Bladder Transplantation of Amniotic Fluid Stem Cell may Ameliorate Bladder Dysfunction After Focal Cerebral Ischemia in Rat

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INTRODUCTION

Cerebral stroke is ranked as one of the leading causes of death, and the poststroke neurological injury is the most important problem to cause disability worldwide. In addition to neurological disability, voiding dysfunction is common in the patients with stroke and impairs life quality severely in the long term. Bladder dysfunction related to stroke is characterized by a plethora of urologic symptoms, such as overactive bladder, voiding difficulty, and urinary incontinence [1, 2]. Neurogenic overactive bladder after cerebral stroke is currently treated with antimuscarinic therapy, detrusor botulinum toxin injection, or sacral neuromodulation; however, these therapeutic methods are not effective and associated with adverse effects [3]. Recently, we have reported that systemic administration of human umbilical cord blood CD34 cells to middle cerebral artery occlusion (MCAO) rats could significantly reduce infarct volume [4]. Treatment with amniotic fluid stem cells (AFSCs) transplantation may facilitate functional recovery in a rodent model of ischemic stroke [5, 6]. Therefore, stem cell transplantation seems a promising treatment for bladder dysfunction after cerebral stroke.

Bladder overactivity induced by MCAO in rats has been found to involve receptors in the brain such as dopamine and glutamate [7]. Nerve growth factor (NGF) and muscarinic receptors in cerebral-infarcted rats are also involved in the regulation of the micturition reflex in central and peripheral nervous systems [8-11]. NGF has an effect on bladder dysfunction by mediating morphological and functional changes in sensory neurons innervating the bladder [10]. A significant increase in the density of muscarinic receptors...
has been found in the urinary bladder after cerebral infarction in rats [9]. In the bladder of animals, there is parasympathetic excitatory cotransmission with the cholinergic and purinergic components acting via muscarinic and P2X1 receptors, respectively, [12].

At present, no study has ever been designed to demonstrate the effect of AFSCs on bladder dysfunction induced by cerebral ischemia in animals. The present study was conducted to investigate whether human amniotic fluid stem cells (hAFSCs) grafting into the bladder wall may influence bladder functional and molecular changes in a rat stroke model. Since it is reported that local injection of stem cells may ameliorate impaired detrusor contractility of injured bladder in rodent models [13, 14], and local injection may cause more stem cells in the submucosal connective tissue and muscular tissue of bladder than intravenous injection [15], so we used local injection into bladder in the present study.

**Materials and Methods**

**Animal Model**

All protocols were approved by the Institutional Ethics Committee for the Care and Use of Experimental Animals and the Institutional Review Board of our hospital. Female Sprague Dawley rats were maintained at 21°C–23°C room temperatures and 47% humidity with a 12-hour light-dark cycle and free access to standard laboratory chow and tap water. Rats (270–320 g) were assigned into 3 groups: (a) sham-operated group (n = 10): injection with 0.3 ml phosphate buffered saline (PBS) at 3 hours after sham operation, (b) MCAO alone rats with no hAFSCs treatment (n = 10): 0.3 ml PBS injection at 3 hours after MCAO, (c) MCAO rats with hAFSCs treatment (n = 10): injection of 1 × 10^6 hAFSCs cells in 0.3 ml PBS at 3 hours after MCAO. Bladder function was analyzed using conscious cystometry at days 3 and 10 after MCAO. Expressions of NGF, M2-muscarinic receptor (M2), M3-muscarinic receptor (M3), and P2X1 were measured by immunohistochemistry, and real-time polymerase chain reaction. The schema of the experimental procedure is present in Supporting Information Figure 1.

