(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 June 2010 (03.06.2010)

PCT

(10) International Publication Number
WO 2010/060928 A2

(51) **International Patent Classification**:
A61K 35/30 (2006.01)

(21) **International Application Number**:
PCT/EP2009/065835

(22) **International Filing Date**:
25 November 2009 (25.11.2009)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
08382067.0 26 November 2008 (26.11.2008) EP

(71) **Applicant** *(for all designated States except US)*: **PROJECH SCIENCE TO TECHNOLOGY, S.L.** [ES/ES]; Parque Científico de Madrid, PTM - c/Santiago Grisolía, 2, E-28760 Tres Cantos - Madrid (ES).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **SALAMA COHEN, Patricia** [ES/ES]; Projech Science To Technology, S.l., Parque Científico de Madrid, PTM - c/Santiago Grisolía, 2, E-28760 Tres Cantos - Madrid (ES). **RODRÍGUEZ CIMADEVILLA, Juan Carlos** [ES/ES]; Projech Science To Technology, S.l., Parque Científico de Madrid, PTM - c/Santiago Grisolía, 2, E-28760 Tres Cantos - Madrid (ES).

(74) **Agent**: **ALCONADA RODRÍGUEZ, Agustín**; ABG Patentes, S.L., Avenida de Burgos, 16D, Edificio Euromor, E-28036 Madrid (ES).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2010/060928 A2

(54) **Title**: COMPOSITIONS FOR THE TREATMENT OF ISCHEMIC TISSUE DAMAGE

(57) **Abstract**: The present invention refers to a conditioned medium from neurospheres or an extract thereof for use as a medicament for the treatment of ischemic tissue damage. In particular, the present invention refers to the use of said conditioned media or extracts thereof for the treatment of brain ischemic damage, more particularly for the treatment of stroke.

COMPOSITIONS FOR THE TREATMENT OF ISCHEMIC TISSUE DAMAGE

FIELD OF THE INVENTION

The technical field of the present invention relates to the therapeutic use of conditioned media from neurospheres or an extract thereof. More particularly, said medium can be used for the treatment of ischemic tissue damage, in particular, brain ischemia.

BACKGROUND OF THE INVENTION

Cerebral ischemia is the third ranking cause of death in the United States, and accounts for half of neurology inpatients. Depending on the area of the brain that is damaged, an ischemia can cause coma, paralysis, speech problems and dementia. The five major causes of cerebral infarction are vascular thrombosis, cerebral embolism, hypotension, hypertensive hemorrhage, and anoxia/hypoxia.

The brain requires glucose and oxygen to maintain neuronal metabolism and function. Hypoxia refers to inadequate delivery of oxygen to the brain, and ischemia results from insufficient cerebral blood flow. The consequences of cerebral ischemia depend on the degree and duration of reduced cerebral blood flow. Neurons can tolerate ischemia for 30-60 minutes, but perfusion must be reestablished before 3-6 hours of ischemia have elapsed. Neuronal damage can be less severe and reversible if flow is restored within a few hours, providing a window of opportunity for intervention.

If flow is not reestablished to the ischemic area, a series of metabolic processes ensue. The neurons become depleted of ATP and switch over to anaerobic glycolysis. Lactate accumulates and the intracellular pH decreases. Without an adequate supply of ATP, membrane ion pumps fail. There is an influx of sodium, water, and calcium into the cell. The excess calcium is detrimental to cell function and contributes to membrane lysis. Cessation of mitochondrial function signals neuronal death.. The astrocytes and oligodendroglia are slightly more resistant to ischemia, but their demise follows shortly if blood flow is not restored.

Evidence is also emerging in support of the possibility that acute inflammatory reactions to brain ischemia are causally related to brain damage. The inflammatory condition consists of cells (neutrophils at the onset and later monocytes) and

mediators (cytokines, chemokines, others). Upregulation of proinflammatory cytokines, chemokines and endothelial-leukocyte adhesion molecules in the brain follow soon after an ischemic insult and at a time when the cellular component is evolving. The significance of the inflammatory response to brain ischemia is not fully understood.

After a stroke has occurred, treatment in the acute setting can consist of thrombolytic therapy, surgical resection of large strokes that cause major mass effect and coma, and rare reperfusion techniques such as extracranial-intracranial bypass. Neuroprotective agents such as glutamate receptor inhibitors or inhibitors of excitatory amino acid release were in clinical trials for treatment within the first six to twelve hours of stroke onset. To date, none of these trials has been successful since it is difficult for the stroke victim to reach the hospital within the narrow (3-6 hours) window during which the neuroprotective agents can rescue damaged neuronal cells. Agents that interfere with nitric oxide synthesis or generation of free radicals have also been tested.

