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(54) Title: MICROSPHERES CONTAINING MESENCHYMAL STEM CELLS

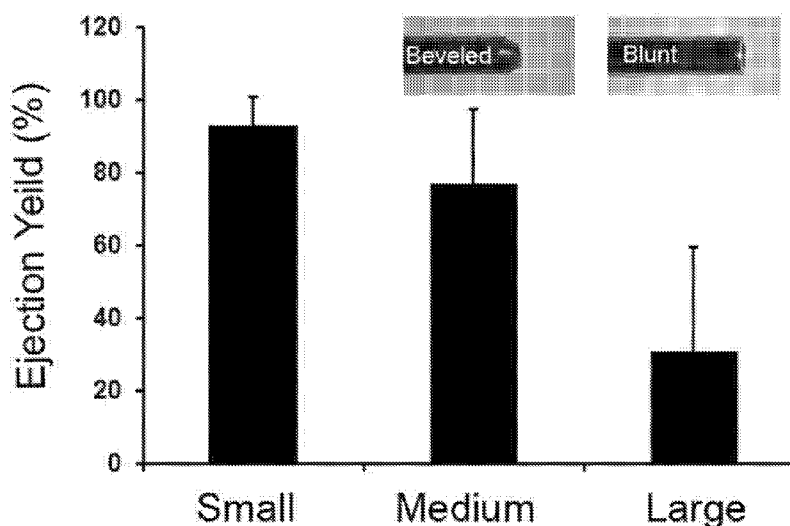


FIG. 3

(57) Abstract: The invention relates to a micro-encapsulation system for immobilizing mesenchymal stem cells (MSCs), methods for delivery of encapsulated MSCs into the central nervous system, and use of the encapsulated cells as cellular transplantation therapies.

MICROSPHERES CONTAINING MESENCHYMAL STEM CELLS

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application No. 62/161,446 filed on May 14, 2015. The content of the application is incorporated herein by reference
5 in its entirety.

GOVERNMENT INTERESTS

The invention disclosed herein was made, at least in part, with Government support under Grant No. T32GM008339 from the National Institutes of Health. Accordingly, the U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

The invention relates to a micro-encapsulation system for immobilizing mesenchymal stem cells (MSCs), methods for delivery of encapsulated MSCs into the central nervous system, and use of the encapsulated cells as cellular transplantation therapies.

BACKGROUND OF THE INVENTION

Mesenchymal stem cells, or MSCs, are multipotent stromal cells that can differentiate into a variety of cell types, including: osteoblasts, chondrocytes, muscle cells and adipocytes. MSCs release many factors that act to suppress multiple inflammatory pathways (Caplan and Correa, 2011; Prockop and Oh, 2012), and also convert pro-
20 inflammatory immune cells (e.g. macrophages) into anti-inflammatory cells by secreting potent anti-inflammatory cytokines that act locally as well as at a distance in vitro and in vivo (Barminko et al., 2011; Prockop and Oh, 2012). Accordingly, MSCs can be used as cellular transplantation therapies. For example, after administration to subjects by intravenous (IV) injection, some MSC become localized in bone marrow as a treatment for
25 acute graft-versus-host disease (GvHD), where they may remain for long times.

While IV injection is the least invasive method for systemic cell delivery, most MSCs delivered by this route become trapped in the liver, lungs and other locations, which reduces dramatically the proportion of cells available to migrate to sites of inflammation associated with injury and/or disease where they may be beneficial to the subject (Wagner
30 and Henschler, 2013). Doses as high as 10^6 MSC/kg are often required to obtain anti-

inflammatory effects and improved outcomes in animal models of spinal cord injury (SCI) (Tetzlaff et al., 2011). Clinical studies for various inflammatory mediated conditions have used similar doses often or higher doses up to ~billions of cells/patient (Wagner and Henschler, 2013). However, even with such high MSC doses, several trials have resulted
5 in only transient improvement and failed to meet their predetermined endpoints. Thus, there is a need for more effective MSC-based therapies.

SUMMARY OF INVENTION

This invention addresses the aforementioned unmet need by providing MSCs encapsulated within alginate microspheres and related methods.

10 Preferably, alginate is used according to present invention as a polymer for the formation of the (spherical) core and/or of the surface coating(s) due to their biocompatibility and their cross-linking properties. From a chemical point of view alginates are anionic polysaccharides derived from homopolymeric groups of beta-D-mannuronic acid and alpha-L-guluronic acid, separated by heteropolymeric regions of
15 both acids. Alginates are water soluble and form high viscosity solutions in the presence of monovalent cations such as sodium or potassium. A cross-linked water insoluble hydrogel is formed upon interaction of single alginate chains with bi- or multivalent cations (such as calcium and barium).

In one aspect, the invention provides a microsphere comprising alginate, and a
20 plurality of MSCs encapsulated by the alginate, wherein the microsphere has a diameter of about 200 μm to about 400 μm . The unpolymerized alginate can have a concentration in the range from about 1.7% (w/v) to about 2.5% (w/v), or a concentration of about 2.2% (w/v). In some embodiments, the microsphere has a diameter of about 200 μm to about 310 μm , about 320 μm to about 400 μm , about 330 μm to about 380 μm , about 340 μm to
25 about 360 μm , about 345 μm to about 355 μm , or about 350 μm . The microsphere can optionally contain nanoparticles. Examples of the nanoparticles includes, but are not limited to, Lanthanide-doped chitosan nanospheres (LDCNs) or lanthanide-Fe(3)O(4)-doped chitosan nanospheres (Fe(3)O(4)-LDCNs) fabricated and show fluorescence, MRI effectiveness and desirable biocompatibility.

30 The invention also provides a pharmaceutical composition comprising the above-described microsphere and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method for promoting tissue protection, repair or regeneration of spinal cord, or for treating SCI or other inflammatory diseases or conditions of central nervous system (CNS) in a subject. The method includes administering to the spinal cord of the subject an effective dose of human MSCs encapsulated within alginate microspheres less than 0.4 mm in diameter (e.g., the
5 microsphere described above) in a location being at a distance (e.g., one or more centimeters) away from an injury site. The encapsulated MSC (eMSC) are capable of releasing anti-inflammatory molecules and neurotrophic factors. The anti-inflammatory molecules cure or ameliorate the SCI or other CNS inflammatory diseases or conditions,
10 and the anti-inflammatory molecules and neurotrophic factors promote tissue protection, repair or regeneration in and around the injury site in the spinal cord or other CNS neural tissues.

In some embodiments, the microspheres can be manufactured using an ejection needle that has a beveled, tapered, tip. The inner diameter of the ejection needle can be
15 0.15-0.25 mm, such as 0.17 mm.

In one embodiment, the said microspheres are injected into a subject with the intrathecal injection needle and the outer diameter can be 0.5 - 1.1 mm and the inner diameter of the needle can be 0.4 -1.0 mm. In one example, the intrathecal injection needle is a thin-walled 23 gauge with an outer diameter of 0.64 mm and inner diameter of
20 0.52 mm. In another example, the intrathecal injection needle is a thin-walled 22 gauge with an outer diameter of 0.72 mm and inner diameter of 0.6 mm.

In the above described method, the MSCs are within an alginate microenvironment. The alginate microsphere can comprise successive layers of poly-L-lysine or poly-L-ornithine, and alginate, which surround the eMSC to provide additional
25 rigidity, diffusion control, and reduced cell adhesivity.

The eMSC alginate microspheres can be formed by crosslinking in a solution only with 20-100 mM barium (e.g., barium chloride or other suitable barium salts). The eMSC alginate microspheres can also be formed by crosslinking in a solution only with 20-100 mM calcium (e.g., calcium chloride or other suitable calcium salts) and 20-100 mM
30 barium (e.g., barium chloride or other suitable barium salts) treated either in combination or sequentially. A clinical grade of alginate (e.g., UP LVG Pronova, from FMC/Novamatrix) can be used.

The method can be used for treating SCI, which is characterized by an inflammatory condition in a spinal cord site of contusion, and the method comprises delivering an effective dose of alginate eMSC intrathecally within the spinal column. For that, the method comprises delivering an effective dose of the eMSC intrathecally into the cauda equine or into the foramen magnum.

The method can also be used for promoting tissue protection, which can occur in the brain or spinal cord in a subject with an inflammatory disease. Examples of the inflammatory disease include neuroinflammation and a neuroinflammatory disease. The tissue protection can also occur in the brain or spinal cord in a subject with trauma in the brain and/or spinal cord, such as a subject with traumatic brain injury, stroke, hydrocephalus, or a neuroinflammatory condition associated with multiple sclerosis, Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis or Parkinson's disease.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objectives, and advantages of the invention will be apparent from the description and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, 1C, 1D, 1D1, 1D2, 1D3, 1D4, 1D5, 1D6, 1D7, 1E, 1E', 1F, 1F', 1G, and 1G' show MRI Visualization of ChMNP capsules in spinal cord injury. Rats without (A) or with (B-E) intrathecal injection of ChMNP capsules were imaged at indicated times after injection. Note the absence of contrast in the spine in panel A by comparison to the signals (black) detected after 2 days (B) and that persist after 42 days (C) in cross sections. Horizontal images at 7 days (D-D7) show localization of ChMNP capsules along the spinal axis (bracket in D1) localized in serial sections from dorsal to ventral encompassing ~2.2 mm. 3-D reconstruction of super-threshold signals superimposed on a spinal X-ray (E) co-localizes with a bright field image of the same spinal cord after dissection from the spine with ChMNP particles in brown (E'). SCI Rats at 42 days after intrathecal injection of ChMNP capsules without (F, F') or with (G, G') eMSC were imaged. The signal extends more caudally to L3-4 (F') but was primarily ventral. Horizontal image (G) with signals of ChMNP particles in eMSC in ventral regions of L3- L5.