**Focal Cerebral Ischemia Model**

The left MCAO was used as an acute ischemic model according to the methods described in our previous work [18]. The cells in culture were trypsinized and stained with phycoerythrin (PE)-conjugated antibodies against CD44, CD73, CD90, CD105, CD117, and CD45 (BD PharMingen, CA). Thereafter, the cells were analyzed using the Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Passage 6-8 hAFSCs were collected and prepared to a final concentration of 1 × 10^6 cells per 0.3 milliliter in PBS. In the hAFSCs-treated groups, 1 × 10^6 collected hAFSCs were transplanted into each rat at 3 hours after MCAO by injection into the five sites of bladder (anterior, posterior, bilateral, and dome) under inhalation anesthesia. Before each local injection, the syringe was pushed backwards to confirm the needle was not present inside the vessel.

**Cystometric Study**

All rats received suprapubic tube implantation under isoflurane general anesthesia 3 days prior to implementation of cystometry. The animals were placed in special metabolic cages (Med Associates, Saint Albans, VT) to perform conscious cystometries at days 3 and 10 after MCAO according to the methods described in our previous study [19]. Briefly, the suprapubic catheter was connected to both the syringe pump and the pressure transducer. Pressure and force transducer signals were amplified, recorded on a chart recorder and digitized for computer data collection. The bladder was then filled with room-temperature 0.9% saline at 5 ml/hour through the bladder catheter, while bladder pressure was recorded. Urine was collected in a beaker on a balance placed beneath each cage. Changes in the weight of the collection were recorded. Saline infusion was continued until rhythmic bladder micturition contractions became stable. All of the cystometric parameters on five representative micturition cycles were collected for analyzing, including peak voiding pressure, intercontraction interval, voided volume, and residual volume. Cystometry Analysis Version 1.05 (Catamount Research and Development, Saint Albans, VT) was used for cystometric analysis.

**Immunohistochemistry**

Animals were euthanized after cystometry, the dissected bladders were fixed in an optimal cutting temperature compound, frozen in powdered dry ice and stored at −80°C. The bladders were then subjected to cryosection (10 μm) at −18°C. Immunostaining against NGF, M2, M3, and P2X1 in fresh-frozen bladder sections was performed with an avidin-biotin peroxidase method. First, fresh-frozen sections were fixed in acetone 10 minutes for NGF and 4% paraformaldehyde 10 minutes for M2, M3, and P2X1 in fresh-frozen bladder sections was performed with an avidin-biotin peroxidase method. First, fresh-frozen sections were fixed in acetone 10 minutes for NGF and 4% paraformaldehyde 10 minutes for M2, M3, and P2X1, air dried and then rinsed with PBS. After blocking with Dako REAL peroxidase blocking solution (code S2023, DAKO Corp, Carpinteria, CA) for 20 minutes, sections were washed and incubated for 18–20 hours at 4°C with a rabbit polyclonal antibody directed against NGF (1:750, OriGene Technologies, Inc.), M2 (1:1,000, Millipore, Temecula, CA), M3 (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA) and P2X1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Then, sections were washed and incubated for one hour using biotinylated secondary antibodies at a 1:500 dilution (Vector Laboratories, Burlingame, CA). Staining was developed with 3,3′-diaminobenzidine plus...
hydrogen peroxide as the chromogen. The ratio of the optical density of MCAO rats with or without hAFSCs treatment to that of sham-operated rat was determined for NGF, M2, M3, and P2X1 analyses. Image-Pro Plus Software (Media Cybernetics, Silver Spring, MD) was used for immunoreactivity measurement.

Immunofluorescence
Immunofluorescent staining was done according to the previous report [20]. The fresh-frozen bladder sections were first fixed with precooled acetone for 10 minutes, and then washed in PBS three times for 5 minutes. The sections were blocked with 10% fetal bovine serum in PBS for 20 minutes at room temperature and then incubated with rabbit polyclonal antibody (PE) directed against human PAX7 (1:50, LifeSpan BioSciences, Seattle, WA) in a humidified chamber overnight at 4°C. The sections were stained with donkey anti-rabbit IgG antibodies (1:500, Santa Cruz Biotechnology, Santa Cruz, CA) for 45 minutes and then stained with 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Santa Cruz, CA). All images were detected by confocal microscope (Leica, Wetzlar, Germany).

