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Original Research Article

Intravenous Versus Intraarterial Transplantation of Human Umbilical Cord Blood Mononuclear Cells for Brain Ischemia in Rats

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ABSTRACT

Cerebral ischemia is among the most common type of stroke seen in patient. Regeneration of death neurons remains questionable. Human umbilical cord blood mononuclear cell (cbMNC) is one of the treatment options for ischemia stroke through their various advantages; availability, pluripotency, and immaturity. One group of healthy rats and three groups ($n = 6$ per group) of male Wistar rats undergone permanent middle cerebral artery occlusion (MCAO). Rats were allowed to recover for 7 days before intraarterial and intravenous injection of 1×10^6 cells/kg of human cbMNC. Behavioral tests were performed before the MCAO, 1 week after MCAO, and at 3, 9, and 14 days after cbMNC injection. Brain infarct area and neurons in hippocampus were evaluated. Spontaneous activity was much significantly improved compared with the placebo group ($p < 0.05$). Comparing the neuron cells in hippocampus, intraarterial and intravenous have more changes in neurons morphology. No effect of cbMNC implantation in decreasing infarct area. Safety of xenogenic was confirmed by this study when the dosage of 1×10^6 cells/kg was used and showed their beneficial effects.

1. Introduction

Stroke is one of the commonest diseases with high mortality and morbidity. Recently, the only treatment approved by the food drug administration for acute ischemic stroke is thrombolysis, even though this treatment has limited golden period. Thrombolysis intravenously can be performed only in ischemic stroke where the occlusion is not in the middle cerebral artery, and regeneration therapy of death cells, unfortunately, remains questionable (Wei *et al.* 2013; Shinozuka *et al.* 2013; Brouns *et al.* 2009).

Many studies have been conducted to show the effectiveness of cell-based therapy by differentiating the route (intraarterial (IA), intravenous (IV), and intraparenchyme) with result intraarterially is more superior and safer compared with the other route. This hypothesis arised because IA route showed larger amount of cells that injected to the site of injury compared with IV route where the possibility of cells to enterapt in the lung was higher and they must be passing through peripheral organs, and the cell amount would be less in the site of injury as consequences (Guzman *et al.* 2008).

From previous studies, cell-based therapy for ischemic stroke, where cord blood mononuclear cell (cbMNC) was used, showed positive results in functional assessment by decreasing apoptosis, inflammation in periinfarct area, and stimulate angiogenesis, whether it was given intraarterially, intravenously, or intraparenchymally. First and second phase of the study have shown that the injection of stem cells for acute and subacute ischemic stroke was safe (Fruchtman *et al.* 2004).

The source of cell itself is another problem to be solved. The past two decades have shown significant progress in basic understanding of adult stem cells biology. Cells derived from the human umbilical cord have been successfully used in the clinic for almost two decades (Hows *et al.*1992; Gluckman *et al.* 1989; Locatelli *et al.* 2003; Rocha *et al.* 2001). Their simple and economic retrieval, enrichment for hematopoietic progenitors, enhanced proliferation rate, expansion potential (Lewis *et al.* 2000; Ringden *et al.* 2008), and low incidence of graft-versus-host disease (Harris *et al.* 2009; Rocha *et al.* 2004) make them a promising cell treatment for

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neurological diseases. Although their therapeutic benefits were initially thought to be limited to hematopoietic disorders, several recent studies have shown the potential of these human umbilical cord–derived cells to enhance regeneration and tissue repair in various pathological disorders, including neurologic diseases (McGuckin *et al.* 2006; Rosenkranz *et al.* 2011).

Various study methodology play an important role because of different route, dosage, time to inject, and the origin stem cells whether from human cord matrix or cord blood are used could make different results. The other advantage of cbMNC is their pluripotency by having a heterogenous mix of immature lymphocyte, monocyte (Sorg *et al.* 2001; Yang *et al.* 2010), hematopoietic (Mayani *et al.* 1998), endothelial (Ingram *et al.* 2004), and mesenchymal (Erices *et al.* 2000; Flynn *et al.* 2007; Lee *et al.* 2004; Secco *et al.* 2008) stem/progenitor cells.

All the cells derived from cbMNC have their contribution to the neurogenesis and angiogenesis. Neurogenesis could be stimulated by inducing endogenous stem cells in subgranular zone, subventricular zone, and subcortical area (Altman *et al.* 1963; Eriksson *et al.* 1998; Kokaia *et al.* 2013). Angiogenesis and neurogenesis is something that could not be separated, where angiogenesis could stimulate neurogenesis and helping migration of progenitor cells to the periinfarct site (Kojima *et al.* 2010).

