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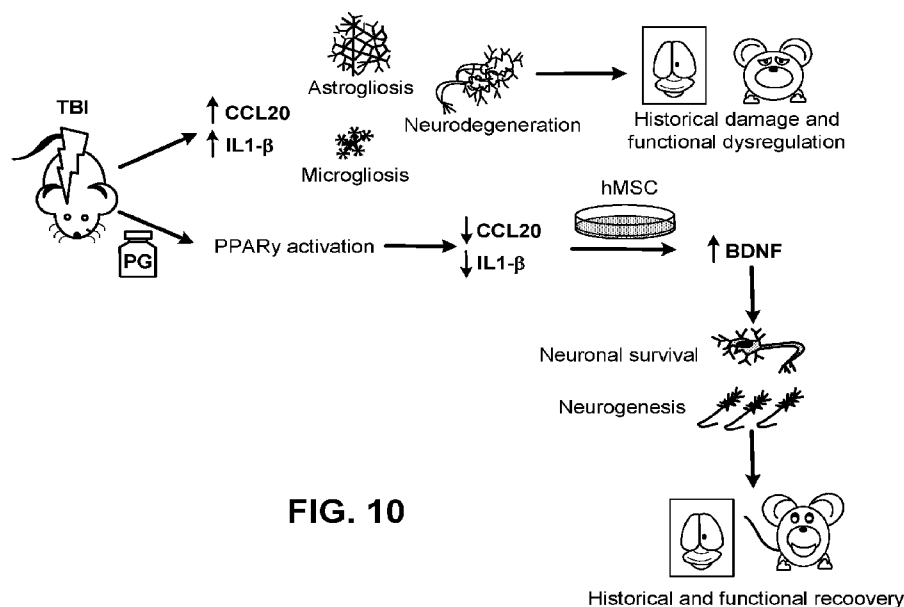


FIG. 10

(57) **Abstract:** The present application is related to methods of treating traumatic brain injury in a subject in need thereof, by administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells to the subject. Also disclosed herein are methods of treating one or more symptoms of traumatic brain injury in a subject in need thereof.



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METHODS OF TREATING TRAUMATIC BRAIN INJURY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/809,283, filed on February 22, 2019; and to U.S. Provisional Application No. 62/912,163, filed on October 8, 2019, all of which are incorporated by reference in their entireties.

STATEMENT OF FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant No. BX002688, awarded by Veterans Affairs. The government has certain rights in the invention.

TECHNICAL FIELD

This invention relates to methods of treating traumatic brain injury, and more particularly to combinations of pioglitazone and mesenchymal stromal cells (MSC).

BACKGROUND

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States and around the world. Approximately 10 million people are either hospitalized or die from TBI globally each year¹. In the United States alone 1.7 million people suffer from TBI and about 52,000 people die annually². TBI affects both children and adults and affects the quality of life of the survivors posing a significant social burden. The most common causes of TBI are related to motor vehicle accidents, sports and battlefield injuries. Falls, especially in children and older people, and physical abuse are among the common causes as well. The National Center for Injury Prevention and Control in the United States recorded per 100,000 people 715.7 TBI related emergency department visits, 91.7 hospitalization and 17.1 deaths in 2010³ with an approximate annual financial burden of \$76.5 billion³. TBI affects the quality of life of the survivors. Even the mild TBI or concussions may have effects later in the life that are difficult to relate to the prior TBI event. The initial injury to the brain damages the cerebral tissues and the BBB and evokes immune response systemically and locally in the brain. This

leads to astrogliosis, immune cell migration, cytokine and chemokine secretion and microglial activation. All these immunological reactions cause further damage to the brain and the secondary injury spreads over time, inducing long term structural, functional, behavioral and psychological deficits.

5 So far the available therapies only provide symptomatic treatments to TBI patients. For example, clinical trials of erythropoietin and progesterone both failed to repair brain damage⁴⁻⁶. Transplantation of mesenchymal stromal cells (MSCs) has been shown to be the most promising regenerative approach. These pluripotent cells have the potential to be transformed to many different cells, are immune suppressive and secrete
10 growth factors to promote tissue regeneration⁷⁻⁹. In addition to time of transplantation, number and quality of transplanted cells, the efficacy of MSC therapy largely depends on the microenvironment of the target tissue. Following TBI, proinflammatory cytokines are secreted in the brain tissues, immune cells migrate and glial cells are activated creating an inflammatory environment which may not favor the survival and functioning of the
15 MSCs¹⁰. Therefore, reducing the inflammation before MSC therapy might help in increasing the efficacy of the therapy.

SUMMARY

The present application is based, in part, on the surprising and unexpected discovery that reducing the proinflammatory cytokine production in the brain tissue with
20 a PPAR γ agonist enhances the efficacy of hMSC treatment in traumatic brain injury. This enhanced efficacy can result in improvement of both the histopathological and the behavioral outcomes in rats after TBI.

In a rat model of lateral fluid percussion injury, combined pioglitazone (PG) and hMSC (combination) treatment showed less anxiety-like behavior and improved
25 sensorimotor responses to noxious cold stimulus. Significant reduction in brain lesion volume, neurodegeneration, microgliosis, and astrogliosis were observed after combination treatment. TBI-induced expression of inflammatory chemokine CCL20 and IL1- β significantly decreased in the combination treatment group. Combination treatment

significantly increased brain derived neurotrophic factor (BDNF) level and SVZ neurogenesis.

Some embodiments described herein provide methods of treating a patient experiencing symptoms associated with traumatic brain injury as substantially disclosed
5 herein.

Some embodiments described herein provide methods of improving the efficacy of stem cells by administering an anti-inflammatory drug as substantially disclosed herein.

Some embodiments described herein provide methods of treating one or more
10 symptoms of traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells.

Some embodiments described herein provide methods of treating traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a
15 pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells.

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

DESCRIPTION OF DRAWINGS

FIG. 1A is a grid of xenogen images showing DiR fluorescence in different organs 7 days after TBI. FIG. 1B is a histogram showing the levels of DiR fluorescence in organs following intranasal and intravenous administration of hMSCs. FIG. 1C
25 depicts brain sections from rats stained with anti-human nuclear antigen (HuNu) antibody (clone 235-1) 35 days after TBI. FIG. 1D is a histogram showing the percentage of HuNu positive cells in the perilesional area of the brains.

FIG. 2A is a set of micrographs of the cortex of a sham animal administered a vehicle, a TBI animal administered a vehicle, sham animal administered pioglitazone,

and a TBI animal administered pioglitazone. FIG. 2B shows a histogram of CCL20 and PPAR γ levels for a sham animal administered a vehicle, a TBI animal administered a vehicle, sham animal administered pioglitazone, and a TBI animal administered pioglitazone.

5 FIG. 3A is a representative image of brain sections after treatment with PG and/or hMSC. FIG. 3B is a series of histograms showing image J quantitation.

 FIG. 4A depicts fluorojade expression in the corpus callosum after TBI-induced neurodegeneration and microgliosis. FIG. 4B depicts representative immunofluorescence images showing the Iba1 positive microglia in different brain regions of the ipsilateral
10 side. FIG. 4C is a histogram of the average number of Iba1 positive microglia (mean $\pm$ SEM) in ipsilateral corpus callosum. FIG. 4D is a histogram of the average number of Iba1 positive microglia (mean $\pm$ SEM) in lateral cortex.

 FIG. 5A depicts representative immunofluorescence images showing the Iba1 positive microglia in different brain regions of the contralateral hemisphere. FIG. 5B is a
15 histogram showing the average number of Iba1 positive microglia (mean $\pm$ SEM) in the ipsilateral cortex. FIG. 5C is a histogram showing the average number of Iba1 positive microglia (mean $\pm$ SEM) in the corpus callosum. FIG. 5D is a histogram showing the average number of Iba1 positive microglia (mean $\pm$ SEM) in the lateral cortex.

 FIG. 6A depicts representative images of immunoperoxidase staining of cortex
20 (adjacent to injury site) showing the CCL20 or IL1- β immunoreactivity under different experimental conditions. FIG. 6B is a histogram showing the numbers (mean $\pm$ SEM) of CCL20 positive cells in the cortex. FIG. 6C is a histogram showing the numbers (mean $\pm$ SEM) of IL1 β positive cells in the cortex.

 FIG. 7A depicts representative immunofluorescence images showing
25 DCX immunoreactivity in the SVZ region or DG region under different experimental conditions. FIG. 7B is a histogram showing DCX immunoreactivity (mean $\pm$ SEM) in SVZ under different experimental conditions measured using image J. FIG. 7C is a histogram showing DCX immunoreactivity (mean $\pm$ SEM) in DG under different experimental

conditions measured using image J. FIG. 7D is a histogram showing serum BDNF level measured by ELISA.

FIG. 8A depicts representative heat maps of the movement of rats in an open field arena. FIG. 8B is a histogram showing latency to the first entry to the center zone of the arena. FIG. 8C is a histogram showing time spent in the center zone of the arena. FIG. 8D is a histogram showing frequency to entry to the center zone. FIG. 8E is a histogram showing latency to first immobility in the arena. FIG. 8F is a histogram showing number of grooming events. FIG. 8G is a histogram showing mean paw withdrawal latency (sec) of the contralateral paw to noxious cold stimulation (mean $\pm$ SEM).

FIG. 9 is a pair of histograms that show the average values (mean $\pm$ SEM) of total distance traveled by rats or mean velocity of movement of rats in the open field arena 35 days post TBI.

FIG. 10 is a schematic representation summarizing the effect of Pioglitazone (PG) and hMSC combination treatment on improving outcomes in rats after traumatic brain injury (TBI).

FIG. 11 is a table showing the primary antibodies and corresponding secondary antibodies used for immunohistochemistry experiments.

DETAILED DESCRIPTION

As used herein, terms “treat” or “treatment” refer to therapeutic or palliative measures. Beneficial or desired clinical results include, but are not limited to, alleviation, in whole or in part, of symptoms associated with a disease or disorder or condition, diminishment of the extent of disease, stabilized (i.e., not worsening) state of disease, delay or slowing of disease progression, amelioration or palliation of the disease state (e.g., one or more symptoms of the disease), and remission (whether partial or total), whether detectable or undetectable. “Treatment” can also mean prolonging survival as compared to expected survival if not receiving treatment.

Traumatic brain injury (TBI) can be diffuse or focal. Symptoms of TBI include, but are not limited to unconsciousness, inability to awaken, headache, vomiting, nausea, convulsions, lack of motor coordination, slurred speech, aphasia, dizziness, difficulty

balancing, lightheadedness, changes in sensory perception (e.g., perceiving a bad taste, blurred vision, tinitis, etc.), fatigue, altered sleep patterns, confusion, deficits in short-term memory, lack of concentration, pupil dilation (one or both), loss of coordination, restlessness and/or agitation, loss of social judgment, muscle weakness, difficulty processing/understanding emotions, and other cognitive defects.

As used herein, the term “subject,” refers to any animal, including mammals such as mice, rats, other rodents, rabbits, dogs, cats, swine, cattle, sheep, horses, primates, and humans. In some embodiments, the subject is a human. In some embodiments, the subject has experienced and/or exhibited at least one symptom of TBI. In some embodiments, the subject has been identified or diagnosed as having TBI (e.g., as determined using a regulatory agency-approved, e.g., FDA-approved, assay or kit, or the standard of care diagnostic, such as the Glasgow Coma Scale and/or MRI). In some embodiments, the subject is suspected of having TBI.

The term “regulatory agency” refers to a country's agency for the approval of the medical use of pharmaceutical agents with the country. For example, a non-limiting example of a regulatory agency is the U.S. Food and Drug Administration (FDA).

Significant tissue loss, degeneration of neurons and neuronal fibers in several brain areas 35 days post-injury (dpi) can be seen in subject with TBI, indicating secondary spread of the damage. Presence of Iba1 positive activated microglia, intense astrogliosis and presence of glial scars along with elevated expression of proinflammatory chemokines CCL20 and IL1- β indicate persistent active inflammation in the brain after TBI in TBI and vehicle treated animals 35 dpi. This is further supported by decreased BDNF in serum after TBI.

MSCs are pluripotent cells that are present in a wide variety of body tissues^{18,19} and can be isolated and cultured, and may ultimately differentiate into many kinds of cells²⁰⁻²². Their immune tolerance⁷, ability to migrate to the site of inflammation²³⁻²⁵, and secretion of growth promoting factors⁷⁻⁹ make them a candidate for regenerative therapies.

The present application demonstrates that treatment with pioglitazone, a PPAR γ agonist, followed by hMSC (human mesenchymal stromal cell) transplantation, improved the behavioral outcome in rats, reduced the brain lesion volume, neuronal death, microglial

activation and CCL20 and IL1- β expression. This treatment also increased the expression of BDNF and neurogenesis. Together these observations indicate an overall trend towards the reduction of post TBI inflammation and regeneration. Increased neurogenesis in the hMSC treated animals shows the regenerative potential of these cells.

5 The efficacy of hMSC treatment depends upon a few factors like number of administered cells, route of administration, time of administration and the microenvironment of the target tissue. Keeping these factors in mind, an optimum number of cells (1×10^6 cells per animal)²⁶ were administered to animals through the intranasal route. Studies have shown that intravenous (i.v.), intra-arterial (i.a.) or intra-cranial
10 (i.c.v.) routes have potential disadvantages. Xiong et al. showed that i.v. administration of MSCs caused systemic distribution while i.a. administration caused cerebral ischemia and i.c.v. administration limits the number of injected cells to a sub-optimal dose²⁷. The intranasal (i.n.) route used in this study, is the preferred non-invasive delivery route. Bypassing the blood-brain barrier, the cells enter the cerebral parenchyma through the
15 nasal mucosa and the cribriform plate^{28,29} and reaches the brain within 1 hour of administration³⁰. 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR)-labelled hMSCs were administered via i.v. or i.n. route. A significantly higher DiR fluorescence was observed in the brain after i.n. administration compared to i.v. group (FIGS. 1A and 1B), indicating higher efficacy of this method. The presence of
20 administered cells in the treated brains 35 dpi was observed, indicating successful transplantation, homing and survival of cells. This method of hMSC administration was used throughout the study.

