Original Article

Anti-HMGB1 Antibody Decreases Surgery-Induced Upregulation of Necroptosis-Associated Proteins in Aged Rats

Hongkang Zhou[1, 2, *], Shuibing Zhang[3, *], Yuan Le[4], Jufang Huang[1], Jianbin Tong[4], and Wen Ouyang[4]

From the [1]Department of Anatomy & Neurobiology, School of Basic Medical Science, [2]Postdoctoral Research Station of Basic Medical Science, Central South University, [3]Department of Anesthesiology, Hunan Children's Hospital, [4]Department of Anesthesiology, Third Xiangya Hospital, Central South University, all in Changsha, China.

*Contributed equally to this work.

Correspondence to Dr. Wen Ouyang at ouyangwen133@vip.sina.com.

ABSTRACT

Background: Systemic high mobility group box 1 protein (HMGB1) plays a pivotal role in mediating development and progression of postoperative cognitive dysfunction (POCD). However, the molecular mechanism on how systemic HMGB1 neutralization improves POCD is not fully elucidated. Necroptosis could cause sterile inflammation and negatively associates with the cognitive score in Alzheimer's disease. Thus we detected the effects of anti-HMGB1 antibody on the necroptosis-associated protein expressions during the POCD of aged rats.

Methods: Aged Sprague-Dawley rats (19-22 months old) were randomly assigned into three groups, (1) control with saline; (2) surgery + immunoglobulin G as control antibody; (3) surgery + HMGB1 neutralizing antibody. A partial hepatolobectomy under sevoflurane anesthesia and analgesia were performed. Immunoglobulin G (1 mg/kg) and HMGB1 neutralizing antibody (1 mg/kg) were injected via tail right before and 6 hours after surgery. The expression of necroptosis-associated proteins (HSP90, CDC37, and RIP3) in the prefrontal cortex of brain was detected by western blot and immunofluorescence. Oxidative stress was measured by dihydroethidium staining.

Results: Systemic administration of anti-HMGB1 antibody decreased the levels of reactive oxygen species (ROS) and reduced the expression of HSP90, CDC37, and RIP3 in prefrontal cortex neurons of brains after surgery ($P < 0.05$, respectively). Moreover, acetylated HSP90 (ACHSP90) which is a negative regulator of necroptosis was significantly decreased by treatments of anti-HMGB1 neutralization antibody ($P < 0.05$).

Conclusion: Systemic administration of anti-HMGB1 antibody may improve POCD through reducing reactive oxygen species and decreasing necroptosis in the prefrontal cortex of the aged brain. (Funded by the National Natural Science Foundation of China and Central South University Postdoctoral Foundation in Changsha, China.)

Postoperative cognitive dysfunction (POCD) usually happens in elderly patients following surgery, especially in patients under critical condition. Most of the elderly patients present impaired memory and damaged thinking skills (1). Up to 74% of elderly patients with critical illness experience POCD in early days following surgery and up to 34% of elderly patients still demonstrate symptoms of POCD one year after surgery (2). POCD is a disorder complicated with multifactors and its occurrence can be affected by multiple risk factors that may present before, during, and after surgery. Among these risk factors, the increased vulnerability of brain is an essential factor intends to induce POCD (3-7). Neuroinflammation is the major pathological mechanism (8, 9). Possession of POCD largely extended the length of hospital stay and cost more expenses for patients. Even after discharged from hospital life independence is significantly decreased. Combined with fast-growing geriatric population, these consequences of POCD put a heavy burden on patients, their families, and the society (10). Therefore, exploring mechanisms and developing treatments for POCD is one of the priorities in geriatric research (11).

High mobility group box 1 protein (HMGB1) which is a crucial member of damage-associated molecular pattern molecules (DAMPs), is quickly released after tissue trauma and plays an essential role in immune cell recruitment and activation, cytokine release, and cell death (12). HMGB1 had been identified as an important mediator for sterile inflammation (13). In previous research, we find out HMGB1 significantly elevated in the peripheral blood of elderly patients after gastrointestinal surgery and the elevated levels of HMGB1 were positively associated with increased level of human POCD (14). Furthermore, systemic neutralization of HMGB1 in peripheral blood of aged rats after surgery remarkably decreased the level of postoperative neurocognitive dysfunction (15). Meanwhile, Maze and colleagues find out single-dose injection of HMGB1 caused memory decline and blocking HMGB1 with monoclonal antibody before surgery reduced postoperative memory decline (16). These data revealed the pivotal role of systemic HMGB1 in mediating development and progression of POCD. However-er, the molecular mechanism on how systemic HMGB1 neutralization improves POCD is not fully elucidated.

Necroptosis is a common form of programmed cell death that often seen in ischemic injuries of the nervous system, heart, and kidney. The occurrence of necroptosis causes sterile inflammation (17, 18). The HSP90 / CDC37 / RIP3 signaling pathway is the classic pathway inducing necroptosis (19-24). A recent study has shown that necroptosis was activated in Alzheimer's disease and necroptosis activation inversely correlated with cognitive scores and increased cell loss (25). The prefrontal cortex is closely involved in cognition and memory. Herein, we assessed the effects of systemic HMGB1 neutralization on necroptosis pathway proteins (HSP90, CDC37, and RIP3) in prefrontal cortex of the brain and its role in postoperative cognitive dysfunction in aged rats after surgery. These effects of systemic HMGB1 neutralization may provide some new evidence for pathophysiological mechanisms of POCD.

Methods

Experimental Animals

All experiments performed in this study were in accordance with the Central South University Animal Care and Use Committee guidelines. The protocol [LLSC(LA)2015-003] was approved by the ethics committee of the Third Xiangya Hospital of Central South University. 19-22 months old (aged) female Sprague-Dawley (SD) rats (body weight 450-600 g) were used in this study (purchased from Central South University Department of Laboratory Animals in China). All animals were housed in a pathogen-free environment on a 12-hour light cycle at 25 °C with access to rodent chow and water. The relative humidity was 40%-60%. Cages and beddings were changed every other day to keep dry and clean. Rats had surgery in the diestrus phase when estrogen levels are at their minimum.