Figures 2A and 2B show effects of small eMSC on recovery after SCI. (A) Post-injury locomotor function assessed using BBB scoring was done weekly for 8 weeks by

trained observers in SCI rats injected with 5×10^4 free human MSC (F-MSC), small eMSC, or saline 1 day after SCI. Data is represented as mean $\pm$ SD. The eMSC (n=5) showed consistently higher scores than F-MSC (n=5) and saline group (n=6) and attained significance at week 7 and 8 (*, $p < 0.05$ ANOVA repeated measures with Tukey HSD test). (B) Percent preserved white matter at 8 weeks after SCI was calculated by measuring Eriochrome Cyanine-R staining in cross sections from rats treated with saline (n=4), F-MSC (n=5) and eMSC (n=3) at the indicated positions relative to the injury epicenter; (NC, normal control). Significant differences were detected in the percent of preserved white matter between eMSC vs. saline groups (*, $p < 0.05$) or vs. F-MSC (#, $p < 0.05$), ANOVA repeated measures with Tukey HSD test).

Figure 3 shows efficiency of eMSC ejection through narrow bore 23 gauge needles. Graph shows the effect of capsule size on eMSC ejection efficiency (% mean $\pm$ SD) in 2-3 experiments for three different eMSC sizes (small ~ 0.2 mm, medium ~ 0.35 mm and large ~ 0.5 mm). The injection yield (%) with larger capsules was much lower than the small and medium size capsules due to aggregation. The inserts show tips of 0.17 inner diameter needles with blunt or beveled ends.

Figure 4 shows different sizes of eMSC reduce TNF- α secretion when cultured with activated macrophages. Culture supernatants collected from transwell co-cultures of LPS-activated macrophages with eMSC at 1:1 ratio were assayed for TNF- α by ELISA and normalized to levels in LPS-activated macrophages supernatants without added eMSC. Differences between the LPS only control and each of the three different sized eMSC were significant (*, $p < 0.05$) and the difference between the medium sized capsules and the large and small capsules was also significant (#, $p < 0.05$).

Figures 5A, 5B, 5C, 5C', 5D, 5E, 5F, 5F' and 5G show effect of medium size eMSC transplantation on activated macrophages in SCI. Tissues from SCI rats one week after injection with ~ 0.35 mm eMSC (D-F) or saline as a control (A-C) were cryosectioned in cross sections and were incubated with Isolectin IB4 Alexa-488 conjugate. Confocal imaging showed more robust staining in control than eMSC treated sections in white matter and grey matter regions. Quantitation (G) of IB4 staining regions in equivalent dorsal midline circles drawn in spinal cord cross sections (for example, C' and F' are magnified images from C and F, respectively) were measured as super-threshold areas at ~ 2.2 mm caudal to the injury epicenter. Panels C' and F' represent

magnified images from panels C and F, respectively, that were used to quantitate IB4 staining. IB4 levels decreased significantly ($p<0.05$) in the eMSC group ($n=3$) as compared to the control ($n=4$) group. Scale bars are 500 μm in panels A-F and 100 μm in panels C' and F'.

5 Figures 6A, 6B, 6C, 6D, 6E, 6F and 6G show that medium size eMSC increased white matter sparing. Spinal cord cross sections in regions adjacent to those analyzed in Figure 5 from spinal cord contusion control (A-C) and ~ 0.35 mm eMSC (D-F) injected groups were stained for ECR. The percent of white matter (G) is significantly higher in eMSC group ($n=7$) as compared to control group ($n=3$). Data is mean $\pm$ SD (*, $p<0.05$).

10 Figure 7 shows permeability of LVG alginate capsules crosslinked with 50 mM barium chloride. Alginate at a final concentration of 2.0% alginate was crosslinked into microspheres in a solution of 50 mM Barium chloride without calcium chloride in the presence of green fluorescent bovine serum albumin (BSA) and red fluorescent immunoglobulin IgG. Confocal images show reduced levels of BSA (middle row) within
15 24 hours while the IgG (top row) remains inside the capsules for at least 48 hours. Bottom row is an overlay of the two top rows.

DETAILED DESCRIPTION OF THE INVENTION

This invention is based, at least in part, on an unexpected discovery that MSCs encapsulated within alginate microspheres of certain sizes are more effective in treating
20 various disorders.

As mentioned above, injection of MSCs reduces inflammation after spinal cord injury (SCI) and improves outcomes. As disclosed herein, while doses of $\sim 10^6$ MSC/kg are required for such results by conventional methods, about ~ 10 -fold lower doses are effective when the MSC are encapsulated in alginate microspheres, which prolong MSC
25 survival and modulate secretion of anti-inflammatory factors. As shown in the examples below, encapsulated MSC (eMSC) are delivered into the cerebrospinal fluid near the base of the spinal cord in a minimally invasive procedure through injection needles, which need to be narrow to prevent complications. Microcapsules of ~ 0.5 mm in diameter have been used widely to encapsulate MSC because of advantageous diffusion of factors compared
30 to larger ones and higher yields of incorporated cells compared to smaller ones. However, these ~ 0.5 mm microcapsules require large inner diameter needles that are problematic for intrathecal delivery in both animals and humans.

As described herein MSCs encapsulated within alginate microspheres of certain sizes (*e.g.*, 200 μm to about 400 μm , about 200 μm to about 310 μm , about 320 μm to about 400 μm , about 330 μm to about 380 μm , about 340 μm to about 360 μm , about 345 μm to about 355 μm , or about 350 μm) are more effective in treating various disorders.

Also described herein is manufacture using a 0.17 mm inner diameter needle with a beveled (tapered) tip of MSC microspheres of ~ 0.35 mm in diameter, which can be injected intrathecally in rat spinal cord through thin-walled 23 gauge needles. These smaller MSC microspheres have anti-inflammatory effects in rat SCI. These needles are more suitable for human intrathecal delivery than wider, smaller gauge, needles because they are associated with lower frequencies of side effects such as post-lumbar puncture headaches (PLPH).

In one embodiment, manufacture of eMSC of ~ 0.35 mm in diameter was achieved using a 0.17 mm inner diameter needles with a beveled (tapered) tips. The medium sized ~ 0.35 mm diameter eMSC showed improved efficacy over smaller and larger diameter eMSC *in vitro* in reducing macrophage secretion of the pro-inflammatory cytokine TNF α and improving locomotor recovery. Intrathecal injection of ~ 0.35 mm eMSC in rat spinal cord through thin-walled 23 gauge needles, represents an appropriate method for human delivery with limited complications. Smaller eMSC cannot be produced in sufficient quantities for scale up and larger one cannot be delivered reproducibly. Intrathecal injection of ~ 0.35 mm eMSC after rat spinal cord reduced pro-inflammatory macrophages, preserved white matter containing axons critical for function, and improved locomotor function.

Encapsulation of MSC in alginate allows cells to survive for weeks to months in microspheres *in vivo* (Goren et al., 2010; Heile et al., 2009). This is in contrast to IV injection where the MSC distribute widely through the body and are difficult to detect even after a few days, thus their activity and fate have been uncertain. Unlike free MSC that can migrate widely, microspheres provide a barrier blocking migration of the encapsulated cells into the host. Microspheres injected in a particular location resist movement, can be detected by imaging (Figure 1), and can be recovered from subjects for analysis. MSC in microspheres can act at a distance by secreting factors that move through body fluids such as cerebrospinal fluid, which flows into inflammatory sites in the CNS (Barminko et al., 2011).

The most common MSC microspheres are ~0.5 mm in diameter, which form when alginate-cell suspensions extruded through needles as microdrops are cross-linked with divalent cations such as calcium. These relatively large microcapsules have been delivered in the CNS through relatively large bore needles and holes of at least 1 mm, which require
5 invasive surgical procedures such as craniotomy (Heile et al., 2009) or laminectomy. Despite studies demonstrating successful delivery of encapsulated cells in vivo with long-term factor secretion providing benefits to animal subjects, no results have been reported for clinical trials in the CNS using MSC encapsulated in alginate; a trial transplanting large microcapsules containing MSC transfected to secrete GLP-1 into the brain has been
10 terminated (NCT01298830).