 The success of hMSC transplantation therapy also depends on the microenvironment of the recipient tissue. For example, Garcia-Olmo et al. have shown that
25 low success rate of the stem cell transplantation therapy owed to the proinflammatory cytokines and reactive oxygen species secreted by the immune cells in the inflammatory microenvironment of the injured tissue^{10,31}. The caspases secreted in this environment caused apoptosis of the transplanted cells, leading to a low success rate of the therapy³². Impaired survival and homing efficacy of stem cells due to post-trauma inflammation has
30 been reported by Malcanyi et al.³³.

Pioglitazone (PG) is a thiazolidinedione (TZD), which are synthetic high affinity ligands of PPAR γ .^{14-17, 35-39}. In TBI animals, PPAR γ expression declined with the increased expression of CCL20; treatment with PG reduced CCL20 expression post TBI (FIGS. 2A and 2B).

5 Animals were treated with PG following TBI and continued the treatment until hMSC transplantation. This therapeutic strategy enhanced the efficacy of hMSC therapy in decreasing post-TBI inflammation, improving neurotrophic factor secretion and neurogenesis. All these effects were reflected in improved behavioral and sensorimotor responses. The overall treatment outcome of the combination treatment was significantly
10 improved over PG only or hMSC only treatments. Although both hMSC and the combination treatment reduced the lesion and neurodegeneration to a similar extent, the combination treatment was more efficient in reducing the microglial activation in all brain regions (FIGS. 3A-3B and FIGS. 4A-4D), even in the contralateral side (FIGS. 5A-5D). Inflammatory mediators like CCL20 and IL1- β were also significantly reduced by the
15 combination treatment indicating the efficacy of this strategy over the other treatment options.

Depression and anxiety disorders are common and devastating after effects of TBI. Although generally considered psychological, these conditions may have neurobiological underpinnings. Open field test in rodents is a simple yet promising test to
20 evaluate such neurobehavioral aspects of TBI. In an open field arena, rats typically prefer to be close to the walls, a behavior called thigmotaxis. They tend to avoid the open, well lit area of the center which is a novel, stressful environment to the animal, with occasional exploratory trips to the center area. When the animals are less anxious they tend to spend more time in the open center zone and also decrease the latency to enter
25 this area (anxiolytic activity). Jones et al. (2008) showed that 1 to 3 months after TBI induced by FPI, rats showed anxiogenic behavior by exhibiting reduced entry and reduced time spent in the center area of an open field arena⁴⁰. Recently, Kim et al. have shown increased activity in the center zone of rats treated with synthetic estrogen 17 α -ethynylestradiol-3-sulfate after FPI-induced TBI⁴¹. In line with these reports, this indicates
30 that TBI and vehicle treated rats showed significant anxiety-like behavior and this

behavior improved in the combination treatment group over other treatment groups. The rats with combination treatment showed lower latency to enter the CZ, they spent more time in the CZ and entered the zone more frequently indicating anxiolytic behavior. They also exhibited the longest latency to first immobility and the least grooming. All these behaviors indicated that the functional recovery was best in the combination treated rats.

Sensory hypersensitivity and persistent pain, including headache, nociceptive pain and neuropathic pain are somatic symptoms of TBI⁴². Pain has been reported as a secondary complication of TBI^{43,44}. Very few models are available that describe post-injury pain and its mechanism^{45,46}. One test is for increased thermal pain sensitivity (decreased withdrawal latency to noxious cold exposure) in the contralateral paw in rats after TBI. This indicates the involvement of central sensorimotor pathway in this phenomenon. Following TBI, synthesis and release of inflammatory cytokines and pro-nociceptive mediators from activated microglia has been reported⁴⁷. Rowe et al. implicated glial activation, central and peripheral inflammatory mediators and T_{reg} dysregulation as potential causes of TBI-induced mechanical hyperalgesia⁴². Combination treatment has shown to reduce the hyperalgesia to the normal level.

Neurotrophic factors are associated with reactive processes occurring as a result of lesions in the CNS. BDNF is a neurotrophic factor which has been shown to be associated with post-TBI depression and cognitive dysfunction^{52,53}. It is also involved in neuronal survival and synaptic plasticity^{54,55}. Following TBI, a decrease in serum BDNF is correlated with injury severity⁵⁶ and poor recovery⁵³. One mechanism by which hMSCs exert their effects in CNS repair and regeneration is by secreting neurotrophins^{52,57,58}. Following transplantation these cells may directly influence neuronal repair or stimulate the glial cells to secrete neurotrophins like BDNF or NGF. On the other hand, the interaction between glial cells and hMSCs may lead to neurotrophin release and subsequent neuronal repair^{58,59}. The combination therapy described herein demonstrated the best recovery of histological, behavioral and sensorimotor parameters, also had significantly increased BDNF level in serum as compared to TBI or vehicle groups.

In stem cell therapy of brain injury, a key step of recovery is neuro-regeneration. *In vitro* differentiation of embryonic stem cells to neural cells has been shown by Guan et

al.⁶⁰. Mahmood et al. have shown that hMSC injected in rat after TBI promotes tissue repair by means of neurogenesis and synaptogenesis⁶¹. Adults have two major neural stem cell niches, the SVZ and the dentate gyrus⁶². Both hMSC and combination treatment enhanced neurogenesis in the SVZ region. But the extent of neurogenesis reached the level of the sham animals only in the presently described combination therapy group. Since BDNF has potent role in neuroprotection and neurogenesis⁶³ it is possible that hMSC induced neuroprotective and neurogenic effects were mediated, at least partially through BDNF. BDNF, along with other neurotrophic factors has been shown to utilize canonical β -catenin pathway to promote hMSC neurogenesis and synaptogenesis⁶⁴. The specific mechanism of PPAR γ activation in improvement of hMSC functioning and hMSC-induced neurogenesis needs further investigation.

FIG. 10 is a schematic representation summarizing the effect of Pioglitazone (PG) and hMSC combination treatment on improving outcomes in rats after traumatic brain injury (TBI). The top path is the degenerative pathway, and the bottom path is the regenerative pathway. TBI induces neurodegeneration and evokes inflammatory reactions including elevated cytokines CCL20 and IL1- β , microgliosis and astrogliosis. These lead to histological and functional deficits. PG treatment after TBI activates PPAR γ and reduces CCL20 and IL1- β . In the reduced inflammatory microenvironment hMSCs increased BDNF secretion which at least partially improves the histological and functional recovery. CCL20, Chemokine ligand protein 20, IL1- β , Interleukin 1 beta, hMSC, human mesenchymal stem cells, BDNF, brain-derived neurotrophic factor.

As shown in FIG. 10, FPI induced injury in rat brains with significant tissue loss and neurodegeneration which persisted 35 dpi. TBI in rats induced microglial and astroglial activation, increased secretion of proinflammatory cytokines and caused behavioral and sensorimotor deficits. PG reduced neuroinflammation in the brain by decreasing inflammatory cytokine production prior to hMSC transplantation. Reduction of local cerebral inflammation improved the efficacy of transplanted hMSC which was evident by increased neurogenesis, reduced anxiety-like behavior and pain sensation in combination treated rats. Possibly, in a reduced inflammatory microenvironment, hMSCs helped in histological and behavioral recovery through enhanced production of

neurotrophic factor like BDNF.

Some embodiments provide a method of treating traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells. Other embodiments provide a method of treating one or more symptoms of traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells.

In some embodiments, (a) is administered prior to (b). In other embodiments, (a) is administered prior to, and concurrently with, (b).

In some embodiments, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 24 hours prior to the administration of (b) mesenchymal stromal cells. For example, in some embodiments, the pioglitazone, or a pharmaceutically acceptable salt thereof, is administered about 30 minutes, about 45 minutes, about 1 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4 hours, about 4.5 hours, about 5 hours, about 5.5 hours, about 6 hours, about 7 hours, about 8 hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, or about 24 hours, prior to the administration of (b) mesenchymal stromal cells, or any value in between. In other embodiments, the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 12 hours prior to the administration of (b) mesenchymal stromal cells. For example, in some embodiments, the pioglitazone, or a pharmaceutically acceptable salt thereof, is administered about 30 minutes, about 45 minutes, about 60 minutes, about 90 minutes, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4 hours, about 4.5 hours, about 5 hours, about 5.5 hours, about 6 hours, about 6.5 hours, about 7 hours, about 7.5 hours, about 8 hours, about 8.5 hours, about 9 hours, about 9.5 hours, about 10 hours, about 10.5 hours, about 11 hours, about 11.5 hours, about 12 hours, prior to the administration of (b) mesenchymal stromal cells, or any value in between. In still other embodiments, the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 2 hours

prior to the administration of (b) mesenchymal stromal cells. For example, about 30 minutes, about 40 minutes, about 50 minutes, about 60 minutes, about 70 minutes, about 80 minutes, about 90 minutes, about 100 minutes, about 110 minutes, about 2 hours, prior to the administration of (b) mesenchymal stromal cells, or any value in between.

5 In some embodiments, between about 5 mg and 100 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject. For example, about 5 mg, about 10 mg, about 20 mg, about 30 mg, about 40 mg, about 50 mg, about 60 mg, about 70 mg, about 80 mg, about 90 mg, about 100 mg, of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject, or any value in
10 between. In other embodiments, between about 10 mg and 75 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject. For example, about 10 mg, about 20 mg, about 30 mg, about 40 mg, about 50 mg, about 60 mg, about 70 mg, about 75 mg, of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject, or any value in between. In still other embodiments, between about 25 mg
15 and 50 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject. For example, about 25 mg, about 30 mg, about 35 mg, about 40 mg, about 45 mg, about 50 mg, of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject, or any value in between.

 In some embodiments, the pioglitazone, or a pharmaceutically acceptable salt
20 thereof is administered orally. In other embodiments, the pioglitazone, or a pharmaceutically acceptable salt thereof is administered intravenously.

 In some embodiments, the mesenchymal stromal cells are administered intranasally. In other embodiments, the mesenchymal stromal cells are administered intravenously. In still other embodiments, the mesenchymal stromal cells are administered
25 intra-arterially. In further embodiments, the mesenchymal stromal cells are administered intra-cranially.

 Some embodiments further comprise administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, after the administration of (b) mesenchymal stromal cells. In some embodiments, the pioglitazone, or a pharmaceutically acceptable
30 salt thereof, is administered from about 1 hour to about 4 weeks after the mesenchymal

stromal cells, for example, about 1 hour, about 2 hours, about 3 hours, about 4 hours, about 5 hours, about 6 hours, about 7 hours, about 8, hours, about 9 hours, about 10 hours, about 11 hours, about 12 hours, about 14 hours, about 16 hours, about 18 hours, about 20 hours, about 22 hours, about 24 hours, about 36 hours, about 48 hours, about 60 hours, about 72
5 hours, about 96 hours, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, about 10 days, about 2 weeks, about 3 weeks, about 4 weeks, or any value in between. In some embodiments, the pioglitazone, or a pharmaceutically acceptable salt thereof, is administered for between about 1 day to about 7 days after the mesenchymal stromal cells.

In some embodiments, about 1×10^6 to about 1×10^7 mesenchymal stromal cells are
10 administered to the subject. For example, about 1×10^6 , about 1.1×10^6 , about 1.2×10^6 , about 1.3×10^6 , about 1.4×10^6 , about 1.5×10^6 , about 1.6×10^6 , about 1.7×10^6 , about 1.8×10^6 , about 1.9×10^6 , about 1×10^7 , mesenchymal stromal cells are administered to the subject, or any value in between.

Some embodiments provide a method of treating traumatic brain injury in a
15 subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells; wherein the (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is administered orally prior to intranasal administration of the (b) mesenchymal stromal cells. Other embodiments provide a method of treating one or more symptoms of traumatic brain injury in a subject in need thereof,
20 comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells; wherein the (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is administered orally prior to intranasal administration of the (b) mesenchymal stromal cells.

In some embodiments, the one or more symptoms are selected from:
25 unconsciousness, inability to awaken, headache, vomiting, nausea, convulsions, lack of motor coordination, slurred speech, aphasia, dizziness, difficulty balancing, lightheadedness, fatigue, altered sleep patterns, confusion, deficits in short-term memory, lack of concentration, pupil dilation, loss of coordination, restlessness and/or agitation, loss of social judgment, muscle weakness, and difficulty processing/understanding emotions.

30 In some embodiments, the one or more symptoms is unconsciousness. In some

embodiments, the one or more symptoms is inability to awaken. In some embodiments, the one or more symptoms is headache. In some embodiments, the one or more symptoms is vomiting. In some embodiments, the one or more symptoms is nausea, convulsions. In some embodiments, the one or more symptoms is lack of motor coordination. In some
5 embodiments, the one or more symptoms is slurred speech. In some embodiments, the one or more symptoms is aphasia. In some embodiments, the one or more symptoms is dizziness. In some embodiments, the one or more symptoms is difficulty balancing. In some embodiments, the one or more symptoms is lightheadedness. In some embodiments, the one or more symptoms is fatigue. In some embodiments, the one or more symptoms is
10 altered sleep patterns. In some embodiments, the one or more symptoms is confusion. In some embodiments, the one or more symptoms is deficits in short-term memory. In some embodiments, the one or more symptoms is lack of concentration. In some embodiments, the one or more symptoms is pupil dilation. In some embodiments, the one or more symptoms is loss of coordination. In some embodiments, the one or more symptoms is
15 restlessness and/or agitation. In some embodiments, the one or more symptoms is loss of social judgment. In some embodiments, the one or more symptoms is muscle weakness. In some embodiments, the one or more symptoms is difficulty processing/understanding emotions.

In some embodiments, the one or more symptoms comprise two or more
20 symptoms. In other embodiments, the one or more symptoms comprise three or more symptoms. In still other embodiments, the one or more symptoms comprise two to five symptoms.

Some embodiments provide a method of treating a patient experiencing symptoms associated with traumatic brain injury as substantially disclosed herein. Other
25 embodiments provide a method of improving the efficacy of stem cells by administering an anti-inflammatory drug as substantially disclosed herein. In some embodiments, the anti-inflammatory drug is pioglitazone.

In some embodiments, the subject has been previously determined to have a traumatic brain injury. In some embodiments, the subject has been previously diagnosed
30 as having a traumatic brain injury. In some embodiments, the subject is currently suffering

from a traumatic brain injury. In some embodiments, the subject is suspected to have a traumatic brain injury.

In some embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 3 and 8, prior to treatment. In other embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 6 and 11, prior to treatment. In still other embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 9 and 14, prior to treatment. In some embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 4 and 9, prior to treatment. In other embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 7 and 12, prior to treatment. In still other embodiments, the subject has been determined to have a Glasgow Coma Scale score of between 10 and 14, prior to treatment.

In some embodiments, the subject's Glasgow Coma Scale score improves between about 1 point to about 6 points, or any value in between, within about 2 weeks to about 2 months, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 1 point, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 2 points, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 3 points, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 4 points, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 5 points, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment. In some embodiments, the subject's Glasgow Coma Scale score improves about 6 points, within about 2 weeks to about 2 months of treatment, or any value in between, after treatment.

One skilled in the art will recognize that both *in vivo* and *in vitro* trials using suitable, known and generally accepted cell and/or animal models are predictive of the ability of a test compound to treat or prevent a given disorder.

One skilled in the art will further recognize that human clinical trials including first-in-human, dose ranging and efficacy trials, in healthy subjects and/or those suffering from a given disorder, can be completed according to methods well known in the clinical and medical arts.

Provided herein are pharmaceutical kits useful, for example, in the treatment of TBI, which include two or more containers containing (a) pioglitazone, or a pharmaceutically acceptable salt thereof; and (b) mesenchymal stromal cells. Such kits can further include, if desired, one or more of various conventional pharmaceutical kit components, such as, for example, containers with one or more pharmaceutically acceptable carriers, additional containers, etc., as will be readily apparent to those skilled in the art. Instructions, either as inserts or as labels, indicating quantities of the components to be administered, guidelines for administration, and/or guidelines for mixing the components, can also be included in the kit.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

EXAMPLES

Animals and TBI induction

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of South Florida. 40, male Sprague-Dawley rats

(Envigo, USA) weighing 250 to 300 g were housed in a climate-controlled facility with food and water available *ad libitum*. Animals in this study were divided into 6 groups: 1. Sham (n = 8); 2. TBI (n = 6), 3. Vehicle treated (TBI+DMSO, n = 5), 4. PG treated (TBI+PG, n = 8), 5. hMSC treated (TBI+hMSC, n = 6), 6. Combination treatment (Combo) (TBI+PG+hMSC, n = 7).

Animals were anesthetized using a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) (intraperitoneal, i.p.). Carprofen (5 mg/kg, sub cutaneous, s.c., pre-operative and post-operative up to 48h) and buprenorphine (5mg/kg, s.c., pre-operative) were administered to all animals. Body temperature was monitored and maintained using a heating pad during the entire surgical procedure. Using a stereotaxic frame, 1 mm diameter craniotomy was performed with 0.7 mm drill bit 2 mm lateral and 2.3 mm caudal to the bregma on the right side of the midline¹¹. In case of accidental disruption of the meninges, the animal was excluded from the study. A female luer-lock hub was implanted at the craniotomy site and secured with dental cement and connected to fluid percussion injury (FPI) device. An impact ranging from 2.5 – 3.0 atm. was administered. Sham animals underwent all surgical procedures except the impact delivery. The luer-lock was detached, the craniotomy hole sealed with bone wax and the scalp was sutured. Rats were returned to their home cages and allowed to recover for 35 days prior to subsequent experiments.

Drug and cell administration.

Pioglitazone hydrochloride (PG) (Tocris, Inc.) dissolved in dimethyl sulfoxide (DMSO) at a dose of 2mg/kg in 100uL or equal volume of vehicle was injected intraperitoneally (i.p.) once a day for 5 days after TBI.

hMSCs were obtained from the Institute for Regenerative Medicine, Texas A&M Health Science Center. Cells were cultured in α Minimum Essential Medium (α MEM) (Gibco, cat#12561-056) supplemented with 16.5% FBS, 2mM L-glutamine and 1% penicillin and streptomycin at 37°C and 5% CO₂. At 90 - 95% confluence, cells were detached, washed and re-suspended in sterile PBS. The final volume of the cell suspension was adjusted so that 1×10^6 live cells were present in 100 μ L of cell suspension. On 5-day post injury (dpi), hyaluronidase was applied to each nostril (100U) and after 30 min

hMSCs were instilled through the nostrils (50 μ L/nostril) under isoflurane anesthesia. Rats were allowed to recover on a heating pad and returned to their home cage. hMSCs labeled with 1 μ g/mL DiR (XenoLight DiR, Caliper Lifesciences) were washed and resuspended in required volume of sterile PBS. Rats were injected with 1×10^6 hMSCs either through tail vein (i.v.) in 200 μ l or by i.n. route 50 μ l per nostril. Animals were imaged after 7 days by IVIS system using 710 nm excitation and 760 nm emission. Radiant efficiency of the region of interest (ROI) was plotted for each organ and each method of administration.

Open field test (OFT).

After 7 days of acclimation and gentle handling, baseline activity in the open field arena (90 cm(W) x 90 cm (D) x 40 cm (H) cm enclosure) was recorded for 10 min using Noldus Ethovision XT 10 software. Open field test (OFT) was recorded for 10 min on 35 dpi. Rats were gently placed in the center of the arena and allowed 2 min to acclimatize in the arena before start of the recording. The arena was cleaned with 70% alcohol and dried in between rats. Activities in the peripheral and central zone were analyzed. Grooming and rearing behaviors were analyzed independently by 3 people blinded of the experimental conditions and averaged.

Cold plantar assay.

The Cold plantar assay was performed to assess the sensorimotor behavior. On 35 dpi rats were held gently in the lap by one experimenter with the hind paws hanging freely. A small portion of powdered dry ice compacted in the shape of a stick was pushed out from the cylinder of a syringe with the plunger and wrapped in a nitrile glove. The wrapped dry ice was touched to the plantar of the freely hanging hind paws, one paw at a time and the time of paw withdrawal was recorded. Care was taken that the paw movement was not hindered by any means. An additional group of naïve animals served as controls.

Euthanasia, tissue harvest and processing.

On 35 dpi animals were deeply anesthetized with ketamine (75 mg/kg) and xylazine (7.5 mg/kg). Blood was collected and rats were perfused with 0.9% saline

followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4). The brains were post-fixed in 2% PFA, saturated with sucrose solution, frozen and 30 µm coronal cryo-sections were generated.

Thionin and Fluor Jade staining.

5 Sections were treated with 1.25% thionin acetate solution (Sigma Aldrich, USA) for 45 seconds followed by rinsing with deionized water for 1 min., dehydrated with graded ethanol, cleared with xylene and coverslipped with DPX mounting medium (Electron Microscopy Sciences, Ft. Washington, PA). For fluor jade (FJ) staining^{65,66} slide mounted sections were hydrated in graded ethanol and then oxidized with 0.06% 10 KMnO₄ solution for 15 minutes and stained in a 0.001% solution of FJ (Histochem, Jefferson, AR) in 0.1% acetic acid for 30 min. Slides were rinsed, dried at 45°C for 20 min, cleared with xylene, and cover-slipped using DPX mounting medium.

Immunohistochemistry.

For immune-peroxidase staining, sections were incubated in 3% hydrogen peroxide 15 for 20 min, washed with PBS and heated in antigen unmasking solution (1:100; Vector Laboratories Inc., Burlingame, CA) for 40 min at 90°C, cooled, permeabilized for 1 hr in permeabilization buffer (10% host serum, 0.1% Triton X-100 in PBS) and incubated overnight at 4°C with primary antibody in antibody solution (5% host serum, 0.05% Triton X-100 in PBS). Next day, sections were washed with PBS, incubated sequentially with 20 biotinylated secondary antibody (2h, RT) and avidin-biotin complex mixture (ABC, 1:100; Vector Laboratories, Inc., Burlingame, Ca) (1h, RT) and developed using DAB/peroxide solution (Vector Laboratories, Inc.). After 3 washes, sections were dried, dehydrated, cleared with xylene and cover-slipped with DPX. For fluorescence immunohistochemistry, no peroxide blocking was performed. After primary antibody 25 incubation sections were incubated with fluorescent secondary antibody, washed with PBS, dried and cover slipped with vectashield anti-fade mounting medium with DAPI. Detailed information on antibodies used in this study is shown in the table in FIG. 11. Sections were viewed with Olympus IX71 microscope using appropriate filters. Images were captured using Olympus DP70 imaging system. The low magnification (4x) collages

of the entire brain sections were taken and processed with a Keyence BZ-X800 microscope and associated software (Keyence America).

Enzyme Linked Immunosorbent Assay (ELISA).

Serum samples were thawed on ice for ELISA development using Picokine rat BDNF ELISA kit from myBiosource (San Diego, USA, Cat# MBS175935) following manufacturer's instruction. Briefly, standards or samples were added to wells of a 96 well ELISA plate pre-coated with anti-BDNF antibody and incubated overnight at 4°C. The plate was washed, and incubated sequentially with biotinylated anti-rat BDNF antibody for 1 h, avidin-biotin-peroxidase complex for 30 min and color development reagent for 25 min at room temperature. Reactions were stopped with 2N H₂SO₄. The absorbance readings were taken at 450nm using a synergy H4 hybrid reader (BioTek). Serum BDNF concentration was expressed as pg/mL.

Image analysis and quantitation

Cell count or intensity was calculated using the NIH Image J software. Images (100x or 200x) were taken at the same exposure and digital gain settings for a given magnification for all the sections in order to eliminate differential background intensity and/or false positive signal. The RGB channels of fluorescent images were split and either the red or green channel was used for Quantitation. The bright field images were converted to grey scale. The brightness and contrast were adjusted to discard the noise pixels. The threshold of the binary images was adjusted in between 0 and 255 to highlight all positive cells to be counted. In the set measurement tool, the particle sizes were adjusted to exclude the small noise pixels or the large clumps from the count. Circularity was adjusted in between 0 and 1 to discard any cell fragments, cell processes, or tissue aggregates that can create false results. The same specifications were used to quantitate across the board. The number of cells were counted using Analyze>Particle tool. The fluorescence intensity (integrated density) was measured using Analyze>Measure tool. For lesion volume measurement, the scale was calibrated using set scale tool and lesion area was measured from each section. Lesion volume of each section was calculated by multiplying the lesion area of each section with corresponding section thickness. The

values were then added up to obtain total lesion volume from each brain and expressed as mm³.

Statistical analysis

All data are presented as mean $\pm$ Standard Error of Mean (S.E.M.). Statistical significance was evaluated by one-way ANOVA with Tukey's or Fisher's post-hoc test or student's t-test. A p value of less than 0.05 was considered statistically significant for all comparisons.

Example 1: Intra-nasally administered hMSCs were delivered to the brain post TBI.

Intranasal (i.n.) delivery of hMSCs to the brain was confirmed in a sub set of rats. DiR labeled hMSCs were delivered through i.n. or intra-venous (i.v.) routes. FIG. 1A depicts xenogen images showing DiR fluorescence in different organs 7 days after TBI. FIG. 1B is a histogram showing the significantly higher level of DiR fluorescence in brain and spleen following intra-nasal (i.n.) administration as compared to intra-venous (i.v.) administration (left bar = naïve, middle bar = i.n., right bar = i.v.). FIG. 1C depicts brain sections from rats after 35 days of TBI were stained with anti-human nuclear antigen (HuNu) antibody (clone 235-1). The immunostaining indicates the presence of hMSCs (arrows) in the ipsilateral cortex close to the lesion site. *p<0.01. The i.n. delivery showed significantly higher DiR signal in the brain compared to i.v. delivery (Fig 1B). IVIS imaging showed the presence of DiR fluorescence in brain, lung, liver and spleen after 7 days of hMSC administration. DiR fluorescence indicate that a large portion of the hMSCs delivered by i.v. route was cleared by the system in 7 days, whereas the i.n. delivered hMSCs stayed in the system. Presence of hMSCs in the brain 35-days post impact (dpi) was confirmed by immunohistochemical staining of the brain sections with anti-human nuclear antigen (clone 235-1) antibody (HuNu). The immunostaining showed the existence of HuNu positive nuclei in the rat brain cortex close to lesion area in hMSC or combination treated rats (FIG. 1C). FIG. 1D is a histogram showing the percentage of HuNu positive cells in the perilesional area of the brains. HuNu positive cells which were also DAPI positive were counted from the perilesional area of each section located 120 μ apart. Also, 50 DAPI

positive cells were counted from the same area of the sections. % of HuNu positive cells was calculated from 7 dpi and 35 dpi brains as shown in the histogram. A paired t test did not show any significant difference between the % of HuNu positive cells present in the brains of 7 dpi or 35 dpi. $n = 3$ (7 dpi), and 7 (35 dpi). A few scattered HuNu-DAPI double positive cells were observed in the treated brains. The number of HuNu positive cells in the perilesional area of the brains observed at 7 dpi did not change significantly at 35 dpi.

Example 2: Combination treatment reduced the pathological changes and neurodegeneration in the rat brain cortex.

Thionin staining shows tissue loss and decreased cellularity in the TBI and vehicle treated animals as compared to sham. Significant cortical tissues loss was evident after 35 dpi. Lesion volume in the vehicle treated group was not significantly different from the TBI alone group. FIGS. 3A and 3B show that hMSC or PG+hMSC combination treatment reduces the brain damage and neuroinflammation 35 days after TBI. FIG. 3A is a representative image of brain sections after treatment. Thionin images are low magnification images (scale bar 500 μ), showing tissue loss. Remaining panels in FIG. 3A show degenerating cells and fibers (FJ panel), microgliosis (Iba1 panel), or astrogliosis (GFAP panel) in the ipsilateral cortex 35 days after TBI. FIG. 3B depicts histograms showing image J quantitation. The histograms show quantities of lesion volume, therefore expressing the average tissue loss (FIG. 3B; first (left) bar represents sham, second bar represents TBI, third bar represents vehicle, fourth bar represents PG, fifth bar represents hMSC, and sixth bar represents combo). FJ and Iba1 was measured by counting positive cells and GFAP was measured by integrated density using image J. PG, pioglitazone, hMSC, human mesenchymal stem cells, combo., PG+hMSC combination treatment. $*p < 0.01$, $**p < 0.001$ vs TBI, $***p < 0.0001$. Scale bar 100 μ . FIGS. 3A and 3B show that PG treated animals showed some

reduction in the lesion volume but the average was not statistically significant. On the other hand, lesion volume decreased significantly after hMSC or combination treatment.

