Effect of dexmedetomidine combined with etomidate on IL-17A and S-100β expression levels in rats with postoperative cognitive dysfunction

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Abstract. The present study aimed to explore the effects of dexmedetomidine combined with etomidate on the expression levels of interleukin (IL)-17A and S-100β in rats with postoperative cognitive dysfunction (POCD). A total of 50 SD rats were randomly allocated into the control group, model group, etomidate group (Eto group), dexmedetomidine group (Dex group) and dexmedetomidine combined with etomidate group (Dex-Eto group). Inhalation anesthesia was used in all five groups. Apart from the control group, partial lobectomy was performed to construct a rat model of cognitive dysfunction. The rats of the model group received no intravenous anesthesia, except general anesthesia with intubation. Morris water maze test was performed before injection (T1), at the 1st day (T2), the 3rd day (T3) and the 5th day (T5) after operation to assess the memory ability of the rats. At the end of T5, the expression levels of IL-17A, S-100β, TNF-α, IL-6 and IL-1β in serum were detected by ELISA and the expression of NF-κB p65 by western blot analysis. Compared with the control group, the model group showed an increased escape latency and swimming distance, decreased number of times of crossing the platform and target quadrant residence time, and increased expression levels of IL-17A, S-100β, TNF-α, IL-6, IL-1β and NF-κB p65. Compared with the model group, the escape latency and swimming distance in the Dex, Eto and Dex-Eto groups were reduced, whereas the number of times of crossing the platform and the target quadrant residence time were increased. In addition, the expression levels of IL-17A, S-100β, TNF-α, IL-6, IL-1β and NF-κB p65 were decreased in the Dex, Eto and Dex-Eto groups, compared with the model group. Among the Dex, Eto and Dex-Eto groups, the escape latency and swimming distance in the Dex-Eto group were the shortest, the number of times of crossing the platform and the target quadrant residence time were the highest, and IL-17A, S-100β, TNF-α, IL-6, IL-1β and NF-κB p65 expression levels were the lowest. In conclusion, dexmedetomidine combined with etomidate can effectively improve POCD.

Introduction

Postoperative cognitive dysfunction (POCD) is one of the most common complications in patients undergoing general anesthesia (1), whose clinical manifestations are usually progressive weakening of cognitive function, decline in self-care ability, increased length of hospitalization and delayed recovery (2). POCD may also develop postoperative delirium with a risk of long-term cognitive impairment (3). However, the pathogenesis and effective treatment for POCD remain unclear (4). Previous studies have shown that the expression levels of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6 and TNF-α, are increased in patients with POCD (5-7). Therefore, systemic inflammation induced by surgery may be the cause of POCD (8). IL-17A is the first member of the IL-17 protein family, which can induce the secretion of pro-inflammatory cytokines to exacerbate inflammatory response (9). S-100β protein is secreted by astrocytes in the central nervous system (CNS) (10). The high concentration of S-100β in the serum suggests the damage of the brain and therefore can be used as a biomarker to assess the severity of brain damage (11).

Dexmedetomidine is a highly selective α2 adrenergic receptor with dose-dependent sedative and hypnotic effects (12). Dexmedetomidine binds to the nicotinic acetylcholine receptor 7 subunit to promote the release of acetylcholine, thereby suppressing the secretion of inflammatory cytokines (13,14). Dexmedetomidine has been reported to exert neuroprotective effects in early POCD animal models (2), thereby preventing POCD (15). It has been shown (16) that dexmedetomidine could improve the neuronal apoptosis caused by isoflurane in neonatal rats. In addition, Etomidate is a type of drug used for general anesthesia and sedation (17), which has the advantages of quick effect, short acting time and little effect on circulation.
According to Liu et al (18), etomidate inhibited the production of pro-inflammatory cytokines in rat macrophages.

In the present study, a POCD rat model was constructed by partial hepatectomy in aged rats to observe the effect of dexmedetomidine combined with etomidate on the expression levels of IL-17A and S-100β, and evaluate the therapeutic effect of dexmedetomidine combined with etomidate, in order to provide future reference for clinical application.

Materials and methods

Main reagents and animals. Dexmedetomidine hydrochloride injection was purchased from Sichuan Guorui Pharmaceutical Co., Ltd. (approval no. H20110097; 2 ml/0.2 mg). Etomidate fat emulsion injection was purchased from Jiangsu Nhwa Pharmaceutical Co., Ltd. (approval no. H20020511; 10 ml/20 mg). Rat IL-17A (ELISA) kit was purchased from Abcam (item no. ab214028). Rat S-100β protein (ELISA) kit was purchased from Abcam (item no. ab234573). TNF-α, IL-6 and IL-1β (ELISA) kits were all purchased from Abcam (items no. ab236712, ab234570, ab255730). Rat tissue protein extract was purchased from Best Biotechnology Co., Ltd. (batch no. BB1801). BCA protein assay kit was purchased from Beyotime Institute of Biotechnology (batch no. P0012). NF-κB Pathway Sampler kit, goat anti-mouse IgG (H+L) and β-actin were purchased from Shanghai Abcam. Morris water maze system was purchased from Beijing Zhongshi Dichuang Science and Technology Development Co., Ltd.

A total of 50 healthy male SD rats, 19-23 months of age and weighing 510-695 g, were acquired from Hunan Sliaike Jingda Experimental Animal Co., Ltd. The study was approved by the Ethics Committee of Jiangxi Provincial People’s Hospital Affiliated to Nanchang University (Nanchang, China) and all procedures were carried out in strict accordance to the Guidelines of the Nursing and Use of Laboratory Animals (published by the National Institutes of Health and revised in 1996; no. 85-23) (19). It was confirmed that there was no obvious abnormal behavior in all the rats enrolled and the rats were randomly allocated to the control group, model group, etomidate group (Eto group), dexmedetomidine group (Dex group) and dexmedetomidine combined with etomidate group (Dex-Eto group), with 10 rats in each group.

POCD model construction. All rats were maintained at 22-23°C, with a 12-h light/12-h dark cycle. All rats were fasted overnight before surgery with free access to water. After anesthesia with 2% sevoflurane, the rats in the five groups were ventilated and intubated with 1.5-2% sevoflurane continuously. Dose conversion calculations between laboratory animals and humans were based on the FDA recommendation to use surface area standardization (20). Since the surface area per unit body area of rats is ~6 times that of a human body, the commonly used regimen for normal clinical dexmedetomidine sedation is load dose 1.0 g/kg (for ≥10 min), and thus, the rats were given 6.0 µg/kg (for ≥10 min); etomidate induced dose is 0.2 mg/kg, so the rats were given 1.2 mg/kg. The rats of the model group received no intravenous anesthesia, except general anesthesia with intubation, and no treatment was given in the control group. In the Dex group, 6 µg/kg dexmedetomidine were injected intravenously for 10 min. In the Eto group, 1.2 mg/kg etomidate were injected intravenously for 10 min. In the Dex-Eto group, 6 µg/kg dexmedetomidine with 1.2 mg/kg etomidate were injected intravenously for 10 min. Except for the control group, partial lobectomy was performed after 30 min in the rest of the four groups in order to construct the rat POCD model. The procedures were as follows: the rats were placed on a sterile pad. After sterilizing and removing part of the hair, the rats were subjected to longitudinal incision along the lower edge of the xiphoid process. The left lobe of the liver was separated, and part of the left lobe of the liver was removed. The incision was then soaked with 2% lidocaine and then sutured with thread so that the surgical procedure should be aseptically controlled. All these procedures should be completed within 30 min. Ventilation and appropriate temperature should be maintained at the end of the operation. After the operation, the rats were placed in the previous feeding room and fed separately, and anti-infection measures were taken.

Morris water maze test. Cognitive evaluation was performed using Morris water maze system 24 h after anesthesia. Morris water maze was a cylindrical pool, which was separated into four quadrants, all containing water. A quadrant platform was selected randomly.

