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Research report

Systemic multipotent adult progenitor cells improve long-term neurodevelopmental outcomes after preterm hypoxic-ischemic encephalopathy

Melinda Barkhuizen[a,b,c,1], Ralph van Mechelen[a,b], Marijne Vermeer[a,b], Peter Chedraui[d,e], Dean Paes[b], Daniel L.A. van den Hove[b,f], Bart Vaes[g], Robert W. Mays[g], Harry W.M. Steinbusch[b], Nicola J. Robertson[h], Boris W. Kramer[a,b,h,i], Antonio W.D. Gavilanes[a,b,d,i,*]

[a] Department of Pediatrics, Maastricht University Medical Center (MUMC), Maastricht, the Netherlands
[b] Department of Psychiatry and Neuropsychology, School of Mental Health and Neuroscience (MHeNs), Maastricht University, Maastricht, the Netherlands
[c] DST/NWU Preclinical Drug Development Platform, North-West University, Potchefstroom, South Africa
[d] Instituto de Investigación e Innovación de SaludIntegral, Facultad de Ciencias Médicas, Universidad Católica de Santiago de Guayaquil, Guayaquil, Ecuador
[e] Facultad de Ciencias de la Salud, Universidad Católica "Nuestra Señora de la Asunción", Asunción, Paraguay
[f] Department of Psychiatry, Psychosomatics, and Psychotherapy, University of Würzburg, Würzburg, Germany
[g] Department of Regenerative Medicine, Athersys Inc., Cleveland, OH, USA
[h] UCL Institute of Women's Health, London, United Kingdom
[i] School of Oncology and Developmental Biology, Maastricht University, Maastricht, the Netherlands

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ABSTRACT

There is an urgent need for therapies that could reduce the disease burden of preterm hypoxic-ischemic encephalopathy. Here, we evaluate the long-term effects of multipotent adult progenitor cells (MAPC) on long-term behavioral outcomes in a preterm rat model of perinatal asphyxia. Rats of both sexes were treated with two doses of MAPCs within 24 h after the insult. Locomotor, cognitive and psychiatric impairments were evaluated starting at 1.5 (juvenile) and 6 months (adult). Hypoxia-ischemia affected locomotion, cognition, and anxiety in a sex-dependent manner, with higher vulnerability observed in males. The MAPC therapy partially attenuated deficits in object recognition memory in females of all tested ages, and in the adult males. The hypoxic insult caused delayed hyperactivity in adult males, which was corrected by MAPC therapy. These results suggest that MAPCs may have long-term benefits for neurodevelopmental outcome after preterm birth and global hypoxia-ischemia, which warrants further preclinical exploration.

1. Introduction

Preterm birth is a major worldwide healthcare challenge, with 11.1% of all births occurring before 37 weeks of gestation, and 1–2% before 28 weeks [1,2]. Preterm infants often complicate with encephalopathy, due to a combination of immaturity and injurious events around birth - such as hypoxia-ischemia (HI), inflammation or infection. Pathologically encephalopathy is characterized by diffuse injury to the white matter of the brain, accompanied by secondary injury to the gray matter [3–5]. This has devastating consequences, especially at a very low gestational age (VLGA), below 28 weeks at birth, where encephalopathy leads to lasting functional impairments in almost half of survivors [6].

Unfortunately, therapeutic options for preterm encephalopathy are limited to supportive care, however neuroprotective strategies such as bone-marrow derived stem cell therapies are under evaluation for this group of patient [7]. More precisely, previous studies reported that multipotent adult progenitor cells (MAPCs) or mesenchymal stem cells (MSCs), reduce acute neurological injury after HI in the preterm sheep brain [8,9]. MAPCs have the advantages of a lower immunogenicity and a higher proliferation capacity as compared to MSCs, which are important for clinical translation [10]. Several studies have shown that MSCs and MAPCs can improve functional outcomes in rodent models of late-preterm neurological injury [11–15]. However, it has not yet been established whether these cells are also beneficial for an insult in the VLGA brain.