Neurodegeneration is evident in the TBI animals 35 dpi. FJ staining in the rat brain shows damaged FJ positive cells and fibers in the ipsilateral cortex around the impact site and in the lateral part of the cortex, around primary somatosensory area in TBI and the vehicle treated groups. White matter damage was also visible in these brains in the ipsilateral corpus callosum and striatum. The treated groups (PG, hMSC or combination) showed significantly fewer degenerating cells or fibers than the TBI or vehicle groups, but there was no significant difference among the treatment groups (FIG. 3A-FJ panel, B). FJ positive fibers were also observed in corpus callosum and striatum in TBI animals. Contralateral brain did not show cellular or fiber damage in animals from any of the groups.

Example 3: Effect of PG + hMSC combination treatment on TBI induced neurodegeneration and microgliosis.

FIG. 4A depicts fluorojade expression in the corpus callosum. FIG. 4B depicts representative immunofluorescence images showing the Iba1 positive microglia in different brain regions of the ipsilateral side. White dotted lines indicate the boundaries of the corpus callosum. Scale bar 100 μ . FIG. 4C is a histogram of the average number of Iba1 positive microglia (mean $\pm$ SEM) in ipsilateral corpus callosum. FIG. 4D is a histogram of the average number of Iba1 positive microglia (mean $\pm$ SEM) in lateral cortex. In FIGS. 4C and 4D, first (left) bar is sham, second bar is TBI, third bar is vehicle, fourth bar is PG, fifth bar is hMSC, and sixth bar is combo. C.C., Corpus callosum, L.Cort., lateral cortex, PG, pioglitazone, hMSC, human mesenchymal stem cells, Comb., PG + hMSC combination treatment. Numbers in the parentheses indicate number of animals in each group. * $p < 0.01$. ** $p < 0.001$, *** $p < 0.0001$.

Example 4: Effect of combination treatment on neuroinflammation.**Microgliosis:**

Microgliosis, as indicated by increased number of Iba1 positive microglia in the cerebral tissue, was observed 35 dpi in TBI as well as vehicle treated animals. The number of Iba1 positive microglia significantly increased in TBI animals as compared to sham. Increased number of microglia were observed in the ipsilateral cortex (adjacent to the injury site) (FIG. 2A - Iba1 panel, FIG. 2B - Iba1+ cells), lateral part of the cortex and corpus callosum (FIG. 4A). All the treatment groups showed significantly fewer Iba1 positive microglia in these areas compared to TBI or vehicle treated groups in the cortex, the combination treatment group showed significantly fewer Iba1 positive cells as compared to TBI, vehicle or PG treated animals (FIG. 2B-Iba1+ cells).

FIGS. 5A-5D show the effect of combination treatment on microglial activation. FIG. 5A depicts representative immunofluorescence images showing the Iba1 positive microglia in different brain regions of the contralateral hemisphere. White dotted lines indicate the boundaries of the corpus callosum. Scale bar 100 μ . FIGS. 5B-5D show average number of Iba1 positive microglia (mean $\pm$ SEM) in ipsilateral cortex (FIG. 5B), corpus callosum (FIG. 5C) and lateral cortex (FIG. 5D). C.C., Corpus callosum, L. Cort., lateral cortex, PG, pioglitazone, hMSC, human mesenchymal stem cells, Combo., PG + hMSC combination treatment. Numbers in the parentheses indicate number of animals in each group. * $p < 0.01$. ** $p < 0.001$, *** $p < 0.0001$. In FIGS. 5B-5D, first (left) bar is sham, second bar is TBI, third bar is vehicle, fourth bar is PG, fifth bar is hMSC, and sixth bar is combo. The number of Iba1 positive cells also increased in the contralateral hemisphere of these areas but not as much as the corresponding areas of the ipsilateral hemisphere (FIGS. 5A-5D). In the contralateral corpus callosum also, combination treated animals showed significantly fewer Iba1 positive cells as compared to TBI, vehicle, PG or hMSC groups.

Astrogliosis:

Increased GFAP immunoreactivity with scar formation was observed in all other groups as compared to sham (FIG. 3A - GFAP panel, FIG. 3B - GFAP integrated density)

indicating activation of astrocytes 35 dpi. In the TBI and vehicle groups, astroglial activation and glial scars were still observed after 35 dpi. PG treatment reduced the GFAP immunoreactivity significantly as compared to TBI or vehicle treated animals. In hMSC treated animals the GFAP immunoreactivity increased significantly as compared to TBI, vehicle or PG treated groups although close observation did not reveal glial scar formation in these animals. In the combination treated animals GFAP immunoreactivity was high, although not significantly as compared to TBI or vehicle groups, and no glial scars were observed in these brain sections. On the other hand, in this group GFAP immunoreactivity was significantly reduced from the hMSC treated group (FIG. 3B - GFAP integrated density).

Cytokine expression.

Expression of CCL20 is a landmark of neuroinflammation after TBI. FIG. 6A depicts representative images of immunoperoxidase staining of cortex (adjacent to injury site) showing the CCL20 or IL1- β immunoreactivity under different experimental conditions. Scale bar 100 μ . FIGS. 6B and 6C are histograms showing the numbers (mean $\pm$ SEM) of CCL20 (FIG. 6B) or IL1 β (FIG. 6C) positive cells in the cortex. PG, pioglitazone, hMSC, human mesenchymal stem cells, combo., PG+hMSC combination treatment. * $p < 0.01$, ** $p < 0.001$. In FIGS. 6B-6C, first (left) bar is sham, second bar is TBI, third bar is vehicle, fourth bar is PG, fifth bar is hMSC, and sixth bar is PG + hMSC. According to FIGS. 6A and 6B, after 35 dpi, TBI or vehicle treated animals express significantly more CCL20 in the cortex as compared to sham. FIGS 2A and 2B show results of pioglitazone treatment on sham and animals subjected to TBI. FIG. 2A is a set of micrographs of a sham animal administered a vehicle, a TBI animal administered a vehicle, sham animal administered pioglitazone, and a TBI animal administered pioglitazone. FIG. 2B shows a bar graph of immunoreactivity analysis of a sham animal administered a vehicle, a TBI animal administered a vehicle, sham animal administered pioglitazone, and a TBI animal administered pioglitazone. In FIG. 2B, the left bar in each pair of bars shows the CCL20 level, while the right bar in each pair of bars shows the PPAR γ level. FIGS. 2A and 2B show that CCL20 expression was up-regulated and PPAR γ was downregulated in the

brain 48h post TBI. Also, PG treatment reduced the CCL20 expression and increased the PPAR γ expression 48h post TBI. In the present study, PG treatment reduced the CCL20 expression 35 dpi. Both hMSC treatment as well as combination treatment reduced the CCL20 expression in the cortex. Importantly, CCL20 expression in the ipsilateral cortex in the combination treatment group was significantly lower than that in hMSC treatment group indicating better efficacy of the combination treatment (FIGS. 6A and 6B). The expression of IL1- β in the ipsilateral cortex was also increased in the TBI or vehicle treated groups and decreased after PG, hMSC or combination treatment. IL1- β expression decreased significantly in the combination treated group as compared to hMSC treated group (FIGS. 6A and 6C). This cytokine expression profile indicates that PG treatment prior to hMSC therapy reduced the proinflammatory microenvironment facilitating the performance of hMSC.

Example 5: PG-hMSC combination treatment increased neurogenesis.

Neurogenesis is an important indicator of recovery from TBI. FIG. 7A depicts representative immunofluorescence images showing DCX immunoreactivity in the SVZ region or DG region under different experimental conditions. Scale bar 20 μ . FIG. 7B is a histogram showing DCX immunoreactivity (mean $\pm$ SEM) in SVZ under different experimental conditions measured using image J. FIG. 7C is a histogram showing DCX immunoreactivity (mean $\pm$ SEM) in DG under different experimental conditions measured using image J. FIG. 7D is a histogram showing serum BDNF level measured by ELISA. IntDen, integrated density, PG, pioglitazone, hMSC, human mesenchymal stem cells, SVZ, sub ventricular zone, LV, lateral ventricle, combo, PG+hMSC combination treatment. In FIGS. 7B-7C, first (left) bar is sham, second bar is TBI, third bar is vehicle, fourth bar is PG, fifth bar is hMSC, and sixth bar is PG + hMSC. According to FIGS. 7A-7C, neurogenesis occurs at a basal level in the adult, uninjured animals (sham) in the sub-ventricular zone (SVZ) area of the brain as observed in the experiment. In TBI or vehicle treated animals, neurogenesis significantly decreased as indicated by decreased DCX immunoreactivity. PG or hMSC treatments increased

neurogenesis but not significantly compared to TBI or vehicle treated animals, whereas combination treatment significantly increased neurogenesis in the SVZ region compared to TBI or vehicle groups. In the combination group, neurogenesis was restored to the same extent as observed in sham animals.

5 PG-hMSC combination treatment increased brain derived neurotrophic factor (BDNF) secretion.

The neurotrophic factor BDNF supports neurogenesis. Serum BDNF level as measured by ELISA decreased in TBI and vehicle treated groups. PG treatment restored the level close to sham animals while hMSC and combination treatment significantly
 10 increased serum BDNF level. BDNF level after combination treatment was significantly higher than hMSC treated group (FIG. 7C).

Example 6: Combination treatment reduced the anxiety like behavior of TBI rats in the open field.

15 FIGS. 8A-5G depict results of open field or sensorimotor behavior of rats under different experimental conditions. FIG. 8A depicts representative heat maps of the movement of rats in the open field arena. Baseline behavior (Day 0) was recorded before TBI or sham surgeries. FIGS. 8B-8G are histograms that show the average values (mean $\pm$ SEM) of anxiogenic behaviors of rats in the open field arena. FIGS. 8B-8F are
 20 anxiety like behavior of rats in the open field. FIG. 8B is a histogram showing latency to the first entry to the center zone of the arena. FIG. 8C is a histogram showing time spent in the center zone of the arena. FIG. 8D is a histogram showing frequency to entry to the center zone. FIG. 8E is a histogram showing latency to first immobility in the arena. FIG. 8F is a histogram showing number of grooming events. FIG. 8G is a histogram showing
 25 mean paw withdrawal latency (sec) of the contralateral paw to noxious cold stimulation (mean $\pm$ SEM). PG, pioglitazone, hMSC, human mesenchymal stem cells, Combo, PG+hMSC combination treatment. * p <0.05, ** p <0.01, *** p <0.001. In FIGS. 8B-8G, first (left) bar is sham, second bar is TBI, third bar is vehicle, fourth bar is PG, fifth bar is hMSC, and sixth bar is PG + hMSC.

Behavior in the Center Zone (CZ).

Rat behavior in the open field arena before and after TBI was recorded and analyzed using Noldus Ethovision software as described in the methods section. A heat map was generated showing the activities in the arena (FIG. 8A). As a general tendency, rats preferred to stay close to the walls of the apparatus. The baseline (Day 0) behavior showed that the rats were visiting the CZ frequently while staying mostly to the peripheral zone close to the wall of the open field box. The 35 days recording showed changes in the open field behavior of the rats. Both TBI and vehicle treated animals avoided the CZ of the arena, did not spend time in the CZ and when explored for the first time had long latency (FIGS. 8A-8C). On the other hand, as seen in the heat maps, PG, hMSC or combination treated rats explored the CZ more frequently (FIG. 8A), had lower latency to the first entry to the CZ (FIG. 8B), spent more time in the CZ (FIG. 8C) and entered the CZ more frequently (FIG. 8D).

In the open field arena rats were busy in travelling, rearing and occasional grooming. Significant differences were not observed in their locomotor activities in the open field. The distance traveled or the movement velocity were not significantly different between groups indicating that TBI did not affect the locomotor behavior of the rats. FIG. 9 is a pair of histograms that show the average values (mean $\pm$ SEM) of total distance traveled (top histogram) by the rats or mean velocity of movement (bottom histogram) in the open field arena 35 days post TBI. First (left) bar is baseline, second bar is sham, third bar is TBI, fourth bar is vehicle, fifth bar is PG, sixth bar is hMSC, and seventh bar is PG + hMSC combination treatment. No significant difference in the distance traveled or the movement velocity was observed. Numbers in the parentheses indicate number of animals in each group.

Immobility in the open field arena.

Analysis of other anxiety like behaviors in the open field showed that TBI and vehicle treated animals had shorter latency to become immobile (FIG. 8E) exhibiting anxiety-like behavior in the open field. This behavior improved significantly after the combination treatment as the combination group showed the longest latency to become

immobile indicating that they were least anxious in the open field. Combination treatment significantly improved the anxiety like behavior over the hMSC or PG treated groups (FIG. 8E).

Grooming and rearing behavior.

5 TBI and vehicle treated rats showed a significantly increased number of grooming events as compared to sham. PG or hMSC treatment did not alter this behavior. However, combination treatment reduced the number of grooming events significantly as compared to TBI. The number of grooming events was significantly lower from the hMSC group indicating an improvement in behavior (FIG. 8F).

Example 7: Combination treatment improved the TBI induced hyperalgesia.

10 The cold plantar assay showed significant decrease in the withdrawal latency of the contralateral paw in TBI or vehicle treated rats when compared with sham (FIG. 8G). The combination treatment group indicated significant increase in withdrawal latency as compared to control groups (TBI and vehicle). Also, in the combination treatment group withdrawal latency increased significantly from the hMSC treated group (FIG. 8B). However, no significant difference in withdrawal latency in the ipsilateral paw was observed. This observation indicates that the combination treatment helped in recovering from TBI-induced hyperalgesia.