Place navigation test: one day before the experiment, the aged rats became familiar with the environment in Morris system, so that they could learn to swim for a platform within 120 sec. The rats that did not find the platform in 120 sec were guided to the platform to stay for 30 sec. Only rats that could swim were included in this experiment. Morris water maze test was carried out 24 h after anesthesia. The aged rats were randomly placed into water with their backs to the wall of the pool. The time spent in finding a fixed platform is called escape latency, and the escape latency and swimming distance of the rats were recorded. If the platform was not found within 2 min, it was recorded as 120 sec.

Spatial probe test: the platform was removed from the water and the rats were placed in the water from any point in the corresponding quadrant of the platform area. The target quadrant residence time and the number of times of accurately crossing the platform within 2 min were recorded. The poorer the cognitive function, the longer the escape latency and the swimming distance, the less target quadrant residence time and times of crossing the platform.

ELISA detection. At the end of T3, tail vein blood samples (1 ml) of the rats in all five groups were collected and placed into anticoagulant tubes. The samples were centrifuged at 4°C at 3x10³ rpm for 30 min to collect the supernatant. The expression levels of IL-17A, S-100β, TNF-α, IL-6 and IL-1β in the supernatant were detected by the corresponding kit. All operations were strictly carried out according to the manufacturer’s instructions of the respective kit.

Detection of NF-κB p65 protein expression by western blot analysis. At the end of T3, pentobarbital sodium (150 mg/kg body weight) was injected intraperitoneally until breathing and heartbeat stopped. The right hippocampus tissues (20 mg) were isolated from the rats, mixed with 100-200 µl lysates and homogenized in a glass homogenator at 4°C for 15 min, 1.2x10⁴...
rpm. The supernatant was taken. We used the BCA kit (Abcam) to detect protein concentration. SDS-PAGE electrophoresis was used to distinguish the proteins before transferring to the nitrocellulose membrane, and then the proteins were blocked at room temperature for 1 h with 5% PBS solution. Next, NF-κB p65 (1:1,000) was added and maintained overnight at 4˚C. Membranes were washed with PBS solution. This operation was repeated 3 times before a secondary antibody (HRP cross-linking, 1:10,000) was added, and the mixture was allowed to stand at room temperature for 1 h. The membrane was finally washed with PBS solution and protein bands were visualized using an electrochemiluminescent substrate kit (cat. no. ab133406; Abcam). The internal reference protein was β-actin. The relative protein expression of NF-κB p65 was calculated: Relative expression level of protein=(gray value of protein band)/(gray value of β-actin band). We used ImageJ (National Institutes of Health) to measure the gray value.

Statistical analysis. The experimental data were processed by SPSS 20.0 software package (AsiaAnalytics; formerly SPSS China) for statistical analysis. The measurement data were expressed as the mean ± SD and one-way analysis of variance with Tukey's HSD post hoc test were used for their comparison. Pearson's correlation analysis was used to study the correlation of IL-17A and S-100β with NF-κB p65, TNF-α, IL-6 and IL-1β. The confidence interval was 95%. P<0.05 was considered to indicate a statistically significant difference.

Results

Place navigation test. In order to investigate the effect of dexmedetomidine combined with etomidate on the cognitive function in rats with POCD, the place navigation test was used to assess the cognitive ability of the rats by recording the escape latency and swimming distance (Fig. 1). The shorter the escape latency or the longer the swimming distance, the stronger the cognitive function.

Compared with the control group, the number of times of crossing the platform and the target quadrant residence time in the model group decreased, and the differences were statistically significant (P<0.001). Compared with the model group, the escape latency in the Dex, Eto and Dex-Eto groups during the same period, among which the Dex-Eto group presented the shortest time, and the difference was statistically significant (P<0.01) (Fig. 1A).

Compared with the control group, the swimming distance in the model group increased during the same period, but the difference was not statistically significant. Compared with the model group, the swimming distance in the Dex and Eto groups during the same period, among which the Dex-Eto group presented the shortest swimming distance (Fig. 1B).

Spatial probe test. Spatial exploration experiments were performed to evaluate the cognitive function of the rats by recording the times the rats crossed the platform and the time of stay at the target quadrant (Fig. 2). The more times the rats crossed the platform or the longer the target quadrant stays, the stronger the cognitive function of the rats.

