

Intra Arterial Heparin Injection Increases Number of Neural Stem Cells and Very Small Embryonic-Like Stem Cells in Acute Ischemic Stroke Patient

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Abstract

Background: Stroke is the second leading cause of adult mortality and long term disability. Our body has a response for any injury which occurs including stroke. There is endogenous neurorepair mechanism to response stroke event. Stem cells play critical role in endogenous neurorepair. Heparin has been used for a long time in therapy of acute ischemic stroke. Although use of heparin become controversy, few prospective studies support the use of heparin during the acute phase of ischemic stroke. Heparin is a glycosaminoglycan well known for its anticoagulant and anti inflammatory properties. In addition, injection of heparin through intra arterial has been associated with increased cerebral blood flow. Our study aims to investigate stem cells profile after injection of intra arterial heparin

Methods: Our study is restrospective study. We enrolled ischemic stroke patient in The Central Hospital of the Army (RSPAD) Jakarta with onset of stroke less than 7 days (< 7 days). We collected patient's blood 6 hours, 7 days and 30 days after injection of heparin intra arterial. Mesenchymal Stem Cell (MSC), Hematopoetic Stem Cells (HSC), Very Small Embryonic-Like Stem Cells (VSEL), Endothelial Progenitor Cells (EPC) and Neural Stem Cells (NSC) were measured with flow cytometer.

Results: After injection of heparin intra arterial, the number of Mesenchymal Stem Cells (MSC) are around 233.88 ± 105.27 and appears highest in acute stroke patient after 15 days of heparin injection. Our study show that the difference of NSCs before injection heparin is around 8.65 ± 8.65 and 6 hours after injection around 168.16 ± 305.71 ($p = 0.039$).

Conclusion: Our study show that the number of Neural Stem Cells (NSCs) and Very Small Embryonic-Like Stem Cells (VSEL) changes before and after intra arterial heparin injection in acute ischemic stroke patient.

Keywords: Stem Cells Profile; Intra Arterial; Heparin; Acute Stroke Ischemic; Neural Stem Cells (NSCs); Very Small Embryonic-Like Stem Cells (VSEL)

Introduction

Stroke remains a major health problem [1]. Globally, stroke is also known as production and economic burden [2,3]. According to the World Health Organization, 15 million people in the world suffering stroke event each year. 5 million of those pass away and another 5 million are permanently disabled [4,5]. After the onset of stroke, a series of time dependent pathophysiological response are activated. These reactions not only include excitotoxicity, apoptosis, leakage of the blood—brain barrier (BBB), inflammation cell death but also endogenous neural repair [6]. Many study show evidence supporting the capacity of bone marrow derived stem cells to mobilize from bone marrow into peripheral blood [7]. Stem cells plays role in endogenous neural repair [5].

For many years, anticoagulant have been used for emergency treatment of patients with acute ischemic stroke [8]. Heparin has long been contested for therapy in acute ischemic stroke [9]. Although the use of heparin in acute ischemic stroke may be one of the most controversial topics in stroke literature [9]. Since its introduction, controversy has existed about the administration of intravenous heparin for the treatment of acute ischemic stroke [10]. Guidelines issued in 2007 by the American Heart Association/American Stroke Association mention the use of anticoagulation is not recommended for the treatment of patients with acute ischemic stroke. Similarly, guidelines from the American College of Chest Physicians (ACCP) recommend the use of full-dose anticoagulation for patients with acute ischemic stroke. The ACCP noted that some experts recommend early anticoagulation for various ischemic istroke subtypes, including cardioembolic stroke [8].

Behind the controversy, many study show that besides anticoagulation properties, heparin has an anti-inflammatory properties [11,12]. The mechanisms responsible for the anti-inflammatory effects of heparin are complex and incompletely understood [12]. Heparin also can increase cerebral blood flow around infarct area and probably offers some benefit for stroke [3].

A better understanding of correlation of heparin and mobilization of stem cells from bone marrow into peripheral blood is likely to aid in optimizing heparin injection for stroke patient. The aim of our study is to observe the number of circulating stem cells after intra arterial heparin injection in acute ischemic stroke.

Materials And Methods

Subjects

Subjects in this study were part of cohort study of ischemic stroke in The Central Hospital of Army (RSPAD) Gatot Subroto Jakarta. Diagnosis of ischemic stroke was made using clinical examination and magnetic resonance imaging (MRI) interpreted by neurologist. We are grouping the subjects into 3 group. Group A, subjects with onset stroke less than 7 days (< 7 days), group B, subjects with onset stroke between 7 – 15 days and group C subjects with onset stroke more than 15 days. This study was approved by ethics committee of Faculty of Medicine, Hasanuddin University Makassar, Indonesia with register number: UH14110578.

