

Mini Review

Application of Human Umbilical Cord Blood-Derived Mononuclear Cells in Animal Models of Ischemic Stroke

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Abstract

Complex pathology of ischemic stroke warrants a combination treatment approach that targets multiple pathways in order to provide a general effective therapy for stroke. Human umbilical cord blood-derived cells have been used in experimental models of injury and disease over the past several years and show encouraging leaps toward development of such an all-inclusive treatment. The purpose of this review is to highlight the potential use of human umbilical cord blood-derived cells in ischemic stroke and to discuss the animal studies which were conducted to date in that direction.

Keywords: Ischemic stroke; Umbilical cord blood; Stem cells; Infarct size; Neurological recovery

Abbreviations

HUCB: Human umbilical cord blood; HUCBCs: Human umbilical cord blood cells; HSCs: Hematopoietic stem cells; nHSCs: non-hematopoietic stem cells; MSCs: Mesenchymal stem cells; EPCs: Endothelial progenitor cells; SSEA: State-specific embryonic antigen; TRA: Tumor rejection antigen; MCAO: Middle cerebral artery occlusion; tPA: tissue plasminogen activator; ROA: Route of administration; BBB: Blood brain barrier; SH: Spontaneously hypertensive; BDNF: Brain-derived neurotrophic factor; VEGF: Vascular endothelial growth factor; IFN: Interferon.

Introduction

Globally, fifteen million people suffer from a stroke each year and five million stroke patients die with another five million left permanently disabled [1]. Stroke is the leading cause of disability and has become one of the major challenges to health. Approximately 85% of the strokes are ischemic and occur due to thrombosis, embolism or stenosis. However, many ischemic strokes occur without a well-defined etiology and are labeled as cryptogenic. Cryptogenic stroke accounts for 30 to 40 percent of ischemic strokes.

Stem cell transplantation offers a promising therapeutic strategy for ischemic stroke. In addition to preventing the ongoing damage, which has been the focus of conventional therapy, stem cell transplantation actually repairs the injured brain. It has emerged as a potential regenerative treatment to reduce post-stroke handicap. In addition to ethical and moral concerns, limited availability of embryonic, fetal, and adult brain-derived neural stem cells has prompted the search for alternative sources of stem cells. Human umbilical cord blood (HUCB) has emerged as an alternative stem cell sources. HUCB is the blood left over in the placenta and in the umbilical cord after the birth of the baby. HUCB contains a highly heterogeneous mixture of cells, which includes red blood cells (erythrocytes), white blood cells (leukocytes), thrombocytes (platelets), stem cells, etc. HUCB contains at least three types of stem cells; hematopoietic stem cells (HSCs), non-hematopoietic stem cells (nHSCs), and mesenchymal stem cells (MSCs). HSCs, nHSCs, MSCs,

and agranulocytes of leukocytes (lymphocytes and monocytes) constitute the mononuclear cells of HUCB (HUCBCs).

HUCB-HSCs are characterized by their differential expression of hematopoietic markers CD34, CD45, and CD133 [2,3]. HUCB-HSCs can be selectively induced into specific hematopoietic lineages. HUCB-nHSCs are characterized by their expression of pluripotency markers (Sox2, Oct4, and Nanog), state-specific embryonic antigen markers (SSEA-3 and SSEA-4), tumor rejection antigen markers (TRA1-60 and TRA1-80), and lacking of hematopoietic markers CD34 and CD45 [4]. HUCB-nHSCs possess multipotent differentiation potential and have been shown to differentiate in various cell types representing the three germ layers. HUCB-MSCs show high morphological and molecular similarities to bone marrow MSCs including the lacking of hematopoietic surface antigens CD34, CD45, and CD133 [5-8]. HUCB-MSCs are characterized by their expression of MSC-specific surface markers CD29, CD44, CD73, CD105 and vimentin [9]. HUCB-MSCs possess several advantages over other types of stem cells including those derived from bone marrow [10]. HUCB-MSCs possess multipotent differentiation potential and thus can be induced to differentiate into cells of multiple lineages such as adipocytes, osteocytes, chondrocytes, myocytes, hepatocytes, neurons, astrocytes and oligodendrocytes [10-17]. Recent investigations suggested that the HUCB-MSCs harbor a small unique population of cells that express pluripotent stem cell markers such as Sox2, Oct4, Nanog, ABCG2, and nestin along with MSC markers [9]. Although few stem cell types obtained from HUCBCs show the expression of pluripotency markers, these cells do not form teratomas after transplantation in immuno-compromised mice, the current gold standard for determining the pluripotency of human cell lines. Therefore, these cells do not satisfy the current criteria for defining them as pluripotent stem cells [18].

