Postconditioning of stellate ganglion block improves intestinal barrier function by inhibiting autophagy in conscious rats following hemorrhagic shock and resuscitation

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To the Editor: Hemorrhagic shock is a critical pathological process characterized by microcirculation dysfunction and hypoperfusion, with severe consequences of cell damage and organ dysfunction. Intestinal barrier dysfunction is a critical link of distant organ injury caused by hemorrhagic shock. Prophylactic treatment with stellate ganglion block (SGB) significantly reduces hemorrhagic shock-induced intestinal barrier damage.[1] However, it is not clear whether SGB postprocessing can also reduce intestinal barrier damage after hemorrhagic shock, which is pivotal to expand the clinical application of SGB. Autophagy is a highly conserved catabolic process that occurs through intracellular lysosomes and plays a unique and essential role in maintaining cell homeostasis and survival. Previous studies have shown that the excessive activation of autophagy aggravates ischemic intestinal injury, whereas autophagy inhibition reduces intestinal mucosal barrier damage.[2,3] Thus, the role of autophagy in SGB protection of the intestinal barrier is unclear. Therefore, we speculated that SGB postprocessing suppresses the intestinal barrier damage caused by hemorrhagic shock by inhibiting autophagy activation. To verify this hypothesis, we investigated the effects of SGB postprocessing on the survival rate, intestinal blood flow, intestinal tissue morphology, intestinal barrier function, and autophagy-related mechanisms in rats that underwent hemorrhagic shock.

Eighty-eight healthy Wistar male rats were selected and purchased from SiPeiFu Biotechnology Co., Ltd. (Beijing, China), weighing 300 ± 20 g. All surgeries were performed under anesthesia, and the experiments were approved by the Animal Ethics Committee of Hebei North University (No. 2019-1-9-15). According to the conventional method in our laboratory,[1] the left side stellate ganglion was blocked by injecting 0.5% ropivacaine hydrochloride (AstraZeneca AB, Sodertalje, Sweden). Under the induction of 2% isoflurane inhalation anesthesia, all rats underwent surgery for establishing a conscious hemorrhagic shock model [Supplementary File, http://links.lww.com/CM9/A918]. As a control, the left stellate ganglion of the Sham SGB group was given an equal amount of normal saline.

A total of 52 animals were used to observe the effect of SGB post-treatment on the survival time and rate of rats that underwent hemorrhagic shock, whereas the survival time was determined by video surveillance. Survival for >72 hours is considered long-term survival. Subsequently, another 36 rats were used to investigate the effects of SGB postprocessing on the gut barrier following hemorrhagic shock and the mechanism of autophagy ($n = 6$/group). The hemorrhagic shock model in conscious rats was created in the various shock groups. The same procedure was implemented in the sham and sham + SGB groups but without bleeding or resuscitation. One hour after shock or at the corresponding time point, SGB or sham SGB was administered under inhalation anesthesia. During the recovery period, an intravenous injection of 3-methyladenine (3MA) (autophagy inhibitor) (30 mg/kg,[4] M9281, Sigma, Missouri, USA) and intraperitoneal injection of rapamycin (RAPA) (autophagy agonist) (10 mg/kg,[5] V900930, Sigma, MO, USA) were given to the Shock + 3MA and Shock + SGB + RAPA groups, respectively. Three hours after resuscitation or at the corresponding time point, all rats were anesthetized via inhalation and intramuscularly injected with 10% pentobarbital sodium (40 mg/kg, P11011, Merck, Darmstadt, Germany) for the observation of intestinal blood flow, permeability,

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morphology, the wet/dry ratio, and protein expression. These detailed methods are shown in Supplementary File, http://links.lww.com/CM9/A918.

