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Neuroprotective effects of lipopolysaccharide and naltrexone co-preconditioning in the photothrombotic model of unilateral selective hippocampal ischemia in rat

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Preconditioning with lipopolysaccharide (LPS) or opioid antagonists has a neuroprotective effect in ischemic insults. However, the co-preconditioning effect of toll-like receptor ligands and opioid antagonists has not been investigated. In this study we examined the neuroprotective effect of LPS and naltrexone (NTX) preconditioning and co-preconditioning in unilateral selective hippocampal ischemia in rats to assess for possible synergistic protective effects. LPS and NTX were injected unilaterally into the left cerebral ventricle of male rats. Forty-eight hours after LPS and twenty-four hours after NTX injection, ipsilateral selective hippocampal ischemia was induced using a modified version of the photothrombotic method. Protective effects for LPS and NTX were assessed by evaluating infarct volume (using 2,3,5-triphenyltetrazolium chloride staining), and cognitive function (using radial arm water maze and passive avoidance tests). Animals in the ischemic group had an infarct lesion and considerable cognitive impairment, compared with the sham group. LPS or NTX preconditioning significantly reduced the infarct size and improved cognitive function. Moreover, co-preconditioning with LPS and NTX increased the protective effect compared with preconditioning with LPS or NTX alone. Our data showed that LPS and NTX preconditioning resulted in a neuroprotective effect in hippocampal ischemia. Furthermore, co-preconditioning with LPS and NTX resulted in a synergistic protective effect.

Key words: naltrexone, lipopolysaccharide, preconditioning, hippocampal ischemia, TLRs

INTRODUCTION

Ischemic stroke is the second most frequent cause of death in the world and is one of the primary causes of morbidity worldwide (Secades et al., 2016). However, the number of strokes declined by approximately 10% in high-income countries, while increasing by 10% in developing countries, from 1990 to 2010 (Feigin et al., 2014). Because of a substantial increase in global life expectancy over the past 40 years, the prevalence of ischemic stroke has also increased (Secades et al., 2016). Stroke is often due to a transient or permanent reduction of cerebral blood flow. Many studies have been designed in an attempt to find a neuroprotective strategy for the prevention and treatment of ischemic brain injury. Currently, administration of thrombolytic drugs, to restore blood flow, is the most common approach in cases of ischemic brain injury. However, restoration of blood flow due to spontaneous reperfusion or thrombo-

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lytic therapy results in the production of reactive oxygen species (ROS), a rise in intracellular calcium levels, glutamate excitotoxicity, and the release of inflammatory mediators (Gong et al., 2014). In general, the existing approaches for treating stroke are either ineffective or associated with adverse effects (Vann and Xiong, 2016).

Toll-like receptor 4 (TLR4) is a member of the pattern-recognition receptors (PRRs) family, which are homologous to the cytosolic domain of a *Drosophila melanogaster* protein called Toll (O'Neill et al., 2013). TLRs are expressed on microglia and astrocytes (Gurley et al., 2008; Schaafsma et al., 2015). These receptors recognize and respond to specific molecular patterns including pathogen-associated molecular patterns (PAMPs) from exogenous pathogens, e.g. lipopolysaccharide (LPS) or damage-associated molecular patterns (DAMPs) from damaged tissue, (e.g. heat shock proteins, fibrinogen, RNA, and methylated DNA). Therefore, glial cells would be activated upon TLR and DAMPs or PAMPs interaction (Gurley et al., 2008; Chen and Nuñez, 2010). Normally, after stroke, DAMPs activate TLR4 expressed on glial cells and worsen the injury. However, brief activation of TLR4 before an ischemic attack results in protection against the negative consequences of ischemia (Marsh et al., 2009).

Currently, there is significant interest in the investigation of endogenous protection mechanisms against ischemic injury or any other deleterious condition. Preconditioning is a technique that is used to study endogenous protection. In preconditioning the subject receives a harmful stimulus near to but below the threshold for damage. This procedure modulates several endogenous mechanisms that initiate protection against future same, similar, or even different, but more intense, harmful stimuli. Different types of preconditioning include immunological, pharmacological, anesthetic, mimetic, and remote ischemic preconditioning (Dirnagl et al., 2009). Immunological preconditioning is established by administration of low doses of the gram-negative bacterial cell wall component LPS, which can provide resistance against a probable subsequent damaging ischemic insult. Unfortunately, because of the toxic nature of LPS, it has a narrow therapeutic window (Elliott, 1998).

Naltrexone (NTX) and naloxone are both opioid receptor antagonists. Naloxone preconditioning has been investigated in many different studies (Tang et al., 2005; Lu and Liu, 2009). The bioavailability and biological half-life of NTX is higher than that of naloxone, but these two agents have a similar pharmacology (Verebey and Mule, 1975). Thus, NTX preconditioning may have a protective effect similar to naloxone.

Many different mechanisms have been described in explaining the protective effects of preconditioning. However, TLRs have not been thoroughly considered, despite their apparent involvement in the development of LPS and opioid receptor antagonist preconditioning (Medvedev et al., 2002; Rosenzweig et al., 2007; Watkins et al., 2007). To assess the synergistic protective effect of LPS and NTX preconditioning against an ischemic attack we established co-preconditioning of these two agents in the photothrombotic model of unilateral selective hippocampal ischemia in rat.

METHODS

Animals

Male albino Wistar strain rats (250±25 g mean body weight; 10 weeks of age) were purchased from the Faculty of Pharmacy, Shahid Beheshti University of Medical Science, Tehran, Iran. All animal groups were housed in Plexiglas cages until surgery, were given ad libitum access to tap water and chow, and maintained on a 12 hour dark/light cycle. Temperature and relative humidity were controlled at 22±2°C and 40-60%, respectively. Procedures were conducted according to the Shahid Beheshti University of Medical Science Animal Ethics Committee.