Macrophages secrete inflammatory factors and effector cells, which have been classified into at least two different types as M1 pro-inflammatory and M2 anti-inflammatory. After SCI there is a dramatic and persistent increase in M1 pro-inflammatory and a decrease in M2 anti-inflammatory macrophages. The pro-inflammatory M1 human macrophages secrete high levels of pro-inflammatory cytokines including TNF α , which can be cytotoxic and prolong inflammation. Co-incubation of eMSC with M1 macrophages in vitro reduced the levels of TNF α secretion (Barminko et al., 2011). Encapsulated MSC also convert M1 to M2 macrophages insofar as they decrease expression of cellular markers for M1 macrophages and increase markers for M2
15 anti-inflammatory macrophages in culture and in vivo (Barminko et al., 2011). Injection of MSC microcapsules in a rat model for SCI modulated inflammation significantly better than MSC alone, demonstrating the efficacy of eMSC in vivo (Barminko et al., 2011). When doses of MSC/kg are compared among different published studies, MSC in microcapsules are effective at lower doses than free MSC in promoting recovery from
20 SCI. Thus, lower doses of human MSC may be required for efficacy in vivo when they are encapsulated in microspheres. In addition, MSC within microcapsules do not contact host cells directly, only through fluids that permeate across the capsule walls, thereby protecting immunologically the MSC from the host and the host from potential deleterious effects of the MSC.

Commonly used ~0.5 mm MSC microcapsules require large inner diameter needles for intrathecal delivery in both animals and human, which can cause side effects including leakage of cerebrospinal fluid (CSF). Lumbar puncture needles of 20 gauge used for
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human spinal taps have been associated with a higher incidence of PLPH than narrower 22 gauge needles (Kleyweg et al., 1998). A clinical trial (NCT01481922) is currently testing additional potential benefit of even narrower needles. MSC encapsulated in ~ 0.2 mm capsules injected intrathecally in rat SCI through 23 gauge needles modulated inflammation and improved locomotor activity (Barminko et al., 2011). However, current methods for manufacturing small (~ 0.2 mm diameter) MSC microcapsules yield limited amounts, making them impractical for scale-up to perform well-controlled pre-clinical or clinical trials. Medium-sized MSC microspheres of ~0.35 mm in diameter are advantageous for pre-clinical studies and clinical applications because they can be injected through relatively narrow bore needles (23 gauge) that are associated with a lower incidence of post-lumbar puncture headache (PLPH) than occur with the larger needles. Thus scalable methods are needed to manufacture MSC capsules in sufficient quantities with relatively small diameters, which can be injected intrathecally for treatment of SCI. The manufacture of MSC microspheres of ~0.35 mm in diameter and their intrathecal delivery through thin-walled needles is described herein.

Companies are testing human MSCs in human CNS clinical trials for various indications using doses as high as 10^9 /patient with limited success despite efficacy in animal models. The methods described herein for manufacturing and delivering MSCs allow one to lower the dose required and prolong the efficacy of MSC transplants in SCI (Barminko et al., 2011). A unique feature of this invention is the encapsulation of MSC in alginate microspheres, which protects the host from the cells and the cells from the host. This results in prolonged survival of MSCs *in vivo* and prolonged delivery of soluble cytokines and growth factors that modulate inflammation and protect tissue in the host. The efficacy at lower doses and prolonged cell survival *in vivo* make this technology valuable to companies involved in or planning human CNS clinical trials because it increases the likelihood for success in human CNS clinical trials.

The ~0.35 mm eMSC can be used for treating a wide range of inflammatory conditions associated with cytokine storm, a condition in which pro-inflammatory cytokines persist abnormally and inflammation is not resolved as it normally would. In addition to SCI, this technology has CNS applications for damage (traumatic brain injury, hydrocephalus and ischemic stroke), as well as for chronic conditions (multiple sclerosis,

amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, and Alzheimer's disease).

The term "pharmaceutical composition" refers to the combination of an active agent (*e.g.*, cells) with a carrier, inert or active, making the composition especially suitable for diagnostic or therapeutic use *in vivo* or *ex vivo*. A "pharmaceutically acceptable carrier," administered to or upon a subject, does not cause undesirable physiological effects. The carrier in the pharmaceutical composition must be "acceptable" also in the sense that it is compatible with the active ingredient and can be capable of stabilizing it. One or more solubilizing agents can be utilized as pharmaceutical carriers for delivery of an active agent. Examples of a pharmaceutically acceptable carrier include, but are not limited to, biocompatible vehicles, adjuvants, additives, and diluents to achieve a composition usable as a dosage form. Additional suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences.

As used herein, a "subject" refers to a human and a non-human animal. Examples of a non-human animal include all vertebrates, *e.g.*, mammals, such as non-human mammals, non-human primates (particularly higher primates), dog, rodent (*e.g.*, mouse or rat), guinea pig, cat, and rabbit, and non-mammals, such as birds, amphibians, reptiles, etc. In one embodiment, the subject is a human. In another embodiment, the subject is an experimental, non-human animal or animal suitable as a disease model. The term "animal" includes all vertebrate animals including humans. In particular, the term "vertebrate animal" includes, but not limited to, humans, canines (*e.g.*, dogs), felines (*e.g.*, cats); equines (*e.g.*, horses), bovines (*e.g.*, cattle), porcine (*e.g.*, pigs), as well as in avians.

As used herein, "treating" or "treatment" refers to administration of a compound or agent to a subject who has a disorder or is at risk of developing the disorder with the purpose to cure, alleviate, relieve, remedy, delay the onset of, prevent, or ameliorate the disorder, the symptom of the disorder, the disease state secondary to the disorder, or the predisposition toward the disorder. An effective amount refers to the amount of an active compound/agent that is required to confer a therapeutic effect on a treated subject. Effective doses will vary, as recognized by those skilled in the art, depending on the types of conditions treated, route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

As disclosed herein, a number of ranges of values are provided. It is understood that each intervening value, to the tenth of the unit of the lower limit, unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither, or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention. The term “about” generally refers to plus or minus 10%, 5%, 2%, or 1% of the indicated number. For example, “about 10%” may indicate a range of 9% to 11%, and “about 1” may mean from 0.9-1.1. Other meanings of “about” may be apparent from the context, such as rounding off, so, for example “about 1” may also mean from 0.5 to 1.4.

EXAMPLES

Example 1 Manufacture of MSC Microcapsules

MSC were detached using trypsin-EDTA (Gibco), washed and mixed with 2.2% (w/v) alginate (Sigma Aldrich, USA) solution to obtain an initial cell density of $4\text{-}6 \times 10^6$ cells/ml (Barminko et al., 2011; Maguire et al., 2007). An electrostatic bead generator (NISCO) was used to form microdroplets, which were subsequently cross-linked in a 200 mL bath of CaCl_2 (100 mM) (Sigma-Aldrich). Microcapsules of ~ 0.35 mm and ~ 0.50 mm microcapsules both were manufactured within 1 h using 0.17 mm inner diameter extrusion needles, the former using a beveled tip (PE-00940, Nisco) and the latter a blunt tip PEQ00515, Nisco) (Fig. 3). The beveled needle has a smaller outer diameter at the tip of slightly greater than 0.17 mm, which is narrower than a blunt needle, resulting in the formation of smaller diameter micro-drops that become crosslinked into smaller microspheres in the CaCl_2 bath. Microcapsules were washed with phosphate buffered saline (PBS, Gibco) and incubated in 5 mL of poly-L-lysine (PLL) (Sigma-Aldrich, MW: 68,600 g/mol) (0.05% w/v) for 10 min in PBS and then by a second incubation with alginate for 10 min. Encapsulated cells were re-suspended in cell culture medium and transferred to 25 cm^2 tissue culture flasks (Fig. 3). Flow rates were similar for manufacture

of ~0.35 mm and ~0.50 mm microcapsules using 0.17 mm inner diameter extrusion needles with or without a beveled tip, respectively, and were more than 10-fold higher than through the 0.12 mm inner diameter extrusion needles used to manufacture the smaller ~0.2 mm microcapsules. The much lower flow rates with the narrow 0.12 mm inner diameter extrusion needles yielded much fewer MSC in ~0.2 mm microcapsules during a 1 h encapsulation (Table 1).

Table 1. Analysis of MSC Encapsulation Parameters.

MSC Capsules	Diameter (μm) +/- Std.	Viability (%)	Live Cells/capsule	Total Live Cells Encapsulated	Encapsulated/Input (%)
Large	450 ± 65	88 ± 7	124 ± 43	~10 ⁶	~25
Medium	378 ± 29	83 ± 7	73 ± 15	~10 ⁶	~25
Small	214 ± 27	83 ± 13	14 ± 5	~50,000	~1.25

Example 2 Viability of MSC in Microcapsules in Vitro

MSC viability in the capsules was assessed using a calcein and ethidium homodimer assay (Molecular Probes, USA) (Maguire et al., 2007). Capsules were imaged in an inverted fluorescent microscope (IX81, Olympus, Tokyo, Japan) and diameters were measured using SlideBook image analysis software version 5.0 (Intelligent Imaging Innovations, USA). Microcapsules were manufactured using 0.17 mm inner diameter extrusion needles with a beveled tip (PE-00940, Nisco) and encapsulations were performed within 1 h to maximize viability. On average 73 MSC were incorporated in ~0.35 mm microcapsules (Table 1).

