SUPPLEMENTAL MATERIAL

METHODS

Ischemic stroke induction followed by reperfusion

Rats from all the groups were subjected to a 2-hour middle cerebral artery occlusion (MCAO) using an intraluminal suture model, as previously described by our group [14]. Briefly, a ventral midline incision was made in the neck under isoflurane anesthesia, and the right common carotid artery and its internal and external branches were surgically exposed. To induce focal cerebral ischemia a silicone-coated monofilament suture (Doccol Corporation, CA) of the recommended size based on the animal body weight was introduced into the internal carotid artery via the external and common carotid arteries and then advanced superiorly until it reached the origin of the middle cerebral artery. After two hours of ischemia the monofilament suture was withdrawn to restore blood flow, and the neck incision was closed with suture. At the time of the MCAO the age of the animals was between 8 and 10 weeks, and the weight of the animals was 260 ± 5 g for the males and 220 ± 5 g for the females.

Stem cell culture and treatment

Cryopreserved human umbilical cord blood-derived mesenchymal stem cells (MSC) obtained from Vitro Biopharma (Golden, CO) were used to establish cultures in MSC-GRO low serum, complete MSC medium according to the manufacturer's instructions. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 with a change of culture medium twice a week. When the cell cultures were about 80% to 90% confluent, cells were split and subcultured. From the subcultures of less than eight passages MSC were detached, washed twice in sterile phosphate-buffered saline (PBS), counted, suspended in sterile PBS, and administered to the appropriate groups of stroke-induced rats at one day after reperfusion.

TTC staining

One day after reperfusion male and female rats from the untreated ischemic cohort were deeply anesthetized with sodium pentobarbital and then decapitated. Brains were collected, placed in an adult rat brain matrix (Kent Scientific Corporation, USA), chilled in a freezer at -80 ˚C until hardened, and then cut into 2-mm thick coronal sections. The sections were stained in the dark for 30-45 min at 37 ºC with a freshly prepared 2% solution of triphenyl-tetrazolium chloride (TTC). Non-ischemic (stained red) and total ipsilateral areas as well as the total contralateral area of each coronal brain section were traced and measured using Image J analysis software (NIH).

Modified neurological severity score (mNSS) assessment

The mNSS is a widely accepted standardized method of assessing the severity of post-stroke injury and recovery and is a composite of reflex, balance, sensory (tactile, visual, and proprioception), and motor (abnormal movement and muscle status) tests [29]. A cumulative score obtained from all the tests determines the severity of post-stroke injury in each animal. Scores between 1-6 indicate mild injury; 7-12, moderate injury; and 13-18, severe injury. The mNSS assessment was performed on each cohort of MSC or vehicle-treated rats prior to ischemia (baseline) and at regular intervals (day 1, 3, 5, 7, and 14) after ischemia.

Modified adhesive removal (sticky tape) test

This test provides an assessment of post-stroke somatosensory dysfunction and can reliably quantify the degree of focal sensory impairment in animals without requiring any prior training [30]. For this test a strip of adhesive tape was wrapped around the rat's forepaw, and the animal's

interaction time with the taped limb (i.e., licking/biting or shaking) was recorded during a 30-sec observation period. The sticky tape test was conducted on the different cohorts of rats both before ischemia (baseline) and at regular intervals (day 1, 3, 5, 7, and 14) after ischemia. For each animal three trials were performed on both the affected (left) limb and the unaffected (right) limb at each time point, and the average of the three trials was then used to calculate the sticky-tape ratio (affected limb/unaffected limb). This ratio provides a relative index of post-stroke somatosensory limb functioning within each animal.