Experimental design

The animals were randomly divided into five groups of six each (except for the groups that were allocated for behavioral assessment, which included eight animals each): sham group (underwent all surgical procedures without induction of hippocampal ischemia), ischemic group (intracerebroventricular, i.c.v., injection of 5 µl/rat DMSO and 5 µl/rat normal saline, 48h and 24h prior to hippocampal ischemia induction, respectively), ischemia plus LPS preconditioned group (i.c.v. injection of 5 µl/rat LPS (Schaafsma et al., 2015) and 5 µl/rat normal saline, 48h and 24h prior to hippocampal ischemia induction, respectively), ischemia plus NTX preconditioned group (i.c.v. injection of 5 µl/rat DMSO and 5 µl/rat NTX (Braida et al., 1997), 48h and 24h prior to hippocampal ischemia induction, respectively), and ischemia plus LPS and NTX co-preconditioned group (i.c.v. injection of 5 µl/rat LPS and 5 µl/rat NTX, 48h and 24h prior to hippocampal ischemia induction, respectively). LPS was dissolved in DMSO and NTX was dissolved in normal saline to reach a 1 mg/ml concentration. For i.c.v. injection and hippocampal ischemia induction, rats underwent stereotaxic surgery. After recovery from stereotaxic surgery, preconditioning was achieved by administering a sin-

gle i.c.v. dose of LPS (48h before hippocampal ischemia induction) and/or NTX (24h before hippocampal ischemia induction). Forty-eight hours after LPS injection and/or 24h after NTX injection, unilateral selective hippocampal ischemia was induced through a modified version of the photothrombotic model (Schmidt et al., 2012). Respectively, 24h and 48h after hippocampal ischemia induction, infarct size (n=6) and learning and memory function (n=8) were assessed in separate experimental groups.

Stereotaxic surgery

Rats were anesthetized via intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg) and subjected to stereotaxic surgery. The level of anesthesia was assessed by withdrawal reflex. The animal was mounted in a standard stereotaxic frame and the incisor bar was adjusted until the dorsal surface of the skull was level. The scalp was incised with the use of a scalpel, and two small holes were drilled. According to the Paxinos atlas (Paxinos and Watson, 2007), one guide cannula was implanted for i.c.v. injection at a coordinate of 1.6 mm lateral to the midline and 0.6 mm posterior to bregma, and 3 mm below the skull (i.c.v. cannula); the other was implanted for unilateral selective hippocampal ischemia induction at a coordinate of 5.2 mm lateral to the midline and 5.64 mm posterior to bregma, and 2.6 mm below the skull (hippocampal cannula). Dental cement attached to two stainless steel screws was used to fix the two 27 gauge stainless steel guide cannulas in place. To avoid cannula clogging, a 29 gauge stainless steel stylet was placed into the cannula.

Intracerebroventricular injection

After a one-week recovery period, the stylet was removed from the i.c.v. guide cannula and the microinjections were administered into the cerebral ventricle of conscious rats. The injection was carried out using a 10 µL Hamilton syringe connected by PE-10 polyethylene tubing to a 29 gauge injection cannula. The tip of the injection cannula extended into the lateral ventricle 1 mm beyond the guide cannula. The injection was performed at a constant rate (0.5 µl/min) using an infusion pump (New Era Pump Systems, Inc, NE-1000). The volume of the i.c.v. injection was 5 µl/rat. The injection cannula was withdrawn 1 min after injection to minimize backflow. The accuracy of the i.c.v. injection was verified by injecting 10 µl of 0.25% trypan blue (Stempniak et al., 1995).

Unilateral selective hippocampal ischemia induction

The hippocampal formation blood supply is provided by branches of the basilar and internal carotid arteries which terminate at the longitudinal hippocampal artery (Dorr et al., 2007; Barth and Mody, 2011). To achieve a unilateral selective hippocampal ischemia, we targeted the hippocampal fissure, which is in close proximity to the longitudinal hippocampal artery, by using a modified version of the photothrombotic method (Barth and Mody, 2011). To access the position of the artery, we used the Paxinos atlas and then a hippocampal guide cannula was implanted above the ascending part of the hippocampal fissure, at the same time as i.c.v. guide cannula implantation.

Forty-eight hours after the i.c.v. administration of LPS (or its vehicle) and/or twenty-four hours after the i.c.v. administration of NTX (or its vehicle), general anesthesia was achieved by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Rose Bengal (2 ml/kg body weight, 20 mg/ml saline) was injected intravenously through left femoral vein cannulation with PE-10 polyethylene tubing (Schmidt et al., 2012). The rats were placed into the stereotaxic frame, the 29 gauge stainless steel stylet was removed from the hippocampal guide cannula, and then the optical fiber (200 µm core diameter) coupled to a 523 nm laser diode was placed into the hippocampal guide cannula that extended 1 mm beyond its tip. The longitudinal hippocampal artery was illuminated by the optical fiber through the hippocampal cannula for 25 min. During the first two minutes of illumination, Rose Bengal was injected slowly through the previously cannulated left femoral vein. Systemically injected Rose Bengal photoactivates under optical fiber illumination, which in turn can cause an ischemic insult. After 25 min of illumination, the femoral vein catheter was withdrawn and the skin was sutured.

Infarct volume measurement

Twenty-four hours after hippocampal ischemia induction the rats were sacrificed by chloroform anesthesia. Within 5 min of sacrifice, brains were removed and sectioned coronally into 2 mm-thick slices in a stainless steel brain matrix. Brain slices were immersed in a 2% solution of triphenyltetrazolium chloride (TTC) in normal saline at 45°C for 15 min. The uncolored areas of the brain slice were considered non-viable tissue (necrotic area) (Hua et al., 2009). Viable cells are stained by tetrazolium because in intact cells the succinate dehydrogenase enzyme reduces tetrazolium salts produc-

ing insoluble formazan pigments. After 15 min, images were taken using a digital camera and infarct volume was measured by image J 1.50i software (Wayne Rasband, National Institutes of Health, USA). To determine the actual volume of the ischemic insult, considering post-ischemic edema, the following formula was used:

The infarct size (%) = [(volume of the left hemisphere – non-infarct volume of the right hemisphere)/volume of the left hemisphere]×100% (Ji et al., 2012; 2017).

Learning and memory impairment evaluation

A separate experimental group was allocated for learning and memory impairment evaluations (n=8). Two behavioral tasks that assess spatial (radial arm water maze test, RAWM) and non-spatial (passive avoidance test, PA) memory function were used for this aim.