Example 3 Ejection of MSC in ~0.35 mm Microcapsules through Intrathecal Delivery Needles in Vitro

Ejection efficiency of eMSC through 23 gauge thin walled needles (0.52 mm inner diameter, NIPRO) used for intrathecal delivery was measured as the number of ejected capsules recovered divided by the calculated number of capsules (the average number of capsules/ml in suspension) x (the volume ejected in ml). The number of ~0.35 mm diameter MSC microcapsules recovered after ejection was ~80% of the expected numbers of capsules (Fig. 3). Recovery was at least as good or better with the smaller ~0.20 mm diameter MSC microcapsules but not with the larger ~0.5 mm diameter MSC

microcapsules where the yield was much lower and the variability was much greater (Fig. 3).

Example 4 MSC Capsules Inhibit Secretion of TNF- α from Activated Macrophages in Vitro

5 Macrophages were isolated and co-culture assays were performed as described (Barminko et al., 2014) except with adaptations made for the use of capsules. Peripheral blood mononuclear cells were collected from blood of healthy donors (Blood Center of New Jersey) after centrifugation in Ficoll density gradients (GE Healthcare, USA). Monocytes were isolated by magnetic cell sorting using anti-CD14 coated beads (Miltenyi
10 Biotec, USA), and CD14⁺ monocytes were cultured in 175 cm² flasks (BD Biosciences, USA) at 10⁷ cells/flask in Advanced RPMI 1640 medium (Gibco) supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (Gibco). Macrophages were incubated with 5 ng/mL GM-CSF (R&D Systems, USA) for 7 days and replated at 1x10⁴ cells/ml in 96 well tissue
15 culture dishes (Corning, USA) and allowed to attach overnight. To activate macrophages, the medium was replaced with medium containing 1 μ g/mL LPS (Sigma-Aldrich). Transwell inserts containing eMSC in medium with 1 μ g/mL LPS were then added to the wells in a 1 to 1, macrophage to MSC, ratio. After 48 hours, co-culture supernatants were collected, stored at -20°C and then analyzed for secreted tumor necrosis factor (TNF)- α by
20 ELISA (Biolegend, USA) (Barminko et al., 2011). TNF- α levels were normalized to the LPS control without eMSC and statistical analysis was determined by one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) *post hoc* test, with $p < 0.05$ considered to be significant. (*) MSC microcapsules of ~0.35 mm diameter were more effective (#, $p < 0.05$) than larger and smaller diameter capsule in decreasing TNF- α
25 secretion levels (Figure 4).

Example 5 SCI and Transplantation of MSC in ~0.2 mm Microcapsule after SCI

For SCI surgery, adult female Sprague-Dawley rats (77 $\pm$ 2 days old, Taconic, USA) were anesthetized with 2% isoflurane (IsoFlo; Abbott Laboratories, USA), the spinal cord was exposed by laminectomy at T9–T10, and then contused by dropping a 10-g rod on the
30 exposed cord from a height of 12.5 mm using a Multicenter Animal Spinal Cord Injury Study (MASCIS) Impactor as described (Barminko et al., 2011; Hasegawa et al., 2005).

Following contusion, the muscles and skin were closed separately. Cefazolin (25 mg/kg) was administered daily for 7 days by subcutaneous injection after SCI to all rats. Food and water were provided *ad libitum*. One day after SCI, saline, microcapsules without MSC, 5×10^4 free MSC or 3×10^4 eMSC were injected intrathecally into the lumbar (L4-L5) spine over a period of 30 seconds using a thin-walled needle (23G X 1, inner diameter = 0.52 mm, outer diameter = 0.64 mm, NIPRO) attached to 250 microliter Hamilton syringe. The needle was left in place for another 60 seconds to prevent leakage. After the injection, the muscles and skin were sutured separately.

Example 6 Localization of Microcapsules by MRI in the Spinal Cord

Alginate capsules were prepared with 100 nm chitosen coated magnetite nanoparticles (ChMNP, 6 mg/ml) and $\sim 4 \times 10^3$ ChMNP-containing ~ 0.2 mm diameter capsules were implanted intrathecally into the rat cauda equina as described in Example 5. Magnetic resonance imaging (MRI) was performed under isoflurane anesthesia using a T1-weighted fast spin echo sequence with the M2™ Compact High-Performance MRI. Imaging performed at 2 – 42 days after ChMNP capsule implantation to track the capsules in the spinal cord over time showed that microcapsules were localized by MRI to cauda equina for 6 weeks after injection (Fig. 1.). Cross-section images at day 2 and 42 indicated that the capsules were restricted to the spinal column (Fig. 1A-C) and horizontal imaging showed that they remained close to where they were injected in the cauda equina at 1-6 weeks after injection (Fig. 1D, E.). The most intense mass of capsules was localized within ~ 1 cm along the rostro-caudal axis of the spinal cord within ~ 3 spinal segments after 1-6 weeks in the live rat (Fig. 1E). Imaging of the spinal cord after sacrifice (Barminko et al., 2011) showed that most capsules were found in this same region as in the MRI after the spinal cord was removed from the spinal column (Fig. 1E, E'). SCI Rats at 42 days after intrathecal injection of ChMNP capsules without (F, F') or with (G, G') eMSC were imaged (n=2, 3, respectively). A bracket marks the SCI site at T9-10 in the horizontal image (F) with signals of ChMNP particles in dorsal regions of T13- L2; the signal extends more caudally to L3-4 (F') but was primarily ventral (not shown). Horizontal image (G) with signals of ChMNP particles in eMSC in ventral regions of L3- L5; note the signal is in the spine above the vertebral body (G').

Example 7 Locomotor Recovery after SCI and Transplantation of MSC in ~0.2 mm Microcapsules

Locomotor recovery was assessed weekly using the 21-point BBB score (Basso et al., 1996) by a BBB scoring team that was unaware of experimental treatments using SCI rats prepared as in Example 6. Post-injury locomotor function assessed using BBB scoring was done weekly for 8 weeks by trained observers in SCI rats injected with 5×10^4 free human MSC (F-MSC), 0.2 mm diameter eMSC (3×10^4), or saline 1 day after SCI. Data is represented as mean $\pm$ SD. The eMSC (n=5) showed consistently higher scores than F-MSC (n=5) and saline group (n=6) and attained significance at week 7 and 8 (*, $p < 0.05$ ANOVA repeated measures with Tukey HSD test) (Figure 2A).

Example 8 MSC in ~0.2 mm Microcapsules Capsules Promotes White Matter Sparing in SCI

Cryosections from SCI rats (Hasegawa et al., 2005) ranging from -3 mm (rostral) to +3 mm (caudal) relative to injury epicenter at 1 mm intervals were stained with Eriochrome Cyanine-R (ECR) as described (McEwen and Springer, 2005) using SCI rats prepared as in Example 6 at 8 weeks after intrathecal injection of treatments. Sections were dehydrated at room temperature in a series of graded ethanol solutions for 5 min each and cleared in histoclear (National Diagnostics, USA) solution for 5 min. The sections were then rehydrated in a reverse-graded ethanol series. Sections were stained for 10 min with a solution of 0.16% ECR, 0.4% sulfuric acid, 0.4% iron chloride, and 0.12% HCL. The section was then rinsed with water for 10 min, mounted in Permount media (Fisher Scientific, USA) and scanned with Super Coolsan 8000 (Nikon, Japan). Images were analyzed using NIH ImageJ software to obtain super-threshold areas for each section using a constant threshold for all sections that were stained at the same time with ECR. Percent white matter is defined as the super-threshold ECR area divided by the total area outlined in each section. Averages of spared white matter tissue were calculated. Quantitation of ECR staining showed larger superthreshold areas of white matter with eMSC treatment compared to controls (Fig. 2B).

Example 9 MSC in ~0.35 mm Microcapsules Reduced Levels of IB4+ Macrophage staining in SCI

Rat SCI was performed as in Example 6 except intrathecal injections 1 day after SCI consisted of saline, or ~0.35 mm microcapsules with no cells or with 3×10^4 eMSC (Fig. 5). Cryosections were prepared from the SCI rats after 1 week later as described in Example 8. Sections at ~2.2 mm distal to the SCI epicenter were blocked with 10% normal goat serum at room temperature and stained with Isolectin IB4 Alexa-488 conjugate (1:100, Invitrogen) for 2 h. Confocal images were collected using a Zeiss 510 confocal laser scanning microscope and analyzed using LSM software. IB4 binding, which is a marker for activated macrophages (Tabor et al., 1989) and microglia (Lee et al., 2010), was more robust in the control (Fig. 5A-C) treatments than with eMSC (Fig. 5D-F) as observed in the micrographs. Quantitation of superthreshold signals using Zeiss LSM Imaging Software within circles of 1.9 mm circumference (Fig. 5C' vs. F') showed significantly lower average levels in the eMSC rats by comparison to controls (Fig. 5G).