Materials And Methods

Blood Sampling and Cell Isolation

10 mL of blood was drawn from antecubital veinn of subjects and collected in a vacutainer containing 3.8 % buffered sodium heparin. Mononuclear cells (MNCs) were then isolated by density-gradient centrifugation of histopaque (Histopaque-1077, Sigma-Aldrich). Add 3 mL of whole blood onto 3 mL histopaque. Centrifuge for exactly 30 minutes at room temperature. After centrifugation, carefully aspirate the upper layer with a Pasteur pipette within 0,5 cm of the opaque interface containing mononuclear cells. Discard upper layer. Carefully transfer the opaque interface with a pasteur pipette into a clean conical centrifuge tube. Wash the cells by adding 10 mL of isotonic phosphate buffered saline (PBS) solution and mix by gently drawing in and out of the pipette. Centrifuge for 10 minutes. Aspirate the supernatant and discard. Resuspend cell pellet with 5 mL of isotonic phosphate buffered saline solution and mix by gently drawing in and out of the pipette. Erythrocytes and granulocytes should pellet to the bottom of centrifuge tube. Mononuclear cells should band at the interface between the histopaque and plasma.

Flow Cytometry

All samples were analyzed using a BD FACS Canto flow cytometer with 3 fluorescent parameters: fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP). The forward scatter (FSC-H) and side scatter (SSC-H) of cells were measured using a linear scale. A stop criterion of 1 million events was used for all data acquisition.

We measured the number of HSC, MSC, NSC and EPC using flow cytometer with gating strategy (see figure 1) and surface marker HSC with CD34 antibodies – FITC (130-081-001, Miltenyi Biotec GmbH) and CD45 antibodies – PerCP (130 – 094 - 975, Miltenyi Biotec GmbH),

MSC (CD105⁺/CD73⁺/CD90⁺/CD34⁻/CD45⁻) with human MSC analysis kit (562245, BD Biosciences), NSC with CD133 antibodies – PE (130-080-801, Miltenyi Biotec GmbH), CD34 antibodies – FITC (130-081-001, Miltenyi Biotec GmbH) and CD45 antibodies – PerCP (130 – 094 - 975 Miltenyi Biotec GmbH) and EPC with CD34⁺ CD34 antibodies – FITC (130-081-001, Miltenyi Biotec GmbH), CD133 antibodies PE and KDR antibodies (Sigma), VSEL with CXCR4 antibodies PE, CD45 antibodies – PerCP (130 - 094 - 975, Miltenyi Biotec GmbH) and gating strategy below:

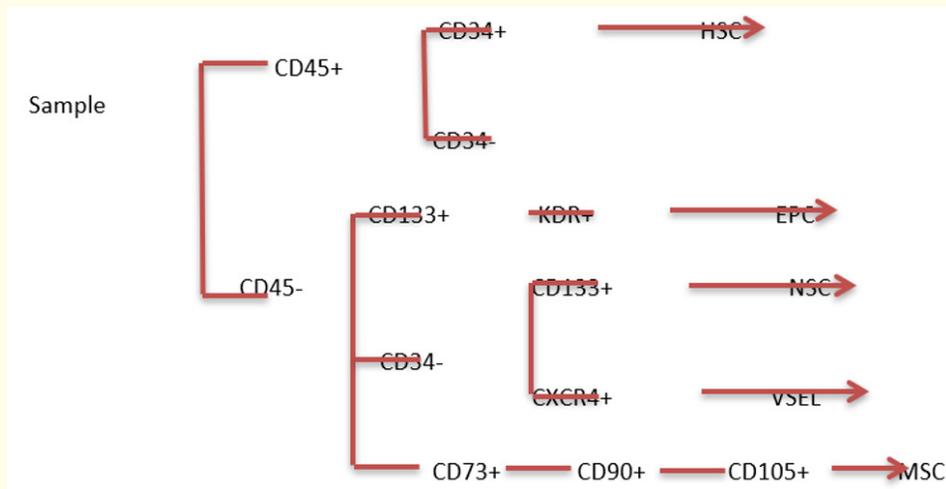


Figure 1: Gating Strategy for MSC, HSC, VSEL, EPC and NSC.