2. Materials and Methods

2.1. Permanent middle cerebral artery occlusion model

One group of healthy rats and three groups ($n = 6$ per group) of 250–300 g of male Wistar rats undergone permanent middle cerebral artery occlusion (MCAO), where group 2 was treated with physiological fluid intraarterially, group 3 with cbMNC intraarterially, and group 4 with cbMNC intravenously. Behavioral tests were performed before the MCAO, 1 week after MCAO, and at 3, 9, and 14 days after cbMNC injection. Brain infarct area, neurogenesis in hippocampus, and neovascularization in infarct area were evaluated. One week after occlusion, rats were injected by cbMNC with 1×10^6 cells/kg that has been characterized by cd34+ (7%) intraarterially and intravenously. Two weeks after implantation, all rats were euthanized and functional assessment in day 3, 9, and 14 after implantation were evaluated. Histopathology confirmation by hematoxylin eosin (H&E) and cresyl violet staining were marked (Hunter *et al.* 2000; Lubjuhn *et al.* 2009; Rosell *et al.* 2013) (Figure 1).

2.2. cbMNC isolation

Cord blood sample obtained from cryopreservation was not used by the Cellsafe International Corporation. All cord blood units tested negative for human immunodeficiency virus, hepatitis C virus, hepatitis B virus, human T-cell lymphotropic virus, and syphilis. Cord blood suspension was processed using gradient centrifugation method as follows: Use aseptic technique procedures and biosafety cabinet operation. Cryopreserved cord blood samples were thawed and washed using PBS and centrifuged at 1500 rpm for 10 minutes. Washing was done two times. Ficoll-Paque solution was put in 15 mL tube. Pipette carefully the cleaning results of cord blood into a tube containing Ficoll-Paque. The volume ratio of bone marrow suspension: Ficoll-Paque = 1:1. Centrifugation was performed at 2200 rpm for 10 minutes at 20°C, centrifugation termination did not use brakes (to prevent disorganization of fractions of separate components). Buffy coat layer (the layer contains nucleated cells) was taken using a pipette slowly and transferred to a 15-mL centrifuge tube. Clean the buffy coat of erythrocytes using lysis buffer as much as 3 mL. Clean the buffy coat of lysis buffer using NaCl as much as 4 mL, and the number of cells was counted with a counting chamber and trypan blue staining.

2.3. Implantation of cbMNC procedures

On the 7th day after ischemia condition, the experimental animals were randomly assigned and received a transplant of 1×10^6 cells/kg of cbMNC in 1 mL of fluid. In the group treated with implantation intravenously, cbMNC was inserted slowly through the tail vein of rats. In the group treated with implantation

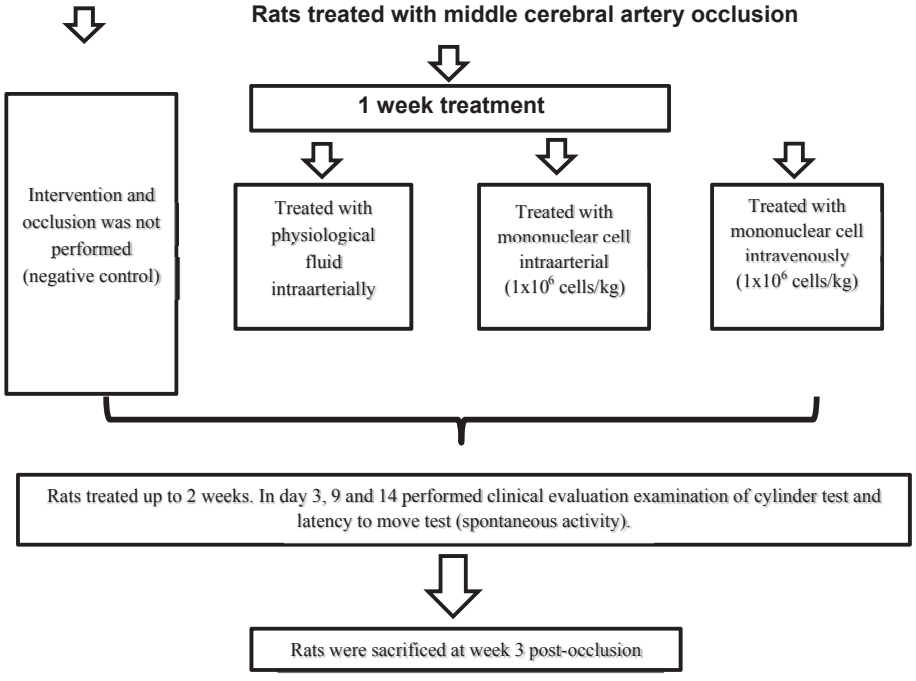

Figure 1. Study design.

intraarterially, cbMNC was slowly inserted through the common carotid artery on the contralateral side by using a 32 gauge size needle.