Drug Administration

Aged rats were randomized into 3 groups: (1) control with intravenous (i. v.) saline injection only; (2) surgery + immunoglobulin G as control antibody (S + IgG) (1 mg/kg, i. v.); (3) sur-

gery + HMGB1 neutralizing antibody (S + anti-HMGB1) (1 mg/kg, i. v.). For each group, five aged rats were used. Treatments were implemented through tail vein injection right before surgical incision and 6 hours after surgery; dosage and timing were previously described by Okuma et al (26). Mouse IgG2b (Sigma, M1395-5MG) was used as an isotype control. Anti-HMGB1 monoclonal antibody (2G7, mouse IgG2b) was kindly provided by Dr. H. E. Harris's laboratory, Stockholm, Sweden. The HMGB1 neutralizing efficacy and efficiency of Anti-HMGB1 monoclonal antibody have been extensively characterized in a series of in vitro and in vivo studies (26-32). The 2G7 anti-HMGB1 monoclonal antibody neutralizes both HMGB1-stimulated cytokine/chemokine releases and chemotactic activities.

Partial Hepatolobectomy

Rats were first rapidly anesthetized with 4.5% sevoflurane (Maruishi Pharmaceutical Co., Ltd., Japan) mixed with high flow of pure oxygen (6 L/min). When each individual rat lost sensation of right reflex, a 14G catheter was inserted into its trachea. 2%-2.5% sevoflurane were supplied with oxygen (80-85%) to maintain anesthesia and analgesia. The constant gas supplied was under surveillance by a multi-functional monitor (Datex-Ohmeda, Helsinki, Finland). Rat's vital sign parameters such as respiratory rate (R), Pet-CO_2, FiO_2, and FiSev were continuously recorded. The depth of anesthesia was modulated according to the R (30-50 cycles/min) and the body movement of the rats. The partial hepatolobectomy was carried out with strict aseptic procedures and performed as previously described with some modifications (33, 34). In brief, an incision about 2 cm long was made below the xyphoid; the left lobe of the liver was carefully isolated, ligated, and then removed. Finally, muscles and skin were closed with sterile sutures and 0.2 ml of 0.25% bupivacaine was subcutaneously administered to provide local postoperative analgesia. Animals were then allowed to recover for further investigation.

Immunofluorescence

For the immunofluorescence experiments, the localization and expression level of necroptosis associated proteins (HSP90, CDC37, and RIP3) were assessed on day 3 after surgery. Rats were euthanized with chloral hydrate (10%) and perfused transcardially with ice cold 0.01 M phosphate-buffered saline (PBS). The brain was dissected immediately following termination. One hemisphere was used for immunofluorescence and the other for western blot. The hemisphere used for immunofluorescence was immersed in 4% paraformaldehyde for fixation at 4 °C for 24 hours. The brains were dehydrated with 15% sucrose for one day and 30% sucrose for two days respectively and later embedded in OCT (Sakura, Tissue-Tek, USA). Cross-sections were consecutively cut at 20 μm of thickness in Leica cryostat (CM1860, Leica Biosystems Inc., IL, USA). Totally thirty sections were prepared. Three sections of prefrontal cortex were randomly picked from 3 sets of serial sections from each rat at + 1.70 mm to + 1.10 mm anteroposterior to the bregma for immunostaining. Sections were washed three times with 0.01 M PBS (10 min/each time). After three washes, sections were blocked with 5% bovine serum albumin (BSA) in 0.01 M PBS plus 0.3% Triton X-100 for 50 min at room temperature and then incubated with primary antibodies HSP90 (1: 100, Proteintech, Rosemont, IL, USA), Acetylated HSP90 (AC-HSP90, 1:100, Rockland Immunochemicals Inc., Limerick, PA, USA), CDC37 (1: 200, Cell Signaling Technology, Danvers, MA, USA), RIP3 (1: 500, Sigma-Aldrich, St. Louis, MO, USA), Neuron (1:300, Abcam, Cambridge, MA, USA), and GAPDH (1:300, Abcam, Cambridge, MA, USA) at 4 °C overnight. More details about the antibodies used are summarized in Table 1. Following three washes in 0.01 M PBS, the sections were incubated with secondary antibodies (Biotinylated Goat Anti-Rabbit IgG, 1:200, Vector, USA) for 2 hours at room temperature. Sections were finally washed three times with 0.01 M PBS and then covered with permount containing DAPI (Vector, USA, H-1200). For each stained section, pictures containing prefrontal cortex area of the brain were taken under the same magnification (40 X objective lens) by a fluorescent microscope (DS-Ril, Nikon, Japan). The mean fluorescence density of target proteins (AC-HSP90, CDC37, and RIP3) was measured and analyzed by using Image Pro-Plus

6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Western Blot

Western blot analysis was employed to further detect the expression of HSP90, CDC37, and RIP3 (necroptosis-associated proteins) in the prefrontal cortex of brain. In brief, frozen prefrontal cortex was homogenized in lysis buffer with protease inhibitors (cat. no. P8340; Roche, Germany) and nylmethanesulfonylfluoride (cat. no. p7626; PMSF, Sigma, USA). Protein samples were centrifuged at 14,000 g for 10 min at 4 °C and supernatant was collected. The protein concentration was quantified by using a BCA protein assay kit (CWBio, China) according to the manufacturer's instructions. Equal amount of protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene fluoride membranes. Membranes were blocked with 10% skim milk in TBST buffer for 1 hour and then incubated with primary antibodies, HSP90 (1:1000, Proteintech, USA), CDC37 (1:1000, Cell Signaling Technology, USA), RIP3 (1:1000, Sigma-Aldrich, USA), and GAPDH (1:2000, Proteintech, China) overnight at 4 °C (Table 1). The next day, membranes were washed by TBST for three times, and then secondary antibodies (cat. no. CW0102; goat anti-mouse IgG, HRP conjugated, 1:2000, CWBio, China or cat. no. CW0103; goat anti-rabbit IgG, HRP conjugated, 1:2000, CWBio, China) were added to the membranes and incubated at room temperature for 2 hours. After three washes by TBST, immunoblotted bans were visualized with ECL PlusTM Western Blotting Detection kit (GE Healthcare Life Sciences, NJ, USA). Densitometry analyses of western blot bands were determined by image J to quantify the relative protein levels of necroptosis associated proteins (HSP90, CDC37, and RIP3) versus GAPDH.

