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Curcumin-loaded embryonic stem cell exosomes restored neurovascular unit following ischemia-reperfusion injury

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ABSTRACT

We tested whether the combined nano-formulation, prepared with curcumin (anti-inflammatory and neuroprotective molecule) and embryonic stem cell exosomes (*MESC-exo*^{cur}), restored neurovascular loss following an ischemia reperfusion (IR) injury in mice. IR-injury was created in 8–10 weeks old mice and divided into two groups. Out of two IR-injured groups, one group received intranasal administration of *MESC-exo*^{cur} for 7 days. Similarly, two sham groups were made and one group received *MESC-exo*^{cur} treatment. The study determined that *MESC-exo*^{cur} treatment reduced neurological score, infarct volume and edema following IR-injury. As compared to untreated IR group, *MESC-exo*^{cur} treated-IR group showed reduced inflammation and *N*-methyl-D-aspartate receptor expression. Treatment of *MESC-exo*^{cur} also reduced astrocytic GFAP expression and alleviated the expression of NeuN positive neurons in IR-injured mice. In addition, *MESC-exo*^{cur} treatment restored vascular endothelial tight (claudin-5 and occludin) and adherent (VE-cadherin) junction proteins in IR-injured mice as compared to untreated IR-injured mice. These results suggest that combining the potentials of embryonic stem cell exosomes and curcumin can help neurovascular restoration following ischemia-reperfusion injury in mice.

1. Introduction

According to the recent reports, stroke is the fifth major cause of death and a leading cause of disability in adults of United States (Kochanek et al., 2014; Mozaffarian et al., 2015). Each year about 800,000 US people experience stroke and on average, one American death was reported every 4 min (Mozaffarian et al., 2015). After attempting decades of efforts in developing neuro-restoration therapy that could also help reducing ischemic lesion volume, only thrombolytic therapy had shown beneficiary effects (Li et al., 2014). However, there remains always a need for powerful and innovative therapy that can limit cascades of ischemia and associated pathology.

Exosomes are the secretory nano-vesicles (<200 nm) that recently acquired great scientific attention because of their ability to transfer cellular and biological information, serving as biomarkers and their potential role in therapeutics (Kalani et al., 2014d; Kalani and Tyagi, 2015). Exosomes possess intrinsic ability to cross blood-brain barrier (BBB) and hence, suitable to overcome the problems associated with powerful and potential drugs that cannot reach to clinical trials because of their BBB impermeability (Pardridge, 2012). Of most interest, exosomes possess paracrine properties, special cargos of miRNA, mRNA, proteins and lipids, of the cell type from where they are released (Gangoda et al., 2015; Zhang and Grizzle, 2014). In this regard, stem cell derived exosomes have been studied to possess enormous rejuvenating powers that can reprogram the target cell to augment the repair/regeneration processes (Khan et al., 2015; Lai et al., 2012). Stem cell-derived exosomes are not only found equally beneficial as stem cells but can also overcome limitations associated with cell-based therapy

Abbreviations: AchE, acetylcholinestrase; BBB, blood brain barrier; BSA, bovine serum albumin; cDNA, complimentary DNA; DAPI, 4,6-diamidino-2-phenyl-indole HCl; FIU, fluorescence intensity units; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; ICAM, intra cellular adhesion molecule-1; IHC, Immunohistochemistry; IR, Ischemia Reperfusion; LEA, Lycopersicon Esculentum agglutinin tomato lectin; LPS, Lipopolysaccharide; *MESC-exo*, Mouse embryonic stem cell exosomes; *MESC-exo*^{cur}, curcumin loaded mouse embryonic stem cell exosomes; NeuN, neuronal nuclei; NMDARs, *N*-methyl-D-aspartate receptors; NTA, nanoparticle tracking analysis; PBS, phosphate buffer saline; QPCR, quantitative PCR; RIPA, radioimmunoprecipitation assay buffer; ROS, reactive oxygen speies; TBS, tris-buffered saline; TBS-T, tris-buffered saline with Triton X-100; TNF-α, tumor necrosis factor; TSG101, tumor susceptibility gene101.

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at ischemic area (Khan et al., 2015). A large body of evidences suggests that mesenchymal stem cell (MSC) derived exosomes possess a myriad of beneficiary effects against stroke by promoting functional recovery, neurovascular plasticity, neuroprotection, neuroregeneration and modulating peripheral post-stroke immune responses (Doeppner et al., 2015; Xin et al., 2013). The therapeutic power of MSC-derived exosomes was proposed by transferring functional miRNAs to the recipient cells (Chopp and Zhang, 2015; Xin et al., 2012). Recently embryonic stem cell-derived exosomes have been found to promote endogenous repair mechanisms and enhancing cardiac functions following myocardial infarction (Khan et al., 2015). The same study reported enrichment of embryonic stem cell-specific miRNAs in the exosomes population derived from embryonic-stem cells (Khan et al., 2015). However, there is lack of reports that suggest the therapeutic efficacy of embryonic stem cell-derived exosomes against ischemic stroke.