The drugs currently used for preventing and treating ischemic diseases include beta blockers, nitrates, calcium channel blockers, etc., which prevent the outbreak of ischemic diseases by reducing oxygen demand in the ischemic area or sustain a therapeutic effect after the outbreak, thrombolytic agents, antithrombotic agents, anti-platelet agents, etc., which are used for reperfusion to the ischemic area after the ischemic outbreak, and the like. Because these drugs are not satisfactory either in efficiency or safety, there has been a continued need for the development of new drugs for the treatment of ischemic disease with new mechanisms.

Recent studies on ischemic diseases focus on the development of a treatment that can prevent or treat damaged cells and tissues following ischemia better than clot blusters or coagulation inhibitors, which are problematic in safety and acceptability.

Cerebral ischemia induced by stroke leads to rapid death of neurons and vascular structures in the supplied region of the brain. The loss of neurons, arterioles, and capillaries in the infarcted zone is irreversible and results in the formation of scar tissue over time. For this reason, most experimental and clinical therapies have mainly focused on limiting infarct size. Nevertheless, experimental evidence supports the concept that establishing reperfusion alone is not enough to cease ischemic injury.

Majority of the substances which were found to be neuroprotective in animals have failed in clinical trials.

The potential therapeutic applications of CNS stem cells in common degenerative and ischemic diseases have become a major focus on research for the last decade. Attempts to replace the necrotic zone of the brain by transplanting fetal brain cells and other stems cells have been done (Chu et al., Brain Res. 2004, 1016; 145-153; Kelly et al. Proc. Natl. Acad. Sci. USA; 2004. 101; 11839-11844; Imitola et al.; Proc. Natl. Acad. Sci. USA; 2004. 101, 18117-18122; Lee et al., Stem Cells. 2007, 25, 1204-1212) and although these studies have been successful in the survival of many of the grafted cells, most of them have failed to reconstitute healthy neurons and cerebral vessels integrated structurally and functionally with the spared cerebral tissue and the functional recovery promoted by the cell transplant is still very limited.

Document WO/2006/055260 describes that treatment with a stem cell factor (SCF) polypeptide alone and in combination with granulocyte colony stimulating factor (G-CSF) provides a regenerative, as well as protective, effect on neurological function after cerebral ischemia. Said document also provides demonstration that this cytokine treatment actually improves neurological function after an ischemic event.

Document WO2008/024996 describes that mesenchymal stem cell conditioned media, more particularly, adipose stem cell conditioned media can be used to treat or prevent various disorders that involve hypoxia-ischemia (H-I) of the brain including neonatal or adult H-I-induced encephalopathy, stroke and neurodegenerative disorders.

SUMMARY OF THE INVENTION

The inventors have found that, surprisingly, a conditioned media from neurospheres effectively increases cell survival in an ischemic condition, more particularly, in a neural ischemic condition. Thus, said medium can be used for the treatment of ischemic tissue damage. In particular, said conditioned media can be used for the treatment of brain ischemia, and more particular for the treatment of stroke.

Therefore, according to the present invention, the use of said conditioned media from neurospheres may be a promising tool for the treatment of ischemic tissue damage, more particularly, for the treatment of brain ischemia, avoiding the complications of

culturing and transplanting cells, not only for technical reasons but also for undesired side effects when implanted into a patient.

Therefore, in a first aspect, the present invention refers to a conditioned media from neurospheres or an extract thereof for use as a medicament for the treatment of ischemic tissue damage.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Adult neurospheres express the neural progenitor marker nestin. Microphotographs of an adult neurosphere with phase contrast and immunofluorescence nestin staining.

Figure 2. Neonatal neurospheres express the neural progenitor marker nestin. Microphotographs of various neonatal neurospheres with phase contrast and immunofluorescence nestin staining.

Figure 3. Differentiated adult neurospheres express the neuronal markers β-III-tubulin, MAP2 and tau, and the neuroblast marker DCX. Microphotographs of immunofluorescence staining of the four neuronal markers.

Figure 4. Differentiated adult neurospheres express both CNPase (detected with anti-RIP and anti-purified protein) and MBP. Microphotographs of immunofluorescence staining of these oligodendrocyte markers.

Figure 5. Differentiated adult neurospheres give rise to astrocytes. Immunofluorescence microphotograph of the astrocyte marker GFAP staining of a differentiated adult neurosphere.

Figure 6. Immunocytochemical characterization of hippocampal neuronal cultures. