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Review Article

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The Effects of Stem Cells on Cerebral Palsy

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Abstract
Cerebral palsy (CP) is a neurodevelopmental disorder that hinders normal motor activity and muscle coordination. CP typically appears before, during, or soon after birth as the brain is still developing. The severity of this multifactorial condition depends on the damage done to the parts of the brain that control muscle tone and body movement. The insult in the underdeveloped brain prohibits normal growth; neurons and oligodendrocytes will either die or fail to develop, and the white matter tracts that connect various brain regions become malfunctional. Cerebral palsy is classified into different types depending on the areas of the brain that were insulted and the nature of the movement disorder: spasticity, dyskinesia, and ataxia. Abnormal brain development in patients with CP cannot be reversed, but various treatments are shown to improve and normalize the symptoms. Stem cell transplantation, a regenerative therapy that can replace the damaged and non-functional cells of the brains in CP patients, has shown effective results. Embryonic stem cells (ESC), mesenchymal stem cells (MSC), hematopoietic stem cells (HSC), human amnion epithelial cells (hAEC), and neural stem cells (NSC) are infused to reproduce into more specialized cell types. This alternative therapeutic method has shown successful results through preclinical animal research and clinical trials.

Keywords: Brain Damage, Cerebral Palsy, Cellular Therapy, Clinical Trials, Nervous System, Stem Cells

List of Abbreviations
BM-MNC: Bone marrow mononuclear cell
BM-MSC: Bone marrow mesenchymal stem cell
CP: Cerebral palsy
ES-NPC: Embryonic stem cell-derived neuronal progenitor cell
hAd-MSC: Human adipose-derived mesenchymal stem cell
hAEC: Human amnion epithelial cell
hESC: Human embryonic stem cell
HIE: Hypoxic-ischemic encephalopathy
HSC: Hematopoietic stem cell
hUCB-MSC: Human umbilical cord blood-derived mesenchymal stem cell
MSC: Mesenchymal stem cell
NSC: Neural stem cell

Introduction
Cerebral palsy (CP) is a static neurological disorder caused by a permanent lesion in the immature brain [1]. The insult in the brain is usually located in the cerebral cortex, therefore affecting an individual's ability to control the body muscles. Damage to the brain before cerebral development has completed can occur prenatally, perinatally, or even postnatally, given that the human brain continuously develops in the first two years of life [2]. The severity of the condition depends on the extent and location of damage done to the brain [1]. The measure of mental, manual, ambulatory, and visual impairments are strong factors in determining survival rates for CP patients [3]. Chances of survival for CP patients that have mild impairments, are only marginally less than those of individuals without cerebral palsy; with more severe impairments present, the patient's life expectancy decreases in relation to the number and severity of associated complications [3]. For example, an infant of 2 years has a 40% chance of living to age 20 if he or she has severe CP, while one with mild CP has a 99% chance [4]. Hypoxic-ischemic encephalopathy (HIE) is a resulting brain injury from oxygen deprivation to the brain; the mortality rates are 50% in patients with HIE, as 25% of the survivors display CP [5].

Cerebral palsy is the most common motor disorder of childhood [6]. Studies have reported that cerebral palsy affects between 2.0 and 2.5 individuals in every 1,000 live births [7]. The prevalence of CP has remained mostly stable since 1970; however, the risk of CP in preterm infants has consistently increased [7]. The causes of CP occur prenatally 80% of the time, perinatally 10%, and postnatally 10% [8]. The most important risk factor is prematurity [6]. As early as 38 weeks of gestation, there is an increase in risk [6].

As the gestational age at birth decreases, the risk of the infant having CP increases [6]. Detections of CP among premature births are mostly found through white matter damage on brain-imaging modalities [6]. Evidence of single or multiple brain lesions or ventriculomegaly in a pre-term infant leads to a 50% risk of developing CP [6]. The prenatal

causes are prematurity (< 38 weeks' gestation), low birth weight (< 2500 g), intrauterine growth restriction, intracranial hemorrhage, white matter injuries, cerebral malformations, maternal age (> 35 years), severe maternal iodine deficiency, associated birth defects, or maternal injection [8]. The perinatal causes are peripartum asphyxia (oxygen deprivation) or maternal infection [8]. The postnatal causes are head trauma and hypoxia within the first two years of life, meningitis, or intentional injury [8].

CP has various classifications, leading to different ways of diagnosing the disorder. One diagnosis can be from the discovery of anatomical brain defects in the cerebral cortex, pyramidal tract, extrapyramidal system, or cerebellum [9]. Other diagnoses can result from clinical symptoms of spasticity and dyskinesia, the topographical involvement of extremities (as in diplegia, quadriplegia, and hemiplegia), the timing of presumed insult (prenatal, perinatal, post-neonatal), or by the classification of muscle tone as isotonic, hypotonic, or hypertonic [9].

Cerebral palsy is distinguished by three dominant forms, depending on the nature of the disorder: spastic, dyskinetic, and ataxic [10]. Children born prematurely with CP are most likely to have spasticity, a velocity dependent increase in muscle tone [11]. Seventy to 80 percent of patients diagnosed with CP show spastic clinical symptoms [2]. CP is categorized into diplegia, quadriplegia, and hemiplegia, depending on the anatomical distribution of the deficit [10]. Spastic diplegia involves gross motor problems particularly in the lower limbs, with usually retained fine motor functions in the upper limbs, cognition, and speech [12]. The chief cause of spastic diplegia is periventricular leukomalacia (PVL) and periventricular hemorrhagic infarction (PVHI), affecting between 15 and 25% of CP patients [12]. In patients with spastic hemiplegia, the ipsilateral arm and leg are affected [1]. Not only do they suffer from spasticity, but other symptoms include sensory deficit and muscle weakness, disturbing the functions of their upper limb more than the lower limb [13]. Neonatal stroke, prenatal stroke, or cortical malformations are the principal causes of spastic hemiplegia, affecting between 20 and 40% of CP patients [12]. Perinatal stroke is the most common cause of hemiplegic cerebral palsy [14]. The majority of perinatal stroke cases are due to ischemic events [14]. Around 60 percent of perinatal strokes lead to neurological deficits; hemiplegic cerebral palsy is a common adverse motor outcome [14]. Spastic quadriplegic CP affects all four limbs of the patient; the spasticity is present in the flexor muscles of the upper and lower limb extensor [10]. The majority of affected individuals have little speech and language development, along with visual impairment, epilepsy, and feeding difficulty [12]. Perinatal asphyxia, congenital infection, and cerebral dysgenesis are the most common causes of spastic quadriplegia, affecting between 20 and 40% of CP patients [12].

Dyskinetic cerebral palsy affects about 10 to 20 percent of CP patients [2]. The symptoms are characterized by abnormal postures or movements as a result of impaired muscle tone regulation, movement control, and coordination [15]. Dyskinetic CP is comprised of two major movement disorder patterns: dystonia and choreoathetosis [15]. They both affect patients independently, but dystonia is the most common in dyskinetic CP [15]. The chief cause of choreo-athetoid CP is severe hyperbilirubinemia, usually occurring in the preterm and term infant [12]. Symptoms include unpredictable contractions of individual muscles, usually involving the face, bulbar muscles, proximal extremities, and digits [12]. Choreo-athetoid CP individuals will have slow writhing movements, usually involving distal muscles [12]. Dystonic CP is characterized by the co-contraction of agonist and antagonist muscles, often having co-existent pyramidal signs and dysarthria [12]. The chief cause of dystonic CP is perinatal asphyxia, most commonly in term infants [12]. The third type of CP is ataxia, affecting only 5 to 10 percent of cerebral palsy patients [2]. Ataxia corresponds to injury of the cerebellum or its afferent and efferent projections [16]. Patients have delayed motor and language milestones, along with hypotonia (decreased muscle tone) [12].