Curcumin, a natural polyphenol found in the rhizomes of *Curcuma longa* (turmeric), is yellow colored spice and possess remarkable medicinal properties. The therapeutic efficacy of curcumin has been extensively studied against ischemic stroke which is contributed by promoting free radical scavenging, anti-inflammatory, anti-lipidemic and anti-aggregation properties (Kalani et al., 2014c; Soni and Kuttan, 1992; Strimpakos and Sharma, 2008). Extensive medicinal properties led curcumin towards clinical trials to prevent brain diseases; however, phase-I clinical trials were unsuccessful because of its low bioavailability (Anand et al., 2007; Ovbiagele, 2008; Perry and Howes, 2011). Poor absorption, quick metabolism, and rapid systemic elimination are the factors that limit curcumin bioavailability. These problems led other investigators to explore alternative methods of treatment or delivery. Patra and Sleem (2013) have developed a novel method for encapsulation of curcumin by synthesizing microcapsule containing self-assembled nanoparticles using poly (l-lysine), trisodium citrate and silica sol. Mouslmani M et al. (2015) developed hierarchically ordered nanocapsule structures by crosslinking curcumin associated poly (allylamine hydrochloride) with dipotassium phosphate and subsequently congregates with silica nanoparticles (Mouslmani M et al., 2015). Excitingly, curcumin loaded in exosomes was not only found more stable, highly soluble and highly concentrated in the blood but also appeared to express more therapeutic potentials (Sun et al., 2010; Zhuang et al., 2011). In the current report, we sought to determine the neurovascular restoration therapy of *MESC-exo*^{cur} [curcumin loaded in *MESC-exo* (mouse embryonic stem cell-derived exosomes)] following an ischemia reperfusion injury in mouse model.

2. Methodology

All procedures were conducted in compliance with guidelines established by the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the University of Louisville's Institutional Animal Care and Use Committee. 8–10 weeks old male wild-type (WT, C57BL/6J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The experimental mice groups were: 1) sham, 2) sham + *MESC-exo*^{cur}, 3) IR, and 4) IR+ *MESC-exo*^{cur}.

2.1. Animal surgical procedure

Mice were anesthetized with sodium pentobarbital (50 mg/kg body wt.) and operated within 1 h. Anesthetized mice were orally intubated, mechanically ventilated and the body temperature was maintained at $37 \pm 1\,^{\circ}C$ during surgery. A midline neck incision was made and common carotid artery (CCA) was exposed carefully. Silicon-rubber coated monofilament (diameter 0.22, 0.23 mm, size 6–0 or 7–0) was used to obstruct middle cerebral artery after inserting it into internal carotid artery and advancing to internal carotid artery. After 40 min of surgery, the filaments were withdrawn to allow reperfusion. Same anesthesia and surgical procedures were performed in sham groups of mice except the insertion of monofilament (Kalani et al., 2015). After the surgery, mice were tested for the neurobehavioral tests to determine neurological deficit score (Belayev et al., 1996; Kalani et al., 2015). The neurological deficit score was reported on a blind fashion using a scale 0–12 (normal score = 0, maximum = 12). Mice showing high neurological deficit scores (>10) were used in the current study.

2.2. Isolation of mouse embryonic stem cell exosomes and their characterization

Mouse embryonic stem cell line was procured from American Type Culture Collection (ATCC, Menassas, VA, USA) and grown on a fibroblast monolayer as per supplier's step-by-step protocol. The cells were maintained in 25 or 75 cm^2 tissue culture flasks under the atmosphere set at 5% CO_2 and 95% air in an incubator. For exosome collection, the cells were made 50–60% confluent and the media was changed, which was prepared with exosome free serum. After 48–72 h of culture, media was collected and processed as described in our earlier report (Kalani et al., 2014a), with certain modifications. Briefly, culture media was centrifuged at $3{,}000 \times g$ (10 min) and supernatant was re-centrifuged at $10{,}000 \times g$ (15 min). $10{,}000 \times g$ supernatant was collected and ultracentrifuged at 1, $40{,}000 \times g$ for 3 h to concentrate *MESC-exo* in pellet. Characterization of exosome was performed with Western blot with TSG101 antibody (Kalani et al., 2014a), acetylcholinesterase activity (Kalani et al., 2014a), and nano-tracking (NTA) analysis (Sokolova et al., 2011).

2.3. Packing of curcumin in MESC-exo and intranasal delivery

The loading of curcumin (dissolved in ethanol) was achieved by mixing it to *MESC-exos* in a fixed proportion (1: 4). The nanopreparation was incubated for 15 min at room temperature and rapid freeze-thawing was done 2–3 times. The unbound drug was removed by centrifuging the preparation twice at $5000 \times g$. The formulation was precipitated either by ultracentrifugation at $1, 40{,}000 \times g$ for 3 h or using total exosome isolation reagent (ThermoFisher Scientific, Grand Island, NY, USA). Fresh preparations of *MESC-exo*^{cur} were used for intranasal delivery in two groups of mice (sham + *MESC-exo*^{cur} and IR + *MESC-exo*^{cur}). Total 10 μl *MESC-exo*^{cur} was administered, twice a day, by alternate nostrils (2 μl X 5 times) started within an hour of IR and sham surgery and continued till 7 days. Neurological behavior tests for instance, posture relax test, forelimb placing test and motor coordination tests were performed on 1st, 3rd and 7th day post- *MESC-exo*^{cur} administration.

2.4. Collection of brain samples

At the end of the experiment, mice were intracardially perfused with phosphate buffer saline (50 mM PBS, pH = 7.4) under deep anesthesia. Brain samples were carefully harvested after opening the cranium, washed with ice-cold PBS, and used for various experimental procedures.

2.5. Determination of infarct volume (IV)

2 mm size coronal sections were cut using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA, USA). 2% of 2,3,5-Triphenyltetrazolium chloride (TTC; Sigma Aldrich, Taufkirchen, Germany) was used to stain the sections. After staining the sections for 20 min in TTC, sections were fixed in 4% paraformaldehyde.