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WHAT IS CLAIMED IS:

1. A method of treating traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells.

2. The method of Claim 1, wherein (a) is administered prior to (b).

3. The method of Claim 1, wherein (a) is administered prior to, and concurrently with, (b).

4. The method of any of Claims 1-3, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 24 hours prior to the administration of (b) mesenchymal stromal cells.

5. The method of any one of Claims 1-4, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 12 hours prior to the administration of (b) mesenchymal stromal cells.

6. The method of any one of Claims 1-5, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 2 hours prior to the administration of (b) mesenchymal stromal cells.

7. The method of any one of Claims 1-6, wherein between about 5 mg and 100 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

8. The method of any one of Claims 1-7, wherein between about 10 mg and 75 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

9. The method of any one of Claims 1-8, wherein between about 25 mg and 50 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

10. The method of any one of Claims 1-9, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof is administered orally.

11. The method of any one of Claims 1-9, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof is administered intravenously.

12. The method of any one of Claims 1-11, wherein the mesenchymal stromal cells are administered intranasally.

13. The method of any one of Claims 1-11, wherein the mesenchymal stromal cells are administered intravenously.

14. The method of any one of Claims 1-11, wherein the mesenchymal stromal cells are administered intra-arterially.

15. The method of any one of Claims 1-11, wherein the mesenchymal stromal cells are administered intra-cranially.

16. The method of any one of Claims 1-15, further comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, after the administration of (b) mesenchymal stromal cells.

17. The method of Claim 16, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof, is administered for between about 1 day to about 7 days after the mesenchymal stromal cells.

18. The method of any one of Claims 1-17, wherein about 1×10^6 to about 1×10^7 mesenchymal stromal cells are administered to the subject.

19. A method of treating one or more symptoms of traumatic brain injury in a subject in need thereof, comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, and (b) mesenchymal stromal cells.

20. The method of Claim 19, wherein (a) is administered prior to (b).

21. The method of Claim 19, wherein (a) is administered prior to, and concurrently with, (b).

22. The method of any of Claims 19-21, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 24 hours prior to the administration of (b) mesenchymal stromal cells.

23. The method of any one of Claims 19-22, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes and about 12 hours prior to the administration of (b) mesenchymal stromal cells.

24. The method of any one of Claims 19-23, wherein the administration of (a) pioglitazone, or a pharmaceutically acceptable salt thereof, is between about 30 minutes

and about 2 hours prior to the administration of (b) mesenchymal stromal cells.

25. The method of any one of Claims 19-24, wherein between about 5 mg and 100 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

26. The method of any one of Claims 19-25, wherein between about 10 mg and 75 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

27. The method of any one of Claims 19-26, wherein between about 25 mg and 50 mg of pioglitazone, or a pharmaceutically acceptable salt thereof is administered to the subject.

28. The method of any one of Claims 19-27, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof is administered orally.

29. The method of any one of Claims 19-27, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof is administered intravenously.

30. The method of any one of Claims 19-29, wherein the mesenchymal stromal cells are administered intranasally.

31. The method of any one of Claims 19-29, wherein the mesenchymal stromal cells are administered intravenously.

32. The method of any one of Claims 19-29, wherein the mesenchymal stromal cells are administered intra-arterially.

33. The method of any one of Claims 19-29, wherein the mesenchymal stromal cells are administered intra-cranially.

34. The method of any one of Claims 19-33, further comprising administering (a) pioglitazone, or a pharmaceutically acceptable salt thereof, after the administration of (b) mesenchymal stromal cells.

35. The method of Claim 34, wherein the pioglitazone, or a pharmaceutically acceptable salt thereof, is administered for between about 1 day to about 7 days after the mesenchymal stromal cells.

36. The method of any one of Claims 19-35, wherein about 1×10^6 to about 1×10^7 mesenchymal stromal cells are administered to the subject.

37. The method of any one of Claims 19-36, wherein the one or more symptoms are selected from: unconsciousness, inability to awaken, headache, vomiting, nausea, convulsions, lack of motor coordination, slurred speech, aphasia, dizziness, difficulty balancing, lightheadedness, fatigue, altered sleep patterns, confusion, deficits in short-term memory, lack of concentration, pupil dilation, loss of coordination, restlessness and/or agitation, loss of social judgment, muscle weakness, and difficulty processing/understanding emotions.

38. The method of any one of Claims 19-37, wherein the one or more symptoms comprise two or more symptoms.

39. The method of any one of Claims 19-37, wherein the one or more symptoms comprise three or more symptoms.

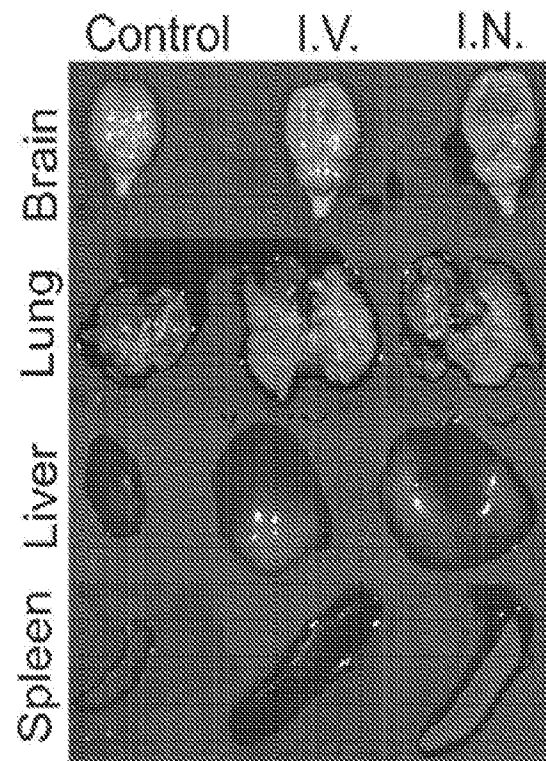
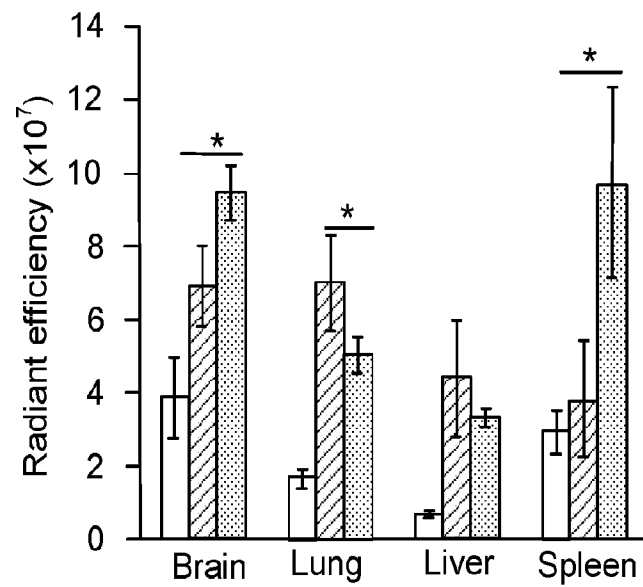
40. The method of any one of Claims 19-38, wherein the one or more symptoms comprise two to five symptoms.

41. A method of treating a patient experiencing symptoms associated with traumatic brain injury as substantially disclosed herein.

42. A method of improving the efficacy of stem cells by administering an anti-inflammatory drug as substantially disclosed herein.

43. The method of Claim 42, wherein the anti-inflammatory drug is pioglitazone.

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**FIG. 1A****FIG. 1B**

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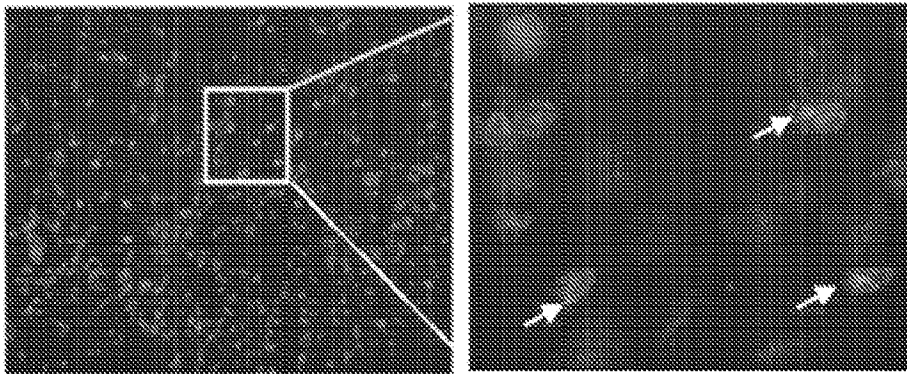


FIG.1C

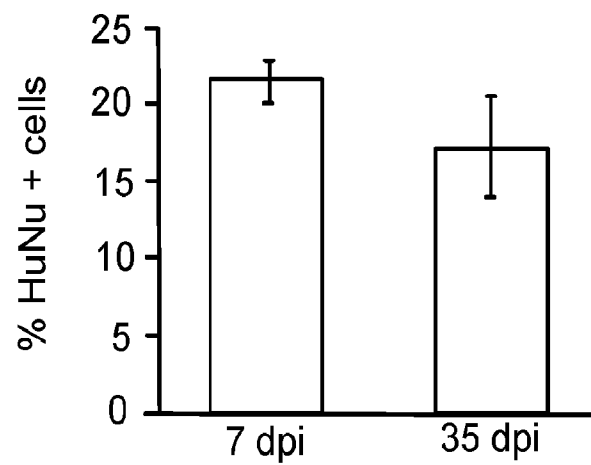


FIG. 1D

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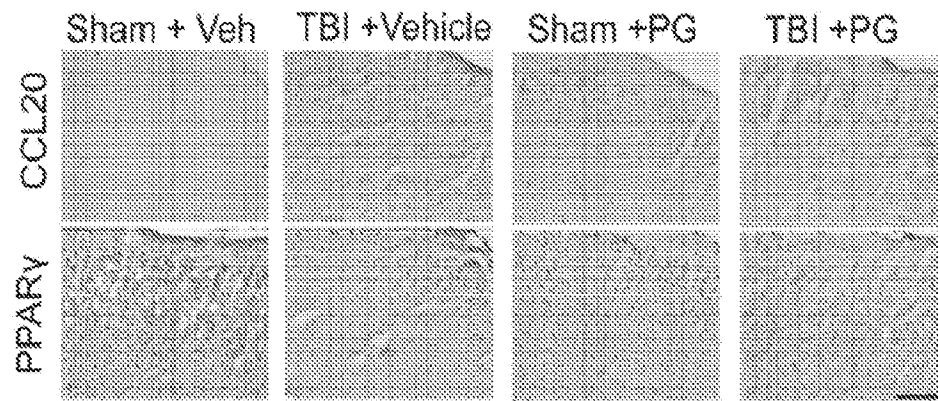


FIG. 2A

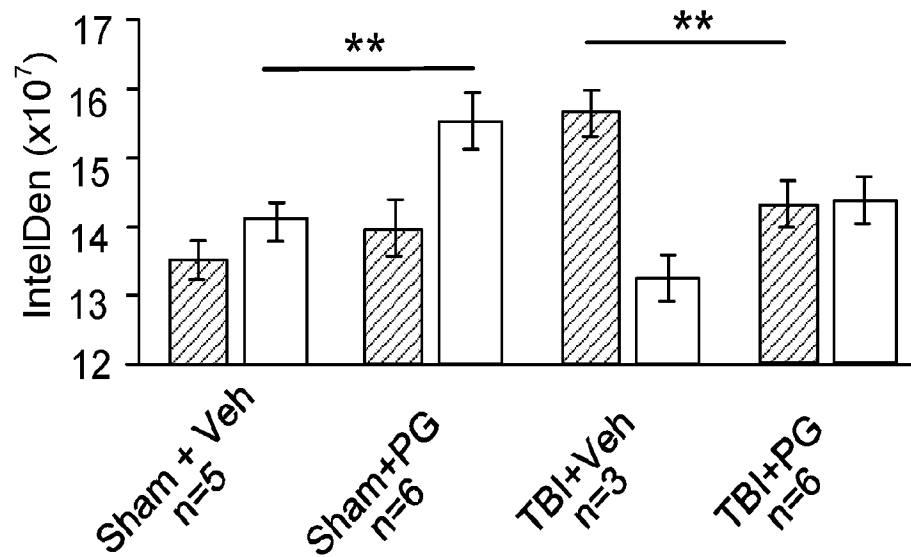


FIG. 2B

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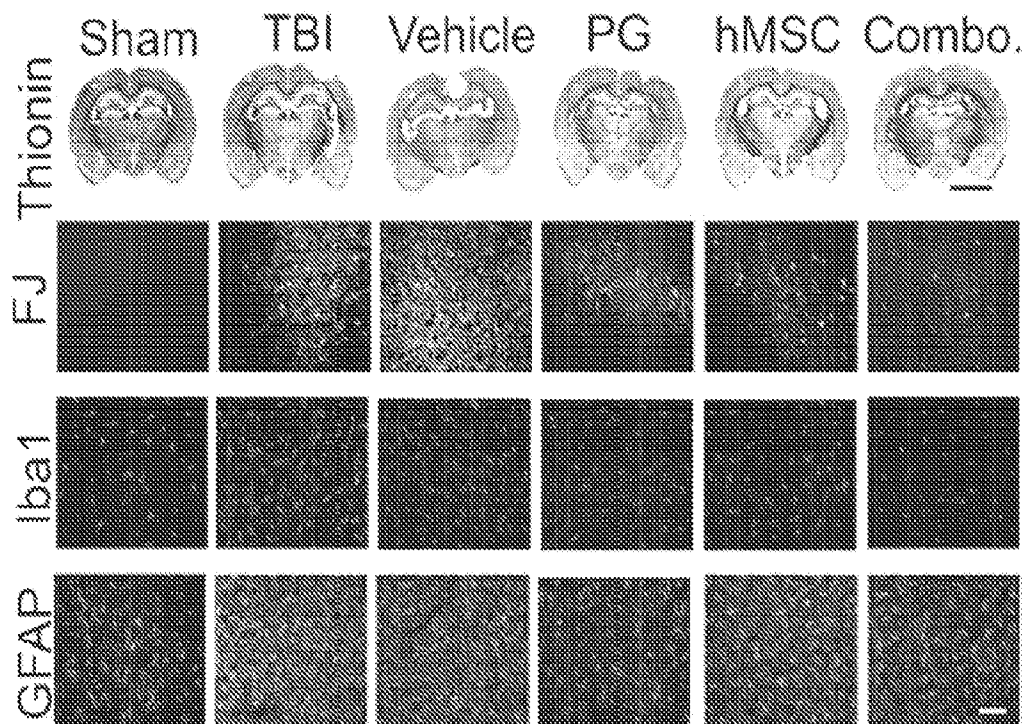