All results were statistically analyzed by SPSS 22.0 software (IBM, Armonk, NY, USA). Except for survival rate which is presented as percentage and analyzed by Chi-squared test, all values were represented as mean ± standard deviation. Kaplan-Meier method was used for survival analysis. The significance of the differences between groups was analyzed by one-way analysis of variance (ANOVA). Subsequent multiple comparisons between groups were analyzed using Tukey's Honest Significant Difference test. When the hypothesis of homogeneity of variance is not satisfied, Welch's ANOVA is used, and then pairwise comparisons between groups are performed through the Games-Howell test. When P is < 0.05, the result is regarded as statistically significant.

The results showed that the rats in the sham and sham + SGB groups all survived. The survival rates of the shock group were decreased compared with those of the SGB + shock group at 24 hours (38.5% *vs.* 92.3%, $\chi^2 = 9.124$, $P = 0.003$), 48 hours (30.8% *vs.* 61.5%, $\chi^2 = 4.481$, $P = 0.034$), and 72 hours (23.1% *vs.* 53.8%, $\chi^2 = 4.579$, $P = 0.032$). The median survival time of the shock and SGB + shock groups was 17.5 hours and 76.9 hours, respectively. Kaplan-Meier survival analysis showed that SGB postprocessing improved the survival rate of rats after hemorrhagic shock, which further proved the effectiveness of SGB. The survival curve is shown in Supplementary Figure 1, http://links.lww.com/CM9/A918. In addition, the blood pressure, heart rate, and pulse pressure results are shown in Supplementary Figure 2, http://links.lww.com/CM9/A918.

The observation of intestinal blood flow [Supplementary Figure 3A, http://links.lww.com/CM9/A918 and 4, http://links.lww.com/CM9/A918] showed that there was a significant difference in the blood flow of the intestinal loop among these groups ($F = 26.227$, $P < 0.001$). This index in the shock group was significantly lower than that in the sham group ($P < 0.001$) and was significantly increased by SGB and 3MA treatments ($P = 0.014$ and $P = 0.005$). In contrast, RAPA offsets the beneficial effect of SGB on improving the intestinal blood flow of rats in the shock group ($P < 0.001$). This result suggests that SGB postprocessing improves the intestinal blood supply after hemorrhagic shock, which is beneficial for reducing intestinal damage and improving tissue perfusion. However, further observations of lactic acid and mixed venous oxygen saturation (SVO_2) need to be made in the future. Based on the distribution of stellate ganglion innervation, we speculate that the potential mechanism of SGB treatment might be related to intestinal neuronal regulation, which should be investigated in the future.

The intestinal morphology results showed that the shock group's intestinal villi were thicker, shorter, and irregular, and morphological damage was apparent. Treatment with SGB or 3MA reduced intestinal injury compared with that in the shock group, whereas RAPA administration worsened intestinal damage in the rats of the shock + SGB group [Supplementary Figure 5, http://links.lww.com/CM9/A918]. We found significant differences in intestinal villus height, submucosal thickness, and muscle layer thickness among these groups ($F = 8.099$, $P < 0.001$ for intestinal villus height; $F = 40.657$, $P < 0.001$ for submucosal thickness; $F = 39.325$, $P < 0.001$ for muscle layer thickness). These measurements in the shock group were dramatically lower than those in the sham group ($P = 0.018$, < 0.001, < 0.001, respectively). SGB and 3MA treatments significantly enhanced these indices compared with those of the shock group ($P = 0.016$, 0.003, 0.004 for SGB; $P = 0.009$, 0.013, <0.001 for 3MA). At the same time, the above measurements in the shock + SGB + RAPA group were substantially lower than those in the shock + SGB group ($P = 0.010$, 0.022, 0.001, respectively) [Supplementary Figure 3B–D, http://links.lww.com/CM9/A918]. In addition, there was a significant difference in the wet/dry ratio of intestinal tissue (2 cm in length, at 15 cm upward from the ileocecal end) among these groups ($F = 32.491$, $P < 0.001$); in particular, this index of shock group was significantly higher than that of the sham group ($P < 0.001$). SGB and 3MA treatment reduced the intestinal wet/dry ratio of rats undergoing hemorrhagic shock ($P = 0.001$ for SBG and $P < 0.001$ for 3MA). However, the combination of RAPA blocked the effect of SGB ($P < 0.001$) [Supplementary Figure 3E, http://links.lww.com/CM9/A918]. These results demonstrated that SGB postconditioning improved the histomorphology and edema of the intestinal mucosa after hemorrhagic shock, which was related to autophagy inhibition.