Passive avoidance test

Forty-eight hours post-injury, animals underwent the PA. Rats were tested in a PA apparatus to evaluate non-spatial fear-based contextual and emotional memory as previously described (Rabiei et al., 2014; Wu et al., 2014). The PA apparatus consisted of a bright and a dark chamber (each 20×20×20 cm) with a grid floor coupled to an electric foot shock generator (1.5 mA for 3 s). These two chambers were separated by a wall containing a guillotine door (8×8 cm). By lifting up the guillotine door, the two chambers become connected and the subject is able to pass between the chambers. Thirty minutes after two habituation sessions separated by a 30 min interval, the training trial was accomplished. In the training trial, the rats were placed in the bright chamber. As soon as the animal passed into the dark chamber, the guillotine door was shut and a brief electric foot shock was delivered at 1.5 mA for 3 s. The animal spent 30 seconds in the dark chamber, and was then removed to its cage. Two minutes later a second training trial was started. When the animal remained in light chamber for two minutes, successful learning of the task was considered achieved, otherwise a rat underwent a third training trial. This procedure was repeated until the animal remained in the light chamber for 2 minutes or, in other words, avoided entering the dark chamber. Twenty-four hours later a test trial was carried out. During the test trial the guillotine door was removed and the animal was placed in the bright chamber. The animal's behavior was observed for 5 minutes. Latency to first entrance (step-through latency: STL) and total time spent in dark chamber (TDC) were then recorded in seconds as the measurement of task recall (Nategh et al., 2016).

Radial arm water maze test

The RAWM test was used to evaluate spatial learning and memory forty-eight hours after hippocampal ischemia induction. The RAWM apparatus consisted of six arms (59×13 cm) placed in a water tank. Visual cues were set relative to each arm. The experimenter stayed in the same place throughout the experiment relative to the visual cues to maintain the same cue pattern throughout testing (Hodges et al., 1995; Chaby et al., 2015). The temperature of the water was constantly monitored and maintained at 25°C at a depth of 50 cm. After every trial the rats were dried by towel and removed to a holding cage that contained a heating pad under dry towels. Each rat was tested on three consecutive days, consisting first of two-day training trials (10 trials per day) followed by a probe trial on the third day. Each rat's ability to locate the maze arm that contained the platform (the goal arm) was assessed. Throughout the two training trial days, the goal arm was identical for each individual rat but was different between rats and the starting arm was randomized, thus rats could not rely on a motor rule and had to learn the spatial location. During the first training trial the platform was 3 cm above the water surface in order to expedite and ease learning of the platform site. After that, it was placed below the water surface to assess spatial memory and learning. To start each trial, a rat was placed at the end of a randomized start arm which did not contain the platform. During the first training trial, if a rat could not find the platform in 1 minute, or after 2 min on all of the following training trials, the rat was gently guided to the goal arm by the experimenter's hand. When the rat found the platform by searching or guiding, and all four feet were on the platform, the animal was allowed to spend 15 s on the platform and then transferred to holding cage. This procedure was repeated 10 times each training trial day. After the first training trial, if a rat could not swim normally or find the platform location within 2 min, it would be excluded. During the first two days of the test (the training trial days), velocity, total distance traveled, latency to locate the platform, number of reference memory errors, and number of working memory errors were recorded. Velocity and total distance traveled were the locomotor measurements for the task. A reference memory error was defined as entering an arm other than the goal arm and working memory errors were defined as any consecutive re-entries into an arm other than the goal arm. An arm entrance was recorded when all four paws of animal were in a maze arm (Chaby et al., 2015). On the third day (probe trial), the animal's behavior was assessed in the RAWM apparatus without the platform for one minute. In the probe trials the animal was placed in a random starting arm and several parameters, including total distance traveled, velocity, number of entries to goal

arm, and time spent in the goal arm were recorded. In all trials the swim path was recorded by the EthoVision video tracking system (Noldus, EthoVision® XT).

Statistical analysis

Data is represented as mean ± standard error of mean (SEM) and were analyzed by one-way analysis of variance followed by *post hoc* Tukey's test. In order to reduce the noise in the RAWM test, the mean of two sequential training trials were calculated for all criteria. Latency to find the platform and the number of reference and working memory errors during the first two days of the test were analyzed using two-way analysis of variance, with drug treatment and time (two-trial means) as fixed effects, followed by *post hoc* Tukey's test. P values <0.05 were considered significant. Statistical analysis was run using GraphPad Prism v. 6.07 software.

RESULTS

LPS and/or NTX preconditioning reduced infarct volume induced by hippocampal ischemia

Infarct volume was assessed 24 hours after unilateral hippocampal ischemia induction through the TTC staining method. As shown in Fig. 1, brain infarct volume in preconditioned groups was reduced compared with the ischemic group (Fig. 1A). By using a standard formula that corrects for post-ischemic edema interference, the infarct volume was reported quantitatively as a percentage of the left hemisphere brain volume (Fig. 1B). The infarct volume in rats preconditioned with 5 µg/rat NTX (p<0.05) or 5 µg/rat LPS (p<0.01) was less ($F_{4,27}$=36.64, p<0.0001) compared with the ischemic group. Co-preconditioning with LPS and NTX (5 µg/rat each) resulted in a greater reduction in infarct volume compared with the ischemic group (p<0.001). Moreover, *post hoc* analysis showed that there was a significant difference between the co-preconditioned group (with LPS and NTX) and each of the preconditioned groups (with LPS or NTX) (p<0.001).

Learning and memory impairment induced by selective hippocampal ischemia was prevented by LPS and/or NTX preconditioning

Passive avoidance

Learning and memory was evaluated 48 hours after hippocampal ischemia. There was a significant difference in step-through latency (STL) ($F_{4,25}$=15.96, p<0.0001) and time in the dark compartment (TDC) ($F_{4,25}$=19.50, p<0.0001) between groups. Animals in the ischemic group (given hippocampal ischemia and treated with vehicle) had low STL. Furthermore, they spent more time in the dark compartment (high TDC) compared with animals in the sham group (which did not undergo hippocampal ischemic induction) on the retention test day (p<0.001). NTX and/or LPS preconditioning and co-preconditioning decreased the level of non-spatial fear-based contextual and emotional memory impairment following hippocampal ischemia. Fig. 2A shows that preconditioning with NTX (p<0.05) or LPS (p<0.05) could increase STL compared with the ischemic group. Fig. 2B shows that preconditioning with NTX (p<0.05) or LPS (p<0.01) could decrease TDC compared with the ischemic group. Co-preconditioning with LPS and NTX resulted in a more efficient protective effect against hippocampal ischemia-induced memory deficits (p<0.001). Moreover, *post hoc* analysis demonstrated that there was a significant difference between the co-preconditioned group (with LPS and NTX) and preconditioned groups (with LPS or NTX) for STL and TDC (p<0.05).

Radial arm water maze

To assess spatial learning and memory, animals underwent the RAWM test 48 hours after hippocampal ischemia induction. During the first two training trial days, there was no significant difference in total distance traveled and velocity between experimental groups (data not shown). However, animals in the ischemic group (given hippocampal ischemia and treated with vehicle) took longer to locate the platform and had a greater number of reference and working memory errors during the training trial days compared with the sham group (which did not undergo to hippocampal ischemic induction) (p<0.001). LPS and/or NTX preconditioning decreased impairments to spatial learning and memory, such that latency to locate the platform and reference and working memory errors in the RAWM test were significantly decreased in the preconditioned groups compared with the ischemic group (Fig. 3A, 3C, and 3E). For latency to locate the platform, a significant interaction was observed between parameters ($F_{36,225}$=3.066, p<0.001; Fig. 3A). Further analysis by Tukey's test revealed a significant reduction in latency to find the platform in the preconditioned groups compared with the ischemic group ($F_{4,25}$=160.0, p<0.001). Also, as shown in Fig. 3C, for number of reference memory errors, a significant treatment effect was seen ($F_{4,25}$=20.02; p<0.0001) with no interaction between factors ($F_{36,225}$=0.6322; p=0.9497). The number of work-

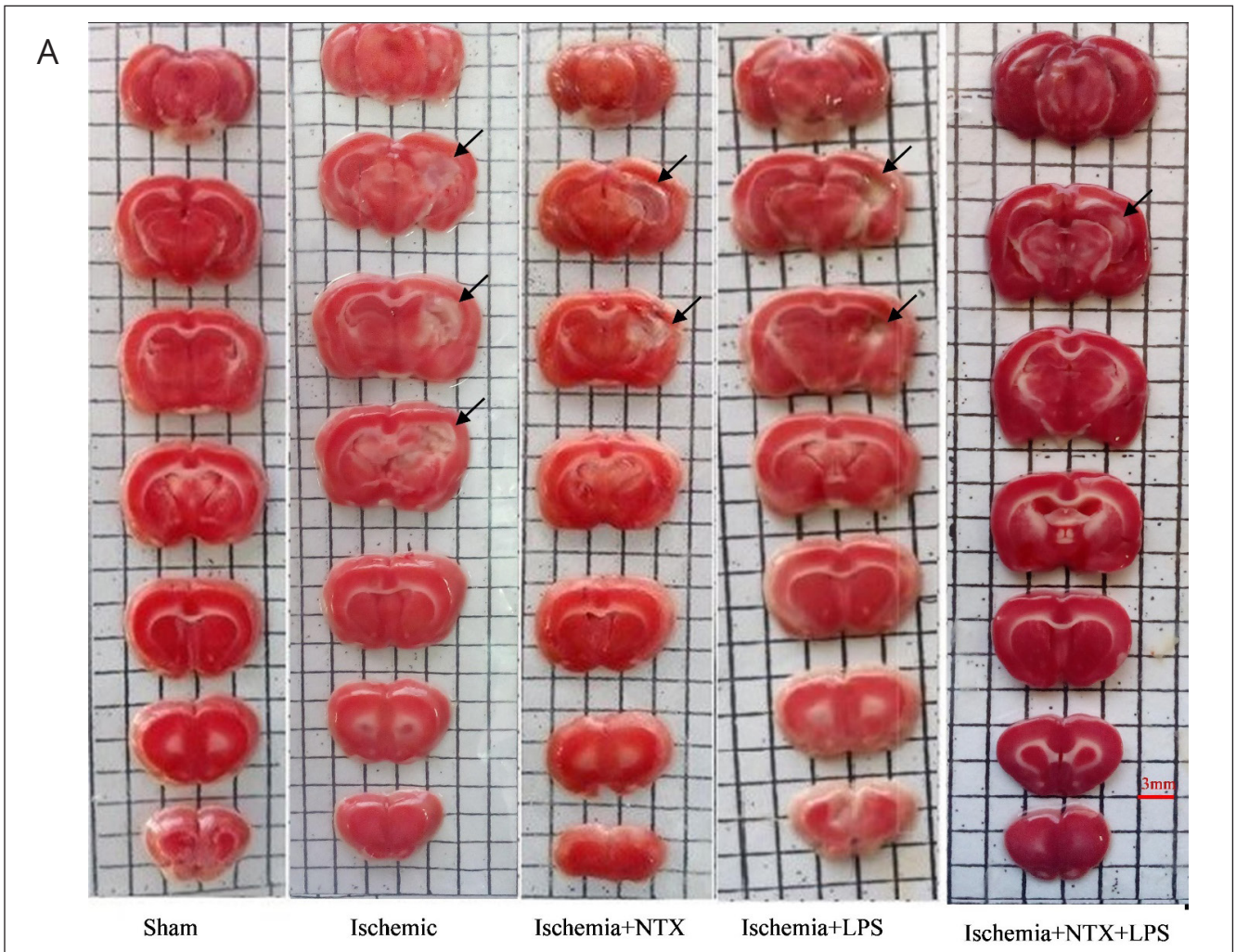

Fig. 1. (A) TTC-stained coronal brain slices 24 h after unilateral hippocampal photothrombotic ischemia. The unstained areas of the brain slice (indicated by an arrow) were considered ischemic lesions. Scale bar: 3 mm. (B) The percentage of actual infarct volume in the right hemisphere brain volume. The infarct size was significantly less in NTX or LPS preconditioned groups and in the LPS and NTX co-preconditioned group compared with the ischemic group; *p<0.05, **p<0.01, and ***p<0.001. The infarction volume in the co-preconditioned group was less than that of the NTX or LPS preconditioned groups; ###p<0.001. Differences were compared by one-way ANOVA. All values were expressed as mean ± SEM (n=6).

ing memory errors also changes significantly among groups ($F_{4,25}$=16.05; p<0.0001; Fig. 3E) with no interaction between factors ($F_{36,225}$=0.2085; p>0.9999; Fig. 3E). To better demonstrate the overall changes during the first two training trial days, the area under the curve (AUC) of these parameters was also calculated. One-way analysis of variance showed that there was a significant difference in latency to locate the platform AUC ($F_{4,25}$=15.43, p<0.0001), reference memory errors AUC ($F_{4,5}$=17.91, p<0.0001), and working memory errors AUC ($F_{4,25}$=19.38, p<0.0001) between groups. Fig. 3B showed that the AUC for latency to locate the platform in the LPS or NTX preconditioned group was less than the ischemic group (p<0.05). Fig. 3D and 3F showed that AUC for reference and working memory errors in the NTX (p<0.05) or LPS (p<0.01) preconditioned group was less compared with the ischemic group. Moreover, the AUC calculations revealed that animals in the co-preconditioned group had a lower latency to locate the platform and less reference and working memory errors compared with the ischemic group (p<0.001) and the NTX or LPS preconditioned groups (p<0.05). On the third day of the RAWM test, velocity and total distance traveled was recorded again. Results showed that there was no significant difference between experimental groups for velocity (Fig. 4A) but there was an effect for total distance ($F_{4,25}$=12.77, p<0.0001) and animals in the ischemic group traveled a greater total distance compared with animals in the NTX or LPS preconditioned groups (p<0.05; Fig. 4B). Co-preconditioned rats traveled a lesser total distance compared with the ischemic group (p<0.001) and NTX or LPS preconditioned groups (p<0.05) (Fig. 4B). Moreover, in the probe trial, one-way analysis of variance showed that there was a significant effect for duration spent in goal arm ($F_{4,25}$=20.39, p<0.0001) and number of entries ($F_{4,25}$=13.85, p<0.0001) between different groups. Animals in the ischemic group spent less time in the goal arm and had fewer en-

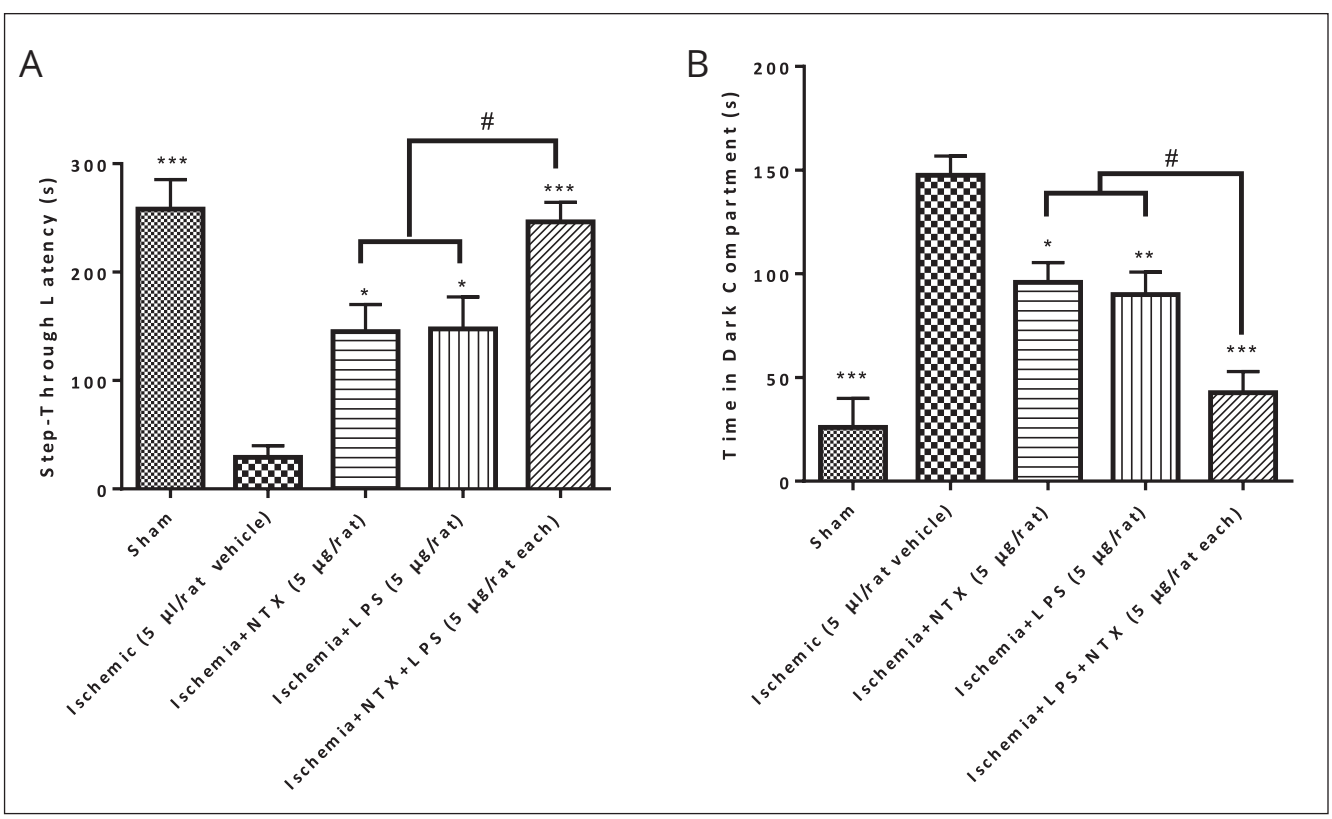

Fig. 2. (A) Step-Through Latency (STL) (s) on the retention test day in the passive avoidance test. Hippocampal ischemia in the ischemic group (5 µl/rat vehicle; i.c.v.) caused a significant decrease in latency to enter the dark compartment compared with the sham group; ***p<0.001. Preconditioning and co-preconditioning with NTX (5 µg/rat; i.c.v.) and/or LPS (5 µg/rat; i.c.v.) resulted in significantly more STL compared with the ischemic group; *p<0.05 and ***p< 0.001. Also, the differences between the co-preconditioned group (with LPS and NTX) and preconditioned groups (with LPS or NTX) in STL were statically significant; #p<0.05. (B) Time in dark compartment (TDC) (s) on the retention test day in the passive avoidance test. Hippocampal ischemia in the ischemic group (5 µl/rat vehicle; i.c.v.) caused a significant increase in time spent in the dark compartment compared with the sham group; ***p<0.001. Preconditioning and co-preconditioning with NTX (5 µg/rat; i.c.v.) and/or LPS (5 µg/rat; i.c.v.) resulted in significantly less TDC compared with the ischemic group; *p<0.05, **p<0.01, and ***p<0.001. Also, the differences between the co-preconditioned group (with LPS and NTX) and preconditioned groups (with LPS or NTX) were statically significant; #p<0.05. Differences were compared by one-way ANOVA. All values were expressed as mean ± SEM (n=8).