Example 10 MSC in ~0.35 mm Microcapsules Promoted White Matter Sparing in SCI

Cryosections from SCI rats were prepared as in Example 9. White matter was imaged after ECR staining as in Example 8. One day after SCI intrathecal injection consisted of saline (Fig. 6A, B), or ~0.35 mm microcapsules with no cells (Fig. 6C) or with 3×10^4 eMSC (Fig. 6D-F). Quantitation of ECR staining (Fig. 6G) performed as in Example 8 yielded larger superthreshold areas of white matter with eMSC treatment (n=7) compared to the control group (n=3). Data is mean $\pm$ SD (*, p<0.05).

Example 11 Locomotor Recovery after SCI and Transplantation of MSC in ~0.35 mm Microcapsules

Rat SCI was performed as in Example 9 with intrathecal injections 1 day after SCI consisting of saline, or ~0.35 mm microcapsules with no cells or ~0.35 mm microcapsules with 5×10^4 eMSC. Locomotor recovery was assessed weekly using the 21-point BBB score (Basso et al., 1996) as in Example 7. Post-injury locomotor function assessed using BBB scoring by trained observers. Data is represented as mean $\pm$ SD. At 6 weeks, the eMSC (n=4) showed higher scores than the ~0.35 mm microcapsules with no cells (n=5) and saline group (n=5).

Example 12 Permeability of LVG Alginate Capsules Crosslinked with 50 mM Barium Chloride

Clinical grade alginate UP LVG (Pronova, FMC/Novamatrix) at a final concentration of 2.0% alginate (A) was crosslinked into microspheres in a solution of 50 mM Barium chloride without calcium chloride in the presence of green fluorescent bovine serum albumin (A488-BSA) and red fluorescent immunoglobulin IgG1 (568-IgG1). Confocal images show reduced levels of BSA within 24 hours while the IgG remains inside the capsules.

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The foregoing examples and description of the preferred embodiments should be taken as illustrating, rather than as limiting the present invention as defined by the claims. As will be readily appreciated, numerous variations and combinations of the features set forth above can be utilized without departing from the present invention as set forth in the claims. Such variations are not regarded as a departure from the scope of the invention, and all such variations are intended to be included within the scope of the following claims. All references cited herein are incorporated by reference in their entireties.

CLAIMS

WHAT IS CLAIMED IS:

1. A method for promoting tissue protection, repair or regeneration of spinal cord, or for treating SCI or other inflammatory diseases or conditions of central nervous system (CNS) in a subject, comprising administering to the spinal cord of the subject an effective dose of human mesenchymal stem/stromal cells (MSC) encapsulated within alginate microspheres less than 0.4 mm in diameter in a location being at a distance away from an injury site, wherein the encapsulated MSC (eMSC) are capable of releasing anti-inflammatory molecules and neurotrophic factors,
wherein said anti-inflammatory molecules cure or ameliorate said SCI or said other CNS inflammatory diseases or conditions, and said anti-inflammatory molecules and neurotrophic factors promote tissue protection, repair or regeneration in and around the injury site in the spinal cord or other CNS neural tissues, wherein the MSC are within an alginate microenvironment.
2. The method of claim 1, wherein the microspheres are made using an ejection needle that has a beveled, tapered, tip and are injected into the subject with an intrathecal injection needle.
3. The method of claim 2, wherein the inner diameter of the ejection needle is 0.15-0.25 mm.
4. The method of claim 2, wherein the inner diameter of the ejection needle is 0.17 mm.
5. The method of claim 2, wherein the outer diameter of the intrathecal injection needle is 0.5 - 1.1 mm and the inner diameter of the needle is 0.4 -1.0 mm.
6. The method of claim 2, wherein the intrathecal injection needle is a thin-walled 23 gauge with an outer diameter of 0.64 mm and inner diameter of 0.52 mm.
7. The method of claim 2, wherein the intrathecal injection needle is a thin-walled 22 gauge with an outer diameter of 0.72 mm and inner diameter of 0.6 mm.
8. The method of claim 1, wherein the eMSC alginate microspheres comprises a layer of poly-L-lysine, which surround the microsphere to provide additional rigidity and diffusion control.

9. The method of claim 1, wherein the eMSC alginate microspheres comprises a layer of poly-L-ornithine, which surround the microsphere to provide additional rigidity and diffusion control.

10. The method of claims 8 or 9, wherein the microspheres comprises a successive
5 layer of alginate, which surround the microspheres to provide additional rigidity, diffusion control, and reduced cell adhesivity.

11. The method of claim 1, wherein the eMSC alginate microspheres are formed by crosslinking in a solution only with 20-100 mM barium.

12. The method of claim 1, wherein the eMSC alginate microspheres are formed by
10 crosslinking in a solution only with 20-100 mM calcium and 20-100 mM barium treated either in combination or sequentially.

13. The method of claim 1, wherein a clinical grade of alginate is used.

14. The method of claim 1, wherein the SCI is characterized by an inflammatory condition in a spinal cord site of contusion, and the method comprises delivering an
15 effective dose of alginate eMSC intrathecally within the spinal column.

15. The method of claim 11, wherein the method comprises delivering an effective dose of said eMSC intrathecally into the cauda equina.

16. The method of claim 11, wherein the method comprises delivering an effective dose of said eMSC intrathecally into the foramen magnum.

20 17. The method of claim 1, wherein said tissue protection occurs in the brain or spinal cord in a subject with an inflammatory disease.

18. The method of claim 14, wherein said inflammatory disease is neuroinflammation or a neuroinflammatory disease.

19. The method of claim 1, wherein said tissue protection occurs in the brain or spinal
25 cord in a subject with trauma in the brain and/or spinal cord.

20. The method of claim 16, wherein said tissue protection occurs in the brain or spinal cord in a subject with traumatic brain injury, stroke, or hydrocephalus.

21. The method of claim 15, wherein said tissue protection occurs in the brain or spinal cord in a subject with a neuroinflammatory condition associated with multiple sclerosis,
30 Huntington's disease, Alzheimer's disease, amyotrophic lateral sclerosis or Parkinson's disease.

22. A microsphere comprising alginate, and a plurality of MSCs encapsulated by the alginate, wherein the microsphere has a diameter of about 200 μm to about 400 μm .

23. The microsphere of claim 19, wherein the alginate has a concentration in the range from about 1.7% (w/v) to about 2.5% (w/v), or a concentration of about 2.2% (w/v).

5 24. The microsphere of any one of claims 17-20, wherein the microsphere has a diameter of about 200 μm to 310 μm , 320 μm to about 400 μm , about 330 μm to about 380 μm , about 340 μm to about 360 μm , or about 350 μm .

25. A pharmaceutical composition comprising a microsphere of any one of claims 17-21 and a pharmaceutically acceptable carrier.

10 26. The microsphere of any claim 17-21, wherein nanoparticles are incorporated within the microsphere.

27. The microsphere of claim 23 wherein the nanoparticles are Lanthanide-doped chitosan nanospheres (LDCNs) or lanthanide-Fe(3)O(4)-doped chitosan nanospheres (Fe(3)O(4)-LDCNs) fabricated and show fluorescence, MRI effectiveness and desirable

15 biocompatibility.