Why ischemic stroke is the primary disease target for stem cell therapy?

The goal of any cell-based therapy is to replace and/or repair dead or diseased cells. Therapy with stem cells can target multiple mechanisms/pathways associated with the pathology of a disease

Table 1: Studies that utilized HUCBCs in animal models of ischemic stroke.

Animal Model	Stroke Model	ROA	Dose	Time of injection	Research Outcome	Reference
Rat	Transient (2 hour) MCAO with a monofilament	Intravenous	3x10 ⁶ cells	1 and 7 days after MCAO	Cells entered brain, survived, migrated and improved functional recovery after stroke.	[36]
Rat	Permanent MCAO	Intravenous	1x10 ⁶ cells	24 h after MCAO	Behavioral improvement indicated by a decrease in spontaneous activity.	[59]
Rat	Permanent MCAO with an embolus	Intrastratial & Intravenous	0.25x10 ⁶ cells (Intrastratial) 1x10 ⁶ cells (Intravenous)	24 h after MCAO	Intravenous delivery may be more effective than striatal delivery in producing long-term functional benefits.	[37]
Rat	Permanent MCAO with a monofilament	Intravenous	1x10 ⁴ to 3-5x10 ⁷ cells in 500 µl over 5 min	24 h after MCAO	Significant recovery noticed in behavioral performance at doses ≥ 1x10 ⁶ cells.	[38]
Rat	Transient (1 hour) MCAO with a suture	Intravenous	0.2x10 ⁶ cells	During MCAO	Administered cells were not detected in the ischemic brains. However, the treatment reduced cerebral infarcts and improved behavioral function.	[42]
Rat	Permanent MCAO with a monofilament	Intravenous	1x10 ⁷ cells in 500 µl	24 h after MCAO	Increased neuroprotection and decreased inflammation	[39]
Rat	Transient (1 hour) MCAO with a thread occluder	Intravenous	1x10 ⁶ cells (CD34-negative)	48 h after MCAO	Reduction in infarct volume and improvement in placement and stepping tests which could be mediated by trophic actions	[40]
Rat	Permanent MCAO with a monofilament	Intravenous	1x10 ⁷ cells in 500 µl	24 h after MCAO	Reduction in infarct size is associated with rescue of the spleen weight and splenic CD8+ T-cell counts as well as increased production of IL-10 while decreasing IFN γ	[41]
Rat	Permanent MCAO with a monofilament	Intravenous	1x10 ⁶ cells in 500 µl	48 h after MCAO	Resulted in both behavioral and physiological recovery with diminished or lack of granulocyte and monocyte infiltration and astrocytic and microglial activation.	[43]
Rat	Transient (2 hour) MCAO with a filament	Intravenous	1-5x10 ⁷ cells	24 h after MCAO	Only few cells were detected in the ischemic brain regions. Neither the histological outcome nor the functional recovery was improved.	[45]
SH Rat	Permanent MCAO by thermo occlusion	Intravenous	8x10 ⁶ cells/kg body weight	24 h after MCAO	Neither the infarct volume nor caspase-3 activity was significantly affected.	[46]
Rat	Transient (2 hour) MCAO with a monofilament	Intravenous	10x10 ⁶ cells (AC133+ EPCs from HUCBCs)	24 h after MCAO	Administered cells migrated to the ischemic brain and exerted therapeutic effect on the extent of tissue damage, regeneration, and time course of stroke resolution.	[44]

HUCBCs-Human umbilical cord blood-derived cells; EPCs-Endothelial progenitor cells; MCAO – Middle cerebral artery occlusion; ROA – Route of Administration; SH-Spontaneously hypertensive

condition. After an ischemic stroke, the affected brain tissue can be described as having two regions, the ischemic core and the penumbra. The tissue damage that occurs in the ischemic core is irreversible and permanent. Therefore, the cells in the ischemic core are considered beyond rescue and the cells in the penumbra are potential targets for therapeutic intervention. To date, clinical treatment had not emerged from 1026 neuroprotective agents deemed successful in animals, reinforcing the perception that “everything works in animals but nothing works in people” [19]. Many neuroprotective agents that failed in clinical trials are aimed at excitotoxicity and oxidative stress. It is quite evident that targeting cell death in the ischemic core will have poor prospects compared to targeting cell death in the penumbra. Further, any approach targeting a single mechanism may not provide a general effective therapy for stroke because of its complex pathology. Hence, the best possible option to replace the dead tissue in the ischemic core and to reestablish the lost neurological function as well as inhibit cell death in the penumbra would be stem cell transplantation.

Current status of drug therapy for ischemic stroke

Although the US FDA approved the usage of certain antiplatelet agents such as aspirin, ticlopidine, clopidogrel and a combination of aspirin and dipyridamole for secondary ischemic stroke prevention,

initial stroke therapy is limited to the FDA-approved clot-busting drug, tissue plasminogen activator (tPA), which must be administered within a four and half hour window from the onset of symptoms [20,21]. Unfortunately, only three to five percent of those who suffer a stroke reach the hospital in time to be considered for this treatment. Moreover, about half of the patients receiving tPA therapy show little or no improvement in functional outcome [22]. In addition, treatment with thrombolytics such as tPA present real safety concerns because of the increased incidence of secondary hemorrhagic transformation and the increased mortality rate in patients especially those who have bleeding disorders [23-25]. The major goal of clot-busting therapy is to reestablish the blood flow to the previously ischemic brain portions and not to address the injury that occurred due to ischemia. Reperfusion of the ischemic brain portion further damages the brain due to reperfusion injury. Despite decades of research, no clinically effective pharmacotherapies exist which can target both ischemia and reperfusion injury as well as facilitate cellular functional recovery after an ischemic stroke.

Rationale for the use of HUCBCs in ischemic stroke

HUCBCs possess several advantages over stem cells from other sources [10]. Recently, we showed that these cells survive, migrate and transdifferentiate to neuronal cells after their transplantation in animal

Table 2: Studies that utilized HUCB-MSCs in animal models of ischemic stroke.

Animal Model	Stroke Model	ROA	Dose	Time of injection	Research Outcome	Reference
Canine	MCAO through injection of thrombotic emboli	Intraarterial	1x10 ⁶ cells in 1 ml	1 day after MCAO	Cell therapy significantly reduced the infarct volume. Cells were differentiated into neurons and astrocytes and secreted BDNF and VEGF.	[47]
Rat	Transient (2 hour) MCAO with a monofilament	Intrathecal & Intravenous	0.5x10 ⁶ / 1x10 ⁶ cells (intrathecal) 0.5x10 ⁶ / 1x10 ⁶ cells (intravenous)	3 days after MCAO	Neurological recovery was better with 1x10 ⁶ dose irrespective of the route of administration. Significant reduction in ischemic damage noticed at 0.5 x 10 ⁶ dose (intrathecal but not for intravenous)	[48]
New-born Rat	Permanent MCAO with a monofilament	Intra-ventricular	1x10 ⁵ cells in 10 µl	6 h after MCAO	Cell transplantation significantly improved survival and functional recovery as well as reduced infarct volume.	[49]
Rat	Transient (2 hour) MCAO with a monofilament	Intravenous	0.25x10 ⁶ / 1x10 ⁶ cells in 500 µl	1 day after MCAO	Treatment with hUCBSCs reduced brain damage by inhibiting apoptosis and down-regulating the apoptotic pathway molecules.	[55]
Rabbit	Transient (2 hour) MCAO with a monofilament	Intravenous	5x10 ⁶ cells in 2 ml	Within few minutes after MCAO	Treatment suppressed the inflammatory responses and neuronal apoptosis.	[54]

HUCB-Human umbilical cord blood; MSCs-Mesenchymal stem cells; MCAO-Middle cerebral artery occlusion; ROA-Route of Administration; BDNF-Brain-derived neurotrophic factor; VEGF-Vascular endothelial growth factor

Table 3: Studies that utilized HUCB-HSCs in animal models of ischemic stroke.