Result

A. Stem Cells Profile Before and After Intra Arterial Heparin Injection

Discussion

Stroke is leading cause of disability and the second cause of death in whole world [8]. Stroke not only induces cell death but also neurorepair. De novo neurogenesis has been found in the subventricular zone after stroke [6]. After onset stroke, many type of stem cells are mobilized at varying degrees into circulation [5]. Mesenchymal stem cells (MSC), Hematopoietic Stem Cells (HSC), Very Small Embryonic-Like Stem Cells (VSEL), Endothelial Progenitor Cells (EPC) and Neural Stem Cells (NSC) were mobilized into peripheral blood [1,5]. Heparin has been long used in therapy of acute ischemic stroke and become a controversy [9]. Behind the controversy, many study show that besides anticoagulation properties, heparin has an anti-inflammatory properties [11,12]. The mechanisms responsible for the anti-inflammatory effects of heparin are complex and incompletely understood [12]. Heparin also known can increase cerebral blood flow around infarct area and probably offers some benefit for stroke3. In vitro study show that heparin can increase the proliferation of MSC [20]. The ability of heparin to interact with many protein, including fibroblast growth factor (FGF) [13], renders it as a potential therapeutic agent beyond its use as an anti-thrombotic [14]. Heparin’s high affinity for protein has resulted in its application in cell culture to enhance the desirable activity of critical extracellular biomolecules used as supplements for the expansion of human stem cells. For example, heparin has been reported to promote both Wnt and FGF signaling in human embryonic stem cells (hESCs), thereby increasing their [14-16]. FGF is an essential growth factor for stem cell. Increase of FGF signaling can improve signaling pathway of stromal derived factor (SDF)-1 [17]. SDF-1 is a chemoattractant for homing stem cells [18]. and mobilizing stem cells from bone marrow into peripheral [19]. In addition, FGF as well as SDF-1 signaling pathways have recently been shown to be involved in control of regeneration [17].

Our study show that stem cells are mobilized after onset stroke and after intra arterial heparin injection (figure 2). Our study also show that MSC is the highest number of stem cells which mobilized into circulation before and after intra arterial heparin injection. MSC are multipotent and self renewing cells [21,22]. MSC transplantation has been reported to improve neurological function following neural injury [22].MSC is needed in successful improvement of neural function recovery [5,7]. The multiple mechanism action of MSC include enhanced angiogenesis, neurogenesis, immunomodulatory and anti-inflammatory effects [22]. MSC can secrete neurotrophic factors to activate endogenous repair mechanisms [7]. These neurotrophic cytokines have been implicated to play an important role in the process of angiogenesis and neurogenesis [24]. MSC provide a nueroprotective effect through cell migration to the infarct region [24]. MSC migration into infarct area through the stromal cell-derived factor-1/C-X-C chemokine receptor (CXCR)-4 signaling pathway [25]. The pathway serve as the homing signalling for MSC [26].

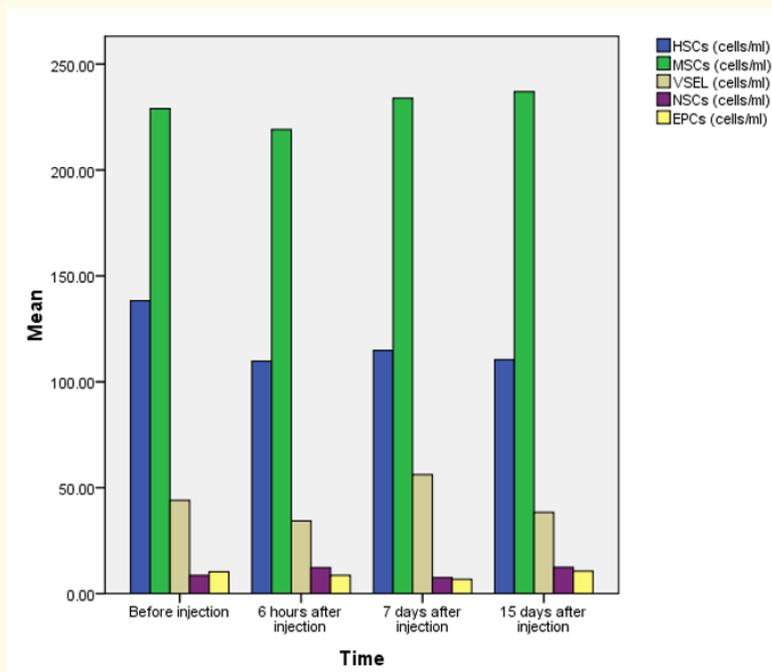


Figure 2: Stem Cells Profile Before and After Intra Arterial Heparin Injection.

