● RESEARCH ARTICLE

Cold water swimming pretreatment reduces cognitive deficits in a rat model of traumatic brain injury

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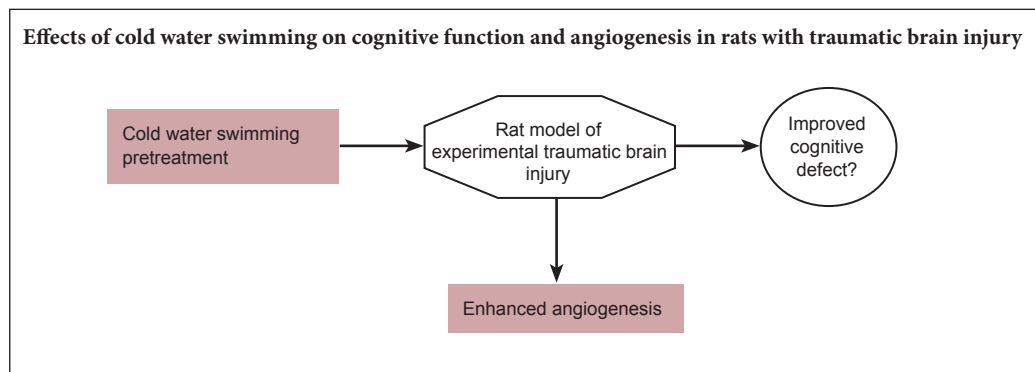5 Intensive Care Units, Tianjin Huanhu Hospital, Tianjin, China
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Graphical Abstract

Effects of cold water swimming on cognitive function and angiogenesis in rats with traumatic brain injury

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Abstract

A moderate stress such as cold water swimming can raise the tolerance of the body to potentially injurious events. However, little is known about the mechanism of beneficial effects induced by moderate stress. In this study, we used a classic rat model of traumatic brain injury to test the hypothesis that cold water swimming preconditioning improved the recovery of cognitive functions and explored the mechanisms. Results showed that after traumatic brain injury, pre-conditioned rats (cold water swimming for 3 minutes at 4°C) spent a significantly higher percent of times in the goal quadrant of cold water swim, and escape latencies were shorter than for non-pretreated rats. The number of circulating endothelial progenitor cells was significantly higher in pre-conditioned rats than those without pretreatment at 0, 3, 6 and 24 hours after traumatic brain injury. Immunohistochemical staining and Von Willebrand factor staining demonstrated that the number of CD34^{+} stem cells and new blood vessels in the injured hippocampus tissue increased significantly in pre-conditioned rats. These data suggest that pretreatment with cold water swimming could promote the proliferation of endothelial progenitor cells and angiogenesis in the peripheral blood and hippocampus. It also ameliorated cognitive deficits caused by experimental traumatic brain injury.

Key Words: *nerve regeneration; cold water swimming; cognitive deficits; endothelial progenitor cells; angiogenesis; neural repair; stress; Morris water maze; fluid percussion injury model; CD34; Von Willebrand factor; neural regeneration*

Introduction

Chronic or severe stress on rodents can result in numerous alterations of their neuroanatomical and neurochemical properties (Buwalda et al., 2005), which have negative consequences in the central nervous system (Adlard et al., 2011). On the other hand, they could be imperative for adapting to changing circumstances. A number of studies have shown the beneficial effects to the lifespan of animals from exposure to stress or harm, including hypergravity, low exposure of toxic substances, heat shock and cold shock (Lindsay, 2005; Shevchuk, 2008; Genchi et al., 2015). There is evidence that stress, such as inescapable shock, can facilitate subsequent learning on such tasks as conditioned eye blinks (Shors et al., 1992; Shors, 2004). It has also been reported that mild stress facilitates learning in mice (Adlard et al., 2011). A moderate amount of electric shock (electroconvulsive therapy) has

long been used to treat drug-resistant depression (Ishihara and Sasa, 1999). These stress-facilitated improvements in neurocognitive functions are believed to be mediated through activating the sympathetic nervous system, increasing blood flow, and enhancing cerebral synaptic release of noradrenaline (Jansky et al., 1996; Jedema et al., 2001; Nutt, 2002). They could also increase production of beta-endorphin (Vaswani et al., 1988), which is known to produce the sense of well-being. Synthesis of growth factors such as vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor increased extensively after cold water immersion and immobilization stress. These growth factors promote angiogenesis and neural repair in injured tissues and organs (Kim et al., 2005; Kondo et al., 2010).

One potential mechanism for the effect of moderate stress is to mobilize stem cells so they are already available to improve a subject's response to injury. Endothelial progenitor cells (EPCs) are the precursor cells of vascular endothelial cells, expressing CD34, CD133, VEGFR-2, that contribute to new vessel formation in postnatal angiogenesis. They are mobilized by physiological and pathological stresses, such as exercise, trauma, tumor, and inflammation (Okazaki et al., 2006; Liu et al., 2007; Schlager et al., 2011). They could increase the rate of angiogenesis and ameliorate substance metabolism to improve tissue perfusion and repair. Increases in circulating EPCs are reported to improve clinical outcomes of stroke, myocardial infarction and diabetes (Fan et al., 2010; Reinhard et al., 2010; Sorrentino et al., 2011). We have previously demonstrated that increasing EPCs, induced by progesterone or atorvastatin, promoted the functional recovery of brain trauma in a rat model (Li et al., 2012; Wang et al., 2012). In this study, we tested the hypothesis that rats pre-exposed to cold water swimming (CWS) improved cognitive defects induced by experimental TBI. We also tested whether this beneficial effect is associated with the level of mobilized EPCs in the peripheral blood.

Materials and Methods

Animals
232 adult male Wistar rats (280–320 g; Experimental Animal Laboratories of the Academy of Military Medical Sciences; Beijing, China) were housed individually in a temperature-controlled (22°C) and humidity-controlled (60%) vivarium, and maintained on a standard 12-hour light/dark cycle (7:00 a.m. to 7:00 p.m. per cycle) with free access to food and water. The study protocol was approved by the Ethics Committee of Tianjin Medical University. The experiment followed the National Guidelines for the Care and Use of Laboratory Animals, and "Consensus Author Guidelines on Animal Ethics and Welfare" produced by the International Association for Veterinary Editors (IAVE). Experiments were designed to minimize the number of animals required and those used were cared for, handled and medicated as appropriate to minimize their suffering.

The 232 rats were randomly divided into four groups (*n* = 58): CWS group: rats were exposed to CWS; sham group: rats were not exposed to CWS or fluid percussion injury (FPI); TBI group: rats were exposed to FPI; CWS-TBI group: rats were subjected to CWS for 1 week before being exposed to FPI.

CWS models
Rats were placed into a tub (50 cm deep, 150 cm diameter) containing water at 4°C and allowed to swim for 3 minutes in the cold water as previously described (Commons, 2003). The rats trembled as they swam for the full 3 minutes. Subsequently, the rats were taken out from the tub and then immediately returned to home cage after drying. This was repeated each day for 1 week. This process lasted for 1 week.

FPI models
Rats with or without the pretreatment (CWS) were subjected to FPI. FPI was performed as previously described (Chen et al., 2009). Briefly, rats were anesthetized with 10% chloride hydrate (3.0 mL/kg, intraperitoneally) and placed in a stereotaxic frame. The scalp was reflected with a single incision and the temporal muscles scraped from the skull. Craniotomy (4.0 mm × 4.0 mm) was performed over the right parietal skull, 2.0 mm lateral from sagittal suture and 3.0 mm caudal from coronal suture, keeping the dura intact. Subsequently, a luer-lock connector (3 mm diameter) was secured to the skull over the opening with cyanoacrylate adhesive and dental acrylic. The skull sutures were sealed with the cyanoacrylate to ensure that the fluid bolus from the injury remained within the cranial cavity. Twenty-four hours after surgery, the rats were subjected to experimental FPI of 2.0–2.2 atmosphere by an FPI device (model 01-B; New Sun Health Products, Cedar Bluff, VA, USA). A rapid bolus of saline from a Plexiglas cylindrical reservoir was introduced into the closed cranial cavity, causing mechanical deformation of the brain. Rat limbs suddenly twitched, and then slowly returned to normal. Immediately after FPI, the incision was suture-closed and the rats were placed on a heating pad until ambulatory and then returned to the home cage.

Morris water maze task
Learning abilities were assessed using a Morris water maze. Rats were trained using a Morris water maze (DMS-2, Chinese Academy of Science, China) according to the protocol by Vorhees and Williams (2006), *n* = 10 per group. Briefly, a tank measuring 150 cm in diameter and 50 cm in height was filled with water at 20–22°C. A target platform (10 cm diameter) was hidden 2 cm below the water surface in a southeast location halfway between the center and the wall of the maze. Rats were allowed to adapt the maze without a platform for 1 minute per day for 3 days before training. Afterwards, the rats were trained to rely on visual distal cues to locate a submerged escape platform. A computerized tracking system (Etho-vision 3.0; Noldus Information Technology, Wageningen, Netherlands) was used to record latency (time to reach the platform) and swim speed. Four
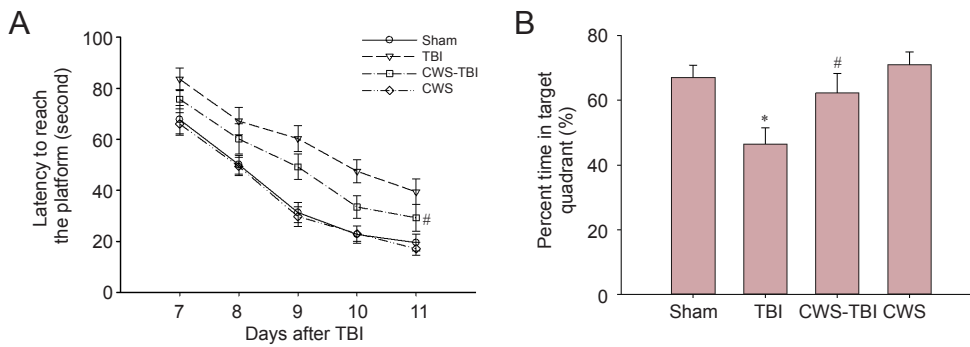
Figure 1 Effects of CWS pretreatment on learning and memory abilities in rats with brain injury.
(A) Escape latency in Morris water maze during training 7 to 11 days post TBI. (B) Percent time in target quadrant of Morris water maze at 12 days post-TBI. Data are expressed as the mean ± SEM (n = 10/group, one-way analysis of variance and the least significant difference test). *P < 0.05, vs. sham group; #P < 0.05, vs. TBI group. Sham group: Rats were not exposed to CWS or fluid percussion injury; TBI group: rats were exposed to fluid percussion injury; CWS-TBI group: rats were subjected to CWS daily for 1 week before being exposed to fluid percussion injury; CWS group: rats were exposed to CWS. The rats subjected to TBI had longer latencies than those without TBI. The CWS-TBI group had a shorter mean latency than the TBI only rats. Compared with the TBI group, rats in the CWS-TBI group spent significantly longer percent times in goal quadrant (P = 0.025). All of this indicated that CWS improved the recovery of cognitive functions after TBI. TBI: Traumatic brain injury; CWS: cold water swimming.
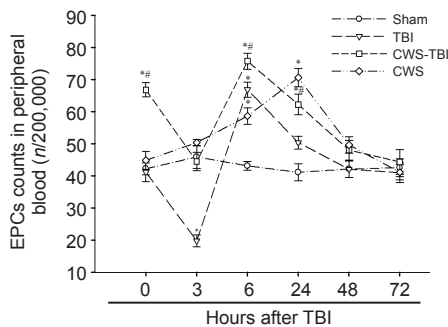
Figure 2 Flow cytometry of EPCs in peripheral blood of rats at 0, 3, 6, 24, 48 and 72 hours after TBI.
The number of EPCs was more in the CWS-TBI group than that in the TBI group at 0, 3, 6 and 24 hours after FPI. Data are expressed as the mean ± SEM (n = 6 in each group at each time point, one-way analysis of variance and the least significant difference test). *P < 0.05, vs. sham group; #P < 0.05, vs. TBI group. Sham group: Rats were not exposed to CWS or FPI; TBI group: rats were exposed to FPI; CWS-TBI group: rats were subjected to CWS daily for 1 week before being exposed to FPI; CWS group: rats were exposed to CWS. TBI: Traumatic brain injury; EPCs: endothelial progenitor cells; FPI: fluid percussion injury; CWS: cold water swimming.

trials from four random start positions (east, north, southeast, and northwest) were tested daily (each trial lasted for 120 seconds with 15 seconds intervals) for 5 consecutive days (from 7 days through 11 days post-injury). Rats that failed to find the platform within 2 minutes were recorded for a maximum latency score of 120 seconds. Latency (seconds) and path length (cm) were recorded over time to generate a spatial learning curve. At 12 days after TBI, the platform was removed and a probe trail was performed with a novel start position, facing the tank wall. The time the rats stayed in the goal quadrant during a 30-second period was recorded.
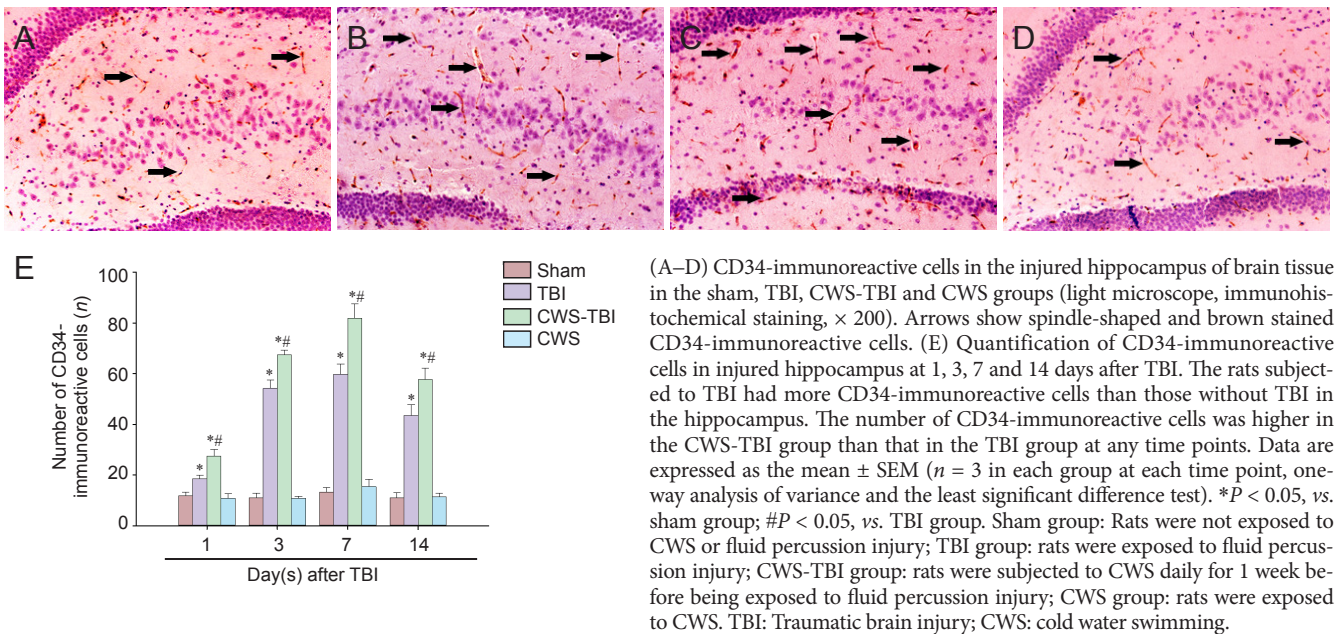
Measurement of EPCs by flow cytometry
Peripheral blood samples (0.5 mL) were collected from retro-orbital venous plexus at baseline (0), 3, 6, 24, 48, and 72

hours after FPI and diluted with PBS (6 rats at each time point). Peripheral blood mononuclear cells were isolated by density-gradient centrifugation using Ficoll-Paque Plus (Chuanye, Tianjin, China). Isolated cells were washed twice with PBS and resuspended in 200 μL of PBS supplemented with 0.5% of bovine serum albumin and 2 mM of ethylenediaminetetraacetic acid. EPCs in peripheral blood were evaluated by first staining with PE-conjugated CD34 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and purified CD133 primary antibody (Abcam, Cambridge, UK) conjugated with FITC (Abcam), then detected by flow cytometry (FACScan, Becton-Dickinson, San Jose, CA, USA). The isotype-matched IgG was used as a control. CD34 and CD133 double positive cells were defined as EPCs. We counted the number of endothelial progenitor cells per 200,000 mononuclear cells.