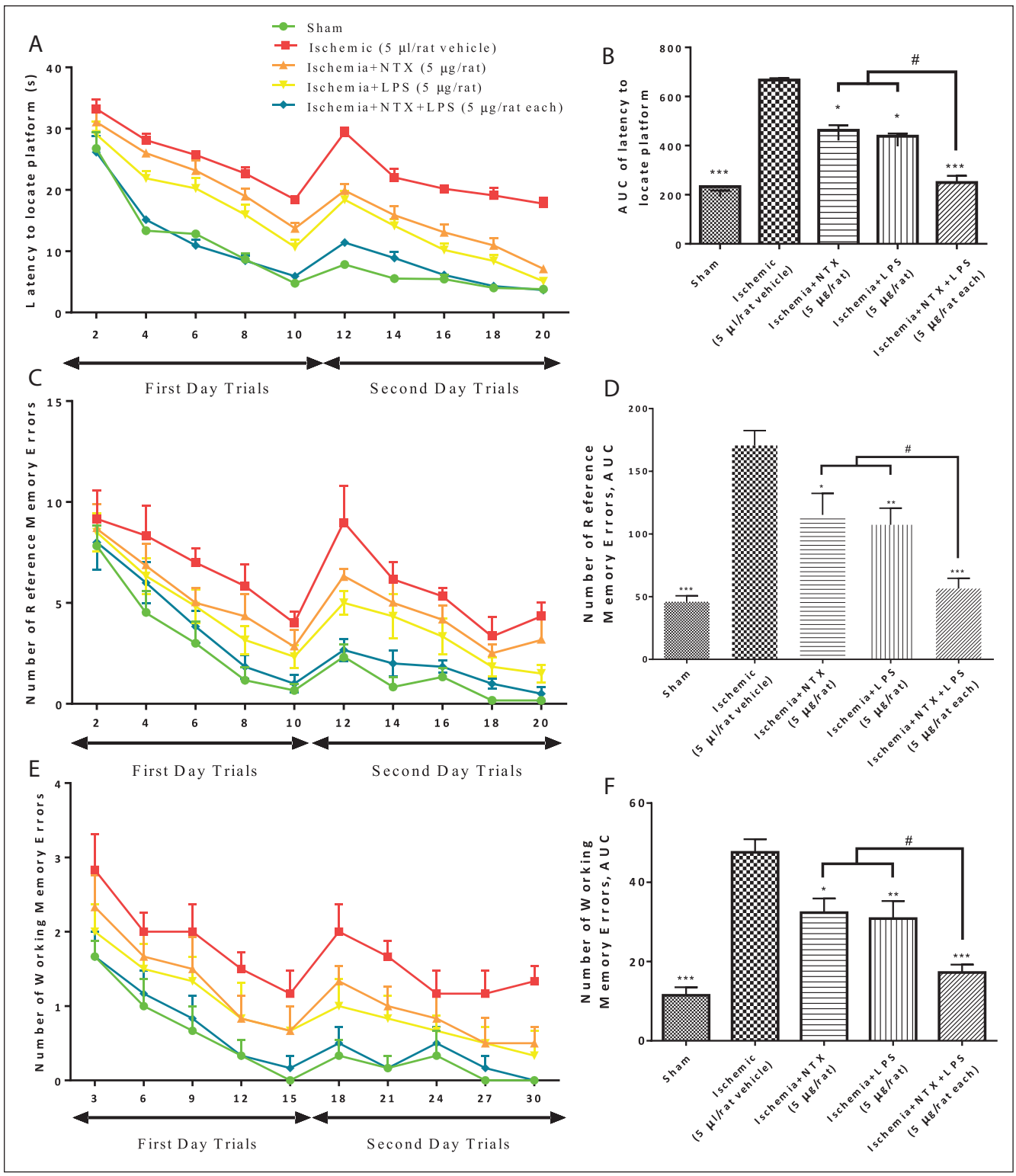

Fig. 3. (A) Latency to locate platform (s) on training trial days in the radial arm water maze test. Differences were compared by two-way ANOVA. (B) Area under the curve for latency to locate platform curve on training trial days in the radial arm water maze test. Differences were compared by one-way ANOVA. (C) Number of reference memory errors on training trial days in the radial arm water maze test. Differences were compared by two-way ANOVA. (D) Area under the curve for reference memory errors on training trial days in the radial arm water maze test. Differences were compared by one-way ANOVA. (E) Number of working memory errors on training trial days in the radial arm water maze test. Differences were compared by two-way ANOVA. (F) Area under the curve for working memory errors on training trial days in the radial arm water maze test. Differences were compared by one-way ANOVA. *p<0.05, **p<0.01, and ***p<0.001 compared with ischemic group; #p<0.05 compared to co-preconditioned group. All values were expressed as mean ± SEM (n=8).