Our study have shown there is a significant difference between the number of Very Small Embryonic-Like Stem Cells (VSEL) ($p = 0.036$) and Neural Stem Cells (NSC) ($p = 0.039$) before injection and after injection intra artery heparin (Figure 3). Neural Stem Cells (NSC) mobilized into vascular after stroke [5]. Many experimental studies have shown an increased proliferation of NSC in animal model of stroke which persist at least for four months after ischemia [27]. NSCs have demonstrated multimodal therapeutic function after transplantation into preclinical animal models of stroke [28]. NSCs are able to protect at-risk neural cells, promote endogenous NSC proliferation and migration, foster synaptic remodeling, stimulate new vessel formation, and/or integrate into host neural circuits, which have been associated with improvements in cognitive and sensorimotor function [28]. After injection intra heparin, the number of NSC increase in 6 hours after injection.

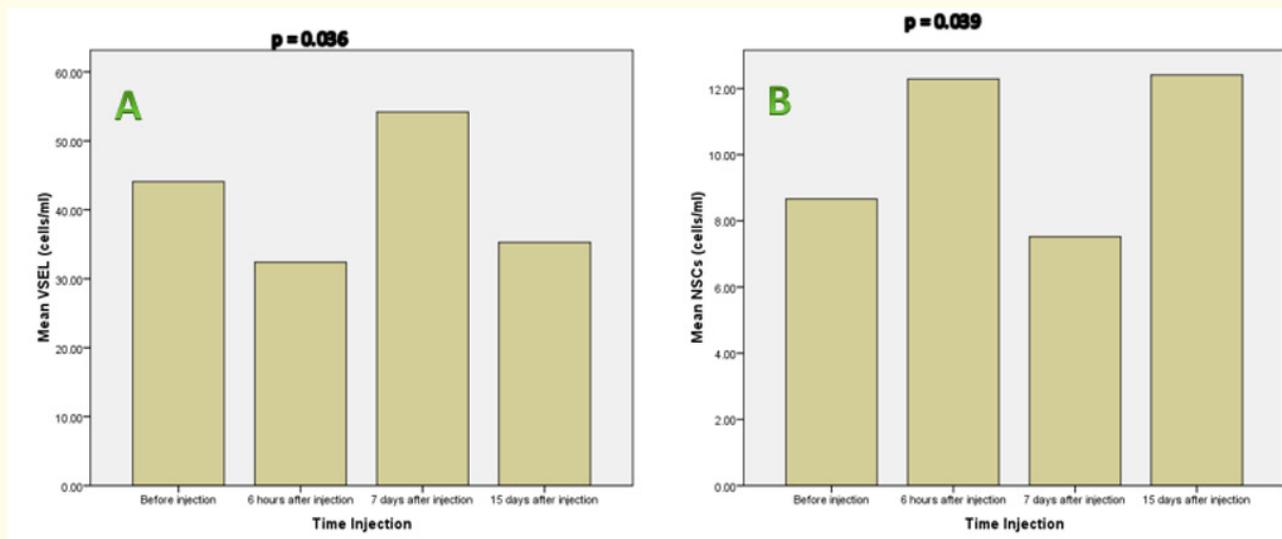


Figure 3: Number of VSEL (A) and NSCs (B) Before and After Injection Intra Arterial Heparin

VSEL is a rare population of stem cells identified in the bone marrow with embryonic characteristics [29]. Under steady-state conditions, VSEL circulate at very low levels in peripheral blood [28]. The underlying reason is that VSELS exist in low numbers, remain dormant under homeostatic conditions, and very small in size [30]. VSELS have been reported in various mouse and human tissues and are actively mobilized from the bone marrow into the peripheral blood under stress conditions including stroke [31,32], myocardial infarction, and burn injury [31]. In vivo study show that the number of VSEL increase 7 – 15 days after stroke [31]. The VSELS maintain life-long tissue homeostasis, serve as a backup pool for adult stem cells and are mobilized under stress conditions. An imbalance in VSELS function (uncontrolled proliferation) may result in cancer [29]. After injection of heparin intra artery, the number of VSEL increase in 7 days after injection.

This is a preliminary case report and we plan to expand this study to monitor more patient over time following onset of stroke to better understand the safety of heparin and perhaps the answer to the controversy of using heparin for stroke patient. However, further studies are needed to determine which type of stem cells is the most effective in restoring neurological function after stroke.

Conclusion

Our study show that the number of Neural Stem Cells (NSCs) and Very Small Embryonic-Like Stem Cells (VSEL) changes before and after intra arterial heparin injection in acute ischemic stroke patient.

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