Rats in each group were sacrificed at 1, 3, 7 and 14 days (3 rats at each time point) after brain trauma and perfused with 0.9% NaCl through the heart to remove blood from the vasculature. After perfusion, brains were collected and fixed in 4% paraformaldehyde for 24 hours and processed as 5 mm coronal paraffin-embedded tissue blocks through the TBI zone. Finally, these blocks were cut into 5–7 μm sections for immunohistochemical staining.

Immunohistochemistry
CD34 immunoreactivity in injured brain tissue were detected by a CD34-antibody (R&D Systems, Minneapolis, MN, USA) as recommended by the manufacturer. Briefly, after deparaffinization and rehydration, non-specific endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide in methanol for 30 minutes. Antigen was recovered by boiling the sections for 20 minutes in 10 mM citrate buffer (pH 6.0). Non-specific binding was blocked with 3% bovine serum albumin in PBS for 30 minutes. The sections were then incubated with a goat CD34 polyclonal antibody (1:200) and Von Wille-

(A–D) CD34-immunoreactive cells in the injured hippocampus of brain tissue in the sham, TBI, CWS-TBI and CWS groups (light microscope, immunohistochemical staining, × 200). Arrows show spindle-shaped and brown stained CD34-immunoreactive cells. (E) Quantification of CD34-immunoreactive cells in injured hippocampus at 1, 3, 7 and 14 days after TBI. The rats subjected to TBI had more CD34-immunoreactive cells than those without TBI in the hippocampus. The number of CD34-immunoreactive cells was higher in the CWS-TBI group than that in the TBI group at any time points. Data are expressed as the mean ± SEM (n = 3 in each group at each time point, one-way analysis of variance and the least significant difference test). *$P < 0.05$, *vs.* sham group; #$P < 0.05$, *vs.* TBI group. Sham group: Rats were not exposed to CWS or fluid percussion injury; TBI group: rats were exposed to fluid percussion injury; CWS-TBI group: rats were subjected to CWS daily for 1 week before being exposed to fluid percussion injury; CWS group: rats were exposed to CWS. TBI: Traumatic brain injury; CWS: cold water swimming.

Figure 3 CD34 immunoreactivity in the injured hippocampus of brain tissue at 1, 3, 7, and 14 days after TBI.

(A–D) vWF-immunoreactive vessels in the sham, TBI, CWS-TBI and CWS groups. Black arrows indicate the vWF-immunoreactive vascular lumen-like structure, which was brown staining (light microscope, immunohistochemical staining, × 200). (E) vWF-immunoreactive vessel density in the injured hippocampus at 1, 3, 7 and 14 days after TBI. Vascular density significantly increased in the rats subjected to TBI. vWF-immunoreactive vascular density in CWS-TBI rats was higher than that in TBI rats at 3, 7 and 14 days after TBI. Data are expressed as the mean ± SEM (n = 3 in each group at each time point, one-way analysis of variance and the least significant difference test). *$P < 0.05$, *vs.* sham group; #$P < 0.05$, *vs.* TBI group. Sham group: Rats were not exposed to CWS or fluid percussion injury; TBI group: rats were exposed to fluid percussion injury; CWS-TBI group: rats were subjected to CWS daily for 1 week before being exposed to FPI; CWS group: rats were exposed to CWS. TBI: Traumatic brain injury; vWF: von Willebrand factor; CWS: cold water swimming.