FIG. 3A

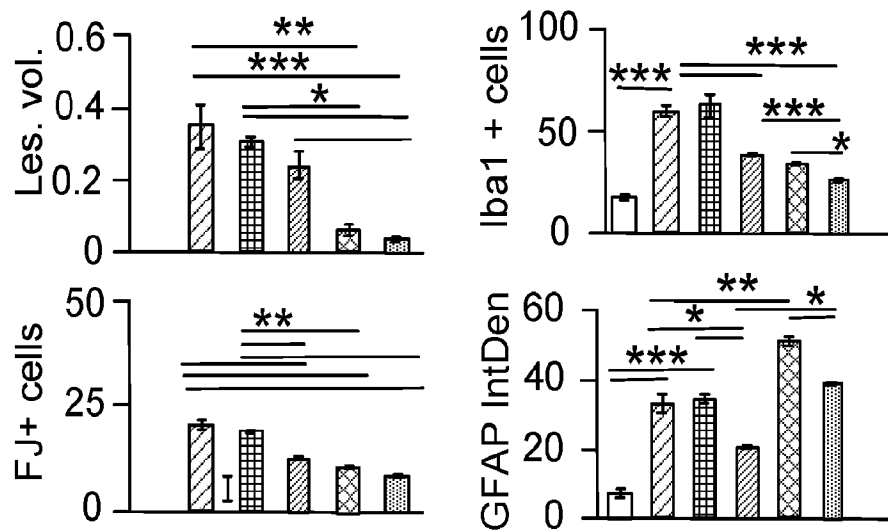


FIG. 3B

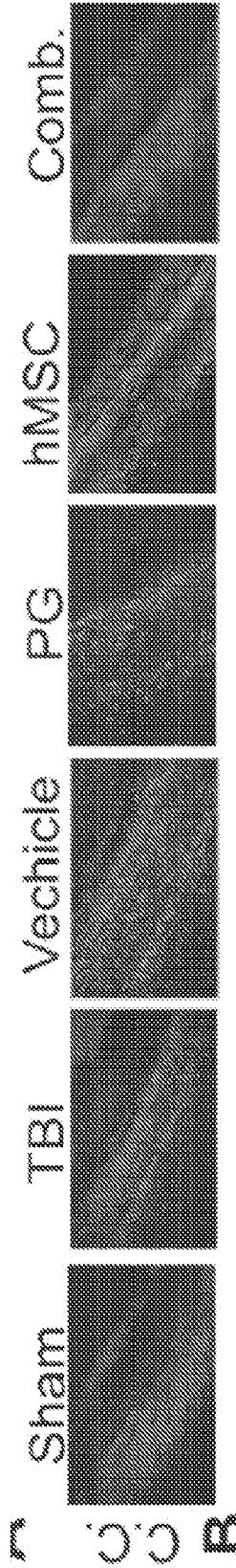


FIG. 4A

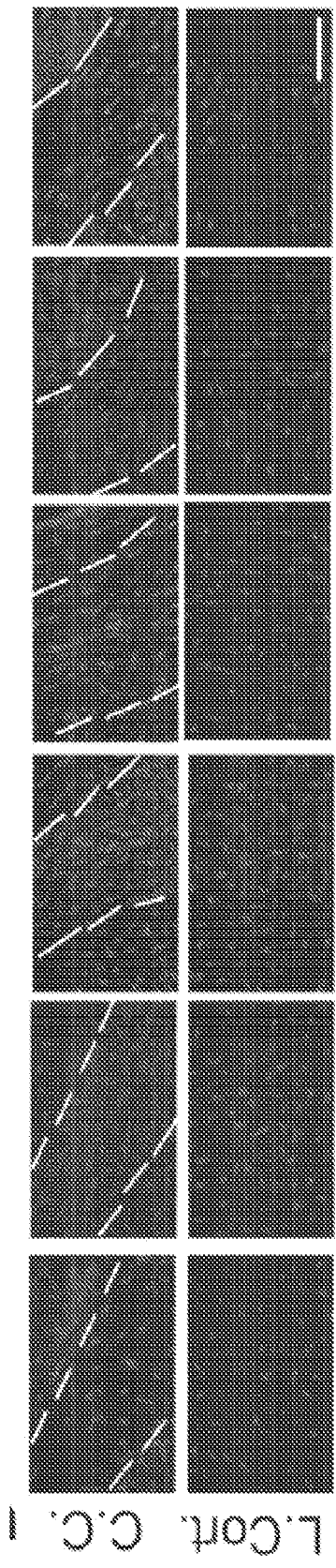
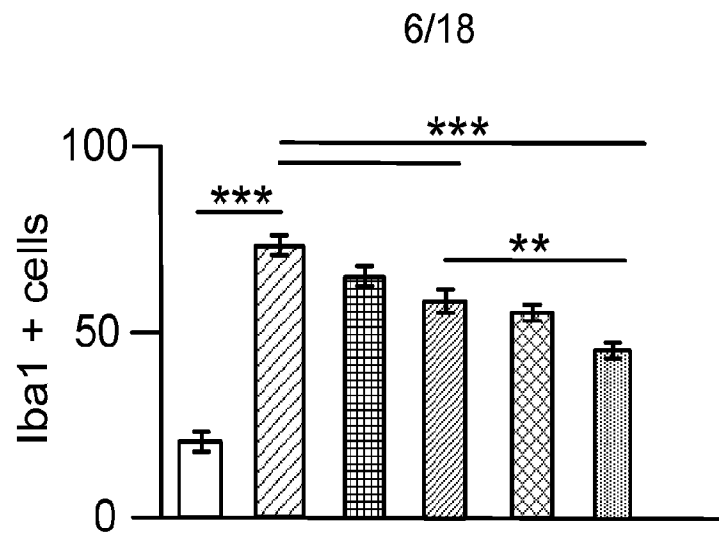
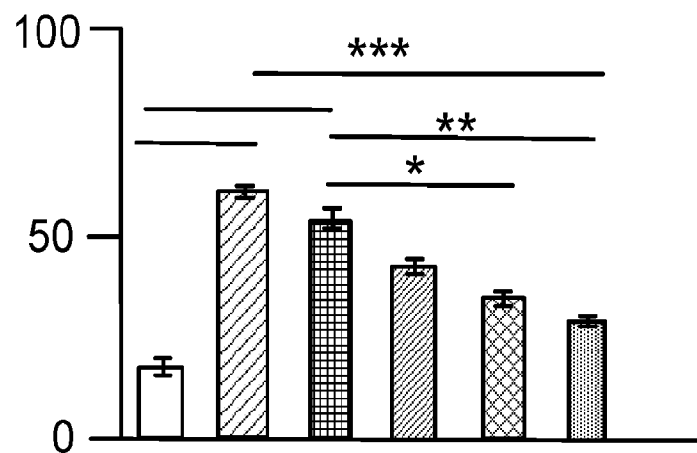


FIG. 4B

**FIG. 4C****FIG. 4D**

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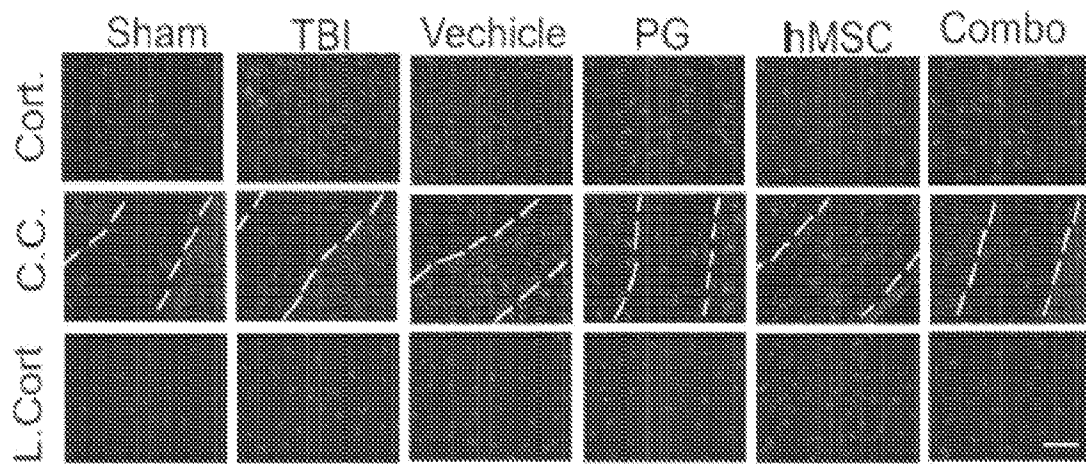


FIG. 5A

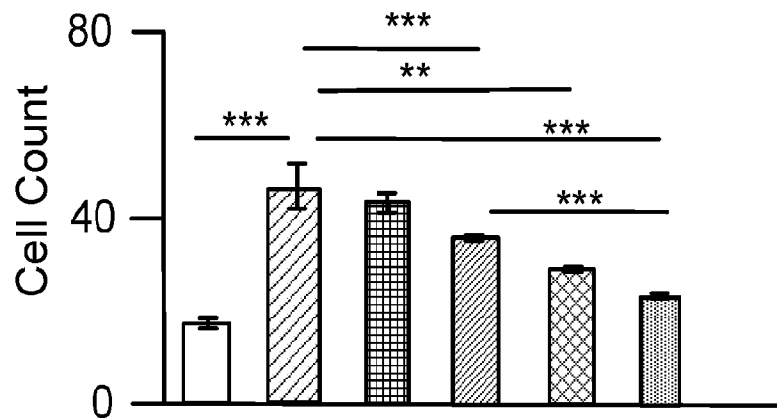


FIG. 5B

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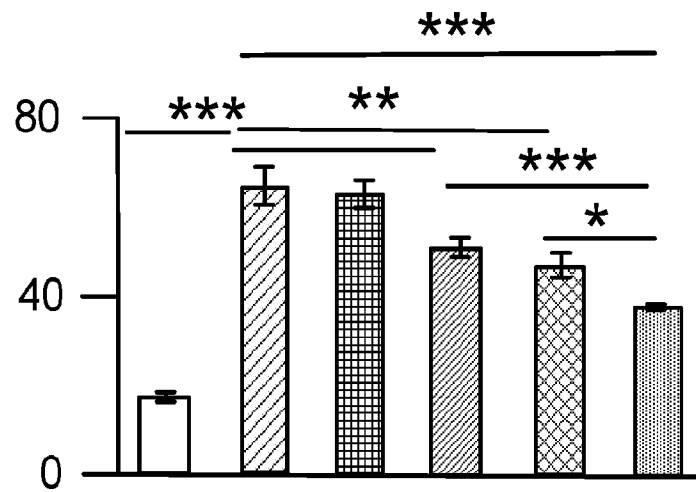


FIG. 5C

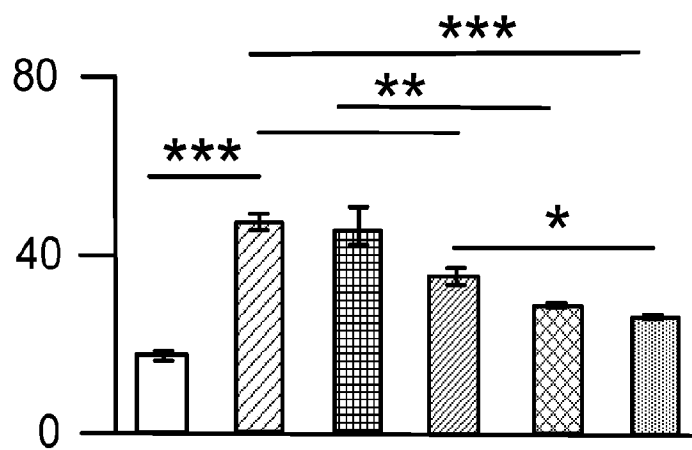


FIG. 5D

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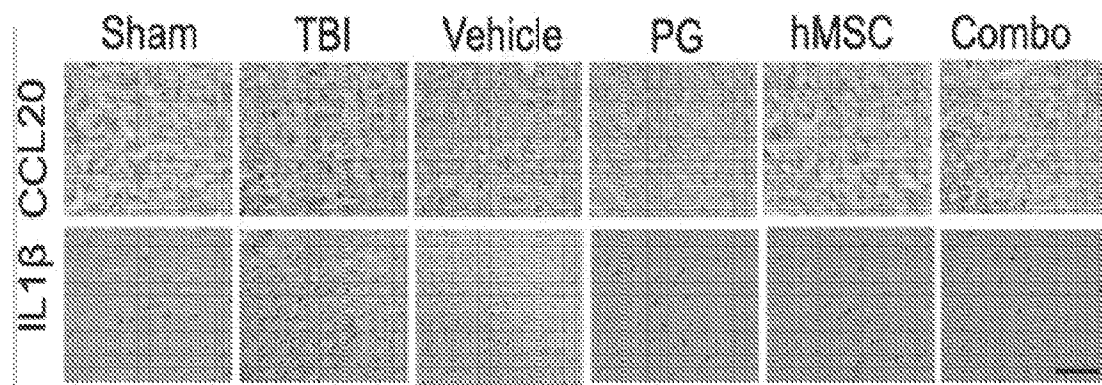