Real-Time Polymerase Chain Reaction
Real-time PCR was carried out according to the manufacturer’s protocol. Total RNAs were prepared using a Trizol reagent (Invitrogen, Carlsbad, CA) and incubated in reverse transcription mixture at 25°C for 5 minutes, 50°C for 1 hour, 70°C for 15 minutes; finally, the tubes were cooled to 4°C for 5 minutes. Gene expression for NGF, M2, M3, and P2X1 in the bladder tissue was analyzed by real-time PCR using inventoried TaqMan assays from Applied Biosystems (Life Technologies, Grand Island, NY). The codes for NGF, M2, M3, and P2X1 assays were Rn01533872-m1, Rn00560986-s1, and Rn00564454-m1, respectively, (Applied Biosystems). GAPDH assays codes (Rn99999916-s1) were used as an endogenous control to allow for quantification of relative gene expression. Thermal cycling and fluorescence detection were performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for one minute. The data were calculated using the 2ΔΔCt method [21]. A ratio of the mRNA level of ischemic rats with or without hAFSCs treatment to that of sham-operated rats was determined. The values were summated and

Figure 1. Urodynamic studies for all groups before and after treatment. Cystometric results in the experimental rats (A–D) are presented (n = 10). MCAO rats show no change in peak voiding pressure (A), but a significant decrease in voided volume (B) and intercontraction interval (C), and an increase in residual volume (D) at days 3 and 10 after MCAO. However, these bladder dysfunctions can be improved following human amniotic fluid stem cells transplantation. *Compared to sham-operated group, p < .05. # Compared to MCAO alone group, p < .05. Abbreviation: MCAO, middle cerebral artery occlusion.
expressed as mean ± SD and were compared statistically among sham operation and each time point in MCAO group.

Statistical Analysis
The data were analyzed statistically using one-way analysis of variance test followed by a Tukey test. Values were considered significant at $p < .05$. Prism 5 software for statistical analysis (GraphPad, San Diego, CA) was used for all data.

RESULTS
Characterization of hAFSC Showing the Stem Cell Markers
We successfully obtained hAFSC as previously described by our group and De Coppi et al. [18, 22] with expressions of CD117 (stem cell markers), CD44 (cell migration marker), and mesenchymal stem cell markers including CD73, CD90, and CD105. These cells did not express CD45 (hematopoietic stem cell marker) (Supporting Information Fig. 2).

Bladder Overactivity Subsequent to MCAO Are Ameliorated by hAFSCs
A total of 6 rats were excluded, including no right-side weakness in 3, death within 2 days after operation in 2, and death during operation in 1. Data of 60 rats were collected and the final number in each group was kept at 10 for statistical analysis.

When compared with sham-operated rats, cystometric studies showed that MCAO alone rats had significant increase in residual volume and decrease in voided volume and intercontraction interval (all $p < .05$) but no difference in peak voiding pressure at days.
3 and 10 after MCAO. When compared with MCAO alone group, hAFSCs group had less residual volume, more voided volume and longer intercontraction interval at days 3 and 10 after MCAO (all $p < .05$; Fig. 1).

**Expressions of NGF, P2X1, and Muscarinic Immunoreactivity and Human PAX7 in Bladder After MCAO**

When compared with sham-operated group, MCAO alone rats had significant decrease in the immunoreactivities of NGF, M3 and P2X1 at days 3 and 10 after MCAO (all $p < .05$; Fig. 1); however, M2 immunoreactivity transiently increased at day 3 ($p < .05$) and then decreased at day 10 after MCAO ($p < .05$). When compared with MCAO alone group, hAFSCs group had significant increase of NGF and P2X1 immunoreactivities at day 3, and increase of M2 at day 10 after MCAO (all $p < .05$; Figs. 2–5). The immunofluorescence of human PAX7 was not seen in the bladder wall of sham-operated rat, but could be seen in that of hAFSCs-treated rat (Supporting Information Fig. 3).