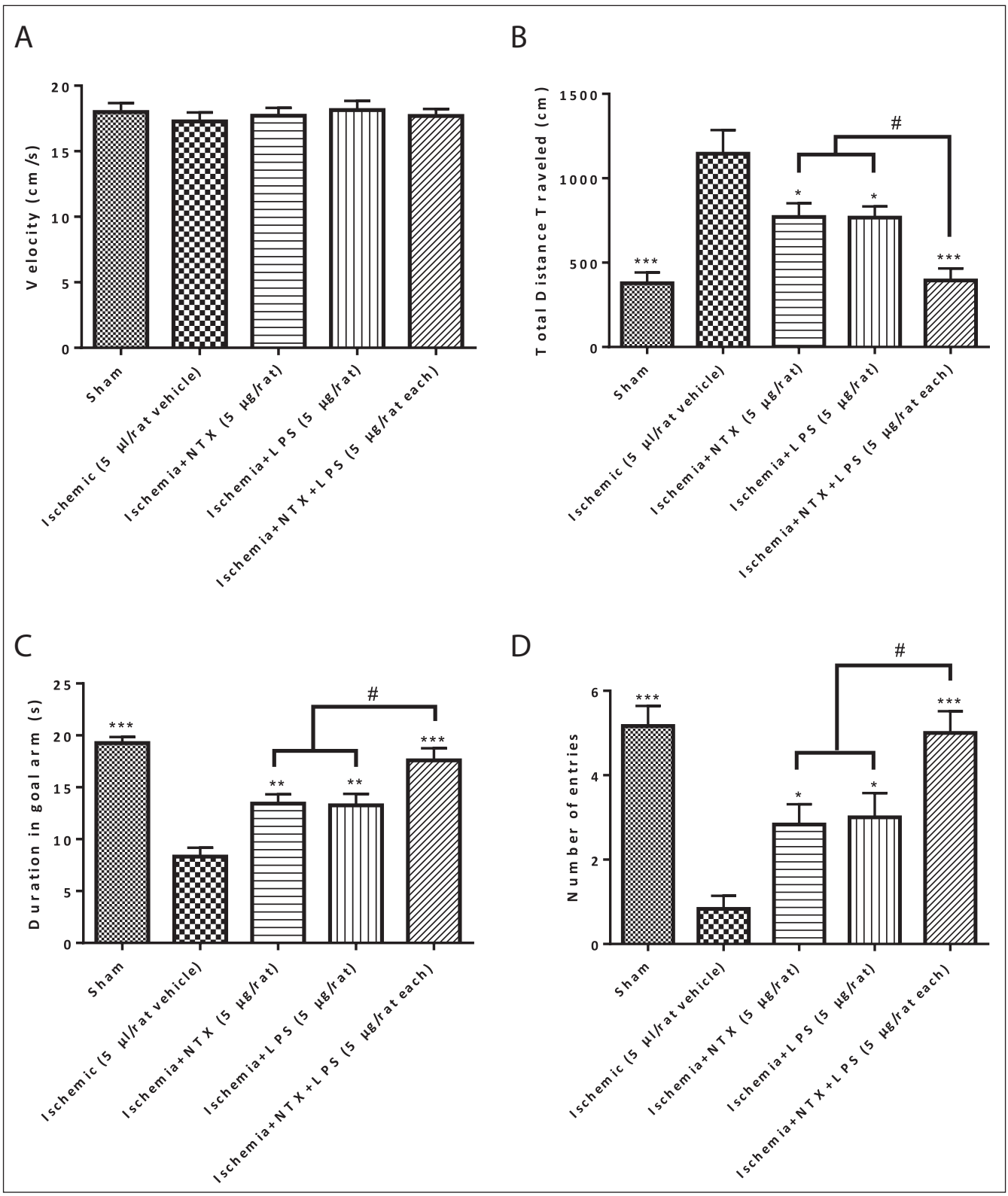

Fig. 4. (A) Velocity (cm/s) on the probe trial day in the radial arm water maze test. (B) Total distance traveled (cm) on the probe trial day in the radial arm water maze test. (C) Duration in goal arm (s) on the probe trial day in the radial arm water maze test. (D) Number of entries into the goal arm on the probe trial day in the radial arm water maze test. Differences were compared by one-way ANOVA. *p<0.05, **p<0.01 and ***p<0.001 compared with the ischemic group; #p<0.05 compared to the co-preconditioned group. All values were expressed as mean ± SEM (n=8).

tries into the goal arm compared with the sham group (p<0.001). LPS or NTX preconditioning resulted in a significant increase in the duration of time spent in the goal arm (p<0.01; Fig. 4C) and the number of entries into the goal arm (p<0.05; Fig. 4D) compared with the ischemic group. Furthermore, co-preconditioning with NTX and LPS resulted in more time spent in the goal arm and more entries into the goal arm compared with the ischemic group (p<0.001) and NTX or LPS preconditioned groups (p<0.05) (Fig. 4C and 4D).

DISCUSSION

In this study we aimed to examine the neuroprotective effect of low dose LPS and/or NTX preconditioning against hippocampal ischemia and the possible synergistic effect of co-preconditioning with LPS and NTX. We showed that both LPS and NTX preconditioning had a neuroprotective effect against hippocampal ischemia and LPS preconditioning's neuroprotection was comparable to or even slightly higher than neuroprotection from NTX preconditioning. Furthermore, there was a synergistic effect of LPS and NTX co-preconditioning, such that the protective effect of LPS and NTX co-preconditioning was significantly higher than that of LPS or NTX preconditioning alone.

Stroke is the most common CNS pathology and represents the second leading cause of death worldwide. The hippocampal neurons are primarily involved in learning and memory, thus any damage in this area may lead to learning and memory deficits (Sadelli et al., 2017). Oxidative stress following some brain injurious incidences, such as stroke, cause serious damage to the hippocampal formation which in turn causes memory deficiency, one of the most prevalent consequences of stroke (Altermann et al., 2017; Ramagiri and Taliyan, 2017). It was suggested that selective hippocampal susceptibility to ischemic incidence is due to massive calcium ion entry into the calcium-sensitive dendritic areas of vulnerable neurons following an ischemic attack (Vibulsresth et al., 1987). Studies showed that delayed dementia would occur in many stroke survivors within three months or after recurrent stroke (Kalaria et al., 2016). In addition, several cross-sectional epidemiological investigations have suggested that 25% of elderly patients suffer from delayed dementia (Desmond et al., 2002). In this study unilateral selective hippocampal ischemia was induced through a modified photothrombotic model to evaluate the neuroprotective effect of LPS and/or NTX preconditioning in stroke. Following photothrombotic ischemia induction, a considerable ischemic lesion developed in hippocampus, which caused learning and memory deficits in the ischemic group compared with the sham group. In the photothrombotic model of ischemia, interaction between systemic photosensitive dye (Rose Bengal) and optical fiber green light causes the generation of ROS. ROS causes endothelial damage followed by platelet activation and aggregation which leads to thrombosis formation in the area that the green light illuminated (Labat-Gest and Tomasi, 2013).