2.4. Assessment of neurologic deficit

Clinical experimental animals were assessed by a cylinder test and latency to move tests (spontaneous activity). Evaluation was assessed before the MCAO, 7 days after occlusion, and at 3, 9, and 14 days after implantation. Cylinder test, in this test, rats were put in glass cylinder tube of 9–10 cm diameter and 15 cm height. Rat will put the limb in a glass tube vertically. Analyzing the results of the test was done by recording and observing which limb was more dominant in contact with the tube. Formula score of asymmetry extremity use = Ipsilateral/(Ipsilateral + Contralateral + Both) − Contralateral/(Ipsilateral + Contralateral + Both) (Balkaya *et al.* 2013; Hunter *et al.* 2000; Zarruck *et al.* 2011). Latency to move test was performed to evaluate the spontaneous activity. The advantage of this technique is easy to perform by putting a rat in a cage (glass box) and count the time the rat takes to move as far as its body length (7 cm) and recorded (Lubjuhn *et al.* 2009; Rosell *et al.* 2013).

2.5. Histopathological confirmation of infarct area and neurons in hippocampus

On day 21 after the MCAO or 14 days after implantation, the experimental animals were euthanized, perfused transcardially with PBS (pH 7.4) followed by 10% buffered formalin. Brains of experimental animals were taken and cut serially between +1 to −1 bregma with three coronal sections (2 mm apart). One coronal section behind bregma (5 μm) was stained with cresyl violet. ImageJ software by the National Institutes of Health was used to measure the total ipsilateral, contralateral, and infarct areas. Infarct area was calculated by using the following formula: infarct size = 100 × [total contralateral hemisphere area − (total ipsilateral hemisphere area − infarct area)/total contralateral hemisphere area] (Chen *et al.* 2001; Vendrame *et al.* 2004). We also calculated the neurons in hippocampus area (Ca1, Ca3, and dentate gyrus). One coronal section in front of bregma was stained by H&E. Preparation was observed by light microscopy and photographed using 400× magnification (Nikon Corporation, Nikon Eeclipse TE 2000-U, Tokyo, Japan) in five visual fields with a consistent location.

2.6. Data processing

Data were analyzed using the Statistical Product and Service Solution (SPSS), IBM Corporation, SPSS Statistics, Version 20.0, New York, US. After normalizing the data, one-way analysis of variance was performed in histopathology evaluation. Kruskal–Wallis analysis was done when the data were not distributed normally. Behavioral evaluations were analyzed using two-way analysis of variance between the periods of time in each group, and least significant difference (LSD) post-hoc analysis was performed when there was a significant difference between groups.

2.7. Research ethics

This research has been approved by the research ethics of medicine/health Medical Faculty of Universitas Indonesia.

3. Results

3.1. cbMNC IA and IV in promoting motor coordination

Before surgery on rats, all groups showed a balanced use of extremity and no significant difference ($p > 0.05$). After the MCAO, there were differences in using their extremity, which in the placebo and treatment groups (3 and 4) used the extremity that was not paralyzed more often than the healthy group ($p < 0.05$). Three days after implantation, significant differences were not found statistically between groups with $p > 0.05$. On day 9 after implantation, there were significant differences between group 3 and other groups with $p < 0.05$. The third group showed no improvement at day 3 or 9 after implantation, but showed positive improvement at 14 days after implantation. Nevertheless, in group 4, at 3 days after implantation obtained better improvement than the groups 2 and 3 (Figure 2).

3.2. cbMNC IA and IV promote improvement in spontaneous activity

In the analysis of spontaneous activity, significant differences occurred at day 7 after occlusion, 3, 10, and 14 days after implantation. A significant difference was found in all groups compared with the normal group ($p < 0.05$). Seeing an existing chart (Figure 2), it is clear that occlusion slowed down spontaneous activity in placebo and treatment groups. Before implantation, placebo group did not showed any improvement until the end of the evaluation day (14 days after implantation). On the other hand, treatment group showed good improvement that close to the value before occlusion with $p < 0.05$ compared with placebo.