Measurement of Reactive Oxygen Species

The level of reactive oxygen species (ROS) was assessed by Dihydroethidium (DHE, cat. no. S0063; Beyotime, China). The DHE can be dehydrogenated by react with ROS and produce ethidium which presents red fluorescence in cells (Et + cells). The detailed procedures of ROS measurement by DHE were described previously (35). In brief, sections of prefrontal cortex were washed three times with 0.01 M PBS and blocked with 5% BSA in 0.01 M PBS plus 0.3% Triton X-100 for 50 min at room temperature. Then sections were incubated with anti-Neuron primary antibodies (1: 300, Abcam, Cambridge, MA, USA) at 4 °C overnight. Following three washes in 0.01 M PBS, the sections were incubated with DHE and secondary antibodies (Biotinylated Goat Anti-Rabbit IgG, 1: 200, Vector, USA) for 30 min at 37 °C. Sections were finally washed three times with 0.01 M PBS and then covered with permount containing DAPI (Vector, USA, H-1200). Pictures containing prefrontal cortex area of brain were taken under the same magnification (40 X objective lens) by a fluorescent microscope (DS-Ril, Nikon, Japan). The mean fluorescence density of Et + cells was measured and analyzed by using Image Pro-Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical Analysis

The data were expressed as mean ± S. E. M. (standard error of the mean). One-way ANOVA was used to analyze the data from immunofluorescence, western blot, and ROS measurements. Bonferroni multiple comparison test was performed to compare selected groups when ANOVA showed significance. Statistical analysis was conducted by using Prism 5 (Graph Pad Software Inc., La Jolla, CA, USA). A $P < 0.05$ was considered as significant.

Results

Expressions of necroptosis-associated proteins are decreased by anti-HMGB1 treatment

HSP90, CDC37, and RIP3 are classic necroptosis associated proteins and play critical roles in necroptosis. Prefrontal cortex is closely involved in cognition and memory. Thus we tried to detect the expressions of HSP90, AC-HSP90, CDC37, and RIP3 in prefrontal cortex by immunofluorescence labeling and western blot analysis.

The immunofluorescent density of AC-HSP90, CDC37, and RIP3 were measured on stained sections of prefrontal cortex, which presenting in situ expression level of these proteins.

Table 1. Antibodies Used in the Study.

| Antibody	Host/Clonality	Immunogen	Company/Category Number	Dilution
HSP90	Rabbit/Polyclonal	HSP90 fusion protein ag3826	Proteintech, 13171-1-AP	1:100
AC-HSP90	Rabbit/Polyclonal	Synthetic peptide corresponding to amino acids surrounding K294 of human HSP90	Rockland, 600-401-981	1:100
CDC37	Rabbit/Monoclonal	Synthetic peptide corresponding to residues surrounding Val297 of human CDC37	CST, 4793	1:200
RIP3	Rabbit/Polyclonal	A peptide corresponding to amino acids 473-486 of murine RIP3	Sigma-Aldrich, PRS2283	1:500
Neuron	Rabbit/Monoclonal	Synthetic peptide within human NeuN aa 1-100 (Cysteine residue)	Abcam, ab177487	1:300
GAPDH	Rabbit/Polyclonal	Full length native protein (purified) corresponding to human GAPDH	Abcam, ab9485	1:300
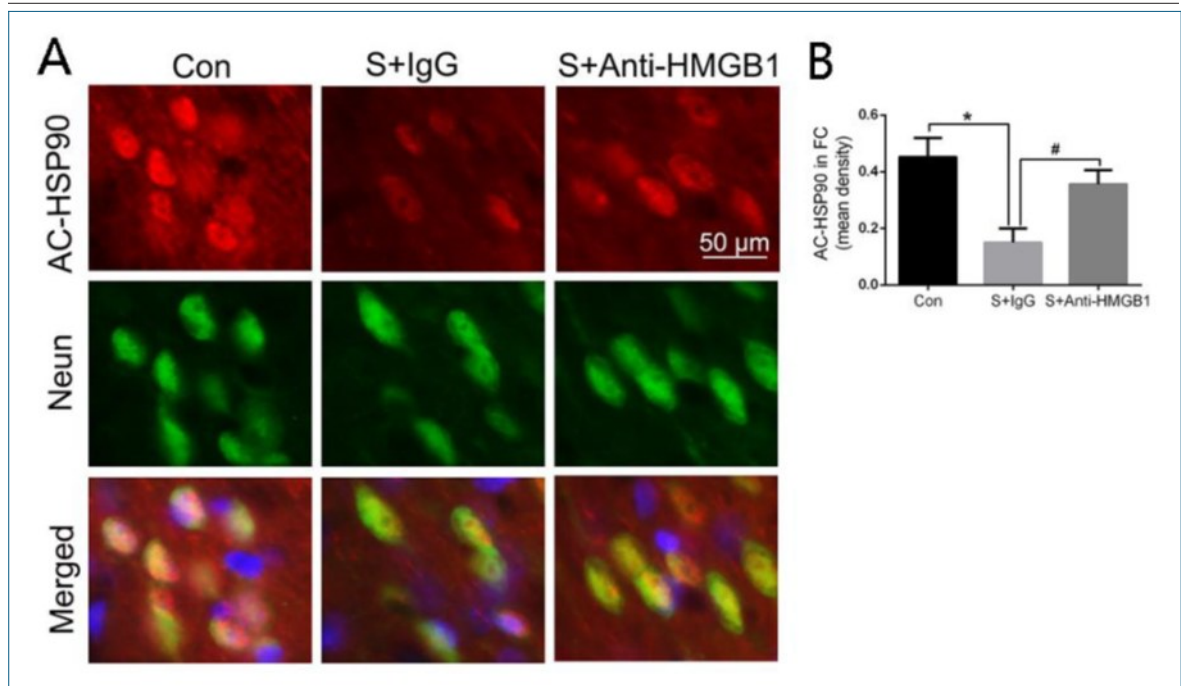
Figure 1. Anti-HMGB1 Treatment Increases Expression of Acetylated HSP90 (AC-HSP90) in Aged Rats After Surgery.
(A) The intensity of AC-HSP90 immunofluorescence (red) was used to show expression level of AC-HSP90 in the prefrontal cortex of the brain on postoperative day 3. Neurons (Neun) were stained with green fluorescence. Merged images of AC-HSP90 and Neun double labeling present blue fluorescence.
(B) The quantified mean density of AC-HSP90 fluorescence in prefrontal cortex (FC) shows distinctively reduced expression of AC-HSP90 in surgery + immunoglobulin G as control antibody group (S + IgG) and significantly elevated ACHSP90 expression level in anti-HMGB1 antibodies treated group (S + anti-HMGB1).
Results are presented as mean ± S.E.M. (n=5). * to normal control group (Con), *P < 0.05; # to S + IgG group, #P < 0.05; by one-way ANOVA. Scale bar = 50 μm.
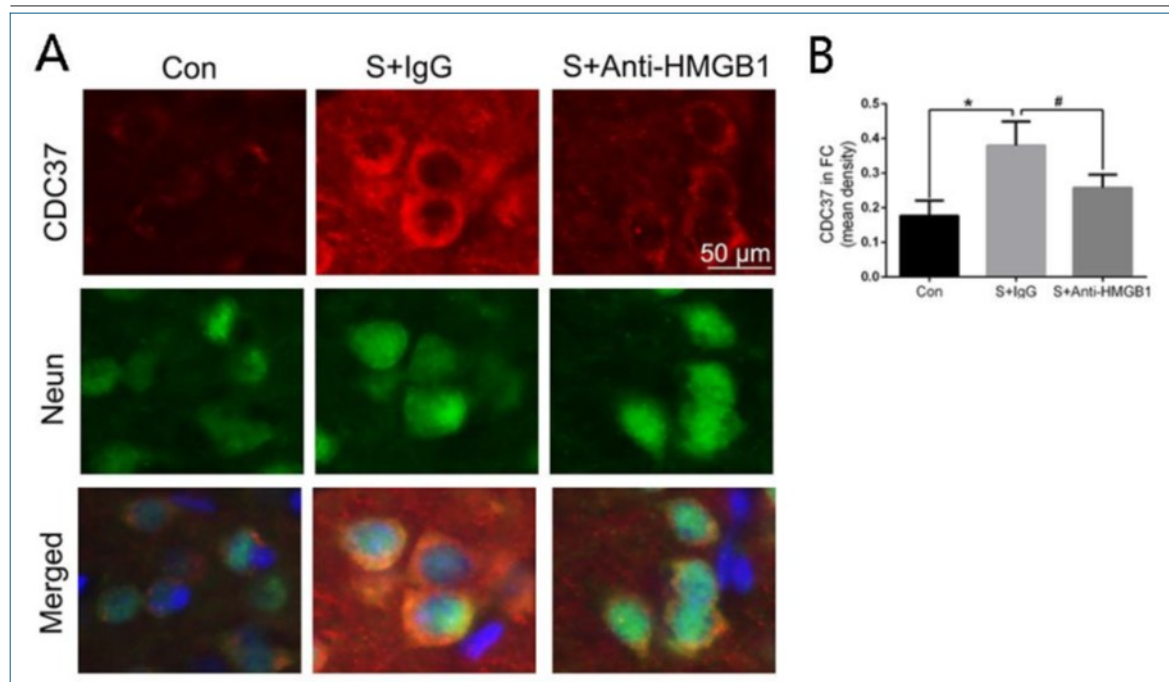
Figure 2. Anti-HMGB1 Treatment Decreases Expression of CDC37 in Aged Rats After Surgery.

