Cytotherapy, 2018; 20: 820–829

International Society ISCT Cell & Gene Therapy

The combination of mannitol and temozolomide increases the effectiveness of stem cell treatment in a chronic stroke model

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Abstract

Background. The blood-brain barrier (BBB) presents a significant challenge to the therapeutic efficacy of stem cells in chronic stroke. Various methods have been developed to increase BBB permeability, but these are associated with adverse effects and are, therefore, not clinically applicable. We recently identified that combination drug treatment of mannitol and temozolomide improved BBB permeability *in vitro*. Here, we investigated whether this combination could increase the effectiveness of stem cell treatment in an animal model of chronic ischemic stroke. *Methods.* Chronic stroke was induced in rats by middle cerebral artery occlusion (MCAo). After then, rats were administered human umbilical cord–derived mesenchymal stromal cells (hUC-MSCs) by intravenous injection with or without combination drug treatment of mannitol and temozolomide. To evaluate the therapeutic efficacy, behavioral and immunohistochemical tests were performed, and the differences among control, stem cell only, combination drug only and stem cell with combination drug treatment were analyzed. *Results.* Although no hUC-MSCs were detected in any group, treatment with stem cells and combination drug of mannitol and temozolomide increased the intracerebral delivery of hCD63-positive microvesicles compared with stem cell only treatment. Furthermore, treatment with stem cells and drug combination ameliorated behavioral deficits and increased bromodeoxyuridine-, doublecortin- and Reca-1–positive cells in the perilesional area as compared with other groups. *Discussion.* The combination drug treatment of mannitol and temozolomide allowed for the efficient delivery of hUC-MSC–derived microvesicles into the brain in a chronic stroke rat model. This attenuated behavioral deficits, likely by improving neural regeneration and angiogenesis. Thus, combination drug treatment of mannitol and temozolomide could be a novel therapeutic option for patients with chronic ischemic stroke.

Key Words: *blood-brain barrier, chronic ischemic stroke, mannitol, mesenchymal stromal cell, temozolomide, umbilical cord*

Introduction

Ischemic stroke is a disease caused by blockade of blood supply to the brain and results in extensive brain damage within a few hours and subsequent severe neurological deficits [1,2]. Although a variety of treatment modalities have been developed, with the exception of recanalization of blocked arteries in the hyperacute phase of ischemic stroke, the others have limitations [1,3,4].

Recently, stem cells have been considered as promising tools for the treatment of central nervous system (CNS) diseases such as stroke, Parkinson's disease and Alzheimer's disease [3,5,6]. Mesenchymal stromal cells (MSCs), in particular, have been used to treat patients with ischemic stroke owing to their anti-inflammatory and neuroprotective effects reported in extensive pre-clinical studies [7–9]. Although the stability of intravenously administered MSCs in CNS diseases has been demonstrated, their therapeutic efficacy is not completely known [10–14]. Some important issues have not yet been resolved, including low engraftment rate into ischemic area due to filtering by peripheral organs and low penetration of the blood-brain barrier (BBB) [15–17], especially in chronic ischemic stroke. Although in acute ischemic stroke, transplanted cells easily pass through the damaged BBB [18,19], in chronic ischemic stroke, the

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(Received 31 January 2018; accepted 25 April 2018)

ISSN 1465-3249
https://doi.org/10.1016/j.jcyt.2018.04.004

BBB recovers, preventing the transplanted cells from entering into the damaged area [20–22]. In support of this, we recently found that human umbilical cord–derived MSCs (hUC-MSCs) had effects in an animal model of acute stroke [23], but not in chronic stroke.

For the aforementioned reasons, various methods have been developed to improve the transplantation efficiency of stem cells by increasing BBB permeability, including osmotic shock, inducing inflammation and ultrasound [17,24–26]. Mannitol is considered to be the most effective treatment option to increase BBB permeability; however, with the exception of a study by Chen *et al.* [27], studies show that it must be administered intra-arterially [16,24,28] to be effective.