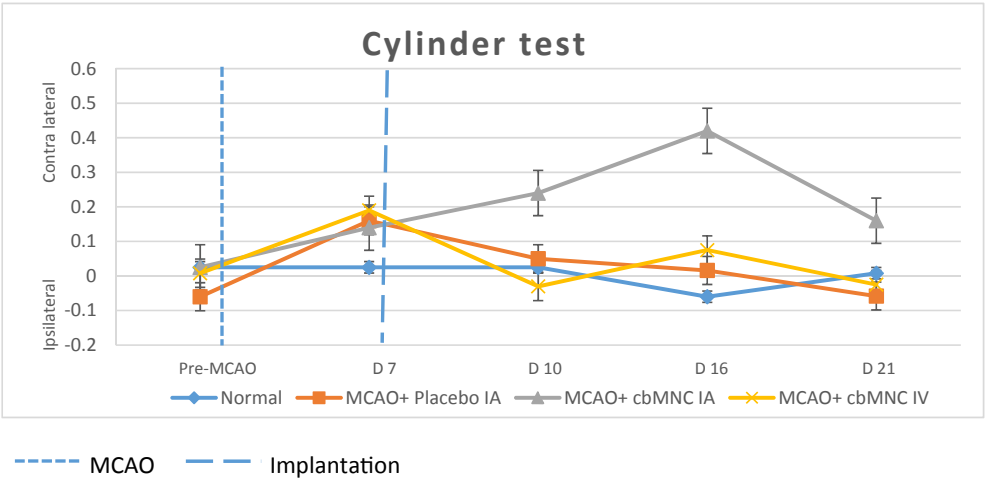

Figure 2. cbMNC intraarterial and intravenous in promoting motor coordination. IA = intraarterial; MCAO = middle cerebral artery occlusion.

3.3. Evaluation of the effect of cord blood mononuclear cell implantation histopathologically

3.3.1. Infarct area reduction

Staining with cresyl violet was used and evaluated quantitatively using the ImageJ software with the following formula: infarction area = 100 × [total area contralateral hemisphere − (total area ipsilateral hemisphere − infarcted area)/total area hemispheric contralateral] (Chen *et al.* 2001; Vendrame *et al.* 2004). One sample was taken from groups 2, 3, and 4 which represented qualitatively. Area taken in this measurement was a coronal slice −1 from bregma with 5 μm thickness. In group 2, area infarction was obtained at 15% wide. Compared with treatment groups 3 and 4, the area of infarction was not reduced and tend to be more, where the infarcted area in group 3 amounted to be 22% and 18% in group 4 (Figure 3).

To ensure quantitatively, the ratio of death neuron cells was calculated with the following formula: ratio of death cells = death cells/(death cells + living cells), which were compared between groups. The calculation of the number of neurons in the cortex area was using H&E staining with a consistent location on each group in five visual fields with a consistent location (Table 1).

3.3.2. Neurogenesis in hippocampus area

In evaluating neurogenesis in the hippocampus as the effect of implantation, the number of neuron cells were compared between groups. Qualitatively, the changes in morphology of neuron cells were also noticed. The calculation in the hippocampus area was counted on three different zones, namely Ca1, Ca3, and dentate gyrus with 400 × magnification (Figure 4). It showed that groups 3 and 4 have more neuron cells in hippocampus area compared with group 2 with $p > 0.05$ (Table 2).

4. Discussion

Based on studies, implantation intraarterially in rat with MCAO obtained cells at the infarct area more than in IV (Li *et al.* 2001; Li *et al.* 2002; Harting *et al.* 2009). Given the evidence of the existence of cells that reach the infarct lesion then functional improvement can occur with paracrine effects possessed by these cells to stimulate endogenous stem cells to be more active in neurogenesis and angiogenesis. The treatment group of rats implanted with cbMNC both intravenously and intraarterially experienced improvement in spontaneous activity. There is an involvement of cognitive, spatial learning, and memory function for rats to do spontaneous activity. Therefore, neurogenesis in hippocampus area is needed to be evaluated, because functions are controlled by hippocampus.

Table 1. The ratio of death cells in the cortex area

Group	Total	Mean	*p*-Value
1 (Negative control)	6	0.36 ± 0.03	0.028[†]
2 (Placebo intraarterial)	6	Minimum: 0.28 Maximum: 0.46[*]	
3 (Intraarterial)	6	0.66 ± 0.06	
4 (Intravenous)	6	0.47 ± 0.11	

[*] Group 2 distribution was abnormal, but overall distribution was normal.;
[†] Using one-way analysis of variance with post-hoc LSD significant groups of 1 and 3, groups 2 and 3.