Beam walk test

This test is often referred to as the foot fault test and was used to assess motor coordination and integration [31]. The beam walk apparatus consisted of a square beam (2 cm x 2 cm cross-section and 152 cm long with a 110 cm walking distance), which was supported 30 cm above the tabletop surface by two stands positioned at opposite ends of the beam. A thick layer of bubble wrap placed under the beam was used to prevent injury to the animal should it slip off the beam. Prior to ischemia induction each cohort of rats was trained for two or three days to traverse the beam, and by the end of the training period all animals had learned the task. The beam walk test was performed on all the rats before ischemia (baseline) and at regular intervals (day 1, 3, 5, 7, and 14) after ischemia. Beam walk performance of rats was rated as follows: 0 - the rat was not able to stay on the beam; 1 - the rat was able to stay on the beam but did not move; 2 - the rat tried to traverse the beam but fell; 3 - the rat traversed the beam with more than 50% foot slips of the affected forelimb and/or hindlimb; 4 - the rat traversed the beam with more than one foot slip, but less than 50% foot slips of the affected forelimb and/or hindlimb; 5 - the rat traversed the beam with only one foot slip of the affected forelimb and/or hindlimb; 6 - the rat traversed the beam

without any foot slips of either the affected forelimb or hindlimb. For each testing session the mean of three trials was used to evaluate the beam walk performance of each rat.

Accelerating rotarod performance test

This test was used to evaluate the balance, grip strength, and motor coordination of stroke-induced rats that had received different treatments. Animals were trained to walk on a rotating rotarod (Rotamex, Columbus instruments; initial speed = 10 rpm; acceleration rate = 0.3 rpm/sec; maximum speed = 80 rpm) for two or three days before ischemia induction. By the end of the training period all rats had learned the task to a satisfactory level (latency to fall ≥ 40 sec). As with the other tests, the rotarod was performed both before ischemia (baseline) and at regular intervals (day 1, 3, 5, 7, and 14) after ischemia. During testing rats were challenged to remain on the accelerating rotarod while walking (maximum period = 300 sec), using the same speed and acceleration parameters employed during training. The latency to fall from the rotarod apparatus was recorded for each rat using a minimum of three trials (each separated by at least 15 min), which were then averaged to obtain a single value. For each animal the rotarod latency was calculated at the various time points and expressed as a percentage of the baseline value, which was considered to be 100%. To prevent injury to the animals during the training and test sessions bubble wrap was placed under the rotating spindles within each lane of the rotarod apparatus.

Data collection and exclusion criteria

One day after reperfusion all animals were evaluated by the mNSS to determine whether or not they qualified for inclusion in the study. The mNSS assessment was performed by trained research personnel who were blind to the subsequent experimental treatment of the animals. Animals that

did not show neurological deficits indicative of stroke or exhibited mNSS < 8 were excluded from the study. In addition, animals that displayed signs of hemorrhage in the vicinity of the MCA at the time of sacrifice were also excluded from the study. Overall, a total of 40 out of the original 100 animals were either excluded or died in the various treatment groups (4 in both the Untreated Males and Untreated Females, 7 in the Vehicle Males, 6 in the Vehicle Females, 8 in the MSC Males, and 11 in the MSC Females).

Statistical analysis

Statistical analysis of the data was carried out using Graph Pad Prism version 6.04 for Windows (Graph Pad Software, San Diego, CA). Clear outliers in the data set were identified by Grubb's test and excluded from the data analysis. Differences in total infarct volume and brain swelling between males and females were analyzed by two-tailed unpaired t-test. In addition, for each neuropathological measure a separate two-way repeated measures ANOVA was performed on the serial brain sections to grossly examine possible regional differences in stroke lesions (with sex as the between-subject factor and section number as the within-subject factor). The neurobehavioral assessment data (mNSS, sticky tape, beam walk, and rotarod tests) were analyzed by two-way repeated measures ANOVA (with treatment as the between-subject factor and time after reperfusion as the within-subject factor). Following a significant ANOVA test result, post hoc comparisons were made using Tukey's multiple comparisons test. Because some animals did not survive the full-time course of the study or had to be eliminated as a consequence of Grubb's test result, it was necessary to perform a separate two-way ANOVA for the final time point of the study (i.e., day 14 after reperfusion). Additional ANOVAs were performed at each reperfusion time interval to directly discriminate possible effects of sex on treatment outcomes in the various

neurobehavioral tests. For these analyses a two-way ANOVA (with sex and treatment as the main factors) was used followed by Sidak's post hoc test. All data are expressed as mean ± SEM. Differences between groups were considered statistically significant at $p < 0.05$.

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