Temozolomide, an ABCB1 transporter inhibitor, has been shown to increase the efficacy of other drugs by blocking efflux through the BBB [29–32]. Interestingly, the combination of temozolomide with MSCs resulted in a prolonged life span in a glioblastoma animal model [33]. Moreover, we recently found that treatment with a combination of mannitol and temozolomide increases BBB permeability through the inhibition of endothelial tight junction proteins [34]. Based on these reports, we hypothesized that combination drug treatment of mannitol and temozolomide would increase the therapeutic efficacy of hUC-MSCs in a rat model of chronic ischemic stroke.

Methods

Ethics

All animal experiments were performed following protocols approved by the CHA animal research center (IACUC160066). Male Sprague-Dawley rats were purchased from Orient Bio. Eight-week-old rats weighing 270–300 g were used in this study.

Middle cerebral artery occlusion model

Rats were housed with free access to food and water under 12-h light-dark cycles throughout the animal experiments. Animal surgeries were performed as previously described [35]. Briefly, rats (n = 97) were anesthetized with ketamine and rompun. An incision was made in the neck and the carotid artery was exposed. The external branch of the carotid artery (ECA) was punctured and filaments (Doccol Corp.) were inserted to block the middle cerebral artery. After 60 min, the ECA/middle cerebral artery (MCA) was reperfused by removing the filaments. During the surgical procedure, body temperature was maintained at 37°C using a heated surgical plate. On the following day, rats were assessed for disease severity based on circling behavior and limb placement tests [36,37]. Referring to previously published values [21,22,34], we determined that the chronic ischemic stroke model is established 28 days after middle cerebral artery occlusion (MCAo). After 28 days, the MCAo rats were again evaluated for behavioral deficits to verify the chronic stroke model and a total 56 rats used in the experiment were randomly divided into four groups: (1) control, (2) combination drug treatment group (CDTG), (3) stem cell treatment group (SCTG) and (4) stem cell with combination drug treatment group (SC + CDTG). Rats in each group were humanely killed after 7 (n = 4) and 28 days (n = 5) for evaluating early- and late-phase changes, respectively, using immunohistochemistry (IHC) analysis.

Culture of hUC-MSCs

hUC-MSCs (CHA Biotech) were maintained as previously described [23]. Briefly, the cells were cultured in Dulbecco's Modified Eagle's Medium high glucose (Hyclone Laboratories Inc.) supplemented with 10% fetal bovine serum (Hyclone), 50 μg/mL gentamycin (Sigma-Aldrich), 1 μg/mL heparin (Sigma-Aldrich) and 25 ng/mL FGF-4 (Peprotech). Experiments were performed at passage 7.

Transplantation of hUC-MSCs and reagent treatment

The *in vivo* experimental design is shown in Figure 1. Clinically relevant time schedule and drug doses were used for the study. Briefly, temozolomide (Sigma-Aldrich) was orally administered once daily at 20 mg/kg for 5 days after the ischemia model was established. A 20% solution of mannitol (Sigma-Aldrich) was then intravenously injected once daily (2.5 mL per rat) for 3 days after temozolomide treatment. To evaluate the toxicity of the combination treatment with mannitol and temozolomide in MCAo rats, we investigated the densities of neurons (number of neuronal nuclei [NeuN]-positive cells) and microglia (number of ionized calcium-binding adapter molecule-1–positive cells) immediately after treatment. There were no differences between the densities of both neurons and microglia in combination-treated rats and nontreated rats (Supplemental Data 1B). Moreover, there were no changes in temperature and body weight in each treatment group. For the transplantation groups, hUC-MSCs (1×10^6 cells in 0.5 mL phosphate-buffered saline [PBS]) were intravenously injected twice on days 2 and 4 using a 31-gauge insulin syringe (injection speed, 0.1 mL/min) 5 min after mannitol injection [23]. No immunosuppressive drugs were used.

Behavioral tests

Behavioral tests were blinded and were performed independently by two investigators. Modified neurological severity scores (mNSS) from 0–28 were assigned. mNSS tests included analysis for motor, sensory, reflex