* Corresponding author at: Department of Pediatrics, Maastricht University Medical Center (MUMC), Maastricht, the Netherlands.
E-mail address: danilo.gavilanes@mumc.nl (A.W.D. Gavilanes).
[1] Current address: Department of Neurology, University of California San Francisco, California, USA.

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We used differences in brain development between rats and humans to address this question. The rat is an ideal model to study the long-term efficacy of therapies for VLGA insults, because the rat brain development at birth resembles the brain at 23 weeks of gestation in the human, and rats can be followed up across key human life stages [16]. Rats reach sexual maturity equivalent to adolescence around 1.5 months of age, but only reach the social maturity of early adulthood around 5–6 months of age [17]. The aim of this study was to explore functional deficits which may be amendable by MAPC administration at birth. We administered two doses of MAPCs within 24 h of a global HI insult at birth [16]. Here we report the effects of this therapy on locomotion, cognition, anxiety during adolescence (1.5–3 months) and adulthood (6–7.5 months) in the rat.

2. Materials and methods

2.1. Perinatal asphyxia (PA) procedure

Sprague-Dawley rats from Charles River (Leiden, The Netherlands) were used for these experiments. Rats were housed at the Central Animal Experimentation Facilities of the Maastricht University, The Netherlands. All experiments were conducted under Dutch Ethical approval according to the guidelines of the EU directive 2010/63/EU. Female rats were time-mated. In the afternoon of embryonic day 21 (expected delivery on E21–E22), the pregnant rat was euthanized by rapid decapitation and the uterine horns, still containing the pups, rapidly removed and submerged in saline solution at 37 °C for 16–18 min according to previously described methodology [18,19], with the groups distributed evenly over different durations. After submersion, the pups were delivered manually, stimulated to breathe and placed in a closed pediatric incubator to recover for an hour (at 37 °C), before being placed with a foster mother that had given birth the day before. Control rats were delivered through C-section from the same litters without going through the submersion process.

2.2. Preparation and administration of MAPCs

Human non-clinical grade MAPCs were obtained from Athersys, Inc. (Cleveland, USA) and stored in liquid nitrogen until resuspension in saline. 200,000 viable cells in 24 µl saline or saline were administered via subcutaneous injection in the neck fold at 6 and 24 h after birth to the HI-exposed groups. The control C-section group did not receive treatment.

2.3. Behavior testing

The rats were housed in pairs after weaning. Two weeks before testing, the day night cycle was reversed. Three groups of rats were tested: MAPC-treated HI rats (12 males, 12 females), vehicle-treated HI rats (12 males, 11 females) and C-section controls (12 males, 13 females). The behavioral tests were performed at 1.5–3 months (juvenile to young adulthood) and at 6–7.5 months of age (older adulthood). The serial testing approach was scheduled from the least stressful to the most stressful.

Locomotion was evaluated with a 40 min open field test in a 100 cm × 100 cm plexiglass arena divided into 4 smaller 50 cm × 50 cm arenas. Total distance moved was tracked with the EthoVision 8.5 software (Noldus, The Netherlands). Object recognition memory was assessed with testing at a delay of 4 h as previously described (Strackx et al., 2010). In the adult group, we used 4 additional novel objects in the second trial. The discrimination index (d2) was calculated from the time spent exploring the new object minus time spent exploring the old object, divided by the total exploration time as scored by two blinded observers. Rats which exhibited total exploration times of less than 8 s in the second trial were excluded from analysis. D2 values significantly greater than zero indicate intact recognition memory [19].

Anxiety-related behavior was evaluated with the elevated zero maze (EZM) as previously described [19] and the home-cage emergence (HCE) test. Prior to the HCE test, the rats were single-housed overnight. The cage was placed in the arena with lighting on the cage but not the part of the arena directly behind the cage. A wire grid was placed in the cage to aid the escape from the lit home cage to the dark arena. An escape was defined as having all four paws on the grid outside the cage. The latency to escape was timed for up to 10 min.

2.4. Statistics

Statistical calculations were performed with Stata10 (Statacorp, College Station, TX, USA). Graphs of average ± S.E.M were made with GraphPad Prism 6 (GraphPad, San Diego, CA, USA). Results were analyzed with a repeated-measures ANOVA with age as a within subjects factor and treatment group (vehicle-treated HI, MAPC-treated HI or C-section), sex as between-group factors. Post-hoc comparisons between groups were done with t-tests. Results from the object recognition test were also analyzed with two-sided paired t-tests against a matrix of zeros. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Mortality and body weights of the rats

The pre-weaning mortality rate for the HI procedure was 39% and it was similar between groups since the majority of deaths occurred before treatment was initiated at 6 h after birth. One additional vehicle-treated HI rat was euthanized due to complications associated with an enlarged thymus gland. Over the study period, the males were consistently heavier than the female rats ($p < 0.001$) and the rats continued gaining weights as they aged ($p < 0.001$), but the HIE- and treatment had no effect on weight ($p = 0.4457$, Supplementary Fig. 1).

3.2. Locomotion

We assessed locomotor deficits with the open field test (Fig. 1). Overall, the juvenile rats moved more than the adult rats (ALL juvenile vs. ALL adult, $p < 0.001$) and females moved more than males (ALL [F] vs. ALL [M], $p < 0.001$).

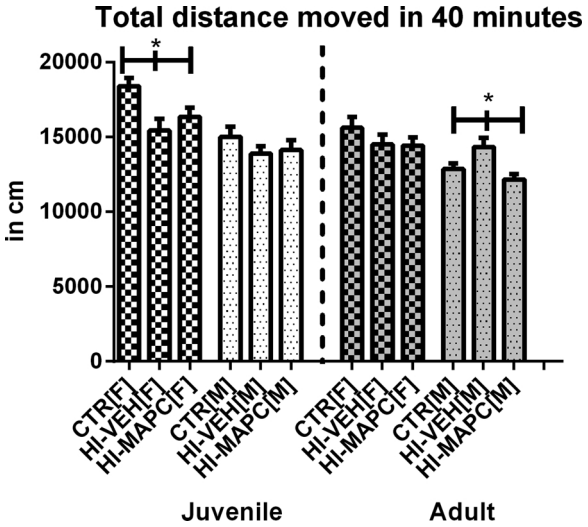

Fig. 1. The total distance moved in 40 min showed an effect of asphyxia in juvenile females, but not of the treatment group. HI-induced hyperlocomotion in the adult male group was corrected by the MAPC therapy (* $p < 0.05$). Group sizes: HI-MAPC-treated rats (12 males, 12 females), HI-vehicle-treated rats (12 males, 11 females) and C-section controls (12 males, 13 females).

In the adult male rats, vehicle-treated HI animals covered a significant greater distance than the C-section control animals (CTR [M] vs. HI-VEH [M], p = 0.022), indicating HI-associated hyperlocomotion in adult males. MAPC treatment significantly reduced hyperlocomotion after global HI in adult males (HI-VEH [M] vs. HI-MAPC [M], P = 0.0021). No significant difference in locomotion was found between the control and treatment group (CTR [M] vs. HI-MAPC [M], p = 0.091) in adult males, which indirectly confirms the treatment effect.

In the juvenile female rats, HI induced significant hypolocomotion (CTR [F] vs. HI-VEH [F], p = 0.011). But, the MAPC therapy did not change HI-associated hypolocomotion in juvenile female rats (HI-VEH [F] vs. HI-MAPC [F] p = 0.191, and CTR [F] vs. HI-MAPC [F] p = 0.011). No significant differences in locomotion between experimental groups were seen in juvenile males or adult females.

3.3. Recognition memory

We assessed deficits in recognition memory assessed with the object recognition test. We evaluated both the presence of an intact recognition memory (d2-discrimination index > 0, p (paired *t*-test) < 0.05) and differences in the extent of recognition memory between groups (ANOVA & post-hoc *t*-test p < 0.05). At the juvenile-time point, the vehicle-treated HI groups and male MAPC-treated HI groups did not recall the original object (HI-VEH [M] & HI-VEH [F] & HI-MAPC [M], d2 > 0, p = not significant), but both male and female control rats, as well as the female MAPC-treated HI group, had an intact recognition memory (CTR [M] & CTR [F] & HI-MAPC [F], d2 > 0, p < 0.05). This indicates that the HI-insult impaired recognition memory relative to the controls, and the MAPC-therapy restored this in the females, but not in the males. In the adult group, all female rats had an intact recognition memory (CTR [F] & HI-VEH [F] & HI-MAPC [F] d2 > 0, p < 0.05). In the males, the vehicle-HI-group forgot (HI-VEH d2 > 0 p = not significant), but both the control and MAPC-treated HI rats remembered (CTR [M] & HI-MAPC [M] d2 > 0, p < 0.05). This indicates that the vehicle treated HI females recovered their memory in the second round of testing, whilst the vehicle treated HI males remained impaired, which the MAPC-therapy restored.

Assessing differences in the magnitude of recognition with an ANOVA showed that the overall d2-scores were not affected by sex or age. Group differences were only significant in the juvenile male rats, where the HI-exposed groups performed worse than the C-section group (CTR [M] vs. HI-VEH [M], p = 0.012, CTR [M] vs. HI-MAPC [M], p = 0.034), but there were no differences between the MAPC-treated and vehicle-treated HI groups (HI-VEH [M] vs. HI-MAPC [M] p = 0.231). There were no significant differences between groups for the adult male rats (p = 0.107–0.265), juvenile female rats (p = 0.068–0.449) or the adult female rats (p = 0.325–0.517) (Fig. 2). This indicates that the benefit of the MAPC-therapy was sufficient to improve the presence of an intact recognition memory (measured with the d2 > 0 test), but not sufficient to improve the magnitude of recognition memory between groups, thus conferring a partial benefit.

3.4. The home-cage emergence test

In the home-cage emergence test (Fig. 3), the females emerged much quicker than males (ALL [F] vs. ALL [M], p < 0.001) and latencies were similar between ages (ALL juvenile vs. ALL adult, p = 0.700). A group effect was only seen in the juvenile females, where the HI-exposed groups were quicker to escape than the C-section control group (HI-VEH CTR [F] p = 0.024; HI-MAPC [F] vs. CTR [F] p = 0.004), but there were no significant differences between the HI-exposed groups (HI-VEH [F] vs. HI-MAPC [F], p = 0.337). Thus, whilst HI had an effect on emergence, there was no effect related to MAPC therapy.

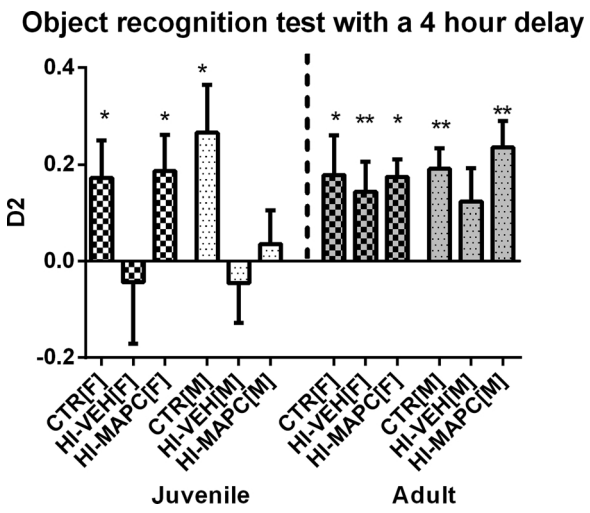

Fig. 2. The discriminatory index after 4 h delay showed a partial rescue of recognition memory in both juvenile and adult females and adult males (* or ** indicates values higher than 0 with a two-tailed paired *t*-test (*p < 0.05, ** p < 0.01)). There were no significant differences observed between HI-MAPC-treated and HI-vehicle-treated rats, as determined with a post-hoc *t*-test at any time point for both sexes. Group sizes: HI-MAPC-treated rats (12 males, 12 females), HI-vehicle-treated rats (12 males, 11 females) and C-section controls (12 males, 13 females).