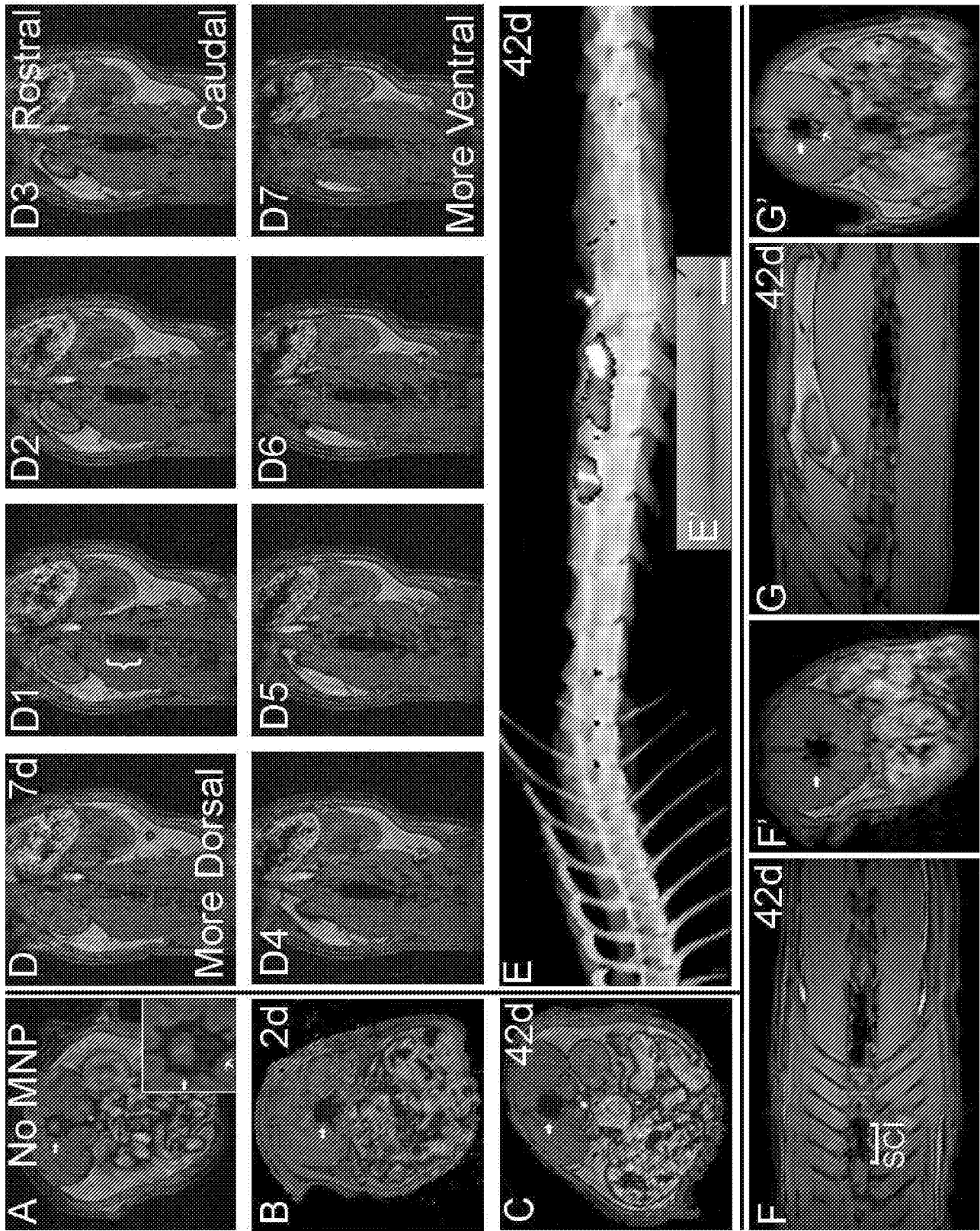


FIG. 1A, 1B, 1C, 1D, 1E, 1F and 1G

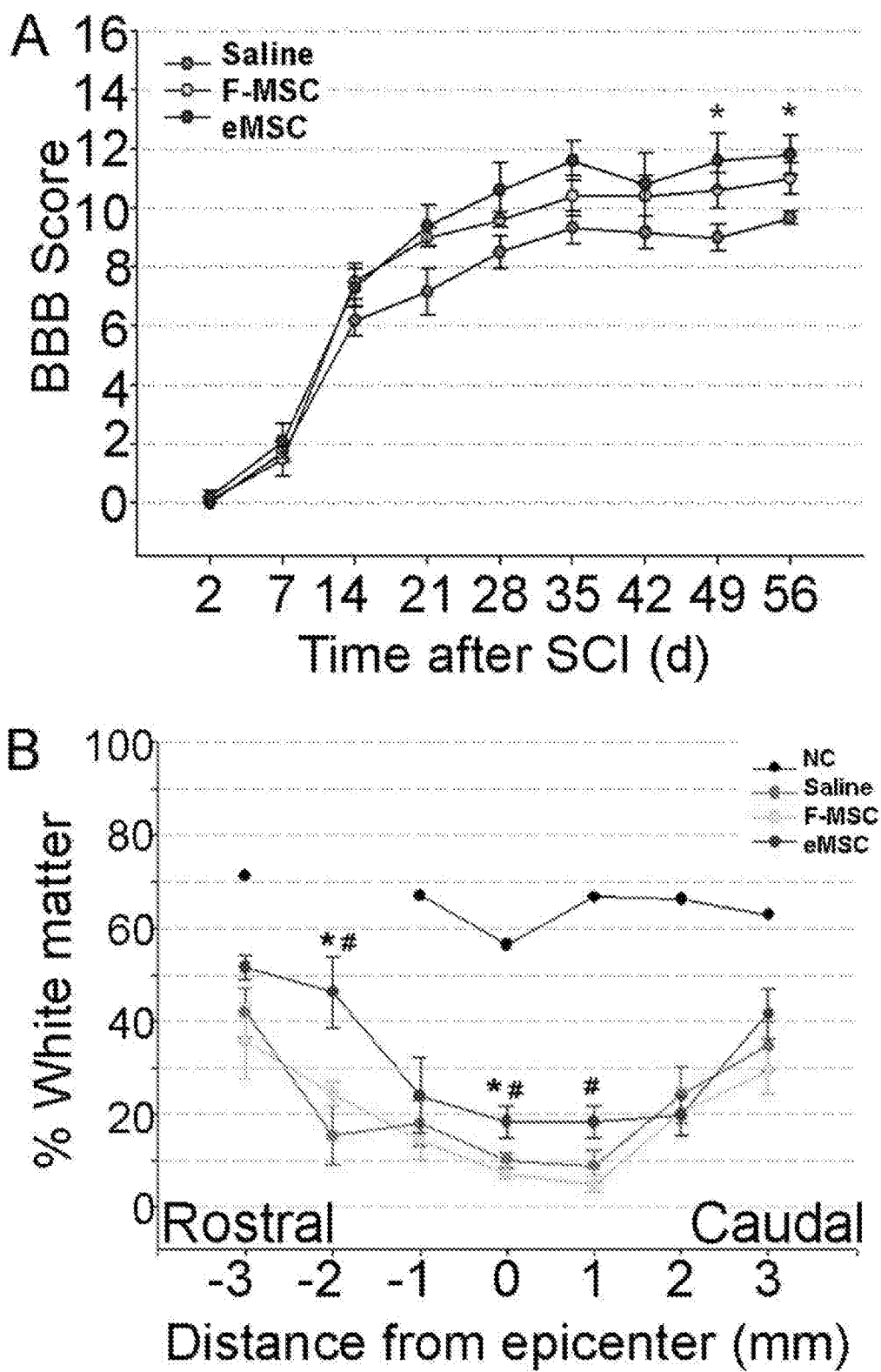


FIG. 2A and 2B

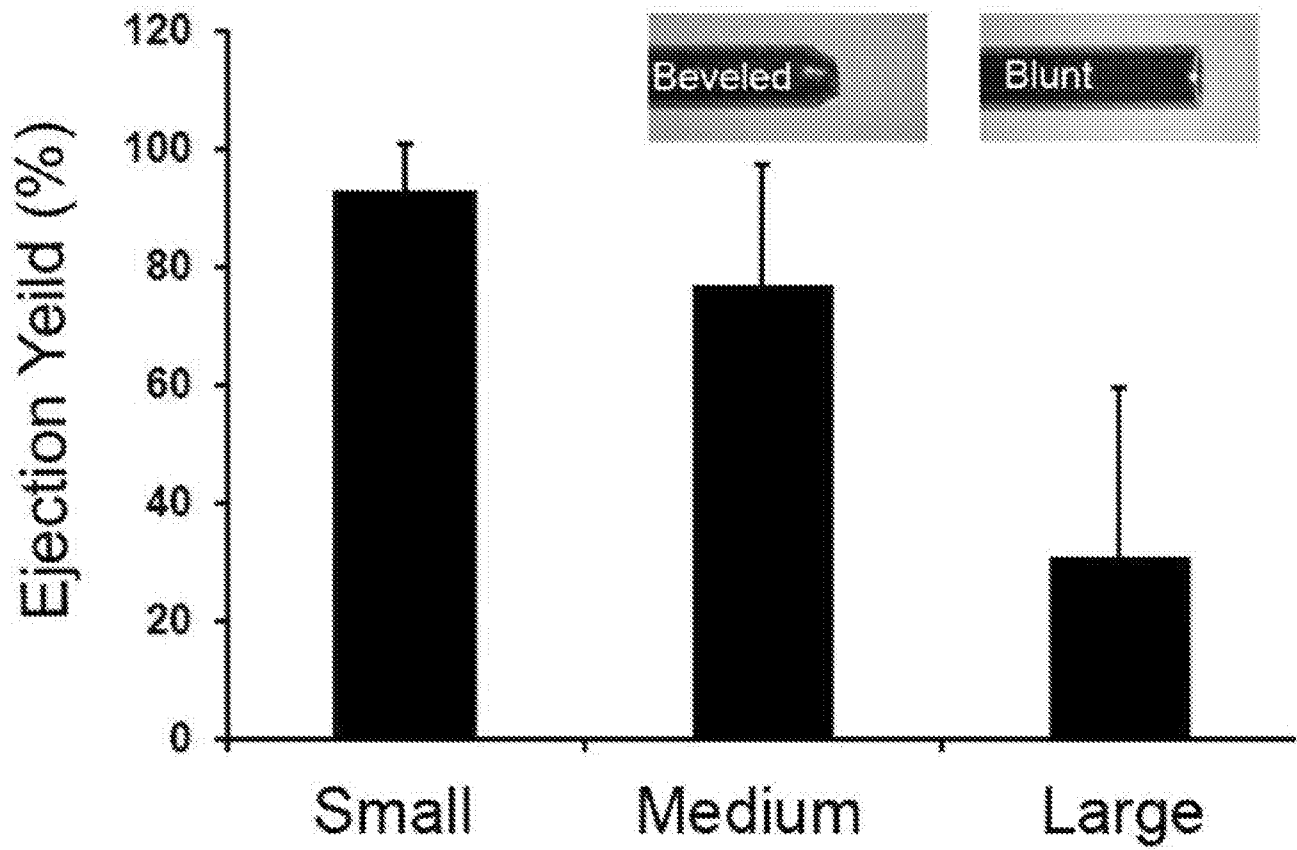


FIG. 3

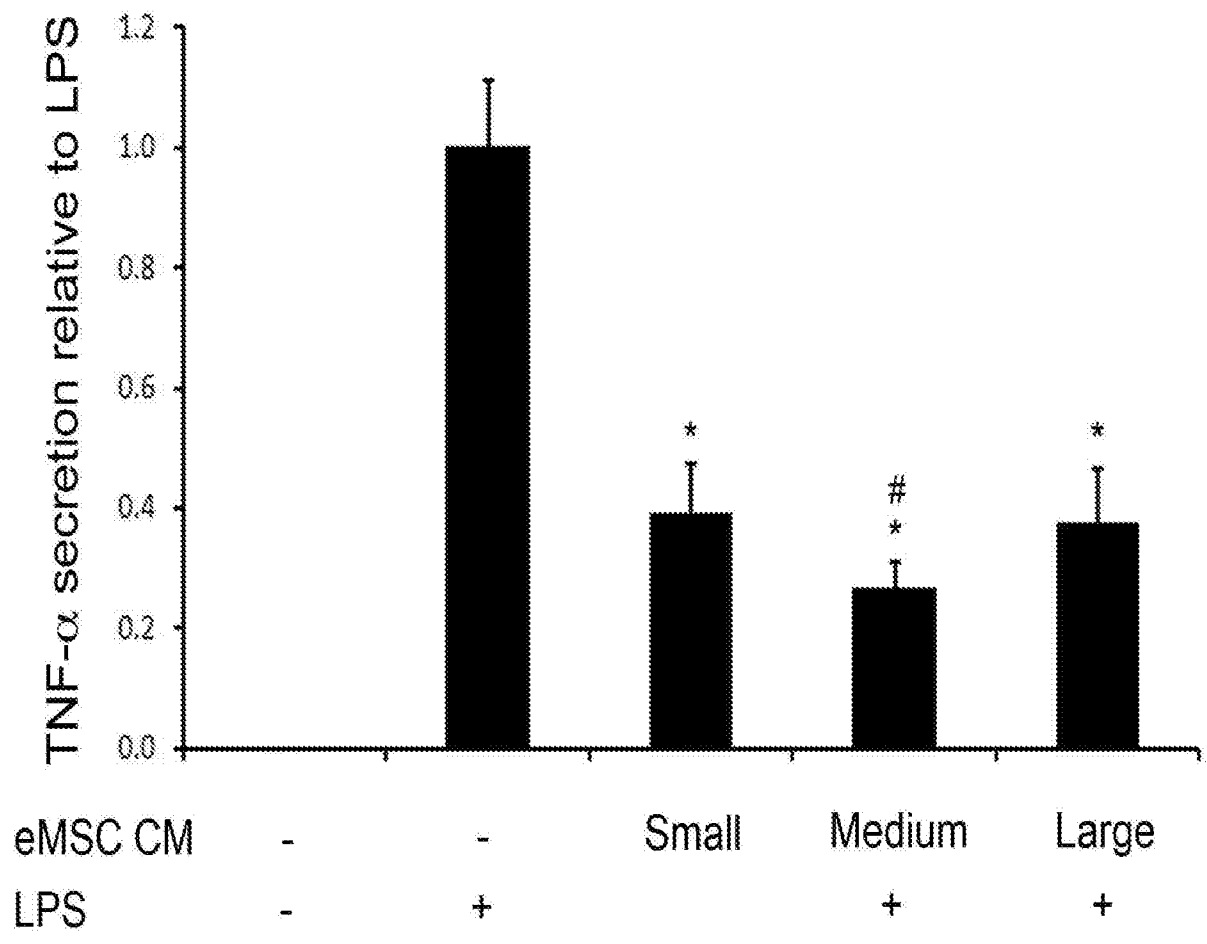


FIG. 4

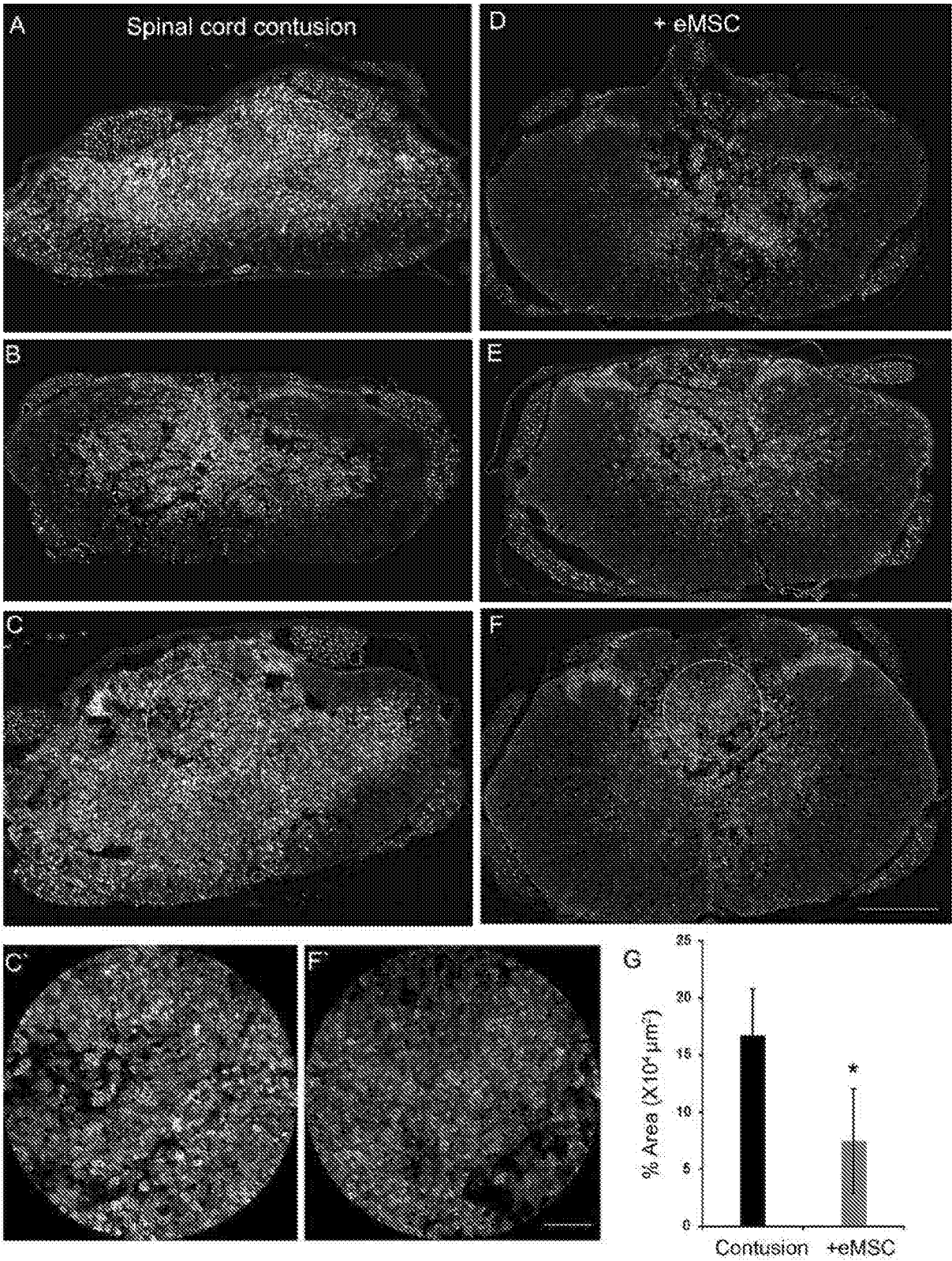


FIG. 5A, 5B, 5C, 5D, 5E, 5F and 5G

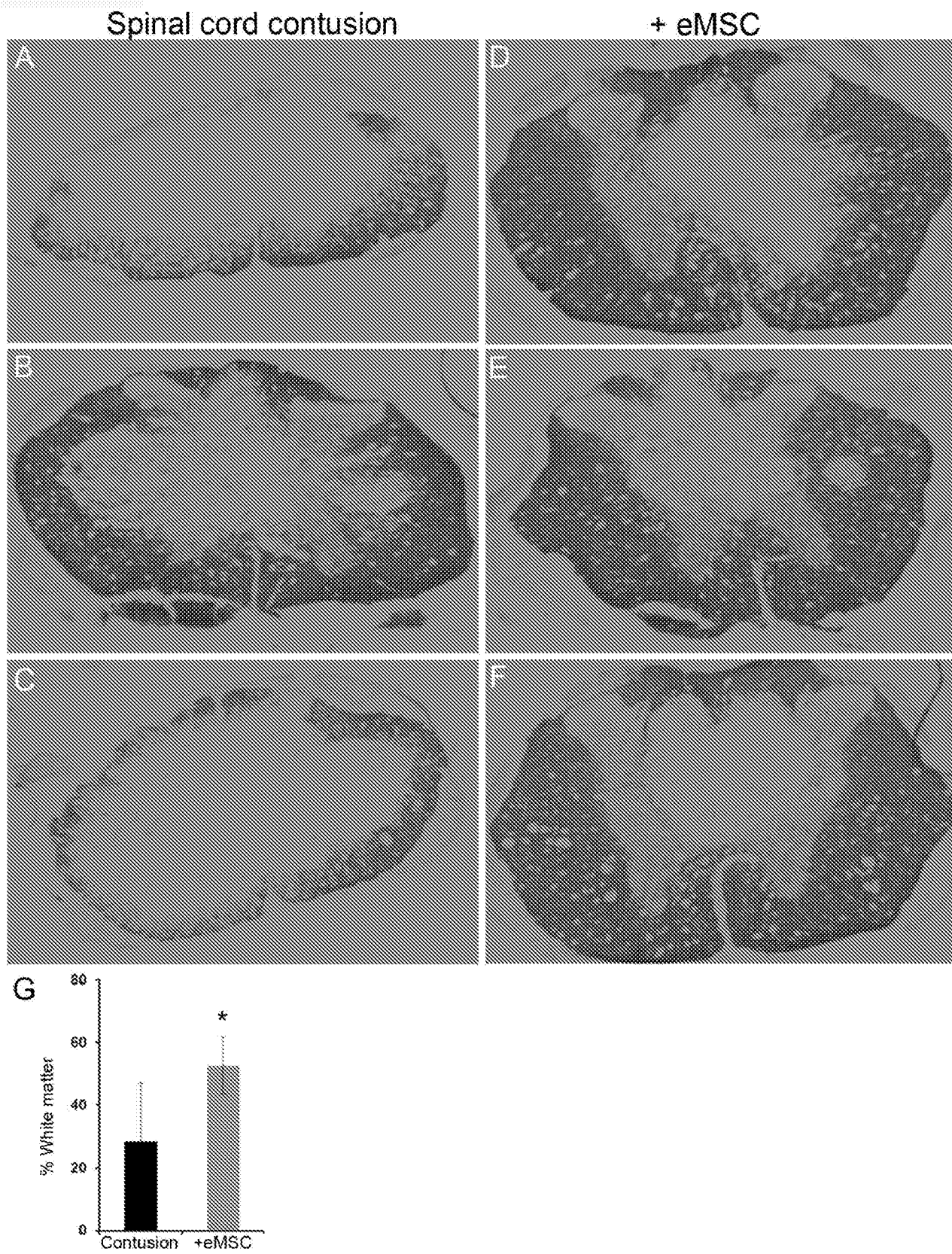


FIG. 6A, 6B, 6C, 6D, 6E, 6F and 6G

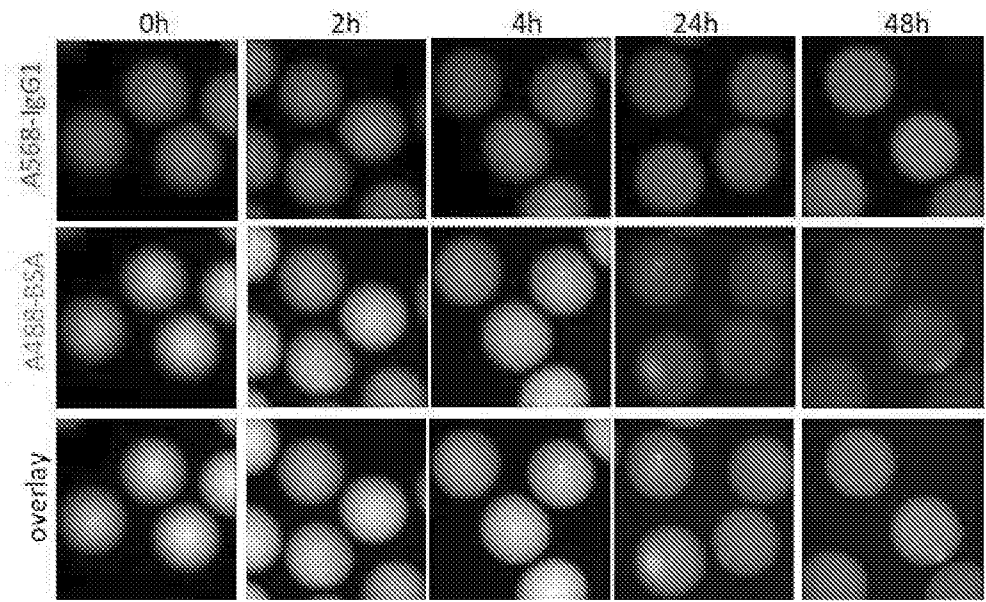


FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/032327

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 9/50; A61K 35/12; A61P 25/00; C12N 5/0775 (2016.01) CPC - A61K 35/28; A61K 2035/122; A61K 2035/128; A61L 27/3834; C12N 2533/74 (2016.05) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC - A61K 9/50; A61K 35/12; A61P 25/00; C12N 5/0775 CPC - A61K 35/28; A61K 2035/122; A61K 2035/128; A61L 27/3834; C12N 2533/74 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC - 424/451; 435/178; 435/325; 435/372; 435/375; 435/377 (keyword delimited) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase, Google Patents, Google Scholar, PubMed Search terms used: alginate microsphere encapsulated mesenchymal stem stromal cell CNS spine		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0020931 A1 (YARMUSH et al) 26 January 2012 (26.01.2012) entire document	1, 8, 13, 14, 17-19, 22-24