Neurogenesis occurs in the hippocampus or subgranular zone (SGZ) have more impact on processes such as learning, memory, and cognitive (Marlier *et al.* 2015). Adult hippocampal neurogenesis generates new excitatory granule cells in the dentate gyrus, whose axons form the mossy fiber tract to CA3 (Kempermann G *et al.* 2015). The connection between dentate gyrus, CA3, and CA1 is interconnected by trisynaptic circuit, and Becker considers neurogenesis in a model of the full hippocampal loop (entorhinal cortex–dentate gyrus–CA3–CA1–entorhinal cortex; Becker 2005). Moreover, the neuroanatomy of dentate gyrus predicts that it carries out a specific information-processing task that receives from the entorhinal cortex and conveys to the area CA3, where they terminate in large synapse- and interneuron-rich structures, the so called "boutons." They also provide excitatory input to the pyramidal cells of CA3.

Adult neurogenesis within the hippocampus is mainly observed in SGZ of dentate gyrus, where it consists of several developmental stages that are characterized by different morphology. Radial glia-like precursor cells are the cell where adult hippocampal neurogenesis originates from where cell belonging to type 1 that are

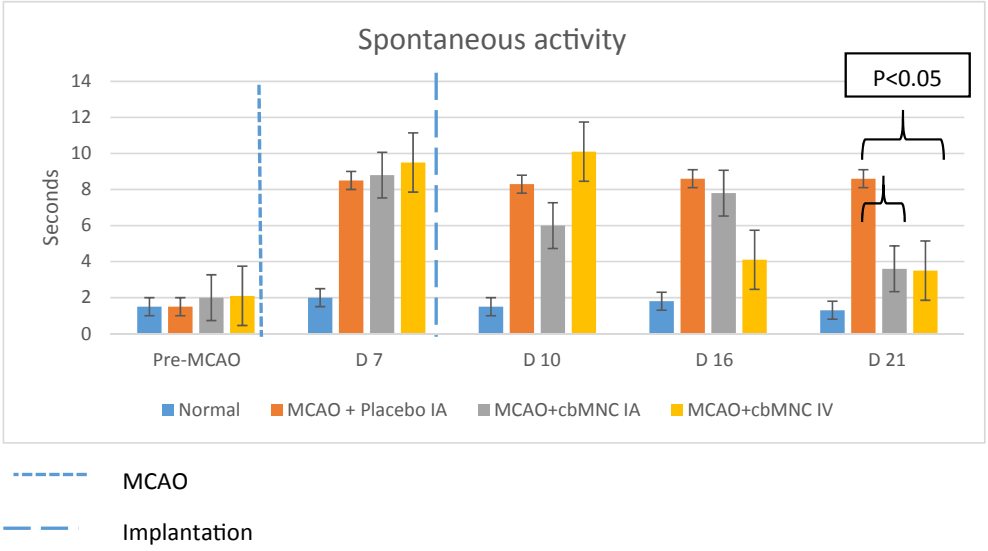

Figure 3. cbMNC intraarterial and intravenous promote improvement in spontaneous activity. cbMNC = cord blood mononuclear cell; IA = intraarterial; IV = intravenous; MCAO = middle cerebral artery occlusion.