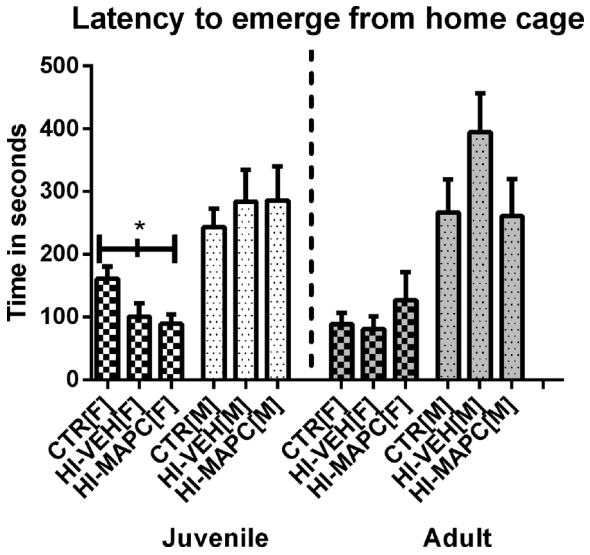

Fig. 3. The home-cage emergence task showed sex differences in the latency to emerge, but no benefit of the therapy (* p < 0.05). Group sizes: HI-MAPC-treated rats (12 males, 12 females), HI-vehicle-treated rats (12 males, 11 females) and C-section controls (12 males, 13 females).

3.5. The elevated zero maze

This test was only conducted in the adult group. There were no overall significant differences in time spent in either arm due to the group (p = 0.360) or sex (p = 0.228) (Fig. 4).

4. Discussion

Encephalopathy of prematurity is a multifactorial insult resulting from a primary insult due to the underdeveloped brain and secondary HI and inflammatory insults [7]. We investigated MAPCs as a therapeutic option for encephalopathy caused by a moderate global HI insult in the newborn immature rat. Brain development in this model resembles the human situation at 23-weeks of gestation in terms of

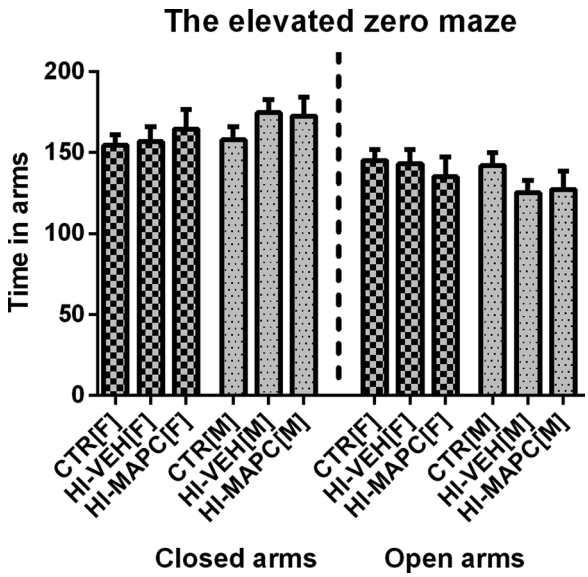

Fig. 4. The results of the elevated zero maze showed no differences between groups or a benefit of the therapy, but the rats spent significantly more time in the closed arm of the test. Group sizes: HI-MAPC-treated rats (12 males, 12 females), HI-vehicle-treated rats (12 males, 11 females) and C-section controls (12 males, 13 females).

oligodendrocyte maturation, immune system development and the establishment of the blood-brain barrier [20]. We showed that MAPC therapy partially attenuated long-term neurodevelopmental deficits with testing ages up to 8 months. We found a persistent cognitive rescue by the MAPCs in the female rats of both age groups, but to a lesser extent in the male rats where only the adult rats responded. For the locomotor outcomes, we found that overall HI reduced movement, and MAPCs did not rescue this. However, the adult male HI-group had a delayed hyperactivity which could represent a delayed onset of a hyperactivity disorder, which was rescued by the MAPC therapy. The cells had no effect on anxiety-related measures. The sex differences we observed in both HI-severity and therapeutic response is reminiscent of the clinical scenario where male infants have more mortality and morbidity after encephalopathy than females, but females have prolonged increased pain sensitivity [18,21,22].