**Expressions of NGF, M2, and M3 mRNA in Bladder After MCAO**

In Figure 6, when compared with sham-operated group, MCAO alone rats had significant increase of NGF, M2, and M3 mRNA at day 3 (all $p < .05$), but decrease of M2 at day 10 after MCAO ($p < .05$). When compared with MCAO alone group, hAFSCs group had significant increase of NGF and P2X1 immunoreactivities at day 3, and increase of M2 at day 10 after MCAO (all $p < .05$; Figs. 2–5). The immunofluorescence of human PAX7 was not seen in the bladder wall of sham-operated rat, but could be seen in that of hAFSCs-treated rat (Supporting Information Fig. 3).
had significant decrease of M2 mRNA at day 3 and increase of
P2X1 mRNA at days 3 and 10 after MCAO (all \( p < .05 \)).

**DISCUSSION**

Previous clinical reports have shown that bladder dysfunction may occur in the acute stage of stroke [23, 24], and around 70% ischemic stroke patients have detrusor overactivity [25]. Animal study also demonstrated that after MCAO, bladder capacity and voided volume were significantly lower in the ischemic than in the sham rats, which was suggested due to detrusor overactivity [9]. The mechanism of reduced bladder capacity in ischemic rats is supposed due to neuronal damage in the forebrain with interruption of tonic inhibitory neuronal pathways toward the pontine micturition center that regulates parasympathetic tone to urinary bladder [26]. The reduced parasympathetic tone may induce compensatory upregulation of muscarinic receptors [9].

In the present study, the ischemic rats showed decrease in voided volume and intercontraction interval and increase in residual volume after MCAO, indicating bladder overactivity. After MCAO, there was a compensatory upregulation of M2 immunoreactivity transiently at day 3 but both M2 and M3 reduced at day 10. There was also a compensatory upregulation of both M2 and M3 mRNAs at day 3 but M2 then reduced and M3 was back to sham level at day 10. This expression disparity between protein and mRNA may be related to the severity of bladder injury. The
direct injection of hAFSCs to bladder wall delayed the compensatory upregulation of M2 immunoreactivity from day 3 to day 10 and recovered the M2 mRNA at day 10. There was also recovery of M3 immunoreactivity and mRNA to sham level from day 3 to day 10 compared to MCAO alone. Similarly, after hAFSCs treatment, NGF immunoreactivity upregulated transiently at day 3 but recovered at day 10 and no change of mRNA. The P2X1 mRNA and immunoreactivity increased at day 3 and P2X1 immunoreactivity recovered at day 10. These data suggest muscarinic and P2X1 receptors and NGF are recovered mostly to sham level at day 10 except M2 and NGF mRNAs, which correlates well to the recovery of bladder function to sham level at day 10. The summary of NGF, M2, M3 and P2X1 expressions in bladder after MCAO is presented in Figure 7.

Bladder contraction mainly depends on the parasympathetic stimulation of the muscarinic receptors in the detrusor smooth muscle, but purinergic receptors also play a certain role in the contraction of bladder [27]. Bladder purinergic activities are elicited predominantly by stimulation of the P2X1 receptors, and this purinoceptor subtype may be expressed in the rat and human bladders [28, 29]. Purinergic receptors are activated by adenosine 5’-triphosphate, which may have a more important role in bladder contraction in patients with overactive bladder [30]. In a rat model, increased P2X1 receptor expression was suggested to contribute to the augmentation of bladder contractile response induced by hypoxia-glucopenia and reoxygenation [31]. A previous immunohistochemical study showed that P2X2 was elevated but P2X1 was significantly decreased by about 60% in the bladders of

Figure 5. Temporal expressions of bladder P2X1 immunoreactivity in MCAO rats (n = 10). Expressions of P2X1 immunoreactivity significantly decrease at days 3 and 10 after MCAO. Following human amniotic fluid stem cells transplantation, the immunoreactivity of P2X1 significantly increases at day 3 after MCAO. Bar indicates 20 μm. *Compared to sham-operated group, p < .05. # Compared to MCAO alone group, p < .05. Abbreviation: MCAO, middle cerebral artery occlusion.
patients with detrusor overactivity [29]. Similarly, our data revealed that when compared with sham-operated rats, bladder P2X1 immunoreactivity was decreased at days 3 and 10 after MCAO, suggesting the involvement of P2X1 in the bladder activity following cerebral stroke.