FIG. 6A

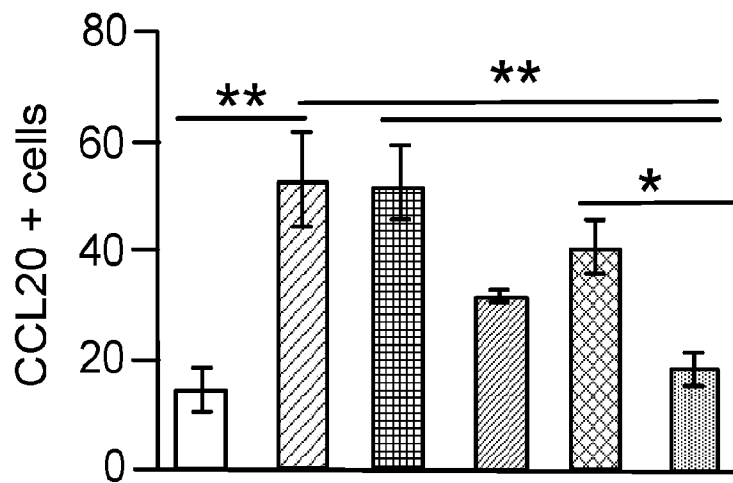
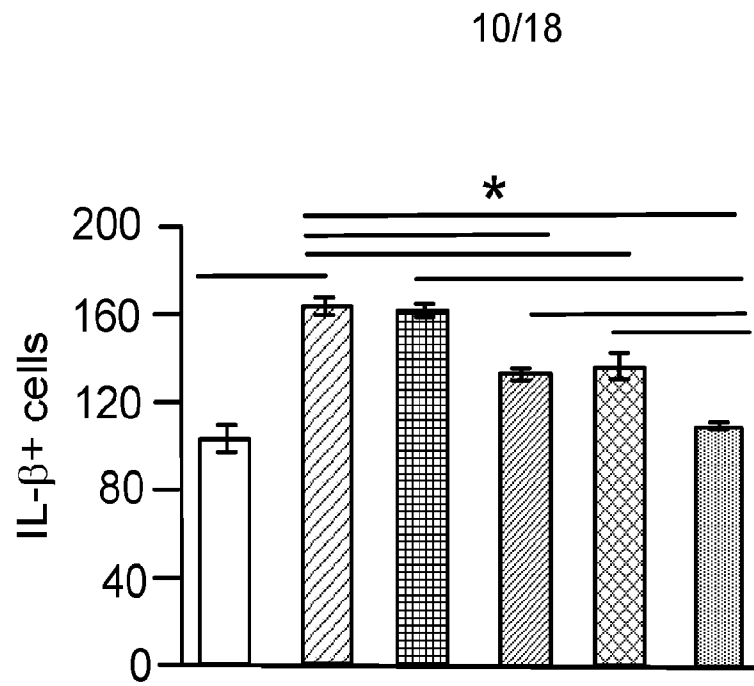


FIG. 6B

**FIG. 6C**

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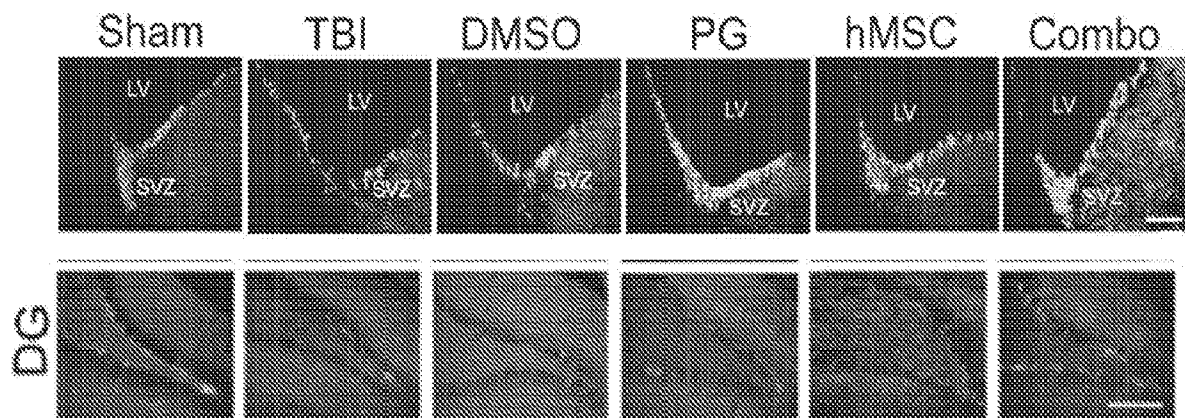


FIG. 7A

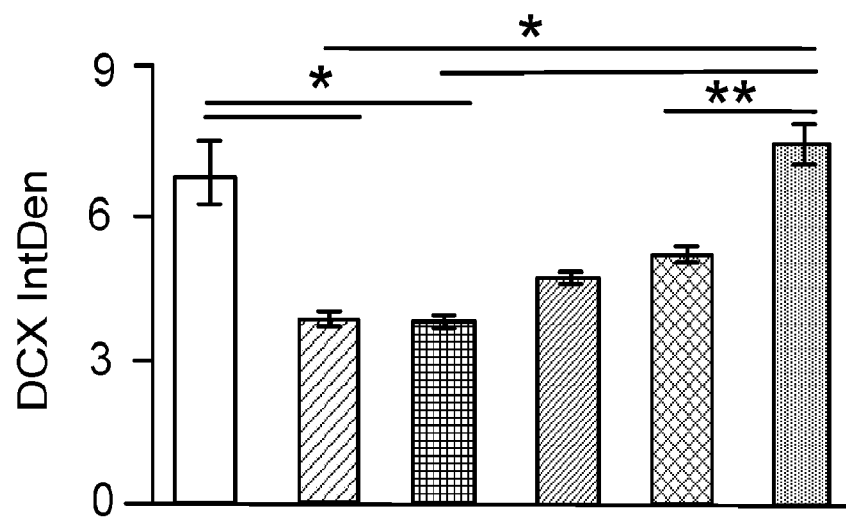


FIG. 7B

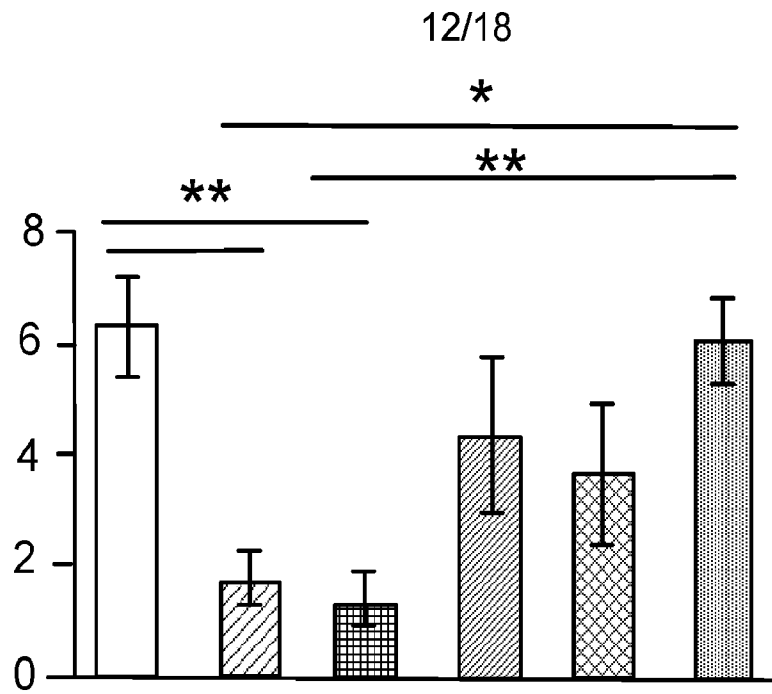


FIG. 7C

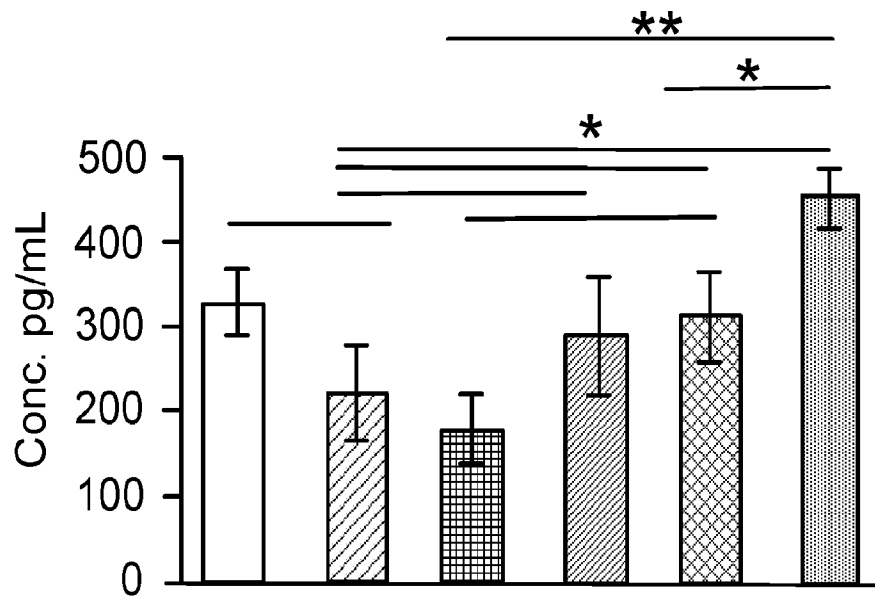


FIG. 7D

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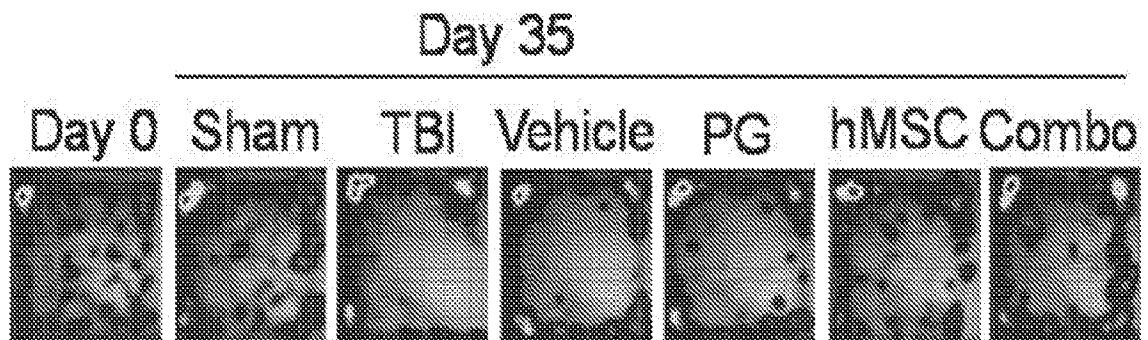