Animal Model	Stroke Model	ROA	Dose	Time of injection	Research Outcome	Reference
Mouse	Permanent MCAO by ligation of the distal portion of MCA	Intravenous	0.5x10 ⁶ cells in 100 µl	48 hours after MCAO	Cell therapy promoted an environment conducive to neovascularization of ischemic brain	[50]
Rat	Transient (1.5 hour) ischemia by three-vessel ligation	Intracerebral	0.2x10 ⁶ cells in 3-5 µl/area in 3 cortical areas	7 days after ischemia	Implantation of the cells that were subjected to hypoxia-preconditioning improved stroke outcome, cerebral blood flow into the ischemic brain via induction of angiogenesis, facilitated proliferation of endogenous neural progenitor cells and promoted neurite outgrowth.	[51]

HUCB-Human umbilical cord blood; HSCs-Hematopoietic stem cells; MCAO-Middle cerebral artery occlusion; ROA-Route of Administration

spinal cords or brains [10,16,17,26]. We also demonstrated the potential of these cells in modulating the spinal cord microenvironment and improving the locomotor recovery of spinal cord injured rats [16,26-29]. Based on our studies, we understand that HUCBSCs treatment inhibited apoptosis, inhibited myelin degradation, remyelinated the damaged axons and thereby contributed to the functional recovery of spinal cord injured rats. Moreover, intracranial implantation of these cells in shiverer mice myelinated the hypomyelinated axons and significantly reduced their shivering [10]. Supported by *in vitro* and pre-clinical studies, HUCBSCs have been utilized in many different clinical trials aiming to treat a wide range of diseases and disorders [4]. The pathophysiology of ischemic stroke is extremely complex and involves numerous processes, including: energy failure, excitotoxicity, oxidative stress, disruption of the blood-brain barrier (BBB), inflammation, necrosis, apoptosis etc. Studies that utilized HUCBSCs improved the stroke outcome in animal models of ischemic stroke by multiple mechanisms. All these studies are discussed in detail in the subsequent sections of this review.

How HUCBSCs are recruited to the infarct site after their systemic administration?

Delivery of viable cells to the damaged brain tissue is the first thing to be considered with any cell-based therapy. Ideally these cells or the neurotrophic factors they secrete will re-establish the damaged host neural connections, either by forming new networks or reconstructing the old pathways.

After intravenous administration, HUCBSCs are recruited to the infarct site possibly by passive diffusion across a damaged BBB. Early

disruption of BBB begins within the first three hours after MCAO [30]. The second peak of BBB disruption is between 24 and 72 hours, in case of transient occlusion model, which is associated with reperfusion injury [31]. In case of permanent MCAO model, however, the second peak of BBB disruption peaks approximately at six days after MCAO [32]. HUCBSCs are also recruited to the site of injury by chemokines when their expression reaches peak level, which usually occur at 48 hours after stroke [33-35]. Administration of HUCBSCs at early time points after ischemia may compromise with the body's early natural attempt to fight against injury and lead to exacerbation of the damage. Treatment with cells that is initiated too early or too late may not help recovery. The timing of treatment with HUCBSCs is more important than the dose. Based on the reports discussed above, it appears that the optimal timing of HUCBSCs treatment is 48 hours after transient focal cerebral ischemia.

Effect of cell type, dose, route and time of administration on stroke outcome in animal models

Application of HUCBSCs in animal models of ischemic stroke has been initiated more than a decade ago. Recent research reports indicated the usage of HUCBSCs, which also include HSCs, nHSCs, and MSCs derived from HUCB. The summary of these studies, including the research outcome is detailed in Table 1. All the studies listed in Table 1 used a rat model of transient or permanent middle cerebral artery occlusion (MCAO) and administered the cells intravenously within seven days after MCAO procedure. The number of cells administered range from 1x10⁴ to 5x10⁷. Intravenously administered HUCBSCs after ischemic stroke entered the rat