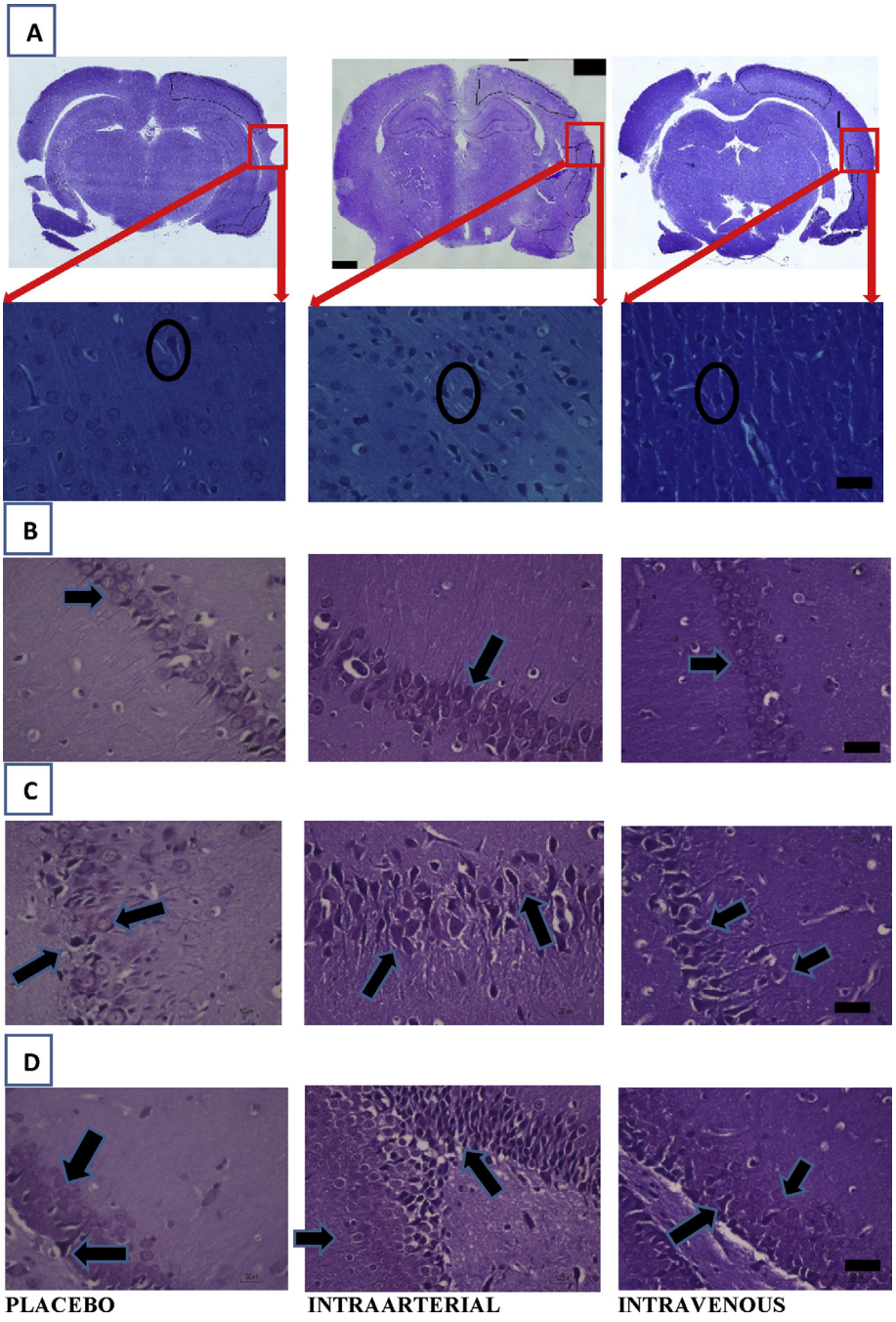

Figure 4. Effect of cbMNC implantation with cresyl violet staining in infarct and hippocampus area. (A) Infarct area in the cortex, that magnified with 400× magnification (within the same area). Black circle marked the death neurons in the cortex. (B) Neuron cells in CA1, (C) CA3, and (D) dentate gyrus. It showed that the number of neuron cells was higher in IA compared with IV and placebo group. Black arrows showed morphology variance changes in neuron cell-shaped that indicates proliferative activity and turnover of the neurons where in IA group was way faster compared with IV and placebo group qualitatively, especially in dentate gyrus and CA3, but not in CA1. *Scale bar* = 50 μm. cbMNC = cord blood mononuclear cell; IA = intraarterial; IV = intravenous.

abundant within SGZ and have astrocytic properties and express glial fibrillary acidic protein (GFAP). To understand the complex multistep processes of neurogenesis, variable marker can be used. The limitation in this study is we did not characterize by any markers to determine if they were CA1 or CA3 pyramidal cells or dentate granule cells. This remains to be performed in future studies. By using morphological changes that are seen in hippocampus, we suggest that the process of neurogenesis is ongoing.