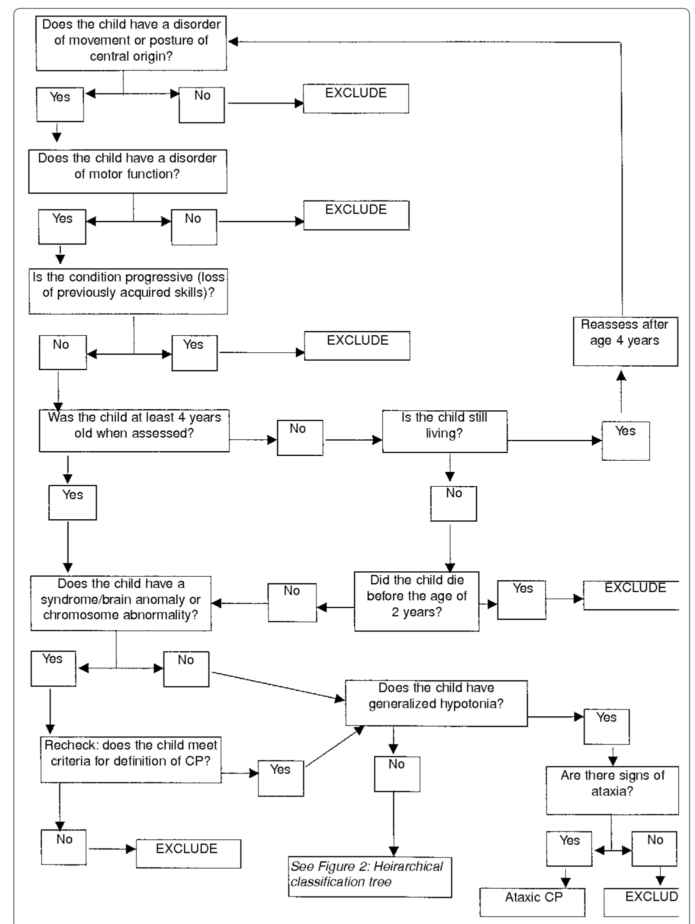

Figure 1: The decision tree for inclusion or exclusion of cases of cerebral palsy on SCPE register [11]

Treatment

Individuals with cerebral palsy usually are diagnosed between 12 and 24 months of age [17]. Numerous functional classification systems have been introduced in order to assess prognosis, communication with parents and other clinicians, and enable objective comparison for research studies [17]. The most commonly used in cerebral palsy are the Gross Motor Function Classification System (GMFCS), the Manual Ability Classification System (MACS), the Communication Function Classification System (CFCS), and the Eating and Drinking Abilities Classification System (EDACS) [17].

Table 1: The five classification levels of the Gross Motor Functional Classification System (GMFCS), the Manual Ability Classification System (MACS), the Communication, Function Classification System (CFCS) and the Eating and Drinking Ability Classification System (EDACS) [18]

Level	GMFCS	MACS	CFCS	EDACS
I	Walks without limitation	Handles objects easily and successfully	Effective sender and receiver	Eats and drinks safely and efficiently
II	Walks with limitations (no mobility aid by 4 years old)	Handles most objects with reduced speed/quality	Effective but slow-paced sender and receiver	Eats and drinks safely but with some limitations to efficiency
III	Walks with hand-held mobility device	Handles objects with difficulty, help to prepare or modify activity	Effective sender and receiver with familiar partners	Eats and drinks with some limitations to safety; there may also be limitations to efficiency
IV	Self-mobility with limitations, may use power	Handles limited number of objects in adapted setting	Inconsistent sender and receiver with familiar partners	Eats and drinks with significant limitations to safety
V	Transported in manual wheelchair	Does not handle objects	Seldom effective sender and receiver with familiar partners	Unable to eat or drink safely, consider feeding tube

The treatment strategy is based on the evaluation and classification of the child's current functional status and possible future prognosis [19]. The GMFCS is the most commonly used out of the four classification systems for prognosis. Children with a level I score can walk and climb stairs without limitations, perform gross motor skills (like running and jumping); however, speed, balance, and coordination are limited [19]. Children with a level II score can walk and climb stairs holding onto a railing, but experience limitations walking on uneven surfaces, inclines, or in crowds and confined spaces [19]. They will usually have a minimal ability to perform gross motor skills [19]. Children with a level III score can walk on a level surface with an assistive mobility device, climb stairs with a support of a railing, and depending on the upper limb function, these children propel a wheelchair manually or are transported when traveling long distances or on uneven ground [19]. Children with a level IV score may maintain levels of function achieved before the age of 6 years or rely more on wheeled mobility; sometimes, the children may achieve self-mobility using a power wheelchair [19]. For children with a level V score, they have no means of independent mobility and are transported [19].

Once the classification level is measured for a patient with cerebral palsy, the subsequent step is to minimize their disabilities while promoting independence and full participation in society [19]. Rehabilitation methods for the child include physiotherapy, occupational therapy, bracing, assistive device, adaptive technology, and sports and recreation [19]. Physiotherapy focuses on developing independent mobility and preventing deformity by bringing the child to an erect position [19]. Occupational therapy focuses on improving hand and upper extremity function in the child through playful and purposeful activity [19]. Braces are devices that CP patients use to keep them upright in a stable position; the purposes of them are to increase function, prevent deformity, keep the joint in the functional position, stabilize the trunk and extremities, facilitate selective motor control, decrease spasticity, and protect the patient from injury in the postoperative phase [19]. The variety of assistive devices, or mobility aids, offers patients the ability to move around and interact with their peers in order for their mental, social, and psychologic skills to develop as much as possible [19].

Another common method of treatment is orthopedic surgery, to prevent or correct specific musculoskeletal problems [19]. Muscle shortening or bony deformities in patients are just two of the many impairments that orthopedic surgery addresses, therefore improving functional ambulation for children with the potential to walk [19]. However, surgery does not eliminate the needs for rehabilitation measures, such as bracing, physiotherapy, *etc* [19]. Despite the various rehabilitative, medical, and surgical interventions that help CP patients maximize functional skills, rigorous standardized assessments of multi-disciplinary outcomes for CP interventions are uncommon [20]. The traditional CP treatments of physiotherapy, pharmacologic therapies, or botulin toxin A injection, have limited effectiveness [21]. Botulinum toxin is a treatment for the controlling of excessive muscle stiffness, spasticity, and dystonia in CP patients; it inhibits the release of inflammatory mediators and peripheral neurotransmitters from sensory nerves [22]. However, reported symptoms in this treatment show unilateral or bilateral ptosis, hematoma, and lower limb weakness and pain [22]. There is not a treatment that cures CP, which encourages researchers to find promising therapies that may lack scientific and clinical evidence of effectiveness [20].

Stem Cell Therapy

Studies have shown that stem cell therapy progresses motor function and reduces the muscle spasticity in children diagnosed with cerebral palsy [23]. Stem cells are multipotent progenitor cells with regenerative, immunomodulatory, and growth stimulating properties [24]. Grown *in vitro*, they have the ability to induce angiogenesis and differentiate into various types of cells, including the cells of the nervous system [24]. The hematopoietic stem cells, the bone marrow mononuclear cells which include the hematopoietic and mesenchymal cells found in the bone marrow in small numbers, embryonic stem cells, human amnion epithelial cells are just few of the stem cells tested for CP treatment [24]. Neural stem cells are applied for targeting neurological diseases and spinal cord injuries [24]. *Chaitinya et al*., showed one patient with CP had improvements in the Gross Motor Function Classification System scale after treatment with autologous bone-marrow-derived mononuclear cells (BM-MNCs) [24]. Autologous stem cells are derived from the patients' own bone marrow [25]. Autologous bone marrow stem cells consist of hemopoietic stem cells ($CD34^+$ $CD38^-$ cells) and stromal mesenchymal stem cells, along with isolated mononuclear cells; they treat neurological diseases, especially spinal cord injury [25].

Stem cell intervention can treat cerebral palsy through different mechanisms. One mechanism is the regenerative mechanism. This takes place when the stem cells replace or repair damaged brain cells as a result of engraftment and proliferation of transplanted cells;

occasionally this can include differentiation of transplanted cells into new microglia or astrocytes to endorse reorganization [26]. Second, is the anti-inflammatory mechanism. This is the weakening of the inflammatory immune response to brain damage through a decrease in the release of excitotoxins, cytotoxins, and oxygen free radicals [26]. This can potentially evoke a defensive response, reducing the magnitude and extent of the white matter injury [26]. Third, there is the trophic mechanism. This mechanism is associated with the release of neurotrophic factors secreted from progenitor cells; this stimulates endogenous cell migration, proliferation, and differentiation to promote cell survival, angiogenesis, and new blood vessel formation [26].

Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are multipotent immunomodulatory cells that can be isolated from the placental tissues, bone marrow, muscle, adipose tissue, and umbilical cord blood [27]. They can be isolated based on their preferential attachment to tissue culture plastic [28]. MSCs exist in the non-hematopoietic $CD34^-$ subset of bone marrow and umbilical cord blood cells [29]. CD34 is a transmembrane phosphoglycoprotein and a common marker for diverse, enhanced progenitor activity [30]. Transplanted MSCs can be an effective treatment for neurological conditions as they secrete an array of neurotrophic and angiotrophic factors for central nervous system repair [31]. Mechanisms of action include immunomodulation, activation of endogenous stem cells, release of growth factors, and anti-apoptotic effects [32]. MSCs can also differentiate into bone (osteoblasts), fat (adipocytes), cartilage (chondroblasts), and periosteum (fibroblasts) [33].