brain, survived, migrated, improved the neurological/functional recovery, and reduced the infarct size [36-44]. In contrast, systemic administration of HUCBCs did not improve histological outcome or functional recovery in MCAO subjected rats [45]. These authors also have reported that only few of the administered cells were detected in the ipsilateral hemisphere. However, we cannot attribute this finding as a reason for the lack of improvement in histological outcome and functional recovery because several other research groups reported the presence of administered cells in ischemic brain regions. Reduced infarcts and improved behavioral function in the absence of administered cells in the ipsilateral hemisphere was also reported [36,42,44]. In addition, intravenous administration of HUCBCs in a spontaneously hypertensive rat MCAO model failed to reduce the infarct volume [46]. However, the absence of positive outcome could be due to the divergent pathophysiological sequences in these rats compared to commonly used rat strains [46]. Therefore, prior to initiating the clinical studies with HUCBCs in stroke patients, their therapeutic potential in animal models that mimic the most common comorbidities of stroke patients should be investigated.

The remaining studies that utilized HUCB-MSCs in ischemic stroke to date are summarized in Table 2. Although these studies have commonly employed the MCAO procedure, different animal models were used including canine, rabbit, adult rat, and newborn rat. Further, in these studies, HUCB-MSCs are administered at different time points (within few minutes to three days after MCAO procedure) by various routes of administration, which include intravenous, intraventricular, intrathecal and intraarterial. The number of cells administered in these studies range from 1×10^5 to 5×10^6 . Despite the differences in animal models, route, and time of administration, HUCB-MSCs treatment significantly reduced the infarct size, improved survival and offered neurological/functional recovery of MCAO-subjected animals [47-49]. Studies that utilized HUCB-HSCs in ischemic stroke to date are summarized in Table 3. Administration of these cells in ischemic stroke models improved stroke outcome despite the differences in pre-treatment, dose, route and time of administration [50,51].

In order to overcome the immune reactions in animals after stem cell transplantation several approaches are currently used, including immunosuppression of the host animal, the use of genetically engineered animals that are immunodeficient, alterations in the stem cells and other approaches. The administration of an immunosuppressant is not common in all the studies discussed in this review. Few research groups administered an immunosuppressant drug to animals that were treated with HUCBCs and others did not. The results from those studies that used an immunosuppressant should be more carefully interpreted because of the involvement of immune component in the pathology of ischemic stroke. The use of immunosuppressant may not be a requirement in case of stem cells obtained from HUCBCs because they are immature as well as elicit a lower incidence of graft rejection, graft versus host disease (GvHD), and post-transplant infections, even though they primarily come from an allogenic origin [52,53].

Possible underlying mechanisms of HUCBCs-mediated neuroprotection after ischemic stroke

Differentiation and paracrine signaling have both been implicated as mechanisms by which stem cells improve tissue repair. Stem cell

differentiation contributes by regenerating damaged tissue, whereas paracrine signaling regulates the local cellular responses to injury such as cell survival, migration and gene expression as well as secretion of known mediators of tissue repair including growth factors, cytokines and chemokines. It is also reported that the central nervous system availability of grafted HUCBCs is not a prerequisite for acute neuroprotection after ischemic stroke provided that the therapeutic molecules such as neurotrophic factors secreted by these cells could cross the BBB [42]. Various studies that utilized HUCBCs which were discussed in the subsequent sections of this review suggest that both differentiation and paracrine signaling mechanisms contribute to the positive stroke outcome in animal models of ischemic stroke.

Administration of HUCBCs inhibited the stroke-induced infiltration by reducing the number of CD45/CD11b-positive cells (microglia/macrophage) and CD45/B220-positive cells (B cells) and abrogated the levels of ischemia-induced pro-inflammatory cytokines such as TNF α and IL-1 β while increasing the concentration of anti-inflammatory cytokines such as IL-10 [39]. The same research group reported another novel immuno-modulatory mechanism by which HUCBCs mediate protection in the rat MCAO model of stroke. According to their report, HUCBCs treatment rescued the spleen weight and splenic CD8+ T-cell counts as well as increased the production of IL-10 while decreasing IFN γ [41]. In another study, HUCBCs treatment offered neuroprotection by trophic actions that could result in the reorganization of host nerve fiber connections within the injured brain [40]. These restorative effects mediated by trophic actions after HUCBCs treatment could be attributed to nHSCs and MSCs present in the HUCBCs, because HUCBCs utilized for this study were CD34-negative. Treatment with HUCB-MSCs in a rabbit model of focal cerebral ischemia suppressed the ischemia-induced increases of IL-1 β , IL-6 and TNF α levels in both the serum and peri-ischemic brain tissues within six hours of ischemia-reperfusion [54]. HUCB-MSCs treatment was also reported to suppress the inflammatory responses and neuronal apoptosis [54]. In another study that specifically utilized HUCB-HSCs, the cell therapy promoted an environment conducive to neovascularization of ischemic brain [50]. In this study, HUCB-HSCs were administered to immunocompromised mice in contrast to other studies, which had utilized a rat model. However, in another study, intracerebral administration of HUCB-HSCs that were subjected to hypoxia-preconditioning improved cerebral blood flow into the ischemic brain via induction of angiogenesis, facilitated proliferation of endogenous neural progenitor cells and promoted neurite outgrowth [51].