Preconditioning was established by single-dose i.c.v. administration of LPS and/or NTX. LPS was administered 48 h before hippocampal ischemia induction and NTX was administered 24 h prior to hippocampal ischemia induction. A protective effect resulting from LPS or NTX preconditioning has been demonstrated in previous study (Anrather and Iadecola, 2016; Hock, 1998). LPS has an agonistic effect on TLR4 (Toshchakov et al., 2002; O'Neill et al., 2013) and one of the most common expression sites for TLR4 is on microglia and astrocytes (Schaafsma et al., 2015). As mentioned earlier, TLR4 as a member of the PRRs, recognizes specific molecular patterns, including PAMPs or DAMPs. Following ischemic attack, DAMPs, such as heat shock proteins (HSPs), fibrinogen, RNA, and methylated DNA, are released from damaged tissue and recognized by TLR4. DAMPs and TLR4 interaction results in glial cell activation and inflammatory response which is associated with more serious and injurious consequences (Chen and Nuñez, 2010). TLR4 has two different signaling adapters, myeloid differentiation primary response gene 88 (MyD88) and TIR domain containing adapter protein inducing IFN-β (TRIF) (Kamigaki et al., 2016). MyD88 mediates a signaling pathway that activates NF-κB which in turn results in a deleterious inflammatory response and ROS production. TRIF mediates a signaling pathway that activates interferon regulatory factor 3 (IRF3) as well as NF-κB, which mainly results in the induction of anti-inflammatory mediators and type I interferons (IFNs). Thus, the MyD88-dependent pathway leads to injurious consequences while the TRIF-dependent pathway leads to neuroprotection (Vartanian et al., 2011; Anttila et al., 2016; Kamigaki et al., 2016). Following cerebral ischemic attack, interaction between TLR4 and injury-associated molecules such as HSP60 lead to the MyD88-dependent pathway. However, studies have shown that LPS preconditioning prior to cerebral ischemia causes TRIF-dependent pathway activation following TLR4 and DAMPs interaction (Marsh et al., 2009). It seems that after the primary interaction between TLR4 and LPS during preconditioning, a brief inflammatory response leads to the expression of some TLR4–NF-κB signaling axis inhibitors, such as Ship-1, IRAK-M, and TRIM30α. This inhibition continues until a second interaction between TLR4 and its ligands, such as DAMPs following stroke, which in turn causes activation of the TRIF-dependent pathway and neuroprotection (Marsh et al., 2009).

NTX HCl has been approved by the FDA for opioid addiction treatment since 1984. It was reported that treatment with low dose NTX results in paradoxical effects, such as analgesia and anti-inflammatory action (Younger et al., 2014). It was shown that NTX at low doses is effective in curing some inflammatory diseases, including Crohn's disease (CD), multiple sclerosis (MS), and complex regional pain syndrome (CRPS) (Cree et al., 2010; Smith et al., 2011; Chopra and Cooper, 2013). The exact mechanisms underlying NTX's anti-inflammatory properties are not completely known. However, it has been suggested that transient opioid receptor blockade following low dose NTX administration may result in both endogenous opioid and opioid receptor upregulation. This effect could augment endogenous analgesia and suppression of critical immune factors (Brown and Panksepp, 2009). It was reported that opioid receptor antagonists could decrease excitatory amino acid, namely glutamate, concentrations during periods of spinal cord ischemia. Moreover, it was shown that naloxone treatment could improve blood flow and outcome during an ischemic event in an animal model of stroke (Lu and Liu, 2009; Tang et al., 2005). Several other studies reported that TLRs are involved in the anti-inflammatory effect of low dose NTX (Watkins et al., 2007). It seems that the antagonistic effect of TLRs may be responsible for the anti-inflammatory properties of opioid antagonists. This anti-inflammatory effect results in the suppression of microglial activation and reduction of ROS production and inflammation (Chang et al., 2000). This hypothesis is further supported in light of dextro-naltrexone's neuroprotective effect (Lewis et al., 2012). Dextro-naltrexone is an NTX stereoisomer which is active at microglia receptors but has no affinity for opioid receptors (Lewis et al., 2012; Valentino et al., 1983). Furthermore, there is strong evidence supporting that NTX inhibits intracellular TLR subtypes, such as TLR7, TLR8, and TLR9, and thereby inhibits the secretion of inflammatory cytokine such as TNF-α and Il-6. Surprisingly, this study showed that NTX did not inhibit TLR4 which is located on the cell surface. This could be explained by the fact that intracellular subtypes (TLR7, TLR8, and TLR9) signal through the MyD88-dependent pathway, although TLR4 signals via both the MyD88-dependent and MyD88-independent TRIF pathway. Therefore, after NTX preconditioning, TLR4 signaling may continue through the MyD88-independent TRIF pathway and lead to IRF3 and anti-inflammatory cytokine induction (Cant et al., 2017).

In this study we showed that LPS or NTX preconditioning has neuroprotective effect against photothrombotic hippocampal ischemia. TTC staining revealed infarct size reduction in LPS ($p<0.001$) and in NTX ($p<0.05$) preconditioned rats. In addition, behavioral testing demonstrated that both LPS and NTX preconditioning decreased learning and memory impairments following photothrombotic hippocampal ischemia. Furthermore, the neuroprotective efficacy against hippocampal ischemia of co-preconditioning with LPS and NTX was significantly higher than that of LPS or NTX preconditioning alone. The differences between co-preconditioned and preconditioned groups in infarct size reduction ($p<0.001$) and memory impairment prevention ($p<0.05$) were statistically significant. These findings may suggest that a synergistic effect occurs upon NTX and LPS co-preconditioning. Previous studies have indicated that LPS preconditioning exerts its neuroprotective effect through TLR4 (Vartanian et al., 2011). NTX's neuroprotective effect against ischemic events is not fully understood, although it has been postulated that the anti-inflammatory effects of NTX, through inhibition of the intracellular TLR subtypes, are causative in the observed synergism. NTX preconditioning by way of blocking the MyD88-dependent pathway of intracellular TLRs (Cant et al., 2017) and LPS preconditioning by way of blocking the MyD88-dependent pathway of TLR4 (Marsh et al., 2009) could suppress the inflammatory response after ischemia and lessen neuronal cell damage. Thus, the co-preconditioning of NTX and LPS and resulting block of the MyD88-dependent pathway for almost all TLR subtypes would result in a synergistic effect and neuroprotection against ischemic insult. However, additional molecular investigations are needed to determine the exact mechanism underlying the observed synergistic effect.

In the RAWM test, during first two training trial days, total distance traveled and velocity between different experimental groups was identical (data not shown). We recorded these two parameters as an index of intact motor ability. Although, surprisingly, on the third test day of the RAWM (in which the platform was removed from the apparatus), animals in the ischemic group traveled a greater total distance compared with the preconditioned groups. This may be due to greater memory impairment in the ischemic group that resulted in more disorientation and vain attempts to find the platform in arms other than the goal arm, while preconditioned rats spent more time in the goal arm, and traveled less distance compared with animals in ischemic group.

CONCLUSIONS

In this study we demonstrated that LPS or NTX preconditioning exerts a considerable neuroprotective effect against photothrombotic hippocampal ischemia.

Inflammatory response following ischemic attack may have been prevented by preconditioning with these agents, resulting in significant infarct volume reduction and prevention of learning and memory impairments. Additionally, a clear synergistic effect occurred upon NTX and LPS co-preconditioning. Further studies are required to determine the exact molecular mechanisms underlying the distinct neuroprotective effects of NTX and LPS co-preconditioning.

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