Y		2-7, 9-12, 15, 16, 20, 21, 25-27
Y	BUGARSKI et al. "Electrostatic droplet generation: Mechanism of polymer droplet formation," Materials, Interfaces and Electrochemical Phenomena, 01 June 1994 (01.06.1994), Vol. 40, Pgs. 1026-1031. entire document	2-7
Y	"Syringes & Needles," Braun, 17 January 2014 (17.01.2014), Pgs. 1-27. Retrieved from the Internet: <http://www.bbraunoem.com/documents/Services/Syringes_and_Needles_en_Edition_01-2014.pdf> on 01 August 2016 (01.08.2016). entire document	2-7
Y	LEPORE et al. "Neural precursor cells can be delivered into the injured cervical spinal cord by intrathecal injection at the lumbar cord," Brain Res. 26 April 2005 (26.04.2005), Vol. 1045, Pgs. 206-16. entire document	2-7
Y	ROKSTAD et al. "Advances in biocompatibility and physico-chemical characterization of microspheres for cell encapsulation," Adv Drug Deliv Rev. 20 July 2013 (20.07.2013), Vol. 67-68, Pgs. 111-30. entire document	9, 10
Y	HARIYADI et al. "Effect of cross linking agent and polymer on the characteristics of ovalbumin loaded alginate microspheres," Int J Pharm Pharma Sci, 2014, Vol. 6, Pgs. 469-74. entire document	11, 12, 15, 16, 20, 21
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 30 July 2016		Date of mailing of the international search report 22 AUG 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300		Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2016/032327