We provided the first long-term follow-up data with minimally invasively administered MAPCs in a neonatal rat model. A previous study using MAPCs in rats with focal neonatal stroke at term showed improvements in neurological scores at 2 weeks after the insult [23]. In preterm global HI, the MAPCs reduced seizure burden, inflammation and disturbances in myelination at 1 week after the insult [8]. Of note, the MAPCs are currently being studied in human clinical trials for adult ischemic stroke, myocardial infarctions, inflammatory bowel disease and graft vs. host disease. The MAPCs are well tolerated in the tested adult populations [24–26]. A clinical study, and previous preclinical studies in adult ischemic stroke and traumatic brain injury, show that the MAPCs have a potent immunomodulatory effect via interaction with the spleen, which translates into a decreased overall hospitalization time for patients [25,27], and reduced lesion size with resulting improved locomotor and neurological scores in rodents [27–29]. MSCs have been more extensively studied in neonatal models. The majority of neonatal MSC studies have focused on focal brain injury, where MSCs improve long-term locomotor, sensorimotor and cognitive outcomes in rodents up to 14 months of age, despite not differentiating into functional neural cells in *vivo* [15,30–32]. Together with our data, this suggests that stem cell therapies, particularly the MAPCs, show beneficial neurological effects.

An acute HI insult damages the brain in three phases [33]. Directly after the insult, during the primary phase, high-energy metabolites are depleted. This leads to progressive depolarization of cells, extracellular accumulation of excitatory amino acids, acidosis and cytotoxic edema. Most neurons survive this stage, only to succumb during the second phase of injury due to cerebral energy failure from 6 to 15 h after birth. The tertiary phase predominantly consists of repair and rewiring. During this period, physiological apoptosis continues to be upregulated which can lead to ongoing cell loss occurring several months after the insult [33,34]. The severity of the secondary phase of HI-injury is closely correlated with later neurodevelopmental impairment [35]. Based on these findings, we choose to administer two doses of 200,000 MAPCs (˜40 million/kg in a 5 g newborn pup) administered within 24 h coinciding with the secondary (6 h) and recovery phase (24 h) after HI-injury [34]. Our dosage selection was based on the previous MAPC data in a rat model of focal neonatal stroke [23]. Previous studies with MAPCs in a model of global HI in the preterm sheep showed that repeated administration has beneficial effects on structural and functional recovery [8] and combining an earlier dose with a dose at 24 h after the insult is beneficial in models of adult traumatic brain injury [36,37]. We hypothesized that a second dose confers additional benefit since stem cells adapt their growth and differentiation factor production according to the needs of the environment at the time of administration, which has been shown with MSCs [31]. We showed partial attenuation of functional deficits at these doses, which could be further explored with different dosage regimens. The limitations of our behavioral testing were that maturation occurred over the testing period and that the juvenile tests were repeated in the adult rats. However, the individual tests were conducted within a week and the rats had a period of 2.5 months between testing sessions to limit the effect of these confounders.

The strength of our study lies on its potential for clinical translation. We utilized a minimally invasive administration route at the clinically feasible time-points of 6 and 24 h after the insult, which gives clinicians time to establish a diagnosis. We further used a model of a moderate global HI insult on top of an already underdeveloped brain, which mimics the often global nature of clinical neonatal brain injury [16]. Our results show that MAPC therapy has promise in attenuating the disability burden of encephalopathy of prematurity which warrants further preclinical development.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bbr.2019.01.016.

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