The probable mechanism of stem cell homing starts as the activated stem cells migrate along the concentration gradient to fulfill regenerative functions [34]. Stem cells can adhere to the vascular endothelium at the tissue of interest and bind to the homing receptors, subsequently initiating the extravasation phase [34]. In a brain that has a hypoxic-ischemic injury, there is an abnormally high expression of the chemokine stromal cell-derived factor 1 (SDF-1) on the astrocytes and glial cells of the injured hemisphere [34]. Human umbilical cord blood (hUCB) cell migration is influenced by SDF-1; transplanted hUCB cells expressing the SDF-1 receptor, C-X-C chemokine receptor type 4 (CXCR-4), are shown to migrate to the damaged area of the brain within 24 hours [34]. The stem cells will then employ paracrine signaling, stimulating the target cells to initiate progenitor cell proliferation and tissue repair [34]. In the paracrine mechanism, stem cells secrete soluble factors that help towards the growth, regeneration, and survival of the neurons; these particular soluble factors include the brain-derived neurotrophic factor (BDNF), the glial cell line-derived neurotrophic factor (GDNF), and the granulocyte colony stimulating factor (GCSF) [34].

In a rodent model of HIE, MSCs exerted neuroprotective effects by the optimization of autophagy through the BDNF signaling pathway [34]. BDNF increases proliferation and differentiation of oligodendrocyte precursor cells, in addition to increasing myelination [34]. GCSF activates STAT proteins and the PI3-K pathway, increasing neurogenesis and decreasing apoptosis [34]. GDNF increases differentiation of neural precursor cells into astrocytes and decreases apoptosis [34]. In addition to secreting these soluble factors, stem cells also secrete extracellular vesicles that can bypass the blood-brain barrier to accumulate in the target brain regions through an inflammation-driven mechanism [34]. The extracellular vesical administration increases brain function and inflammation-induced neuron degeneration, prevents intracerebral inflammation, decreases microglial proliferation, inhibits reactive astrocyte proliferation, and improves spatial learning impairments [34].

The favorable mechanism of MSC transplantation is yet to be determined. MSCs are characterized for their high proliferative activity with confirmed *in vitro* differentiation into osteoblasts, chondroblasts, and adipocytes; however, their differentiation into neurons is debatable [35]. Some studies show results that human umbilical cord-blood derived mesenchymal stem cells (hUCB-MSCs) can differentiate into neural cells *in vitro*, but the main problem is the blood-brain barrier that makes stem cell homing less likely [35]. Other studies show that MSCs can increase the endogenous regeneration of neuronal cells through inhibition of microglial inflammatory activity [27]. Bone marrow stromal cells, another term for bone marrow mesenchymal stem cells, can also migrate to the site of injury, differentiate into multi-lineage cells, and maintain anti-inflammatory properties; the brain tissue will then repair itself through replacement of damaged neurons and oligodendrocytes [36]. On the contrast, reports say that dealing with adult stem cells show only a minimal survival of the transplanted cells with few cells displaying functionality of nervous tissue; the cells often do not develop normal processes and may not function in neuronal circuitry [28]. The stem cells could be differentiating into neurons, but the differentiation is most likely not the cause of neuroprotection if the new neurons are not forming synapses with existing neurons [37].

Human umbilical cord-blood derived MSCs have fewer ethical issues in comparison to rehabilitation [38]. Compared to other MSCs, they have a low immunogenicity; for example, hUCB-MSCs not only have a lower immunogenicity than bone marrow-derived MSCs, but they also have higher proliferative capacity and stronger immunosuppressive potential [38]. Allogeneic (derived from a matching donor or external cell source) hUCB-MSCs can maintain a low immunogenicity *in vitro* and *in vivo*, suggesting the safety of using them in allogeneic clinical applications [38]. It has also been concluded that hUCB-MSCs could be efficaciously transplanted even when they were major histocompatibility complex mismatched [38]. Moreover, cells from umbilical cord blood can tolerate more human leukocyte antigen-mismatches without rejection, possibly due to the immature fetal immune system [33]. With this characteristic, compatibility tests between the donor and recipient are not usually considered before hUCB-MSC infusion [38]. The mononuclear fraction, what is isolated from the cord blood, contains immunosuppressive cells (regulatory T-cells and monocyte-derived suppressor cells) that have the ability to contribute in neuroprotection [33].

In a placebo-controlled, single-blind study, there were 56 children diagnosed with CP and aged between 3 and 12 years old [38]. All of the subjects were randomly and blindly assigned to two groups on a 1:1 allocation [38]. In the infusion group, patients received hUCB-MSC administration with basic rehabilitation; meanwhile, the placebo-controlled group patients received normal saline (0.9% NS) and basic rehabilitation [38]. The gross motor function measure 88 (GMFM-88) scale was applied in evaluating the recovery of gross motor ability in children with CP [38]. The classification system involved 88 questions for five function areas: "lying and rolling,"

"sitting," "crawling and kneeling," "standing," and "walking, running, and jumping" [38]. All patients in the hUCB-MSC infusion group received intravenous infusions and basic rehabilitation [38]. Patients were infused with hUCB-MSCs at day 1 after randomization and then given 3 infusions in each course with an interval of 7 days between administrations [38]. The infusion procedure was carried out twice with a 3-month interval between each course [38]. During each treatment course, the hUCB-MSCs were infused at a fixed quantity of 5 × 107 cells, after being dispersed and mixed in 30 mL of 0.9% NS [38]. In the control group, patients were given 30 mL 0.9% NS and basic rehabilitation with the same procedural steps as those in the hUCB-MSC infusion group [38].

In the same study, two patients dropped out, leaving 54 patients in total to complete all of the required study evaluations at the scheduled time points [38]. Preterm injuries were the causes of CP in 40.7% of patients in the hUCB-MSC infusion group and 37% in the control group. Among the patients, the top three pathogenesis risk factors were hypoxia (29.0%), low birth weight (25.8%), and infection (22.6%) [38]. Other causes for the subjects included neonatal jaundice, trauma, hydrocephalus, and genetic disease [38]. In the results, there was no considerable difference observed in baseline functional assessments between the two groups, including their GMFM-88 scale scores [38]. The improvement, however, versus the baseline status after hUCB-MSC infusion was significantly higher in the infusion group than that in the control treatment group [38].

Table 2: The baseline level and change in total score proportion in GMFM-88 Evaluation [38]

	hUCB-MSC Infusion Group	Control Group
Baseline Level	84.99 ± 0.85	85.03 ± 0.76
Change at 3 months	4.59 ± 0.26	1.74 ± 0.39
Change at 6 months	7.62 ± 0.47	2.96 ± 0.32
Change as 12 months	10.27 ± 0.57	4.75 ± 0.28
Change at 24 months	12.66 ± 0.66	4.81 ± 0.39

The minimum necessary cell dosage for cell engraftment was 1×10^7 cells/kg [38]. At 12 months, improvements in GMFM-88 total score proportion reached an effective level in clinical efficacy evaluation, while it failed to reach an effective level in the control group throughout the whole course of the study [38]. Possible mechanisms of action may include the reducing of proinflammatory cytokine levels, in addition to paracrine effects that stimulate recovery in the injured brain [38]. Nevertheless, researchers assume that the improving of neurological function through neuronal replacement by hUCB-MSC infusion intravenously may not be realistic, considering the limited quantity of MSCs through the hematoencephalic barrier [38].

Following unilateral hypoxic-ischemic injury in neonatal rats, both neural precursor stem cells and MSC transplants from human umbilical cord blood migrate to the lesion site in the brain [14]. Some of the neural precursor stem cells differentiated into glial subtypes, with a few others differentiating into neuronal subtypes [14]. However, mesenchymal stem cells showed little differentiation into either neural or glial subtypes in a neonatal rat ischemic stroke model; even though there was reduced infarct volume and improved functional outcome in the transplanted group, these results were due to the anti-inflammatory effects through the release of trophic factors [14].

Neonatal arterial ischemic stroke is a common cause of spastic hemiplegia in patients with CP, affecting 1 in every 3,500 to 7,700 neonates [39]. Neonates with stroke may exhibit features similar to f, but therapeutic hypothermia is proven to be not as effective for patients with neonatal stroke as it is in HIE [39]. Intravenous administration of UC-MSCs improves damage via attenuating reactive gliosis and hypomyelination in a neonatal mouse model of intraventricular hemorrhage (IVH) [39]. There are no studies on UC-MSC in a neonatal stroke model; a new study, therefore, examined the safety and efficiency of intravenously administered human umbilical cord-derived MSCs in neonatal stroke mice [39].

Human umbilical cord tissues were obtained from women who had cesarean sections [39]. Subjects of the study were female and male postnatal day 12 (P12) pups (n = 90), thought to be equivalents to full-term human newborns at postnatal day 0 [39]. The pups were either divided into a control group without surgery (n = 6), a sham-surgery group (n = 12), or middle cerebral artery occlusion (MCAO) groups (n = 72) [39]. In the MCAO group, a hole was made in the left temporal bone, and the left middle cerebral artery was electrocauterized and disconnected just distal to its crossing of the olfactory tract [39]. In the sham-surgery group, pups had open-skull surgery but no middle cerebral artery electrocoagulation [39].

