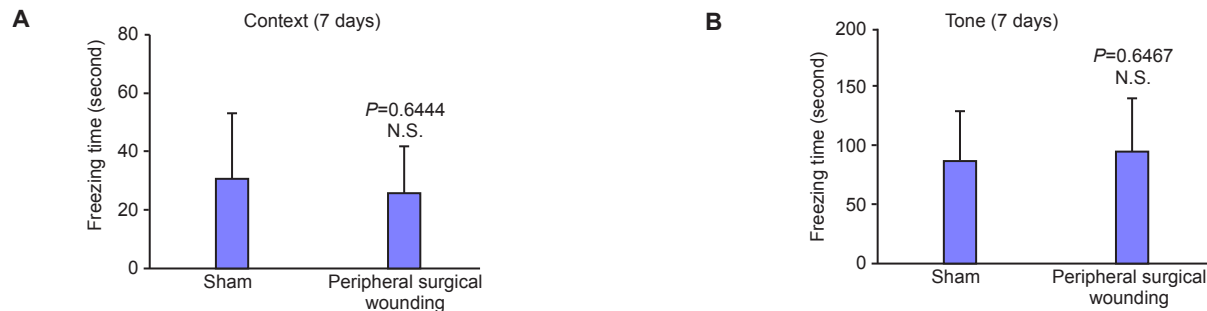


Figure 4: Peripheral surgical wounding did not induce cognitive impairment in aged IL-6 knockout (KO) mice.

Note: The peripheral surgical wounding without the influence of general anesthesia did not significantly alter the freezing time in the context test (A) and tone test (B) of the Fear Conditioning System at 7 days post-procedure in 18-month-old IL-6 KO mice. Data are expressed as the mean \pm SD, $n = 10$ in each group, and analyzed by two-way analysis of variance. IL: Interleukin.

DISCUSSION

Neuroinflammation may contribute to cognitive impairment in rodents following surgery under general anesthesia (Wan et al., 2007, 2010; Cibelli et al., 2010; Terrando et al., 2010a); reviewed in Terrando et al., 2011) or following the surgery without the influence of general anesthesia (under local anesthesia) (Xu et al., 2014a, b). Specifically, pro-inflammatory cytokine TNF- α has been reported as a required cytokine of the cognitive impairment induced by surgery under general anesthesia (Terrando et al., 2010a). We have reported that the peripheral surgical wounding (an abdominal surgery under local anesthesia) without the influence of general anesthesia can also induce neuroinflammation, as evidenced by elevation of the levels of IL-6, TNF- α , CD33 and Iba1 positive cells in the hippocampus of aged WT mice (Xu et al., 2014b). The peripheral surgical wounding also induces cognitive impairment in the aged WT mice (Xu et al., 2014a, b). Elevation of IL-6, one of the pro-inflammatory cytokines, is associated with cognitive impairment in animals and in humans (Braidia et al., 2004; Bjornsson et al., 2007; Katsumata et al., 2007; Huang et al., 2008; Schuitemaker et al., 2009; Cao et al., 2010; Hudetz et al., 2010; Patanella et al., 2010). We therefore investigated the role of IL-6 in the peripheral surgical wounding-induced cognitive impairment via both pharmacological (IL-6 antibody) and genetic (IL-6 KO mice) approaches.

In the aged WT mice, we found that the peripheral surgical wounding without the influence of the general anesthesia increased the IL-6 levels in the hippocampus of the mice

by quantitative western blot analysis, and induced cognitive impairment in the mice. Moreover, we found that IL-6 antibody was able to ameliorate the peripheral surgical wounding-induced cognitive impairment in the aged WT mice. In the IL-6 KO aged mice, we found that the peripheral surgical wounding induced neither elevation of IL-6 levels nor cognitive impairment. These results suggested that elevation of IL-6 might participate to the peripheral surgical wounding-induced cognitive impairment and demonstrated that IL-6 contributes to, at least partially, the underlying mechanism of the POCD, pending further investigations.

Our previous studies showed that peripheral surgical wounding without the influence of general anesthesia could induce A β accumulation, neuroinflammation and cognitive impairment in aged mice (Xu et al., 2014a, b). Our current findings suggested that elevation of IL-6 was required for the peripheral surgical wounding-induced cognitive impairment. Taken together, we postulate that IL-6 may interact with A β metabolism (*via* affecting the generation and degradation of A β), leading to cognitive impairment. The further studies to test this hypothesis are warranted in the future research.

The studies have several limitations. First, we did not measure the effects of IL-6 antibody on the levels of IL-6. However, the findings that the peripheral surgical wounding did not increase IL-6 levels and did not induce cognitive impairment in IL-6 KO mice supports the hypothesis that the peripheral surgical wounding may induce cognitive



impairment through an IL-6-dependent mechanism in aged mice. Second, it remains unknown whether the combination of surgery and general anesthesia still induces cognitive impairment through IL-6-dependent mechanism. Our future studies will systematically compare the effects of general anesthesia alone, surgery without general anesthesia and surgery under general anesthesia on IL-6 levels and cognitive function in both WT and IL-6 KO mice.

In conclusion, we showed that IL-6 antibody (in aged WT mice) and IL-6 KO (in aged IL-6 KO mice) could ameliorate the cognitive impairment induced by the peripheral surgical wounding without the influence of general anesthesia. These findings suggested that the peripheral surgical wounding might induce cognitive impairment through IL-6-dependent mechanisms in aged mice. These findings would promote more research to determine the neuropathogenesis and targeted interventions of POCD.

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Author contributions

ZX, YD, YZ, ZX conceived and designed the project. ZX, YD, and LH performed all the experiments, analyzed the data and prepared the figures. YD and ZX wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors deny conflict of interest.

Plagiarism check

This paper was screened twice using CrossCheck to verify originality before publication.

Peer review

This paper was double-blinded and stringently reviewed by international expert reviewers.

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