Morphology of the cells have their own characterization, where type 1 cells are characterized by triangular shaped and the apical process extend toward the molecular layer of dentate gyrus. From type 1 cells, it will give rise proliferate the intermediate precursors and generate new type 2 cell that is characterized by a small soma, irregular shaped nucleus, and short and horizontally oriented processes (Ehninger & Kempermann 2008; Kempermann G *et al.* 2004). Type 3 cells are in a transition phase from neuroblast to the postmitotic immature neuron and enter a maturation stage, where they extend their dendrite into the molecular layer and the axon to CA3. Under physiological conditions, they have little proliferative activity, but under pathological conditions, they can

Table 2. Effect of cord blood mononuclear cell on neurogenesis in hippocampus area

Group	No	Dosage	Ca1 (mean)	Ca3 (mean)	Dentate gyrus (mean)	Hippocampus (mean)	*p*-Value
1 (Negative control)	6	–	201.00 ± 55.79	121.66 ± 27.65	297.33 ± 15.62	206.66 ± 12.86	<0.001
2 (Placebo intraarterial)	6	–	99.50 ± 60.14	84.33 ± 21.69	153.67 ± 36.22	111.99 ± 33.64	
3 (Intraarterial)	6	1×10^6 cells/kg	112.83 ± 36.42	103.83 ± 16.25	153.33 ± 37.69	123.33 ± 20.65	
4 (Intravenous)	6	1×10^6 cells/kg	104.83 ± 30.07	97.33 ± 22.12	157.50 ± 35.41	119.88 ± 18.66	

increase their proliferative activity. The morphology of type 3 cells is highly variable, but the orientation of the process changes from horizontal to vertical (Kempermann G *et al.* 2011).

Furthermore, neurogenesis in the dentate gyrus appears to be under environmental control, modern stereological studies have shown that the total number of granule cells does not vary in adult animals, and we found there were no significant differences in the number of cells in hippocampus area. This implies that there is a steady-state turnover of granule cells rather than a continuous accretion (Desmond N. 1985). Indeed, we found that many changes in morphology of the cells in hippocampus area qualitatively, especially in dentate gyrus. Thus, findings may suggest that the cbMNC stimulated the turnover of the cells and induced the proliferative of neurons activity. Based on time course, we evaluated that the neurogenesis process was 3 weeks after transplantation and it has been estimated that entire period of adult neurogenesis takes about 7 weeks. The speed of maturation and proliferative activity might differ under pathological conditions and external stimulation (Kempermann G *et al.* 2015). Therefore, we suggest that during 3 weeks after implantation, process of neurogenesis is going on even though maturation of the new neurons may not under complete process of maturation. By comparing the placebo and cbMNC groups, it showed different morphology of neurons in hippocampus, especially in dentate gyrus and CA3, but not in CA1. In contrast to information transfer from CA3 to CA1, the connections between pyramidal neurons are individually weak; and unlike CA3, CA1 contains very few recurrent connections (Bolshakov & Siegelbaum 1995). Based on this result, we suggest cbMNC implantation could induce the proliferative activity and turnover the neurons. However, the number of neuron cells were also higher within hippocampus area in cbMNC groups and it may be the result from neuroprotective effect of cbMNC (Greggio *et al.* 2014; Madeddu *et al.* 2004; Wang *et al.* 2012). This is consistent with the results obtained either functionally or quantitatively in this research.

Unfortunately, in sensory motor function by using cylinder test, the results were very difficult to be used as a benchmark of cbMNC effect. In the placebo group, it turned out to show improvement even without administration of cbMNC. From the previous studies, it was reported that cylinder test may reach the average improvement after 1–2 weeks of occlusion (Balkaya *et al.* 2013; Hunter *et al.* 2000; Zarruck *et al.* 2011). Nevertheless, when we evaluated the Figure 1, even though IA group did not make the improvement as fast as IV group, at the end of the evaluation day, the trend showed positive results. Nevertheless, IV group showed a fluctuative graph after their improvement at day 3 postimplantation, even though it regained positive result at the end of the day. Based on this result, because IA cbMNC will reach faster in the higher amount to the brain area compared with IV, we hypothesize that may be inflammatory factors surged after the implantation that may affect the outcome. After some time, thus inflammatory factors reduced and improvement achieved. On the other hand, there were no cells administered in placebo group, therefore fluctuative graph was not appeared. Moreover, in previous studies, IL6, ILB, and TNF alpha cytokines reached their peak on day 7 postischemia and declined at day 14, and in the administration of cbMNC, there was a nonsignificant decrease of those cytokines, thereby cbMNC capabilities in delivering more improvements to its effect in removing growth factors that can reduce oxidative stress (Karlupia *et al.* 2014). Unfortunately, we did not evaluate further which inflammatory factors that appeared after implantation of cbMNC, because inflammation factors were not the aim of our study and this hypothesis need to be investigated further.