Forty-eight hours after the MCAO procedure, the mice were randomly divided into three groups: vehicle (n = 23), low-dose UC-MSCs (1×10^4 cells, n = 13), and high-dose UC-MSCs (1×10^5cells, n = 36) [39]. The significance in waiting 48 hours after the MCAO was because neonatal stroke is rarely diagnosed on the first day of life, rather diagnosed a few days after the birth in most cases [39]. The researchers included the higher dose of 1×10^5 human UCB CD_{34}+ cells for one group; based on results from their recent study on the same mouse model of neonatal stroke, the dosage was found beneficial [39]. The lower dose of 1×10^4 cells was included to see whether these beneficial effects could be obtained even after lowering the dosage [39].

A cylinder test, a dynamic plantar test, a rotarod test, and an open-field test were all behavioral tests performed on the pups [39]. The cylinder test was performed on P15, the dynamic plantar test on P16, the rotarod test on P16 and P23, and the open-field test on P20-P22 [39]. The cylinder test assessed asymmetry of forelimb use during rearing in a transparent acrylic cylinder. The forepaw use preference was analyzed as follows: (nonimpaired side [left] − impaired side [right])/ (nonimpaired + impaired sides) × 100 (sham n = 12, vehicle n = 18, low-dose n = 13, and high-dose n = 14) [39]. The dynamic plantar test was for measuring responses to von Frey filaments to assess sensory function [39]. Sensorimotor skills were evaluated for the rotarod test. Lastly, locomotor and exploratory behaviors were evaluated for the open-field test [39].

Sixty-two mice survived for two weeks after the insult, while seven died [39]. One mouse from the high-dose UC-MSC group died about 10 minutes after the induction of anesthesia and a few minutes after cell administration; meanwhile, the other six mice died approximately 7 days following the insult, indicating the deaths may have not been directly related to the cell administration, and instead to weakness from the MCAO procedure [39]. In the cylinder test

performed on P15, the sham mice did not have paw preference, but the MCAO caused significant asymmetry to use the left unimpaired forepaw in the vehicle group [39]. With administration of high-dose UC-MSCs, the performance in the cylinder test showed significant improvement in comparison to the vehicle group's performance [39].

For the dynamic plantar test on P16, delayed withdrawal time from the filament was measured in forepaws and hind paws to evaluate the extent of sensory deficit [39]. With changes being more recognizable in forepaw performance, there was significant asymmetrical dullness in the forepaw observed in the vehicle mice group, in comparison to the sham mice group [39]. The mice treated with UC-MSCs did not show notable improvement in performance compared to the performance in vehicle mice; however, the mice administered with the high-dose UC-MSC showed a trend toward improvement in forepaw performance [39]. Between the sham and vehicle groups, there was no significant difference in hind paw sensory preference [39]. At P16 and P23, the rotarod test was performed, where measuring the average number of times fallen from the rotarod cylinder indicates sensorimotor capacity [39]. The spontaneous activity in an open-field test during P20-22 was evaluated as well [39]. However, observations concluded no significant differences between the groups in either behavioral test [39].

In the peri-infarct cortex, high-dose UC-MSCs reduced the ionized calcium binding adaptor molecule 1 (Iba1)-positive percent area, as the low-dose UC-MSCs showed a similar trend toward reduction [39]. These results suggest the high-dose UC-MSCs decreased microglial accumulation in the peri-infarct cortex [39].

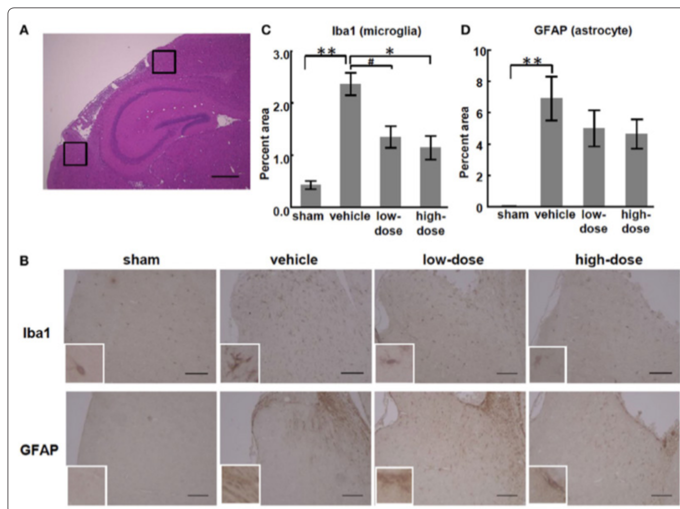

Figure 2: Umbilical cord-derived mesenchymal stem cell administration effects on the expression of glial cell markers in the peri-infarct cortex [39]. **(A)** A representative photomicrograph of a coronal section in the brain, stained with hematoxylin-eosin at P28 [39]. The black squares indicate the regions quantified as the peri-infarct cortex [39]. Bar, 500 μm (× 100). **(B)** Representative photomicrographs of the peri-infarct areas at P28 [39]. Bar, 100 μm (×200). The inset displays higher magnification (×600) [39]. **(C)** In comparison to the sham group, a significantly higher percent area of cells stained positive for Iba1 was found in the vehicle group [39]. There was a significant decrease in the high-dose UC-MSC group with a trend toward a decrease in the low-dose UC-MSC group [39]. **(D)** The percent area of cells that stained positive for glial fibrillary acidic protein (GFAP) was significantly higher in the vehicle group than in the sham group [39]. However, there was not a substantial decrease in the UC-MSC groups in comparison to the vehicle group [39]

Intravenous administration of human UC-MSCs after the middle cerebral artery occlusion in neonatal mice overall was safe to reduce damages in neurodevelopmental behaviors and glial cell reaction following an incident of neonatal stroke [39]. Cerebral blood flow and cerebral hemispheric volume was not restored, but neurological performance was significantly improved [39]. *In vivo* images revealed intravenously injected UC-MSCs were rapidly trapped in the lungs, leading to concerns of a potential pulmonary embolism caused by the administered UC-MSCs [39]. However, since systemic blood flow was not exacerbated by administration in the study, this stability suggests intravenous UC-MSCs does not cause significant blood vessel embolism [39].

In another study, it was indicated that bone marrow stromal cells migrated directly into the boundary of the ischemic area, differentiating into astrocytes and improving recovery from middle cerebral artery occlusion [37]. After stroke, the administration of MSCs promoted angiogenesis around the ischemic insult of the brain; angiogenesis protects nerve cells from secondary cell death [37]. For CP patients, these results show that mesenchymal stem cell transplantation provides neuroprotection by promoting revascularization [37]. The transplantation of MSCs into the striatum of mice after stroke improved the functional recovery [37]. Embryonic rats showed that MSCs displayed functional neuronal characteristics and then differentiated into presumptive neurons in culture [37]. The intracerebral grafts of mouse bone marrow helped regenerate cerebral blood flow and blood-brain barrier after stroke in rats [37].

Liu, *et al*., performed a study on 105 spastic CP patients within the ages of 6 and 150 months [40]. All subjects had a gross motor function classification system (GMFCS) score between levels II and V [40]. All subjects were randomly assorted into three groups: bone marrow mesenchymal stem cell (BM-MSC) group, bone marrow mononuclear stem cell group (BM-MNC), or the rehabilitation group (control) [40]. The Bobath therapy (neuro-developmental treatment) was used for the control group [40]. Autologous BM-MSCs and autologous BM-MNCs are derived from the same source, but vary in the method of culturing; autologous BM-MSCs are made from BM-MNCs; therefore requiring BM-MSCs a different method of culturing [40]. At an interval of 3-4 days, patient subjects, not in the control group, received four transplantations of either BM-MSCs or BM-MNCs; the method of cell delivery was a lumbar puncture in the lumbar 3-4 or lumbar 4-5 intervertebral space [40]. Two mL was injected into the subarachnoid space, inserting a number of cells of 1×10^6/kg body weight [40]. Using the gross motor function measure (GMFM) and fine motor function measure (FMFM), each subject was evaluated at administration and 3, 6 months, and 1 year after the cell transplantation [40].

The results from the study indicated that 3 months after administration, the BM-MSC and BM-MNC groups significantly improved the gross motor function of children with spastic cerebral palsy [40]. However, evaluating the patients six and twelve months after the cell transplantation, the improvement of the gross motor function of the BM-MSC group was more consistent than that of the BM-MNC group; however, with the extension of time, the

BM-MNC group and the rehabilitation group did not show any differences of gross motor function [40]. Results concluded that the bone marrow mononuclear cells had poor persistence in improving gross motor function. In comparison to BM-MNCs, the BM-MSCs are more efficient in both the capacity and ability of paracrine and differentiation [40]. They have a stronger ability to secrete a variety of cytokines, such as neurotrophic factors, anti-inflammatory cytokines, and angiogenic factors [40]. Neurotrophic factors are signaling proteins which encourage neural survival and axonal growth [41]. Anti-inflammatory cytokines are immunoregulatory molecules, responsible for controlling the proinflammatory cytokine response [42]. Angiogenic factors play a significant role in regulating angiogenesis (new blood vessel formation) [43]. BM-MSCs also have an immune regulation mechanism, modulating the body's immune system and reducing the abnormal immune response [40]. Liu, *et al*., believed that the major treatment mechanism for CP patients are the paracrine mechanism and the vascular regeneration mechanism; mesenchymal stem cells express a variety of neuro-regulatory molecules and promote neural cell survival and neurogenesis [40].