(A) The intensity of CDC37 immunofluorescence (red) was used to show expression level of CDC37 in the prefrontal cortex of brain on postoperative day 3. Neurons (Neun) were stained with green fluorescence. Merged images of CDC37 and Neun double labeling present blue fluorescence.

(B) The quantified mean density of CDC37 fluorescence in the prefrontal cortex (FC) shows increased expression of CDC37 in surgery + immunoglobulin G as control antibody group (S + IgG) and significantly decreased CDC37 expression level in anti-HMGB1 antibodies treated group (S + anti-HMGB1).

Results are presented as mean ± S.E.M. (n=5). * to normal control group (Con), *$P < 0.05$; # to S + IgG group, #$P < 0.05$; by one-way ANOVA. Scale bar = 50 μm.

Compared to the control groups, the level of AC-HSP90 in S + IgG was distinctly decreased on day 3 after surgery ($P < 0.05$) (Figure 1A, B). Rats treated with anti-HMGB1 antibodies (S + anti-HMGB1 group) had significantly higher AC-HSP90 expression than the S + IgG group ($P < 0.05$) (Figure 1A, B). In addition, compared to corresponding control groups, the expression level of CDC37 (Figure 2A, B), and RIP3 (Figure 3A, B) were all significantly higher in S + IgG groups at day 3 after surgery ($P < 0.05$, respectively). Treatment with anti-HMGB1 antibodies all apparently decreased CDC37 (Figure 2A, B), and RIP3 (Figure 3A, B) expression at day 3 after surgery ($P < 0.05$, respectively).