Anti-inflammatory and growth factors such as vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) is one of the pluripotency that cbMNC has (Ergul *et al.* 2012), where BDNF if given in IV can reduce infarct area and mediates existing vascular endothelial cell proliferation, migration of neuronal cells, and modulate synapse function (Karlupia *et al.* 2014). In this study, we failed to show a reduction in infarct area (Makinen *et al.* 2006; Zawadska *et al.* 2009). These results provide question whether the dose given quite optimal or required evaluation in a longer time before euthanized the rats to allow new neuron cells to be migrated to infarct area? These results also gave different results to studies that have been done before, where the effective results obtained if given before 48 hours post-MCAO (Newcomb *et al.* 2009). There was possibility that gliosis has occurred and established, because we implanted the cells in subacute period.

In previous studies, CD34+ was given in a large number, but we delivered only 7% of CD34+ to see the effect of pluripotency of cbMNC (Deepti *et al.* 2009; Ergul *et al.* 2012). Human CD34+ cells were shown to secrete numerous angiogenic factors, including VEGF, HGF, and IGF-1 and as a potent regulator of adult neurogenesis (Taguchi *et al.* 2004). In experiments with experimental animal performed by Ohtaki *et al.* (2006), it was reported that on rat after the occlusion and ischemia occurred, a positive reaction for VEGF increases along time. In this study, vascularization appeared more visible in periinfarct area in group was administered by IA and IV route compared with placebo qualitatively (Figure 5).

The cbMNC xenogenic implantation also known can stimulate the body's immune response, even at dosage below 5×10^6 cells was still safely administered and does not cause mortality (Karlupia *et al.* 2014), although immunosuppression was not given which may be neuroprotective so that will give bias outcome (Kaminska *et al.* 2004; Saino *et al.* 2010). In addition, cbMNC has a high percentage in the levels of T lymphocytes regulation (Tregs) than in the peripheral blood. Tregs are known to have the ability to boost the immune system and anti-inflammatory that can be neuroprotective post stroke (Liesz *et al.* 2009). In addition, Tregs also has the ability to reduce the incidence of graft–host reaction (Brunstein *et al.* 2011).Therefore, there was no mortality of rats after implantation in this study, even though we did not evaluate the level of Tregs.

In recent decades, many translational researches with cell-based therapy was performed and produced a very good progress for the future in understanding the basic effect of stem cells in ischemic stroke. This study proved the administration of cbMNC both in IA and IV with doses of 1×10^6 cells/kg showed improvements in spontaneous activity. Histopathologically, cbMNC implantation by IA could induce the proliferative activity and make the turnover of neurons faster compared with IV and placebo. The number of the

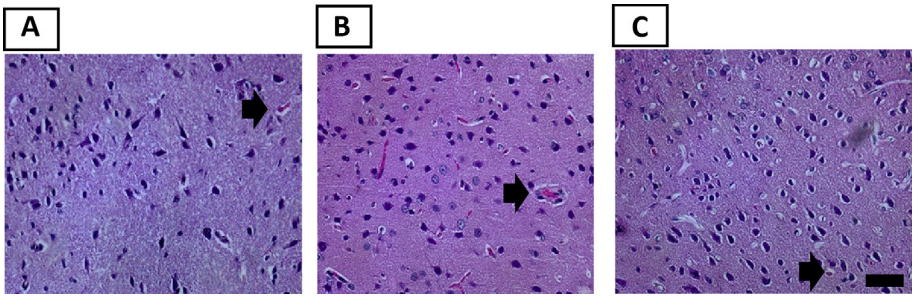

Figure 5. Staining hematoxylin eosin (H&E) with 400× magnification in ipsilateral cortex area: (A) placebo group obtained vessels (black arrow) that formed fewer in periinfarct area compared with group IA (B) and IV (C). *Scale bar* = 50 μm. IA = intraarterial; IV = intravenous.

neuron cells was also higher, although statistically not significant within hippocampus area in cbMNC groups and it may be the result from neuroprotective effect of cbMNC. In addition, the IA administration showed the presence of more vascularization than IV and placebo, although the existing CD34+ is only 7% and it may confirm the pluripotency effect of cbMNC. On the other hand, the improvement of sensory motor function and reduction of infarct area did not show satisfactory results, it may be due to (1) dose given was not optimal, (2) timing of implantation need to be reconsidered, and (3) long-term evaluation was required to provide the opportunity for new neurons to migrate to the periinfarct area. This study also proved the absence of xenograft implantation rejection reactions in both IA and IV cbMNC with a dose of 1×10^6 cells/kg.

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Conflict of Interest Statement

No conflicts of interest declared.

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