Due to their pluripotential properties, human adipose-derived mesenchymal stem cells (hAd-MSC) can differentiate into various types of cells: adipocytes, myocytes, chondrocytes, and osteocytes [44]. One study determined the safety and efficacy of allogeneic hAd-MSC administration in a 7-year-old boy with CP [44]. At the age of 2, he had cerebral infarction due to hemolytic uremic syndrome (HUS) following *Escherichia coli* O-157 infection [44]. HUS involves microangiopathic hemolytic anemia, thrombocytopenia, and renal insufficiency [45]. Many affected individuals develop HUS due to shiga-toxin-producing strains of *Escherichia coli*, most often the O157:H7 subtype [45]. Neurological complications of HUS vary between seizures, alteration of consciousness and coma, transient or permanent hemiparesis, and subarachnoid hemorrhage [46]. These conditions are consequences from a combination of metabolic derangements and cerebral microthrombi [46]. The patient in the study developed CP on the left side of his body because his right thalamus putamen zone was involved in the infarcted area [44].

Adipose tissue was harvested from the peri-umbilical area of the patient's mother [44]. With a total amount of 22 grams of fatty tissue, allogeneic hAd-MSCs were isolated using a combination of enzymatic digestion and centrifugation [44]. Depending on their capacity to adhere to the surface of plastic culture flasks, certain hAd-MSCs were selected [44]. In addition, a positive expression of CD73 and CD90 and a negative expression of CD31 and CD45 defined the hAd-MSCs as stem cells [44]. CD73 is an ecto-5'-nucleotidase with the ability to convert extracellular adenosine monophosphate to adenosine, and CD90 is a glycosylphosphatidylinositol-linked protein involved in cell-cell and cell-matric interactions [47]. Positive expressions in these two markers are useful for identifying MSCs *in vivo* [47]. Determining the expressions of the markers required flow cytometric analysis [44].

Further tests included the determining of cell viability and survival by trypan blue staining and the exclusion test; the rate for cell viability and survival was aimed and set at >80% [44]. The hAd-MSCs selected for the study could differentiate into fat, bone, and cartilage tissue cells, and they could inhibit the proliferation of T cells that are similar in features as mesenchymal stem cells [44].

Depending on the patient's condition throughout the course, the study employed various doses and routes of administration of allogeneic human Ad-MSCs [44]. The efficacy was assessed by observing any changes in the clinical manifestations at each patient visit (1, 2, 3, 6, and 11 months) using the Short Form 8 (SF-8) Health Survey Quality of Life (QoL) questionnaire and the GMFCS [44]. The SF-8 is based off of eight distinct scores that describe the health-related QoL, which are summarized as continuous physical component summary (PCS) and mental component summary (MCS) scores [44]. The eight scores are determined by measuring eight ordinal items: general health, role physical, physical functioning, bodily pain, vitality, social functioning, mental health, and emotional roles [44].

The PCS score decreased by 5.88 at 1 month after hAd-MSC administration, but then gradually increased by 14.51 during the following 4 months [44]. Five months after the administration, the PCS score increased by 8.63 [44]. Using the SF-8 Health Survey QoL, the physical functioning and role physical scores were lower at 1 month after administration in comparison to scores before the treatment [44]. However, four months after, and compared to scores before treatment, the patient had higher scores in all eight variables, including the physical functioning and role physical items [44]. The MCS score increased at 1 month after hAd-MSC administration by 4.12; this score stayed consistent in the following 4 months [44]. Eleven months following the procedure, the patient maintained his recovery with no significant change, and therefore received 75×10^6 hAd-MSCs intramuscularly in the left upper arm [44]. At the 12-month checkup, researchers concluded that the PCS and MCS scores remained stable between 5 and 12 months after treatment [44]. The GMFCS classification of the subject exhibited an improvement from Level II before administration to Level I after administration [44]. Overall, the PCS, MCS, and GMFCS scores improved [44].

Embryonic Stem Cells

Human embryonic stem cells (hESCs) are obtained from the early pre-implantation stage human fertilized ovum [48]. More specifically, these pluripotent stem cells are derived from the blastocytes during the 16-cell stage [49]. Studies prove hESCs have a normal karyotype, expression of high levels of telomerase activity, and specific intracellular and cell surface markers [50]. Embryonic stem cells are self-renewing and can differentiate into any cell type of the human body [48]. These may include neurons, astrocytes, or oligodendrocytes [50]. Because they can differentiate into neural precursor cells and neurons, astrocytes and oligodendrocytes, hESCs have great potential in treating several incurable neurological disorders [51]. hESCs derived precursors are shown to migrate along the olfactory system and play a significant role in neurogenesis [48]. Undifferentiated ESCs are not used in the treating of neurological illnesses due to having immune privilege secondary to reduced major histocompatibility complex antigen expression [50]. This features permits the ESCs to escape immune surveillance in the host to form tumors [50].

Intrauterine infection with cytomegalovirus (CMV) plays an etiological role in CP [50]. In the gestational period, cytomegalovirus has a teratogenous influence during the migration of neural cells from the ventricular zones to the cerebral cortex [52]. A past study reports that ESCs are more resistant to CMV than other cell types because of their lower expression in heparin sulfate, β1-integrin, vimentin, and nuclear pores [50]. This discovery can potentially reduce the ability of CMV to attach to and enter the cellular membrane, translocate to the nucleus, and cross the nuclear membrane in ESCs [50].

Ma, et al., demonstrated in a study that ESCs-derived cells transplanted on hypoxic-ischemic encephalopathy mouse model possess the ability to migrate to the damaged site of the brain, improving learning ability and memory by expressing neural stem cell differentiation markers like Nestin and MAP-2 [48]. CP patients aged 30 days to 18 years old were initially evaluated based on Gross Motor Function Classification Scores Expanded and Revised (GMFCS-E & R; 1-good to 5-bad) [48]. The study consisted of four treatment phases (T1, T2, T3, T4) with gap phases in between each [48]. Ninety-one patients received hESC therapy in T1, 66 patients returned for T2, 38 patients returned for T3, and 15 patients returned for T4 [48]. Patients in the first treatment phase (T1) were administered 0.25 mL (< 4 million cells) hESCs through intramuscular (IM) route once daily for eight weeks; they were also administered 1 mL of hESC (< 16 million cells) twice every 7 days through intravenous (IV) route [48].

The patients entered the second and third treatment phases (T2 and T3) once a gap period of 3-6 months had passed [48]. The same dosage regime as T1 was administered, but both T2 and T3 lasted for 4 weeks each and were separated by another gap phase of 3-6 months [48]. After a gap period of 6-12 months, the T4 treatment phase was performed; the dosage regimen was similar to that of T2 except that the IV dose of hESC was increased by 1 mL [48]. Depending on the condition of the patient, the injections could be increased if required, or the dose of hESCs administered could be changed over a period of time [48].

Table 3: GMFCS-E & R scores of the patients in each treatment phase [48]. The total treatment days were 60 days in T1, 30 in T2, 33 in T3, and 29 in T4 [48]

Treatment phase	Age group	Number of patients									
		GMFCS scores (start of session)					GMFCS scores (end of session)				
		1	2	3	4	5	1	2	3	4	5
T1 (n=91)	≤ 2 yr (n=12)	0	0	2	0	10	0	3	5	4	0
	2-4 yr (n=14)	0	0	1	3	10	0	2	7	5	0
	4-6 yr (n=21)	0	5	2	5	9	1	8	9	3	0
	6-12 yr (n=27)	1	3	8	7	8	3	11	10	3	0
	12-18 yr (n=17)	0	4	2	6	5	2	8	6	0	1
	Overall	1	12	15	21	42	6	32	37	15	1
T2 (n=66)	≤ 2 yr (n=11)	0	3	5	3	0	0	6	3	2	0
	2-4 yr (n=11)	0	1	6	4	0	1	1	7	2	0
	4-6 yr (n=12)	0	5	4	3	0	1	7	2	2	0
	6-12 yr (n=21)	1	8	10	2	0	3	13	5	0	0
	12-18 yr (n=11)	0	5	5	1	0	2	7	1	1	0
	Overall	1	22	30	13	0	7	34	18	7	0
T3 (n=38)	≤ 2 yr (n=6)	0	2	2	2	0	0	3	3	0	0
	2-4 yr (n=10)	0	3	5	2	0	2	5	2	1	0
	4-6 yr (n=7)	0	4	1	2	0	2	3	1	1	0
	6-12 yr (n=9)	1	5	3	0	0	3	6	0	0	0
	12-18 yr (n=6)	2	3	0	1	0	4	1	0	1	0
	Overall	3	17	11	7	0	11	18	6	3	0
T4 (n=15)	≤ 2 yr (n=5)	0	4	1	0	0	2	3	0	0	0
	2-4 yr (n=4)	0	2	2	0	0	2	1	1	0	0
	4-6 yr (n=2)	0	0	2	0	0	0	1	1	0	0
	6-12 yr (n=1)	0	1	0	0	0	0	1	0	0	0
	12-18 yr (n=3)	1	1	1	0	0	1	1	1	0	0
	Overall	1	8	6	0	0	5	7	3	0	0

Results demonstrate that most patients transitioned to a lower score after completion of T4 (Table 1). Overall, 30.2% of the patients gained a score of 1 on the GMFCS-E & R by the end of the fourth treatment phase (338 days) [48]. By using human embryonic stem cell transplantation, 86 out of the 91 patients in the study (94.5%) showed an improvement in GMFCS-E & R score by the end of the fourth treatment phase [48]. Because the GMFCS-E & R score does not consider cognitive skills, they were independently evaluated in the study, showing results of significant improvement in 69% of the patients [48]. The effective mechanism demonstrated by the study was stem celling home of the hESCs at the hypo perfused areas of the brain, driven by migration [48]. In order for the stem cell homing to be successful, there must be an interaction between the transplanted stem cells and the chemokines, cytokines, and growth factors released from the insult in the brain [48].