Figure 4 Detection of vascular density by vWF antibody staining in the injured hippocampus at 1, 3, 7, and 14 days after TBI.

brand factor (vWF) (Abcam, Cambridge, UK) overnight at 4°C. They were then washed with PBS, incubated with a biotinylated anti-goat IgG (1:100; Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 hour at 37°C, washed and incubated in an avidin peroxidase conjugate solution (Beijing Zhongshan Golden Bridge Biotechnology) for 30 minutes. Finally, the sections were developed with diaminobenzidine for 3 minutes. Negative controls were similarly processed without the primary antibody. The number of endothelial-like CD34-immunoreactive cells in each section was counted (per 200×,

IX2UCB; Olympus, Tokyo, Japan) in five fields by two independent observers, each blinded to the experimental conditions, to obtain an average number of CD34-immunoreactive cells per view field (per 200×).

Microvasculature was quantified by counting vWF-immunoreactive vessels in a protocol similar to the CD34 staining. Two independent observers, blinded to the experimental conditions, counted vWF-immunoreactive vessels in five sections under a light microscope (IX2UCB; Olympus). The brown stained vascular lumen-like structure was defined as vessels.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). The data are presented as the mean ± SEM. One-way analysis of variance with *post hoc* least significant difference test was used to analyze data. A value of $P < 0.05$ was considered statistically significant.

Results

Improved recovery of cognitive functions after TBI in rats pretreated with CWS

To assess changes in cognitive function, the spatial memory of rats was tested in Morris water maze, which measures a rat's ability to navigate from a start location in a water maze to a submerged escape platform. As expected, latency was significantly shortened during the 5-day spatial acquisition test ($F = 251.909$, $P = 0.000$), suggesting that spatial memory had developed in all rats (**Figure 1A**). However, the escape latency of all rats was influenced by grouping ($F_{(3, 36)} = 8.31$, $P = 0.000$). The rats subjected to TBI had longer latencies than those without TBI. The CWS-TBI group had a shorter mean latency than the TBI only rats ($P = 0.013$), which indicated that CWS improved the recovery of cognitive functions after TBI.

On day 6 after training, the platform was removed and the ability of rats to look for the removed platform was measured as percent of time they swam in the goal quadrant (reference memory). Our study found that those rats in the TBI group had the lowest percent time (**Figure 1B**). Compared with the TBI group, rats in the CWS-TBI group spent significantly higher percent time in goal quadrant ($P = 0.025$), which represented a better recovery of cognitive defect than TBI without CWS.

EPCs in peripheral blood

To test the changes of EPCs number in rats of each group, blood samples were stained for CD34 and CD133, and measured by flow cytometry. We found that there was no significant change in the EPCs number in the sham group at any time point (**Figure 2**). EPCs levels were higher in the CWS group than that in the sham group at 6 and 24 hours ($P < 0.05$). Compared to the sham group, the number of EPCs in the TBI group decreased rapidly at 3 hours, increased to the peak level at 6 hours, and declined gradually to the normal level thereafter. The CWS-TBI group had more EPCs at 0, 6 and 24 hours compared with the sham group ($P < 0.05$). Although the EPCs number showed similar trends in rats of the TBI group and CWS-TBI group after TBI, the CWS-TBI group had higher numbers of EPCs at 0, 3, 6 and 24 hours than that in TBI group ($P < 0.05$).

Angiogenesis in the hippocampus

CD34 is a marker for the progenitor hematopoietic cells and is expressed on new microvascular endothelial cells. CD34-immunoreactivity therefore indicates angiogenesis by the hematopoietic progenitor cells. In this study, we found that the number of CD34-immunoreactive cells increased in the hippocampus, where FPI occurred, at 1, 3, 7 and 14

days after TBI (**Figure 3**). However, there was no change in the CWS group ($P > 0.05$). The number of CD34-immunoreactive cells in injured hippocampus was significantly higher in rats from the CWS-TBI group as compared to that in the TBI group after TBI ($P < 0.05$).

We also used an antibody to vWF, a vascular marker, to detect the changes in number of vascular vesicles for each group. The vWF-immunoreactivity vessel density of TBI group increased significantly at 3, 7 and 14 days after TBI as compared to the sham group ($P < 0.05$; **Figure 4**). vWF-immunoreactive vessel density increased significantly at 1, 3, 7 and 14 days in CWS-TBI group ($P < 0.05$). As with the results of CD34 staining in the CWS group, vWF-immunoreactive vessel density was not significantly different from the sham group ($P > 0.05$). There was a greater density of vWF-immunoreactive vessel segments in the CWS-TBI group than that in the TBI group at 3, 7 and 14 days ($P < 0.05$).