Compared with the control group, the number of times of crossing the platform and the target quadrant residence time in the model group decreased, and the differences were
statistically significant (P<0.001). Compared with the model group, the number of times of crossing the platform and the target quadrant residence time increased in the Dex, Eto and Dex-Eto groups during the same period, among which the Dex-Eto group presented the highest frequency of crossing the platform and the longest residence time in the target quadrant, and the differences were statistically significant (P<0.05) (Fig. 2).

Expression levels of IL-17A and S-100β. Since IL-17A and S-100β undergo significant changes during the POCD process, the levels of IL-17A and S-100β in serum and brain tissue of rats were detected. The higher the IL-17A, the more obvious the inflammatory response in rats. The higher the S-100β, the more severe the brain damage in rats.

Compared with the control group, the expression levels of IL-17A and S-100β in the model group increased significantly (P<0.001). Compared with the model group, the expression levels of IL-17A and S-100β decreased in the Dex, Eto and Dex-Eto groups, among which the IL-17A and S-100β expression levels in the Dex-Eto group were the lowest, and the differences were statistically significant (P<0.01) (Fig. 3).

Expression levels of TNF-α, IL-6 and IL-1β. In addition to assessing the inflammatory response using IL-17A levels, the three common pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were also detected.

The expression levels of TNF-α, IL-6 and IL-1β increased in the model group compared with those in the control group, with statistically significant differences (P<0.001). Compared with the model group, TNF-α, IL-6 and IL-1β expression levels decreased in the Dex, Eto and Dex-Eto groups, of which the expression levels in the Dex-Eto group were the lowest, and the differences were statistically significant (P<0.01) (Fig. 4).

Expression of NF-κB p65. The NF-κB pathway is closely related to the inflammatory response, so the detection of the
expression of NF-κB pathway-associated protein p65 is also helpful in understanding the mechanism by which dexmedetomidine combined with etomidate affects the inflammatory response.

Compared with the control group, the expression of NF-κB p65 in the model group significantly increased (P<0.001). Compared with the model group, the expression of NF-κB p65 in the Dex, Eto and Dex-Eto groups significantly decreased during the same period, with the lowest expression in the Dex-Eto group (P<0.01) (Fig. 5).

Correlation of IL-17A and S-100β expression levels with NF-κB p65, TNF-α, IL-6 and IL-1β. Since IL-17A, S-100β, NF-κB p65, TNF-α, IL-6 and IL-1β all changed during POCD, the correlation of IL-17A and S-100β with p65, TNF-α, IL-6 and IL-1β in the model group was analyzed. The results revealed that IL-17A and S-100β were positively correlated with p65, TNF-α, IL-6 and IL-1β (Fig. 6), suggesting that a decrease in IL-17A and S-100β may cause decrease in p65, TNF-α, IL-6 and IL-1β expression levels.

Discussion

POCD is one of the most common postoperative complications in elderly patients (21). Tissue damage during surgery stimulates the peripheral immune system activation and causes cytokine cascade and release of inflammatory mediators (22). IL-17 protein family coordinates local tissue inflammation by inducing the release of pro-inflammatory cytokines and neutrophil-mobilizing cytokines (23). S-100β plays an important role in the normal development and injury recovery of CNS (24). For patients with brain injury caused by symptoms, the concentration of S-100β protein in blood and cerebrospinal fluid increases (25). Mercier et al (11) have suggested that S-100β could be used to assess the degree of brain injury and to determine the long-term prognosis in patients with moderate and severe traumatic brain injury. In the present study, dexmedetomidine combined with etomidate was administered to aged rats with POCD and the therapeutic effect of dexmedetomidine combined with etomidate was evaluated by the expression levels of IL-17A and S-100β in aged rats.

Morris water maze test revealed that the cognitive function of aged rats with POCD was impaired, while restored to some extent after dexmedetomidine treatment. Xiong et al (26) studied the effect of dexmedetomidine on POCD aged rats and showed that the rats treated with dexmedetomidine presented a certain degree of cognitive recovery in water maze test, which was consistent with the findings presented in this study. It is worth mentioning that the present study also explored the performance of dexmedetomidine alone and dexmedetomidine combined with etomidate using water maze test, and the results demonstrated that the combination of dexmedetomidine and etomidate has a better therapeutic effect. This may be
due to the fact that both dexmedetomidine and etomidate can protect the brain to some extent (27,28).