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	✓ KNIPPENBERG et al. "Intracerebroventricular injection of encapsulated human mesenchymal cells producing glucagon-like peptide 1 prolongs survival in a mouse model of ALS," PLoS One. 20 June 2012 (20.06.2012), Vol. 7:e36857, Pgs. 1-10. entire document	15, 21
Y	– CHEN et al. "A safety study on intrathecal delivery of autologous mesenchymal stromal cells in rabbits directly supporting Phase I human trials," Transfusion. 21 November 2014 (21.11.2014), Vol. 55, Pgs. 1-15 [Original Pgs. 1013-20]. entire document	16, 20
Y	✓ VU et al. "Meta-analysis of preclinical studies of mesenchymal stromal cells for ischemic stroke," Neurology, 07 March 2014 (07.03.2014), Vol. 82, Pgs. 1277-86. entire document	20
Y	US 2011/0212136 A1 (OSTERHOUT et al) 01 September 2011 (01.09.2011) entire document	25
Y	US 2011/0089375 A1 (CHAN et al) 21 April 2011 (21.04.2011) entire document	26, 27
Y	– WANG et al. "Lanthanide-doped chitosan nanospheres as cell nuclei illuminator and fluorescent nonviral vector for plasmid DNA delivery," Dalton Trans. 31 October 2011 (31.10.2011), Vol. 41, Pgs. 490-7. entire document	27
A	✓ GILL et al. "Does needle size matter?," J Diabetes Sci Technol. 01 September 2007 (01.09.2007), Vol. 1, Pgs. 725-9. entire document	1-27