We also had assessed HSP90, AC-HSP90, CDC37, and RIP3 expression in lysate of pre-frontal cortex of aged rat brains after surgery. Compared to their corresponding controls, Expression of HSP90 (Figure 4A), CDC37 (Figure 4B), and RIP3 (Figure 4C) were all increased in S + IgG groups at day 3 after surgery ($P < 0.05$, respectively). Furthermore, Systemic administration of anti-HMGB1 neutralizing antibodies significantly reduced the upregulation of HSP90 (Figure 4A), CDC37 (Figure 4B), and RIP3 (Figure 4C) in S + anti-HMGB1 groups on day 3 after surgery as compared to the corresponding S + IgG groups ($P < 0.05$, respectively). Acetylation of HSP90 has implicated an inhibitory role in necroptosis (19). Compared to control, the acetylated HSP90 (AC-HSP90) was obviously less in S + IgG group on day 3 after surgery ($P < 0.05$) (Figure 4A), indicating an increased level of necroptosis. However, the anti-HMGB1
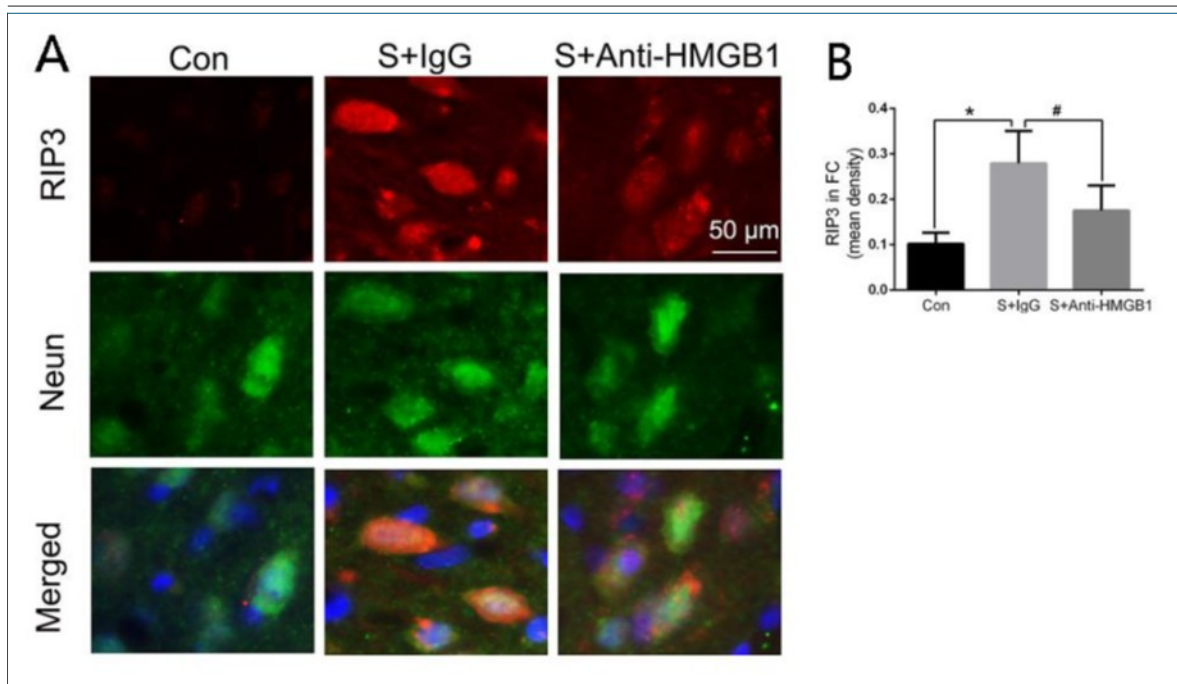
Figure 3. Anti-HMGB1 Treatment Decreases Expression of RIP3 in Aged Rats After Surgery
(A) The intensity of RIP3 immunofluorescence (red) was used to show expression level of RIP3 in the prefrontal cortex of brain on postoperative day 3. Neurons (Neun) were stained with green fluorescence. Merged images of RIP3 and Neun double labeling present blue fluorescence.
(B) The quantified mean density of RIP3 fluorescence in prefrontal cortex (FC) shows increased expression of RIP3 in surgery + immunoglobulin G as control antibody group (S + IgG) and significantly decreased RIP3 expression level in anti-HMGB1 antibodies treated group (S + anti-HMGB1).
Results are presented as mean ± S.E.M. (n=5). * to normal control group (Con), *$P < 0.05$; # to S + IgG group, # $P < 0.05$; by one-way ANOVA. Scale bar = 50 μm.

antibodies significantly increased the AC-HSP90 level in S + anti-HMGB1 group ($P < 0.05$) (Figure 4A). Combined with immunofluorescence data of necroptosis-associated proteins, these results demonstrated a protective mechanism of anti-HMGB1 neutralizing antibodies in necroptosis.

Anti-HMGB1 treatment decreases oxidative stress in the prefrontal cortex of brain after surgery
In order to further investigate the mechanisms of systemic HMGB1 neutralization improving the postoperative neurocognitive decline, the reactive oxygen species (ROS) levels of prefrontal the cortex of aged rat brains after surgery were measured. Compared to the control group, the ROS level of S + IgG group was significantly elevated on day 3 after surgery, showing an higher-than-normal oxidative stress after surgery ($P <$ 0.05) (Figure 5A, B). Notably, Compared to the S + IgG group, the systemic HMGB1 neutralization significantly decreased the ROS level in S + anti-HMGB1 group ($P < 0.05$) (Figure 5A, B). Moreover, the neurons of prefrontal cortex were labeled by immunofluorescence (anti-Neuron primary antibodies). The merged images of DHE and neurons double immunofluorescence labeling indicated that the changes of ROS levels mainly happened in the neurons of prefrontal cortex (Figure 5A).

Discussion

In this study, we investigated the impact of anti-HMGB1 neutralizing antibodies on necroptosis associated proteins of brain prefrontal cortex in aged rats after liver surgery. Our data demonstrate that systemic administration of anti-
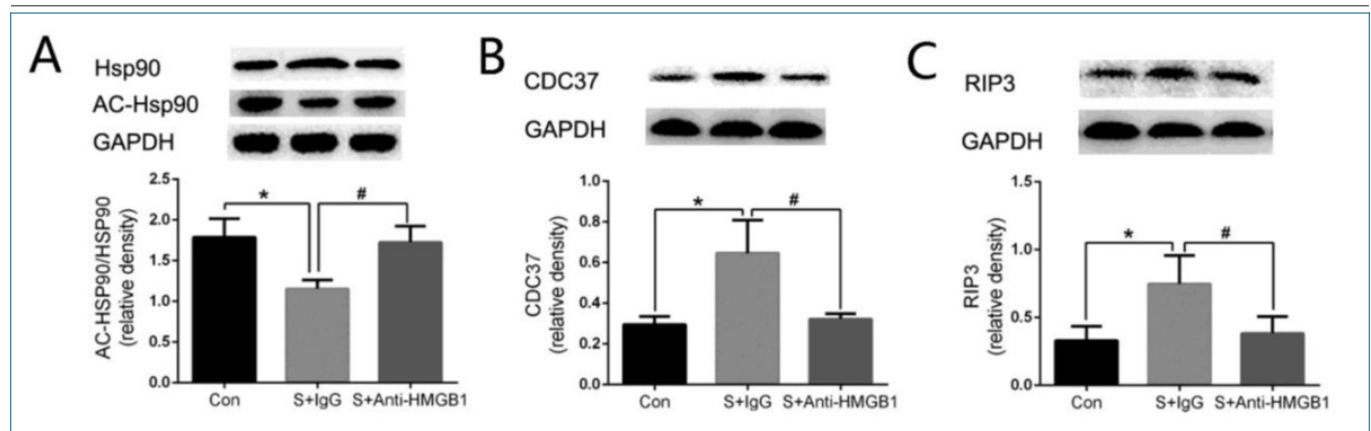
Figure 4. Anti-HMGB1 Treatment Decreases Expression of Necroptosis-Associated Proteins in Aged Rats After Surgery.
(A) The intensity of RIP3 immunofluorescence (red) was used to show expression level of RIP3 in the prefrontal cortex of brain on postoperative day 3. Neurons (Neun) were stained with green fluorescence. Merged images of RIP3 and Neun double labeling present blue fluorescence.
(B) The quantified mean density of RIP3 fluorescence in prefrontal cortex (FC) shows increased expression of RIP3 in surgery + immunoglobulin G as control antibody group (S + IgG) and significantly decreased RIP3 expression level in anti-HMGB1 antibodies treated group (S + anti-HMGB1).
Results are presented as mean ± S.E.M. (n=5). * to normal control group (Con), *P < 0.05; # to S + IgG group, # P < 0.05; by one-way ANOVA. Scale bar = 50 μm.