Studies that employed HUCB-MSCs revealed that these cells differentiated into neurons and astrocytes and secreted several growth factors such as brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) after their intra-arterial administration in a canine MCAO model [47]. In addition, majority of the apoptotic pathway molecules, which were upregulated after focal cerebral ischemia were downregulated after HUCB-MSCs treatment [55,56]. These findings further strengthened our previous research results, wherein HUCB-MSCs inhibited neuronal apoptosis after their transplantation in the injured rat spinal cords [26]. In contrast, HUCBCs treatment did not affect caspase-3 activity in the ischemic rat brain [46]. As stated earlier, the reason could be due to the divergent pathophysiological sequences in spontaneously

Table 4: Studies that utilized HUCBCs in combination with other drugs in animal models of ischemic stroke.

Animal Model	Stroke Model	ROA	Dose	Time of injection	Research Outcome	Reference
Rat	Transient (2 hour) MCAO with a monofilament	Intravenous	1x10 ⁶ cells in 1 ml	24 h after MCAO	Combination therapy with simvastatin increased BDNF/TrkB expression, enhanced cell migration into the ischemic brain, amplified endogenous neurogenesis, synaptic plasticity and axonal growth, and thereby improved functional outcome after stroke.	[57]
Rat	Transient (2 hour) MCAO with a monofilament	Intravenous	1x10 ⁶ cells in 1 ml	24 h after MCAO	Combination therapy with simvastatin increased Ang1/Tie2 and occludin expression in the ischemic brain, amplified endogenous angiogenesis, and enhanced vascular remodeling which in concert may contribute to functional outcome after stroke.	[58]

HUCBCs-Human umbilical cord blood-derived cells; MCAO – Middle cerebral artery occlusion; ROA – Route of Administration; BDNF-Brain-derived neurotrophic factor

hypertensive rats compared to commonly used rat strains. Although the treatment with HUCB-MSCs reduced the apoptotic cell death in ischemic penumbra, as stated earlier in this review, the major goal of stem cell therapy in ischemic stroke is to replace the dead tissue in the ischemic core. Research that investigates the potential of HUCBCs in ischemic stroke to cause neurogenesis, synaptic plasticity, and axon growth as well as determines the underlying molecular mechanisms is still an unmet need. Interestingly, HUCBCs when administered in combination with simvastatin increased BDNF/TrkB, Ang1/Tie2 and occludin expression, enhanced cell migration into the ischemic brain, enhanced vascular remodeling, amplified endogenous angiogenesis, synaptic plasticity and axonal growth and thereby improved functional outcome after ischemic stroke [57,58]. Summary of these studies that utilized HUCBCs in combination with other treatments were listed in Table 4.

Despite the mechanism of action, the clinical relevance of treatment with HUCBCs for ischemic stroke is obvious, although additional research is needed to take this treatment approach from bench to bedside.

Conclusions

Treatment with HUCBCs is an emerging therapeutic approach for ischemic stroke. Unlike other treatment approaches, stem cell therapy with HUCBCs could enhance recovery even when administered many hours/days after ischemic stroke. Although the studies discussed in this review highlight the potential of HUCBCs to offer neuroprotection after ischemic stroke, well-designed preclinical studies in appropriate animal models are still required to investigate the optimum time, dose, route and duration of administration as well as to understand the underlying molecular mechanisms.

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