Another study assessed functional recovery after administration of ESC-derived neuronal progenitor cells (ES-NPCs) into a neonatal HIE model [53]. Researchers wanted to additionally determine whether or not the implanted cells were able to generate deep layer cortex-specific pyramidal neurons and recapitulate an area-specific neuronal network based on axonal projections from the administered cells [53]. Two day-old neonatal mouse pups were randomly distributed to the following four groups: (1) control group with no operative procedure, (2) HI control group (HI only, no transplantation), (3) vehicle-transplantation group (HI and vehicle-transplantation), (4) transplantation group (HI and NPS-transplantation) [53]. On postnatal day 2, the pups were induced with anesthesia and incised at the midline in a linear fashion from the infragnathia to the sternum [53]. The right common carotid artery (CCA) was ligated with 10-0 nylon thread at two points, and between the points, the CCA was cut after treatment with bipolar coagulator [53].

Two days following the HI procedure, a midline linear skin incision was made and the skull bregma was determined; the P4 neonatal mice were administered a single cell suspension (50,000 cells/μ L) of ES-NPCs into 4 sites (1 μ L/site) in the right ischemic hemisphere, targeting to the motor cortex (n = 10) [53]. From the skull bregma, the measurements were (1) antero-posterior (AP) 0 mm, lateral (L)

0.5 mm; vertical (V) 0.5 mm; (2) AP 0 mm, L 1.5 mm, V 0.5 mm; (3) AP 1.0 mm, L 0.5 mm, V 0.5 mm; (4) AP 1.0 mm, L 1.5 mm, V 0.5 mm [53]. For the control group, sham transplantation was conducted using the surgical procedure itself ($n = 10$) [53]. Three weeks after the transplantation, the researchers counted in every third section the number of CTIP2-positive cells in five arbitrary squares (200 × 200 μ m) per randomly selected region of layer V area in each section [53]. The average number of positive cells in the unit area (4.0 × 104 μ m^2) was calculated as well [53].

Balance and motor coordination were evaluated in the mice by testing them at 3 weeks after transplantation for their sensorimotor skills in both the Rotarod and Beam walking tests [53]. The Rotarod test involved placing the mice onto a horizontal rotating rod at 4 revolutions per minute (rpm), with the treadmill then accelerating from 4 to 40 rpm over the course of a 5-minute trial [53]. One complete test began at the time the mouse was placed on the rotating rod until it fell-off or until the 5 minutes had elapsed [53]. The Beam walking test involved a beam 0.6 cm in width and 120 cm in length, suspended about 60 cm above foam pads; the average score (total time spent walking on the beam divided by 5 trials) was measured for each mouse [53].

Twenty-four hours after the HI insult, the lateral side of the ischemic cerebral hemisphere transitioned into a white color, indicating ischemia and edema [53]. TTC staining exhibited an ischemic lesion of the cortex in the same indicated region (Figure 1A) [53]. Microscopic examination of the Kluver-Barrela staining suggested a decrease in pyramidal-shaped neurons in the deep layer (Figure 1C and magnified photograph) in comparison to the contralateral cortex (Figure 1B and magnified photograph) [53]. CTIP2 immunostaining is a certain marker of layers V and VI; it demonstrated that the number of positive neurons in layers V-VI (deep layer) was significantly reduced by nearly 90% in comparison to the contralateral side (Figures 1D, E, H) [53]. The thickness of the cortex of the HIE model ($3.44×10^6$ μ m^2) was significantly attenuated in comparison to the contralateral side ($4.76×10^6$ μ m^2) (Figure 1I) [53].

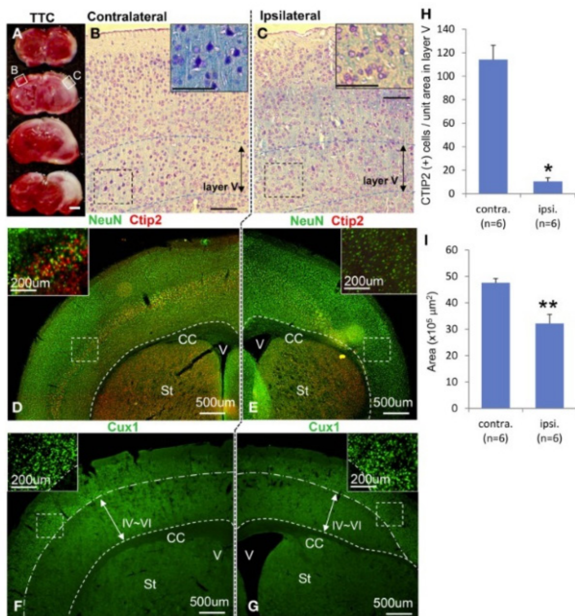

Figure 3: Hypoxia-ischemia encephalopathy and motor function features [53]. The ischemic lesion of cortex in TTC staining 3 weeks after HI exposure is represented in (**A**) with an unstained area [53]. Kluver-Barrela stained sections exhibit a layer of V pyramidal neurons in the contralateral cortex (**B**) and are presented by a square area with solid line in (**A**) [53]. The magnified insert in (**B**) exhibits monopolar and pyramidal-shaped neurons, whereas layer V pyramidal-shaped neurons in the ipsilateral cortex are mainly absent (**C** and magnified insert), shown in the square area with a solid line in (**A**) [53]. The mature neuronal marker NeuN, deep layer marker CTIP2 (**D** and magnified insert), and superficial marker Cux1 (**F** and magnified insert), are shown from the immunostaining [53]. CTIP2-positive neurons in the ipsilateral cortical area significantly lowered in number (**E** and magnified insert), meanwhile the Cux1-positive cells in the superficial area remained preserved (**G** and magnified insert) [53]. CTIP2-positive cells of unit-squared area (200 × 200 μm) in the randomly selected area of layer V cortex are represented by ipsilateral hemisphere (**H**) [53]. The area of cortex is represented by ipsilateral hemisphere (**I**) [53]. Scale bar: (**A**) 1000 μm; (**B,C**) 100 μm; (**D–G**) 500 μm; (**D–G**, magnified insert) 200 μm; CC: corpus callosum, V: ventricle, St: striatum [53]

Three weeks following transplantation, immunohistochemical staining was performed to identify the cells in the ES cell-derived graft [53]. The graft in the HI-injured brain expressed the mature neuronal marker, NeuN, and the deep layer cortical-neuronal marker, CTIP2 [53]. Few of the NeuN-positive and CTIP2-positive presented monopolar morphology, thereby suggesting their differentiation into deep layer neurons [53]. Almost 45% of all cells in the graft were NeuN-positive while 12% were CTIP2-positive; these results indicate that ES-NPS possess the ability to differentiate into cortical deep layer pyramidal neurons due to their morphology and immunohistochemical staining, even if implanted into an impaired cortex [53]. Findings from the study also show that deep layer neuron-specified NPCs migrate into their preferred location, eventually differentiating into region-specific and -functional mature neurons [53]. To assess the efficacy of the ES-NPS engraftment for functional recovery, 2 neurobehavioral tests were performed to evaluate the sensorimotor skills of the mice [53]. In comparison to the sham-transplanted mice, the transplanted HIE mice exhibited significant improvements in their use of limbs in both the Rotarod test and the Beam walking test [53]. As the transplanted animals significantly stayed longer on the rotating rod (30-35 s) than vehicle-transplanted animals (20 s), researchers suggested the functional recovery is due to regeneration of the neuronal networks [53]. NPC's ability to provide neuroprotective support is related to their expression of various neurotrophic factors, such as BDNF or GDNF [53].