HMGB1 antibodies significantly limited surgery-induced up-regulation of HSP90, CDC37, and RIP3 expressions in prefrontal cortex of brain. Meanwhile, oxidative stress in prefrontal cortex of brain also distinctively reduced by anti-HMGB1 treatment in aged rats after surgery. These results indicate that systemic HMGB1 neutralization may provide a protective mechanism of postoperative cognitive dysfunction through reducing ROS level and necroptosis.

The high mobility group box (HMGB) protein family is the most represented protein family among the high mobility group proteins (36). There are four members in the HMGB protein family (HMGB1-4). HMGB1 is the most important member of HMGB protein family. As a discovered nuclear protein, HMGB1 has played a critical role in transcription, replication, DNA repair, recombination, nucleosome assembly, and genomic stability (37, 38). Besides its nuclear function, HMGB1 involves in an essential extracellular activity; that is, HMGB1 belongs to the damage-associated molecular pattern molecules (DAMPs), which are released from damaged cells, causing immunoresponses locally and

systemically (39). Surgical trauma-induced tissue damage leads to a rapid release of HMGB1 and increased systemic levels of HMGB1 have been detected in elderly patients after gastrointestinal surgery. Interestingly, these levels correlated with the development of POCD (14, 16), suggesting that the release of HMGB1 may be critical in initiating inflammatory cascade leading to brain dysfunction. Furthermore, HMGB1 release after surgery has been characterized in some preclinical models of cognitive decline (27, 40, 41).

Increased levels of HMGB1 after surgery have been evidenced to compromising the blood-brain barrier (BBB) and subsequently lead to neuroinflammation (42, 43). The mechanism whereby peripheral HMGB1-induced inflammation contributes to neuroinflammation and cognitive dysfunction had been partially revealed in our previous study. Increased systemic HMGB1 proteins were able to access the brain and causing microglial activation. In the meantime, phosphorylated RREB which is critical in memory and synaptic plasticity of hippocampus was significantly decreased 3 days after surgery. Treat-
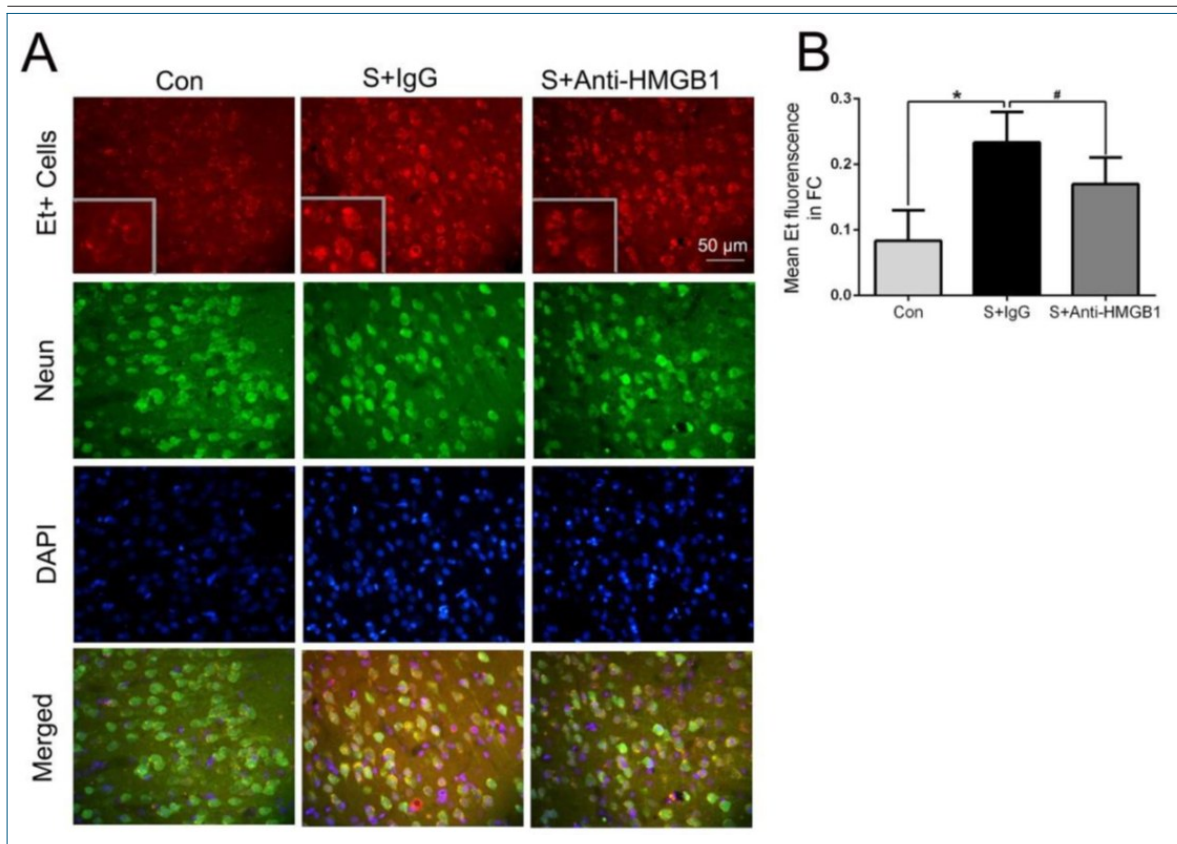
Figure 5. Anti-HMGB1 Treatment Decreases Oxidative Stress in Aged Rats After Surgery.
(A) The immunofluorescence intensity of Et+ cell (red) in prefrontal cortex (FC) of brain on postoperative day 3 was used to show the level of reactive oxygen species (ROS). Neurons (Neun) were stained with green fluorescence and DAPI stained nuclei with blue fluorescence. Merged images of Et+ cells and Neun double labeling present purple fluorescence.
(B) The quantification of mean Et+ fluorescence shows increased ROS level in surgery + immunoglobulin G as control antibody group (S + IgG) and reduced ROS in anti-HMGB1 antibodies treated group (S + anti-HMGB1).
Results are presented as mean ± S.E.M. (n=5). * to normal control group (Con), *P < 0.05; # to S + IgG group, #P < 0.05; by one-way ANOVA. Scale bar = 50 μm.