In addition, the test results revealed that the expression levels of TNF-\(\alpha\), IL-6 and IL-1\(\beta\) were increased in aged rats with POCD, whereas decreased after drug treatment. The lowest expression levels in Dex-Eto group indicated that compared with the simple treatment with dexmedetomidine alone, dexmedetomidine combined with etomidate had more significant inhibition on inflammatory cytokines. In the study of Wang et al (4), dexmedetomidine was reported to be able to reduce TNF-\(\alpha\), IL-6, IL-1\(\beta\) and other expression levels, and thus reduce inflammation; whereas Liu et al (18) reported that etomidate could inhibit the production of pro-inflammatory cytokines in rat macrophages, so dexmedetomidine and etomidate may play a joint role in downregulating TNF-\(\alpha\), IL-6 and IL-1\(\beta\) without eliminating each other.

Furthermore, as IL-17A may be involved in the process of brain injury (29) and S-100\(\beta\) can be used to evaluate the degree of brain injury (11), the expression levels of IL-17A and S-100\(\beta\) in each group were compared in the present study. The results revealed that the expression levels of IL-17A and S-100\(\beta\) in aged rats with POCD increased, but decreased after drug treatment. The IL-17A and S-100\(\beta\) expression levels in the Dex-Eto group were the lowest, indicating that the inhibition effect of dexmedetomidine combined with etomidate on IL-17A and S-100\(\beta\) was the most obvious. Yang et al (29) reported that anti-IL-17A treatment could improve neuroinflammation and oxidative stress, thereby relieving cognitive dysfunction in aged rats, and it was also believed that this mechanism was involved in the reduction of NF-kB pathway. On this basis, the expression of NF-kB p65 in the hippocampus of each group was studied, and the results indicated that the expression of NF-kB p65 was increased in the hippocampus of aged rats with POCD in Morris water maze test, but decreased after drug treatment, with that of the Dex-Eto group being the lowest. This may be due to the fact that dexmedetomidine and etomidate co-inhibit NF-kB in order to alleviate inflammation (18,30), with no elimination effect on each other. In addition, by analyzing the correlation of the above factors, it was shown that the decrease of IL-17A and S-100\(\beta\) may cause the decrease of NF-kB p65, TNF-\(\alpha\), IL-6 and IL-1\(\beta\). Therefore, the treatment of IL-17A and S-100\(\beta\) with dexmedetomidine combined with etomidate can alleviate the condition of rats with POCD.

In the present study, the combined treatment with dexmedetomidine and etomidate in aged rats with POCD was investigated, and its effects on IL-17A and S-100\(\beta\) were explored. However, the test results concerning the expression levels of IL-17A and S-100\(\beta\) were only collected at the end of T3 without the reference results of T1 and T2. Thus, the dynamic effects of dexmedetomidine combined with etomidate on IL-17A and S-100\(\beta\) remain to be further investigated. The purpose of this study was to explore the changes in IL-17A and S-100\(\beta\). Therefore, only the changes of NF-kB were detected by western blot analysis, and the immunofluorescence analysis of NF-kB was not considered. This is the limitation of the present study. Since dexmedetomidine and etomidate can be used to regulate the expression of p65, NF-kB will be systematically analyzed in our future studies. The effect of NF-kB nuclear translocation will be the aim of our future experiments.