The distribution of fluorescein isothiocyanate-dextran (FD4) in the intestinal mucosa is shown in Supplementary Figure 6, http://links.lww.com/CM9/A918. In general, the FD4 concentration in plasma reflects the intestinal mucosa permeability to FD4. The FD4 level in plasma [Supplementary Figure 3F, http://links.lww.com/CM9/A918] showed that there was a significant difference among these groups ($F = 18.714$, $P < 0.001$). The FD4 level in the shock group was significantly higher than that in the sham group ($P = 0.005$) and was decreased in the shock + SGB and shock+ 3MA groups ($P = 0.033$ for SBG and $P < 0.001$ for 3MA). However, the permeability in the shock + SGB + RAPA group was significantly higher than that in the shock + SGB group ($P = 0.016$). Western blotting results [Supplementary Figure 3G–I, http://links.lww.com/CM9/A918] showed that there were significant differences in the expression of the tight junction proteins zonula occludens-1 (ZO-1), occludin and claudin-1 in the intestinal tissue among these groups ($F = 23.591$, $P < 0.001$ for ZO-1; $F = 11.614$, $P < 0.001$ for occludin; $F = 7.098$, $P = 0.003$ for claudin-1). These markers in the shock group were remarkably reduced compared with those in the sham group ($P = 0.002$, 0.004, 0.005, respectively), and SGB and 3MA intervention increased the expression of these proteins ($P = 0.004$, 0.008, 0.003 for SGB; $P = 0.001$, 0.006, 0.001 for 3MA). However, RAPA treatment downregulated the expression of the proteins mentioned above and partially blocked the effect of SGB ($P < 0.001$, $P = 0.003$, $P = 0.008$, respectively). These results demonstrated that SGB postprocessing relieved intestinal barrier

dysfunction after hemorrhagic shock to a certain extent. The effects of 3MA and RAPA also suggested that autophagy is involved in the beneficial effect of SGB on the gut mucosal barrier.

To define the mechanism by which SGB improves the intestinal barrier, we investigated the expression of microtubule associated protein light chain 3-II (LC3-II) (a marker of autophagy activation), Beclin-1 (a key regulator of autophagosome formation) and p62 and found that there was a significant difference in the expression levels among these groups ($F = 7.098$, $P = 0.003$ for LC3; $F = 9.698$, $P = 0.001$ for Beclin-1; $F = 21.874$, $P = 0.000$ for p62) [Supplementary Figure 3J–L, http://links.lww.com/CM9/A918]. The expression of LC3-II and Beclin-1 in the shock group was dramatically higher than that in the sham group ($P = 0.019$ for LC3; $P = 0.016$ for Beclin-1), whereas p62 expression in the shock group was significantly lower than that in the sham group ($P < 0.001$). SGB and 3MA treatments significantly reversed these effects compared with those of the shock group ($P = 0.024$, 0.019, 0.010 for SGB; $P = 0.049$, 0.018, 0.002 for 3MA). At the same time, the autophagy activator RAPA blocked the therapeutic effect of SGB postprocessing ($P = 0.043$, 0.028, 0.007, respectively). These findings showed that hemorrhagic shock enhanced autophagy activation. In contrast, SGB postprocessing inhibits autophagy, which is consistent with the tendency of SGB to repair the intestinal barrier.

Taken together, these findings demonstrated that SGB postconditioning improves the survival rate and intestinal barrier function after hemorrhagic shock by inhibiting excessive autophagy. A more extensive application of SGB and the anti-shock mechanism should be the focus of future research.

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Conflicts of interest

None.

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