ment with anti-HMGB1 antibodies reversed these two impairments in the hippocampus and exerted neuroprotective effects in aged rats after surgery (15). Moreover, increased hippocampal NR2A and NR2B expression had been found in aged rats after surgery. This may relate to acute neurotoxicity (15). Increased neurotoxicity usually causes elevated levels of oxidative stress and potentiates neuronal death (44). In current study, we found significantly increased ROS levels in prefrontal cortex of aged rat brains after liver surgery. Systemic anti-HMGB1 antibody treatment reduced this elevated levels of oxida-tive stress (Figure 5). Increased oxidative stress can induce necroptosis and necroptosis closely relates to sterile inflammation (45, 46). HSP90/CDC37/RIP3 pathway plays a critical role in the process of necroptosis (21-24). Acetylation of HSP90 down-regulates the formation of HSP90-CDC37 complexes and further reduces RIP3 activation and MLKL translocation, which suppresses cell necroptosis (19-24). In the present study, the expression of HSP90, CDC37, and RIP3 were upregulated in aged rats after surgery, treatment with anti-HMGB1 antibodies significantly reduced these expressions (Figure 2-4).

The important discovery of present study is that the acetylated HSP90 (AC-HSP90) was reduced by anti-HMGB1 antibody treatment (Figure 1 and 4A).

Postoperative cognitive dysfunction (POCD) is a multifactorial disorder prominently found in elderly patients after surgery. Previous research showed the ccurrences of POCD were related to elevated levels of oxidative stress and neuroinflammation, phosphorylation of Tau proteins, and loss of NMDA receptors (47). In previous study, our data suggest systemic HMGB1 neutralization improves surgery-induced neurocognitive dysfunction (15) and this process may function through regulating the expression of necroptosis-associated proteins and reducing levels of oxidative stress. These findings provide new evidence for neuroprotective mechanisms of anti-HMGB1 antibody treatment which may be beneficial for discovering defined mechanisms and treatments of POCD.