In this study, researchers used NPCs that grafted into the brains of injured mice, with the motive of uncovering a therapeutic strategy for HIE patients [53]. The results concluded that embryonic stem cell-derived NPCs engrafted into the neonatal cortex and generated pyramidal shaped neurons, demonstrating axonal sprouting into appropriate subcortical-specific areas and therefore recapitulating the normal brain anatomy [53]. The transplanted graft was chiefly located in the motor cortex; because of this, axons extended into the pyramidal tract-related area (i.e., corpus callosum, striatum, and internal capsule) [53]. The study demonstrated that transplanted ES-NPCs expressed both the mature neuronal marker, NeuN, and an additional neuronal marker that was expressed in the deep-layer cortical and axonal projecting neurons, CTIP2 [53]. Because the ES-NPCs in this study were programmed to generate deep layer

cortical-projection neurons, researchers consider the transplantation a compatible cell replacement procedure [53].

Human Amnion Epithelial Cells

On approximately the eighth day subsequent fertilization and before gastrulation, the human amniotic membrane develops from the epiblast and composes the inner layer of the amnion supporting the fetus [54]. The human amniotic membrane plays a crucial role in embryonic development due to its anti-inflammatory and immunological properties [54]. The cell populations that constitute the amniotic membrane display pluripotent properties; in particular, the innermost layer of the human amnion is 8-12 μm thick and contains a single layer of homogeneous cuboidal epithelial cells [54]. These cuboidal epithelial cells, also known as human amnion epithelial cells (hAECs), also share similar characteristics to the cells found in amniotic fluid [54]. This heterogenous population of cells derived from amniotic fluid at amniocentesis, along with hAECs, have the potential to differentiate and offer an alternative therapeutic approach for patients with CP [54].

Human amnion epithelial cells and amniotic fluid-derived stem cells have a positive expression of CD117, a transmembrane protein responsible for functioning as a tyrosine kinase receptor [54]. This receptor is present on embryonic stem cells and somatic stem cells, like neural crest cells [54]. hAECs differentiate from amniotic fluid-derived stem cells in their gene expression patterns assessed by transcriptional profiling [54]. They have low immunogenicity and an ability to reduce an immune response by inhibiting innate and adaptive immune system cells; in addition, hAECs' multi-factorial role in immunomodulation is characterized by their suppression of pro-inflammatory cytokines, regulation of macrophage recruitment, and secretion of factors that inhibit the chemotactic activity of neutrophils and macrophages [54]. These features give hAECs strong potential in cell-based therapies for treatment of brain damage [54].

Human amnion epithelial cells express specific neural marker genes: neuro filament-M, myelin basic protein, microtubule-associated protein 2, and glial fibrillary acid protein [54]. Cultured hAECs that are directed toward a neural lineage can differentiate into neurons and astrocytic cells [54]. Undifferentiated hAECs can synthesize and secrete neurotransmitters, including catecholamines, acetylcholine, and neurotrophic factors [54].

In one study, a rat experimental model was induced by 6-hydroxydopaine (6-OHDA) administration, making multiple lesions and causing the rat to have Parkinson's disease [54]. The results of the study displayed that transplanted hAECs prevented the death of dopaminergic neurons, arbitrated by the active secretion of neurotrophic factors [54]. In another study, adult rats with ischemic stroke, induced by middle cerebral artery occlusion, were given intra-cerebral injection of hAECs; in comparison to non-hAEC treated adult rats, they showed improvement in functional recovery and a decrease in ischemic infarct volume [54]. In general studies and in comparison to post-stroke rats without cell therapy, rats that are treated with stem cells derived from amniotic fluid demonstrated a significant decrease in brain infarct volume, along with a significant increase in endogenous cell proliferation within proliferative zones [54].

Intrauterine infection, which occurs in chorioamnionitis, is one of the main causes of preterm birth and cerebral palsy [55]. One certain study examined hAECs' potential to reduce brain injury induced by intra-amniotic administration of lipopolysaccharide (LPS) in preterm fetal sheep [55]. At 110 days of gestation, surgery was performed on twenty-five singleton-bearing ewes for implantation of fetal polyvinyl catheters into the amniotic cavity, fetal trachea, carotid artery, and jugular vein [55]. At 117 days of gestation, lipopolysaccharide was administered; hAECs were labeled with carboxyfluorescein succinimidyl ester and administered into the fetal jugular vein, trachea, or both, at 0, 6, and 12 hours, following lipopolysaccharide administration [55]. The control group received an equivalent volume of saline [55]. Seven days after the procedure, the brains were collected for histological assessment for brain injury [55]. Microglia were determined using rabbit anti-ionized calcium-binding adaptor molecule (Iba-1) antibody; observations indicated a microglia presence in the brain of all fetuses, but a significant increase in the cortex, subcortical and periventricular white matter in fetuses that received lipopolysaccharide [55]. This indication of inflammation, or the number of activated microglial cells after LPS exposure, was reduced in fetuses that received hAEC administration [55]. The mechanism of protective action of hAECs on the fetal brain is most likely via anti-inflammatory effects, amending the increased numbers of activated microglial cells in all brain areas examined [55].

Hematopoietic Stem Cells

Hematopoietic stem cells are adult precursor cells mostly found in the bone marrow, providing blood cells required for daily blood turnover and for confronting injections [25]. Umbilical cord blood is also a rich source of hematopoietic stem cells, in addition to other stem and progenitor cell types like mesenchymal stromal cells, endothelial progenitor cells, and immunosuppressive cells [31]. Hematopoietic stem cells excrete many types of cytokines that include thrombopoietin and interleukin 11, factors responsible for survival and differentiation neuronal progenitor cells [25]. An additional important hematopoietic cytokine is colony-stimulating factor I that functions as a growth factor in the central nervous system [25]. In one study, 28 patients with cerebral palsy (perinatal hypoxia) and 7 patients with hypoxic brain damage were transplanted intrathecally by their bone marrow stem cells [25]. Transplantation of the hematopoietic stem cells was performed 3-4 hours following bone marrow collection and preparation: between 50 and 200 mL bone marrow was extracted from the patients' posterior iliac crests [25]. The median yield of cells was 2.8×10^7 for CP patients and 2.0×10^7 for hypoxic brain damage patients [25].

For the 28 patients with CP, 20 of them showed clinical improvements after treatment, with more than 90% of all the improvements starting within eight weeks [25]. Patients exhibited better swallowing (n=6), improved neck holding (4 of 4), complete drooling stoppage (4 of 4), decreased spasticity (n=10), improved sitting (n=5), standing (n=10), walking (n=6), posture stability (n=8), improvement in mental function resulting in better communication (n=7), and improvement in speech (n=9) [25]. The average improvement was also 1.3 levels on the Gross Motor Function Classification System with overall cognitive improvements [25]. For the 7 patients with hypoxic brain damage, 6 showed clinical improvements after treatment [25]. Patients exhibited decreased spasticity (n=3), improved sitting (n=2), standing (n=2), posture stability (n=2), better walking (n=1), neck holding (3 of 3), stopped drooling (n=3), and improved mental function (n=3) [25].

CD34 is a hematopoietic stem and progenitor cell marker for human cells; there is a primitive population of $CD34^+$ cells with HSC

potential in human cord blood and adult hematopoietic sources [56]. The phenotype of $CD34^+$ HSCs indicates the concurrent absence of CD38 and the expression of CD133 [56]. Bone marrow-derived hematopoietic stem cells (HSCs) are classified as $CD133^+$ cells, upholding a strong potential for angiogenesis, neuroprotective and neurotrophic factor secretion, a robust capability for proliferation and less immunogenicity potency [57]. Once purified and freed from leukocytes and their progenitors, HSCs can significantly decrease high inflammatory reactions at graft sites [57]. Twelve CP patients from both genders and aged between 4 and 12 years underwent bone marrow aspiration, where approximately 60 mL of bone marrow was aspirated from the patient's iliac crest [57]. Flow cytometry-based quality control measurements were performed in order to determine the amount of $CD133^+$ cells in the unselected bone marrow cell preparations and in the CD133-enriched preparations [57]. Under sterile conditions, $CD133^+$ cells were injected into the subarachnoid space at the L3-L4 level within 24 hours after the bone marrow extraction [57]. The average number of injected cells and viability were $(10.8 \pm 4.8) \times 10^6$ and $94.4 \pm 4.8\%$, respectively, while the purity of transplanted $CD133^+$ cells was $70.8 \pm 8.7\%$ [57].

Table 4: The number, purity, and viability of CD133-positive cells in the subjects [57]

Patient	$CD133^+$ count ($x 10^5$)	Purity (%)	Viability (%)
2	80	63.6	96.0
3	78	89.8	98.0
4	99	65.2	95.2
5	110	67.8	96.0
6	48	65.8	100.0
7	176	72.0	82.5
8	60	60.0	91.0
9	162	81.6	93.0
10	176	73.0	99.0
11	150	76.0	90.0
12	119	61.6	96.2

The Gross Motor Function Classification System (GMFCS) and the Gross Motor Function Measure (GMFM-66) were used in assessment at baseline and 6 months following the procedure [57]. Results of the clinical assessments before and 6 months after administration are summarized in Table 5.