In conclusion, compared with the simple treatment with dexmedetomidine or etomidate, dexmedetomidine combined with etomidate has a better therapeutic effect on aged rats with POCD, which can effectively improve cognitive dysfunction and alleviate stress inflammation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XY conceived, designed the study and drafted the manuscript. XY and YX collected, analyzed and interpreted the experimental data and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University (Nanchang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Zhou C, Zha Y, Liu Z and Ruan L: Effect of dexmedetomidine on postoperative cognitive dysfunction in elderly patients after general anaesthesia: A meta-analysis. J Int Med Res 44: 1182-1190, 2016.
2. Qian XL, Zhang W, Liu MZ, Zhou YB, Zhang JM, Han L, Peng YM, Jiang JH and Wang QD: Dexmedetomidine improves early postoperative cognitive dysfunction in aged mice. Eur J Pharmacol 746: 206-212, 2015.
3. Sprung J, Roberts RO, Weingarten TN, Nunes Cavalcante A, Knopman DS, Petersen RC, Hanson AC, Schroeder DR and Warner DO: Postoperative delirium in elderly patients is associated with subsequent cognitive impairment. Br J Anaesth 119: 316-323, 2017.
4. Wang WX, Wu Q, Liang SS, Zhang XK, Hu Q, Chen QH, Huang HJ, Xu L and Lou FQ: Dexmedetomidine promotes the recovery of neurogenesis in aged mouse with postoperative cognitive dysfunction. Neurosci Lett 677: 110-116, 2018.
5. Cibelli M, Fidalgo AR, Terrando N, Ma D, Monaco C, Feldmann M, Takata M, Lever D, Nanchahal J, Fanselow MS and Maze M: Role of interleukin-1beta in postoperative cognitive dysfunction. Ann Neurol 68: 360-368, 2010.
6. Wright CB, Sacco RL, Rundeck T, Delman J, Rabbani L and Elkind M: Interleukin-6 is associated with cognitive function: The northern manhattan study. J Stroke Cerebrovasc Dis 15: 34-38, 2006.
7. Terrazao N, Monaco C, Ma D, Foxwell BMJ, Feldmann M and Maze M: Tumor necrosis factor-alpha triggers a cytokine cascade yielding postoperative cognitive decline. Proc Natl Acad Sci USA 107: 20518-20522, 2010.
8. Alam A, Hana Z, Jin Z, Suen KC and Ma D: Surgery, neuro-inflammation and cognitive impairment. EBioMedicine 37: 547-556, 2018.
9. Ma X, Reynolds SL, Baker BJ, Li X, Benveniste EN and Qin H: IL-17 enhancement of the IL-6 signaling cascade in astrocytes. J Immunol 184: 4908-4906, 2010.
10. Zuo Y, Zhao L, Zeng M, Yang Q, Chen X and Yang T: The effects of vitamin-rich carbohydrate pretreatment on the surgical stress response and S-100B after splenectomy in elderly rats. BMC Anesthesiol 19: 77, 2019.
11. Mercier E, Boutin A, Lauzier F, Fergusson DA, Simard JF, Zarychanski R, Moore LA, Archambault P, Lamontagne F, et al: Predictive value of S-100B protein for recognition of immediate early genes. Chin Med J (Engl) 129: 48-53, 2016.
12. Li Y, He R, Chen S and Qu Y: Effect of dexmedetomidine on early postoperative cognitive dysfunction and peri-operative inflammation in elderly patients undergoing laparoscopic cholecystectomy. Exp Ther Med 5: 489-494, 2013.
13. Xiong B, Shi Q and Fang H: Dexmedetomidine alleviates postoperative cognitive dysfunction by inhibiting neuron excitation and cognitive function in sevoflurane anesthetized aged rats via activation of NF-κB signaling pathway. BMC Anesthesiol 18: 22-28, 1989.
14. Yang Z and Yuan C: IL-17A promotes the neuroinflammation and cognitive impairment after laparoscopic cholecystectomy in elderly patients. Exp Ther Med 5: 495-499, 2013.
15. Baughman VL, Hoffman WE, Miletich DJ and Albrecht RF: Cerebral metabolic depression and brain protection produced by midazolam and etomidate in the rat. J Neurosurg Anesthesiol 10: 18-26, 1997.
16. Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, Fidalgo A, Sun P, Hossain M, Ma D and Maze M: Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. Anesthesiology 110: 1077-1085, 2009.
17. Lu F, Li W, Xu J, Sun XJ, Qin LZ, Zhang QL, Yao Y, Yu QK and Liang XL: Underlying mechanisms of memory deficits induced by etomidate anesthesia in aged rat model: Critical role of immediate early genes. Chin Med J (Engl) 129: 48-53, 2016.