References

1. Ramaiah R, Lam AM. Postoperative cognitive dysfunction in the elderly. *Anesthesiol Clin* 2017;27:485-96.
2. Pandharipande PP, Girard TD, Jackson JC, Morandi A, Thompson JL, Pun BT, et al. Long-term cognitive impairment after critical illness. *N Engl J Med* 2013;369:1306-16.
3. Shoair OA, Grasso Ii MP, Lahaye LA, Daniel R, Biddle CJ, Slattum PW. Incidence and risk factors for postoperative cognitive dysfunction in older adults undergoing major noncardiac surgery: A prospective study. *J Anaesthesiol Clin Pharmacol* 2015;31:30-36.
4. Silbert B, Evered L, Scott DA, McMahon S, Choong P, Ames D, et al. Preexisting cognitive impairment is associated with postoperative cognitive dysfunction after hip joint replacement surgery. *Anesthesiology* 2015;122:1224-34.
5. Evered L, Silbert B, Scott DA. Pre-existing cognitive impairment and post-operative cognitive dysfunction: should we be talking the same language? *Int Psychogeriatr* 2016;28:1053-5.
6. Nemeth E, Vig K, Racz K, Koritsanszky KB, Ronkay KI, Hamvas FP, et al. Influence of the postoperative inflammatory response on cognitive decline in elderly patients undergoing on-pump cardiac surgery: a controlled, prospective observational study. *BMC Anesthesiol* 2017;17:113.
7. Deiner S, Luo X, Lin HM, Sessler DI, Saager L, Sieber FE, et al. Intraoperative infusion of dexmedetomidine for prevention of postoperative delirium and cognitive dysfunction in elderly patients undergoing major elective noncardiac surgery: a randomized clinical trial. *JAMA Surgery* 2017;152:e171505.
8. Culley DJ, Crosby G. Prehabilitation for prevention of postoperative cognitive dysfunction? *Anesthesiology* 2015;123:7-9.
9. Hovens IB, van Leeuwen BL, Mariani MA, Kraneveld AD, Schoemaker RG. Postoperative cognitive dysfunction and neuroinflammation; cardiac surgery and abdominal surgery are not the same. *Brain Behav Immun* 2016;54:178-93.
10. Vutskits L, Xie Z. Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nat Rev Neurosci* 2016;17:705-17.
11. Inouye SK, Westendorp RG, Saczynski JS. Delirium in elderly people. *Lancet* 2014;383:911-22.
12. Kang R, Chen R, Zhang Q, Hou W, Wu S, Cao L, et al. HMGB1 in health and disease. *Mol Aspects Med* 2014;40:1-116.
13. Tsung A, Tohme S, Billiar TR. High-mobility group box-1 in sterile inflammation. *J Intern Med* 2014;276:425-43.
14. Lin GX, Wang T, Chen MH, Hu ZH, Ouyang W. Serum high-mobility group box 1 protein correlates with cognitive decline after gastrointestinal surgery. *Acta Anaesthesiol Scand* 2014;58:668-74.
15. Terrando N, Yang T, Wang X, Fang J, Cao M, Andersson U, et al. Systemic HMGB1 neutralization prevents postoperative neurocognitive dysfunction in aged rats. *Front Immunol* 2016;7:441.
16. Vacas S, Degos V, Tracey KJ, Maze M. High-mobility group box 1 protein initiates postoperative cognitive decline by engaging bone marrow-derived macrophages. *Anesthesiology* 2014;120:1160-67.
17. Kearney CJ, Martin SJ. An inflammatory perspective on necroptosis. *Mol Cell* 2017;65:965-73.
18. Pyo YH, Seong KS. Effects of Monascus-fermented grain extracts on plasma antioxidant status and tissue levels of ubiquinones and α-tocopherol in hyperlipidemic rats. *Food Chem* 2013;141:428-35.
19. Li D, Xu T, Cao Y, Wang H, Li L, Chen S, et al. A cytosolic heat shock protein 90 and cochaperone CDC37 complex is required for RIP3 activation during necroptosis. *Proc Natl Acad Sci U S A* 2015;112:5017-22.
20. Li D, Li C, Li L, Chen S, Wang L, Li Q, et al. Natural product kongensin A is a noncanonical HSP90 inhibitor that blocks RIP3-dependent necroptosis. *Cell Chem Biol* 2016;23:257-66.
21. Jacobsen AV, Silke J. The importance of being chaperoned: HSP90 and necroptosis. *Cell Chem Biol* 2016;23:205-7.
22. Kim SK, Kim WJ, Yoon JH, Ji JH, Morgan MJ, Cho H, et al. Upregulated RIP3 expression potentiates MLKL phosphorylation-mediated programmed necrosis in toxic epidermal necrolysis. *J Invest Dermatol* 2015;135:2021-30.
23. Jacobsen AV, Lowes KN, Tanzer MC, Lucet IS, Hildebrand JM, Petrie EJ, et al. HSP90 activity is required for MLKL oligomerisation and membrane translocation and the induction of necroptotic cell death. *Cell Death Dis* 2016;7:e2051.
24. Zhao XM, Chen Z, Zhao JB, Zhang PP, Pu YF, Jiang SH, et al. HSP90 modulates the stability of MLKL and is required for TNF-induced necroptosis. *Cell Death Dis* 2016;7:e2089.
25. Caccamo A, Branca C, Piras IS, Ferreira E, Huentelman MJ, Liang WS, et al. Necroptosis activation in Alzheimer's disease. *Nat Neurosci* 2017;20:1236-46.
26. Okuma Y, Liu K, Wake H, Zhang J, Maruo T, Date I, et al. Anti-high mobility group box-1 antibody therapy for traumatic brain injury. *Ann Neurol* 2012;72:373-84.
27. Chavan SS, Huerta PT, Robbiati S, Valdes-Ferrer SI, Ochani M, Dancho M, et al. HMGB1 mediates cognitive impairment in sepsis survivors. *Mol Med* 2012;18:930-37.
28. Qin S, Wang H, Yuan R, Li H, Ochani M, Ochani K, et al. Role of HMGB1 in apoptosis-mediated sepsis lethality. *J Exp Med* 2006;203:1637-42.
29. Wahamaa H, Vallerskog T, Qin S, Lunderius C, LaRosa G, Andersson U, et al. HMGB1-secreting capacity of multiple cell lineages revealed by a novel HMGB1 ELISPOT assay. *J Leukoc Biol* 2007;81:129-36.
30. Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, et al. A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci U S A* 2010;107:11942-7.
31. Ruan X, Darwiche SS, Cai C, Scott MJ, Pape HC, Billiar TR. Anti-HMGB1 monoclonal antibody ameliorates immunosuppression after peripheral tissue trauma: attenuated Tlymphocyte response and increased splenic CD11b (+) Gr-1 (+) myeloid-derived suppressor cells require HMGB1. *Mediators Inflamm* 2015;2015:458626.
32. Valdes-Ferrer SI, Papoin J, Dancho ME, Olofsson P, Li J, Lipton JM, et al. HMGB1 mediates anemia of inflammation in murine sepsis survivors. *Mol Med* 2015;21:951-8.
33. Wan Y, Xu J, Meng F, Bao Y, Ge Y, Lobo N, et al. Cognitive decline following major surgery is associated with gliosis, beta-amyloid accumulation, and tau phosphorylation in old mice. *Crit Care Med* 2010;38:2190-8.
34. Huang C, Zhou H, Tong J, Chen H, Liu YJ, Wang D, et al. FUS transgenic rats develop the phenotypes of amyotrophic lateral sclerosis and frontotemporal lobar degeneration. *PLoS Genet* 2011; 7:e1002011.
35. Kong SS, Liu JJ, Yu XJ, Lu Y, Zang WJ. Protection against ischemia-induced oxidative stress conferred by vagal stimulation in the rat heart: involvement of the AMPK-PKC pathway. *Int J Mol Sci* 2012;13:14311-25.
36. Goodwin GH, Johns EW. Are the high mobility group non-histone chromosomal proteins associated with 'active' chromatin? *Biochim Biophys Acta* 1978;519:279-84.

37. Agresti A, Bianchi ME. HMGB proteins and gene expression. Curr Opin Genet & Dev 2003;13:170-8.

38. Bonaldi T, Längst G, Strohner R, Becker PB, Bianchi ME. The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. *EMBO J* 2002;21:6865-73.

39. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol* 2005;5:331-42.

40. Terrando N, Rei Fidalgo A, Vizcaychipi M, Cibelli M, Ma D, Monaco C, et al. The impact of IL-1 modulation on the development of lipopolysaccharide-induced cognitive dysfunction. *Crit Care* 2010; 14:R88.

41. Mazarati A, Maroso M, Iori V, Vezzani A, Carli M. High-mobility group box-1 impairs memory in mice through both toll-like receptor 4 and receptor for advanced glycation end products. *Exp Neurol* 2011;232:143-8.

42. Terrando N, Monaco C, Ma D, Foxwell BM, Feldmann M, Maze M. Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline. *Proc Natl Acad Sci U S A* 2010; 107: 20518-22.

43. He HJ, Wang Y, Le Y, Duan KM, Yan XB, Liao Q, et al. Surgery upregulates high mobility group box-1 and disrupts the blood-brain barrier causing cognitive dysfunction in aged rats. *CNS Neurosci Ther* 2012;18: 994-1002.

44. Sayre LM, Perry G, Smith MA. Oxidative stress and neurotoxicity. *Chem Res Toxicol* 2008;21:172-88.

45. Liang W, Chen M, Zheng D, Li J, Song M, Zhang W, et al. The opening of ATP-sensitive K+ channels protects H9c2 cardiac cells against the high glucose-induced injury and inflammation by inhibiting the ROS-TLR4-Necroptosis pathway. *Cell Physiol Biochem* 2017;41:1020-34.

46. Ettinger M, Calliess T, Kielstein JT, Sibai J, Brückner T, Lichtinghagen R, et al. Circulating biomarkers for discrimination between aseptic joint failure, low-grade infection, and high-grade septic failure. *Clin Infect Dis* 2015;61:332-41.

47. Vutskits L, Xie Z. Lasting impact of general anaesthesia on the brain: mechanisms and relevance. *Nat Rev Neurosci* 2016;17:705-17.