FIG. 8A

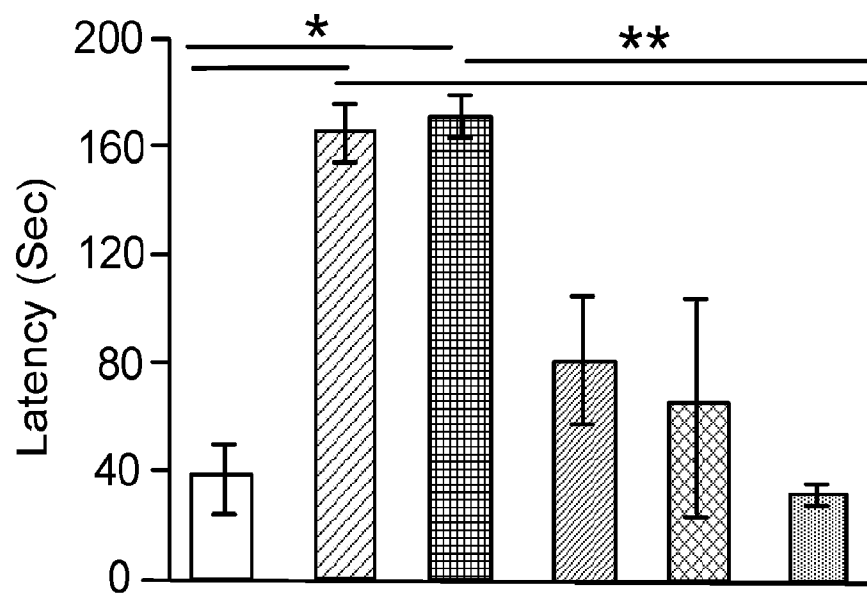


FIG. 8B

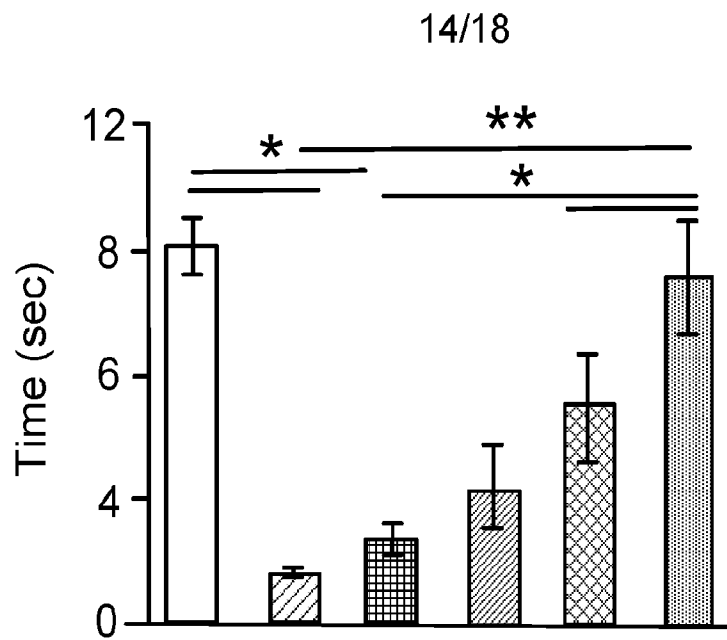


FIG. 8C

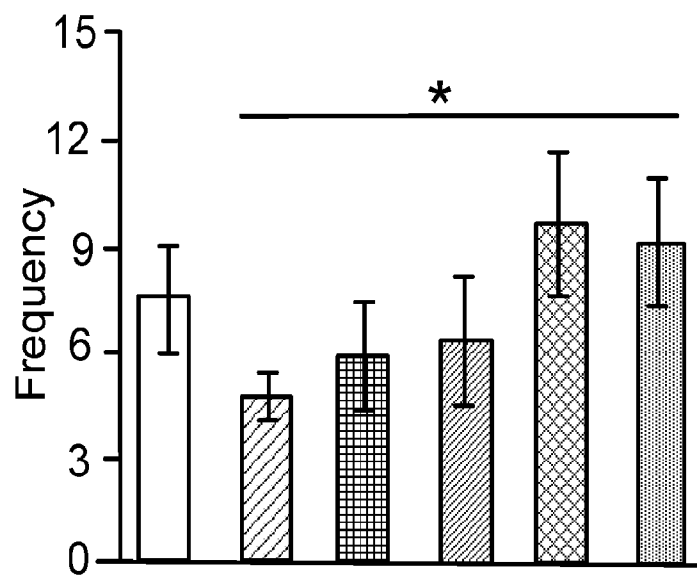
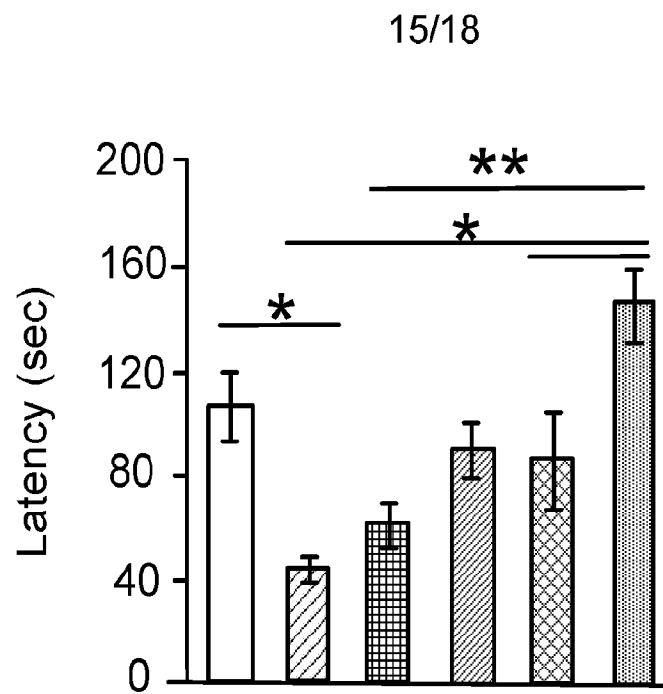
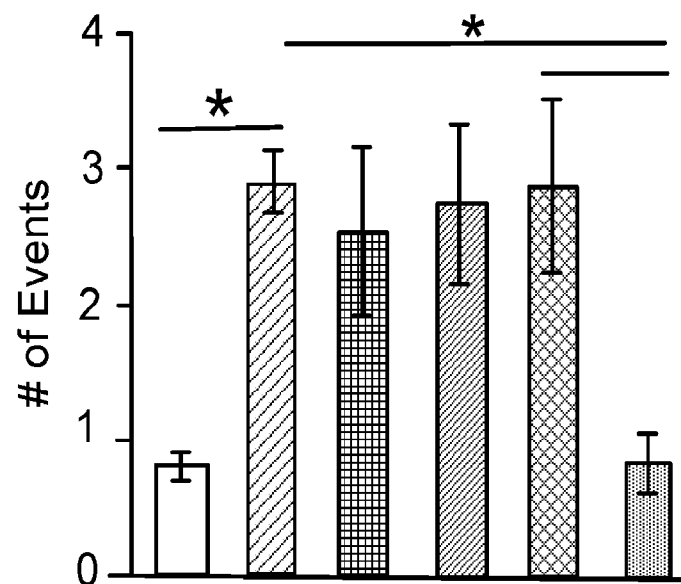


FIG. 8D

**FIG. 8E****FIG. 8F**

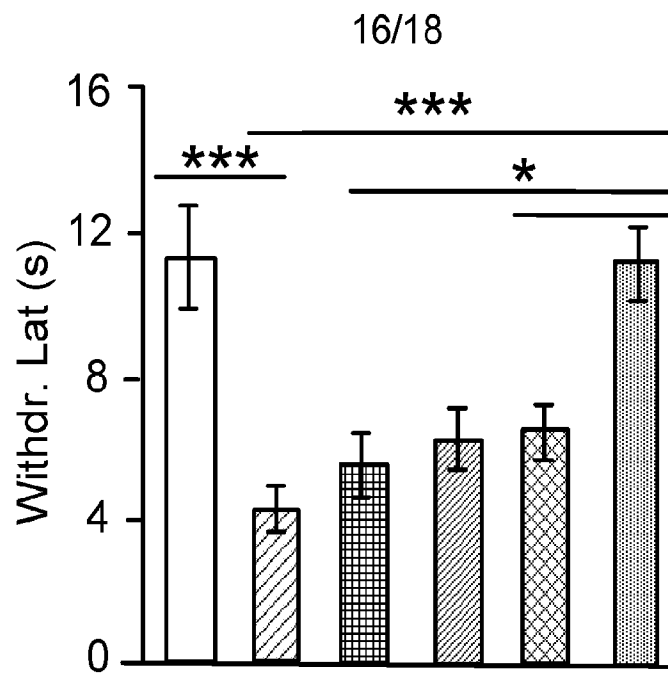


FIG. 8G

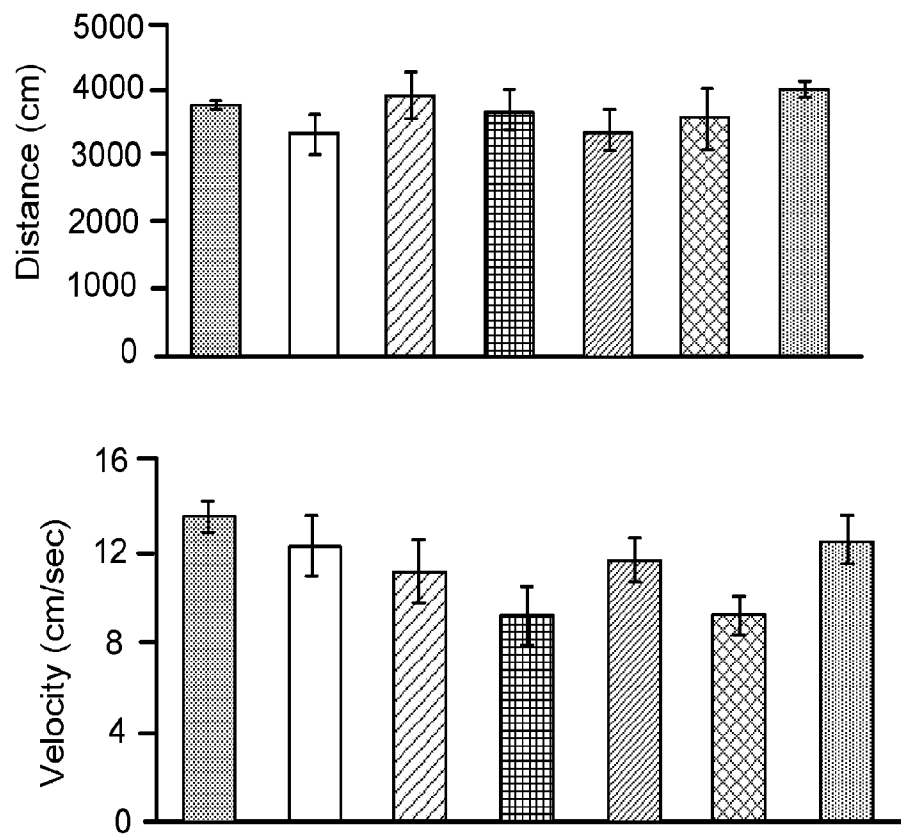


FIG. 9

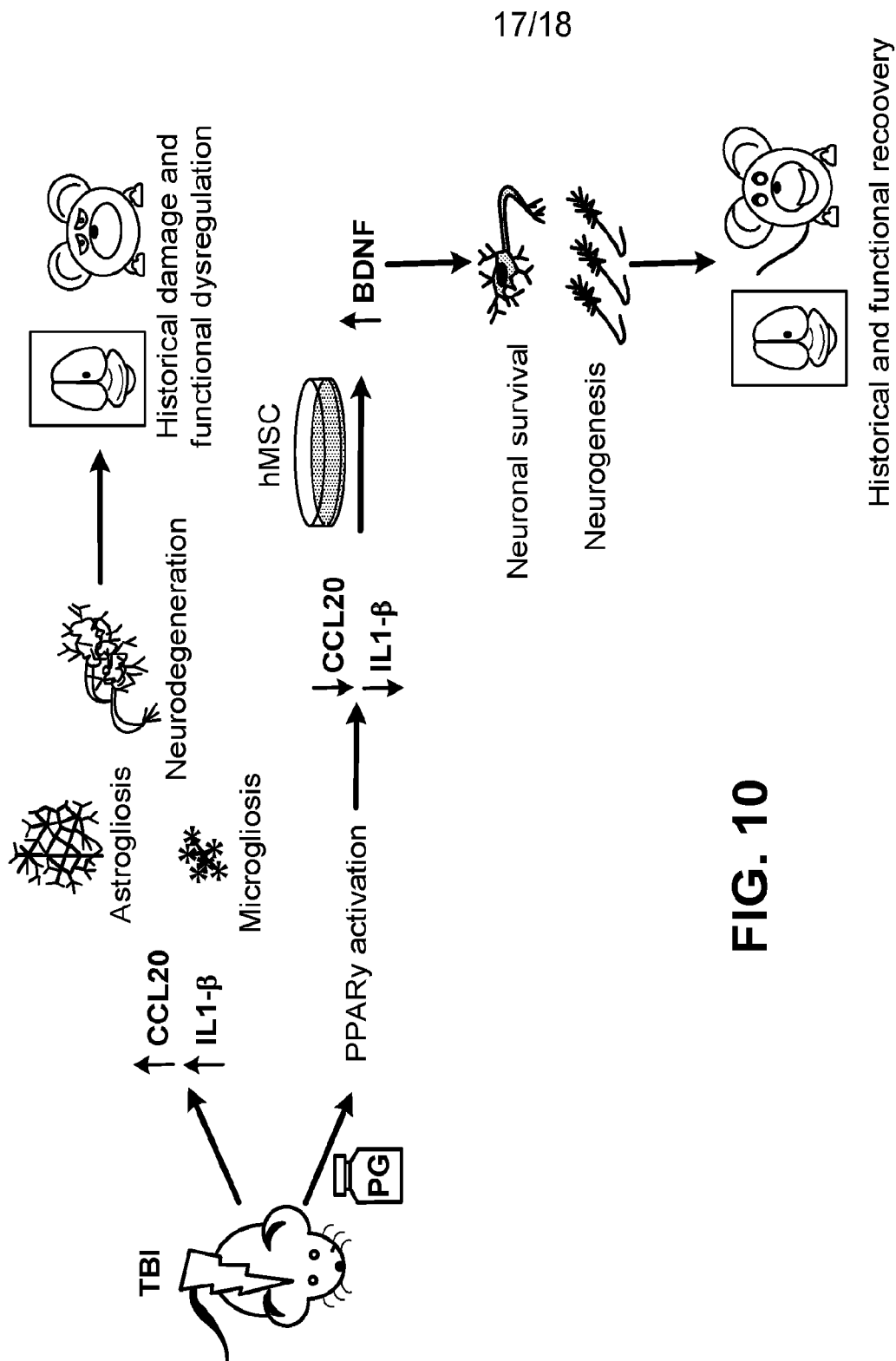


FIG. 10

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Primary antibodies and corresponding secondary antibodies used for immunohistochemistry in the experiment.								
Primary antibody	Source	Catalogue number	dilution	Secondary anti-body	Source	Catalogue number	dilution	development
Anti-human Nuclear Antigen (Clone 235-1)	Abcam, Cambridge, MA	Ab191181	1:100	Alexander 594-anti rabbit	Invitrogen	A11008	1:1000	Fluorescence
rabbit anti-CCL20	Abcam, Cambridge, MA	ab25123	1:1000	Biotinylated goat anti-rabbit Vector Laboratories Inc., Burlingame, Ca	Vector Laboratories Inc., Burlingame, Ca	BA-1000	1:400	DAB
rabbit anti-Iba1	Waco Chemicals, CA	019-19741	1:1000	Alexafluor 594-anti rabbit	Invitrogen	A11008	1:1000	Fluorescence
Chicken anti-GFAP	Invitrogen	AB5541	1:1000	Alexafluor 488-anti chicken	Invitrogen	A11039	1:1000	Fluorescence
rabbit anti-doublecortin (DXC)	Abcam	Sc-8066	1:250	Alexafluor 488-anti rabbit	Vector Laboratories Inc., Burlingame, Ca	A11008	1:1000	Fluorescence
Rabbit ant-PPARY	Abcam	Ab ab19481	1:1000	Biotinylated goat Anti-rabbit Vector Laboratories Inc., Burlingame, Ca	Vector Laboratories Inc., Burlingame, Ca	BA-1000	1:400	DAB

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/19186

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/4439; A61K 9/00 (2020.01)

CPC - A61K 31/4439; A61K 9/0053; A61P 11/00; A61P 17/00; A61P 17/02; A61P 25/00; A61P 25/16

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)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/0338996 A1 (MOHAPATRA et al) 29 November 2018 (29.11.2018), entire document esp abstract, para [0072]	1-4, and 19-22
A	US 2018/0071342 A1 (Creative Medical Technologies, Inc) 15 March 2018 (15.03.2018), entire document	1-4, and 19-22
A	US 2017/0258843 A1 (AngioStem, Inc) 14 September 2017 (14.09.2017), entire document	1-4, and 19-22
A	WO 2013/020136 A2 (Cha Medical University) 07 February 2013 (07.02.2013), entire document	1-4, and 19-22

☐ 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

"D" document cited by the applicant in the international application

"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

01 May 2020

Date of mailing of the international search report

27 MAY 2020

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/19186

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 41-43
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
omnibus claims
3. ☒ Claims Nos.: 5-18, 23-40
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.