Discussion

Using a well-characterized model, we examined cognitive defects in rats subjected to experimentally controlled FPI and correlated such changes with EPCs in peripheral blood and CD34-immunoreactive cells and vascular density in hippocampus. We found that rats developed cognitive defects after TBI, and those pretreated with CWS had a higher tolerance to the brain injury and improved learning ability after TBI.

The number of EPCs in peripheral blood has been associated with angiogenesis (Asahara et al., 1997) and is known to be elevated by physiological and pathological conditions, such as exercise, trauma, tumor, and inflammation (Okazaki et al., 2006; Liu et al., 2007; Schlager et al., 2011). In this study, we further showed that the numbers of circulating EPCs were consistently higher in rats pre-conditioned with CWS as compared to those without the preconditioning after TBI. This is primarily due to a higher baseline level of EPCs after CWS preconditioning that mobilized EPCs from the bone marrow. The high EPCs level may contribute to increased angiogenesis in response to TBI and subsequent improvement in neurocognitive functions. This association is supported by the finding that angiogenesis was more intensive in hippocampus of rats with CWS preconditioning. The finding is consistent with previous reports from other laboratories (Fadini et al., 2009; Rufaihah et al., 2010). Our results also lend support to the notion that endogenously mobilized or exogenous transplanted EPCs may ameliorate the outcome of ischemic or traumatic tissue damages (Fan et al., 2010; Reinhard et al., 2010; Sorrentino et al., 2011). In previous studies, we found that progesterone or atorvastatin could increase circulating EPCs and promote neural regeneration and angiogenesis (Li et al., 2012; Wang et al., 2012). As shown in **Figures 2** and **3**, peak levels of circulating EPCs and the number of CD34-immunoreactive cells and vascularization in ipsilateral hippocampus tissue indicate a sequence of events when EPCs are mobilized during TBI. Circulating EPCs eventually home in on sites of vascular injury and form vascular buds that will eventually develop into new vessels

(Young et al., 2007; Guo et al., 2009). This angiogenesis process has been kick-started by CWS preconditioning which enabled rapid mobilization of EPC from the bone marrow immediately after the TBI. Circulating EPCs may also participate in repairing damaged blood brain barrier (Kaneko et al., 2012). Increase in angiogenesis improves regional blood flow that will deliver such critical repair factors as VEGF and neurotrophic growth factor (Borlongan et al., 2004; Yu et al., 2010). It promotes neural cell survival (Teng et al., 2008), accelerates clearance of apoptotic cells (Gautier et al., 2000), and removes neurotoxic substances such as NAD(P)H oxidase, superoxide and glutamate (Shin et al., 2003; Belin de Chantemele et al., 2009). The increased expression of VEGF after TBI, described in our previous study (Zhang et al., 2013), enables EPC homing to injured brain tissue by the VEGF/VEGFR-2 pathway (Li et al., 2006). Compared with the TBI group, there is a significant increase of CD34- and vWF-immunoreactive cells in the injured brain tissue of rats in CWS-TBI group because more EPCs home in there in the early stage of brain trauma.

VEGF is one of the important factors of angiogenesis. There are some reports that cold stimulation could increase VEGF expression in rat skeletal muscle cells (Sugasawa et al., 2016). Cold water immersion also augments the expression of VEGF mRNA in human skeletal muscle and the adaptive response to acute exercise (Joo et al., 2016). After CWS, the stress hormone glucocorticoid increased immediately (Metz et al., 2005). It has also been reported that intermediate doses of glucocorticoids for a short period of time significantly increase circulating endothelial progenitor cells and promote angiogenesis in active rheumatoid arthritis (Grisar et al., 2007). These effects from CWS may explain how CWS could enhance CD34 and vWF immunoreactivity in injured hippocampus after traumatic brain injury.

In conclusion, CWS pre-conditioned rats have better tolerance to TBI and alleviated TBI-associated cognitive defect. This benefit from CWS preconditioning is associated with improved angiogenesis in the injured brain and a high circulating level of EPCs is critical for angiogenesis.

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