Table 5: Changes in outcome scores 6 months following cell administration [57]

Patients	GMFM-66		GMFCS	
	Before	After	Before	After
1	23.8	30.4	4	3
2	36.4	54.8	3	2
3	26.6	27.0	5	4
4	4.3	4.3	5	5
5	5.8	6.2	5	5
6	18.9	25.7	5	4
7	8.9	14.2	5	5
8	6.3	8.2	5	5
9	13.1	12.0	5	5
10	12.1	18.0	5	4
11	2.6	24.9	5	5
12	4.9	10.8	5	5

The safety and feasibility of the hematopoietic stem cell transplantation was a result from the route of injection and cell transplantation [57]. The intrathecal injection is a noninvasive procedure and a simple direct pathway for cerebrospinal fluid contact [57]. Patients exhibited a significant improvement in motor function, cognitive state (comprehension, expression, social interaction, memory, orientation, and concentration), and spasticity; in addition, patients felt their movements grow more flexible and easier [57]. However, there was not a significant balance improvement with cell administration [57].

Neural Stem Cells

Persisting in the subventricular zone lining the ventricles of the human brain, multipotent neural stem cells (NSCs) generate neurons and glia [58]. The cell proliferation occurs through stages of multiplication, differentiation, migration, and neosynaptogenesis, induced by growth factors like FGF-2 (fibroblast growth factor-2) and EFG-1 (epidermal growth factor-1) [27]. One study reported results that multipotent astrocytic stem cells from the subventricular zone of a CP mouse migrated to the cortical and the periventricular ischemic region; there were signs of neuronal and astrocytic differentiation in CP animals following intracerebral transplantation [27]. In comparison, when the transplantation was performed in healthy animals, the NSCs remained in the region they were administered and retained their astrocytic profile [27]. The results indicate that neural stem cells can migrate and survive in damaged brain regions and can be stimulated to differentiate *in vivo* and *in vitro* into neurons, oligodendrocytes, and astrocytes [27].

Research on the animal models with CP also demonstrate that when the NSCs grafted into the contra lateral hemisphere of the hypoxic brain injury, they migrated across the corpus callosum and other interhemispheric commissures to the infarcted region [27]. When grafted into the ipsilateral hemisphere, however, the NSCs remained at the site of the injury [27]. Another investigation exhibited the intracerebral delivery of NSC in CP rats enhanced the number of axon terminals in the sensorimotor cortex, corpus callosum, and ipsilateral thalamus [27]. The procedure increased the development of axons in the injured hemisphere [27].

NSCs contribute to the repair of neuronal function in brain tissue, but because they are low in number and have a limited distribution range in the brain, NSCs exhibit a limited capacity for repairing neural damage [59]. Vascular endothelial growth factor (VEGF)-mediated effects on vascular endothelial cells include accelerated proliferation and migration of NSCs, the formation of new blood vessels, and an increase in vascular permeability [59]. Studies have reported that transplantation of VEGF-transfected NSCs can be administered to treat cerebral ischemia in adult rats; NSCs have been discovered to migrate to the host brain tissue and express VEGF gene products [59]. This transplantation therapy has a protective effect on local blood vessels and neurons in the brain [59]. In one particular study, a group constructed a recombinant lentivirus vector containing

the $VEGF_{165}$ gene to examine the neuroprotective effect of the transplantation of VEGF-transfected NSCs in neonatal CP rats [59].

Male and female rats (*n* = 150; 7 days old; 12-15 g) were randomly divided into five separate groups (*n* = 30 per group): (1) sham operation (control group), (2) CP model with or without, (3) phosphate-buffered saline (PBS) transplantation, (4) VEGF-NSCs transplantation (VEGF + NSCs), and (4) NSCs transplantation alone (NSCs) [59]. A unilateral occlusion of the carotid artery, followed by exposure to hypoxia, was executed on 7-day-old rats because the surge in brain growth at this age is similar to that occurring in newborn humans [59]. Three days following the CP establishment procedure, and with the transplantation site located on the left sensorimotor cortex, the PBS, VEGF + NSCs, and NSCs groups received a stereotaxic injection of 2 µL PBS buffer, NSCs transfected with recombinant lentivirus vector containing $VEGF_{165}$ gene (lentivirus MOI = 50 TU/cell, 5×10^4 NSCs/µL), and normal NSCs (5×10^4 NSCs/µL), respectively [59].

Observations concluded low immunoreactivity for VEGF in the cerebral cortex of the control group, but significantly higher immunoreactivity in the CP group, indicating hypoxia and ischemia increase VEGF expression [59]. Compared to the cerebral palsy or PBS groups, the number of VEGF-positive cells in the cerebral cortex notably increased in both VEGF + NSCs and NSCs groups [59]. These results suggest that transplantation of either NSCs alone or VEGF-transfected NSCs increases the amount of VEGF in the brain [59]. Additionally, the number of VEGF-positive cells in the VEGF + NSCs group was significantly larger in comparison to the NSCs alone group, therefore indicating VEGF levels are enhanced when neonatal CP rats are transplanted with VEGF-transfected NSCs compared with the transplantation of NSCs alone [59]. Performed on 30-day-old rats, a radial arm water maze test was performed to assess their ability to observe, study, and memorize the environment [59]. Rats from the CP and PBS groups spent a significantly longer time searching, had higher error rates, and a higher number of repetitions; hypoxia-ischemia impairs spatial learning and memory [59]. In comparison, the VEGF + NSCs and NSCs alone groups reached the target faster in the radial maze, with a smaller number of errors and repetitions; transfected NSCs or NSCs alone considerably improves spatial learning and memory in the CP model [59].

Cells in the CP and PBS groups exhibited signs of degeneration, necrosis, and local cystic degeneration, typical of animal models of ischemia-hypoxia-mediated CP [59]. Cells were less prominent and arranged in a disorderly manner [59]. However, in both VEGF + NSCs and NSCs alone groups, degenerated or necrotic cells were not as present, with most cells displaying a relatively normal cell structure with a clear morphology [59]. The cells were more prominent and karyopyknosis, structural areas of cell bodies that were unclear, was not predominantly visible in these groups compared with both CP and PBS groups [59].

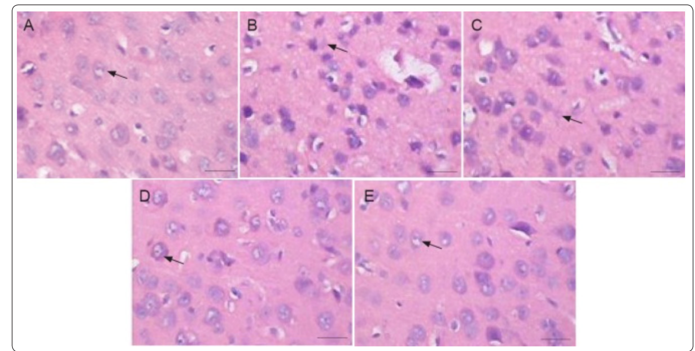

Figure 4: Effect of $VEGF_{165}$ -transfected NSC transplantation on cortical cell morphology in 35-day-old CP rats (hematoxylin-eosin staining) [59]. In the control group (A), cells were neatly arranged and at a high density [59]. In the CP and PBS groups (B, C), cells were less prominent [59]. In the VEGF + NSCs and NSCs groups (D, E), cells were more prominent compared with both the CP and PBS groups, exhibiting a clear morphology [59]. The arrows indicate NSCs. Scale bars: 25 µm

Necrosis and degeneration were alleviated in the NSCs and VEGF + NSCs groups [59]. Results indicate that the transplantation of NSCs or VEGF-transfected NSCs improve neuronal loss and are neuroprotective [59].

Conclusion

Stem cell transplantation is an effective alternative approach in minimizing the severity of symptoms in cerebral palsy patients. There is no current cure for the neurological disorder; however, the stem cells' capacity of self-renewal and differentiation indicate their potential in replacing injured, non-functional cells in the brain. Embryonic stem cells and their unlimited differentiation capacity is promising to a certain extent due to the risk of tumor formation [33]. Mesenchymal stem cells, isolated from several sources of tissue, are beneficial in therapy due to their immunomodulation characteristics; however, whether they can differentiate into neurons is still debatable. Embryonic stem cells have pluripotent properties, allowing them to differentiate into any cell type of the human body; this feature makes them more likely to repair damaged tissue. In addition to their low risk of tumorigenicity and immunogenicity, human amnion epithelial cells promote neural cell survival and regeneration, repair damaged neurons, and reestablish damaged neural connections [59]. Hematopoietic stem cells have self-renewal and multi-lineage differentiation properties, but larger studies need to be conducted in order to confirm these results [25]. Neural stem cells generate the major cell types found within the CNS (astrocytes, oligodendrocytes, and neurons), playing a key role in brain development [60]. However, collecting these cells from the brain or spinal cord is not easily accessible. Even though past studies suggest the great potential of stem cell types in treating patients with CP, highly qualified and larger human clinical trials should be conducted to advance research [61].

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