NGF is normally present in bladder muscle cells and urothelium [32]. NGF administered intramuscularly to the detrusor muscle can induce rat bladder hyperreflexia and neuronal hypersensitivity [33]. Our previous study [19] found NGF immunoreactivity and mRNA in the bladder muscle declined significantly at days 7 and 28 after bilateral common carotid artery occlusion in rats. In the same study [19], we found that the increased NGF expression in bladder one day after cerebral hypoperfusion was associated with bladder hyperactivity which was found to sustain for a certain period even after NGF expression was decreased. Our present results were similar to our previous study [19] that the expressions of NGF protein and mRNA in bladder were decreased after MCAO, except the NGF mRNA increased transiently at day 3 after MCAO.

Our results demonstrated that bladder dysfunctions improved following bladder transplantation of hAFSCs in MCAO rats with less residual volume and improved voided volume and intercontraction interval. The hAFSCs can be obtained from amniotic fluid, grow easily in culture and appear phenotypically and genetically stable, supporting that these cells can act as a novel source for cell transplantation therapy [22, 34]. In our previous studies, hAFSCs had been proved to have therapeutic effects in the mouse models of liver fibrosis and myocardial infarction [35, 36]. Recently, these stem cells have also been used in various tissue repair studies, including bladder injury and neurologic disorders [13, 22]. Soler et al. [34] reported that hAFSCs therapy may ameliorate bladder dysfunction in an animal model of Parkinson disease.

This study has some limitations. First, the present study did not demonstrate the therapeutic effect of hAFSCs on behavior recovery and infarct size after MCAO. However, the amount of hAFSCs needed to treat cerebral infarction through intravenous injection is much larger than that for local injection to treat

![Figure 6](image-url)
bladder dysfunction. The present study used $1 \times 10^6$ cells per 0.3 milliliter injected in five bladder sites, which is much lower than the amount needed for intravenous injection. In such condition, there should be limited effect to the brain. Second, we had examined the effect of hAFSCs in different concentrations and found the concentration in this study was effective. However, it is possible that higher concentration may have better effect on micturition pressure after MCAO. Third, we only examined the effect of hAFSCs given at 3 hours after MCAO, because clinically available acute stroke treatment such as intravenous recombinant tissue plasminogen activator was used in a therapeutic time window within 3–4.5 hours [37]. It is possible that hAFSCs could induce a better effect if given at other time points. Fourth, we used hAFSCs for the present study due to its low immunogenicity, low tumorigenesis, and anti-inflammatory function [33], but xenogenic stem cell transplantation possibly involves host immune reactions. In the future study, these issues should be carefully examined. Fifth, it is possible the proteins we have examined might not be correlated very well with the bladder function. However, we found a relatively correlation of the recovery of muscarinic and P2X1 receptors and NGF with the recovery of bladder function at day 10. Previous reports also found that NGF can be regarded as a potential urinary biomarker for overactive bladder syndrome [38], and there is a parasympathetic excitatory cotransmission with the cholinergic and purinergic components acting via muscarinic and P2X1 receptors, respectively, [12]

CONCLUSION

In conclusion, our data support the inference that transplantation of hAFSCs can improve the bladder dysfunction following MCAO which may act through the regulation of bladder NGF, and muscarinic and P2X1 receptors.

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AUTHOR CONTRIBUTIONS

C.-C.L., S.W.S.S., and Y.-H.H.: conception and design, financial support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Y.-H.L. and T.-H.L.: conception and design, financial support, provision of study material or...
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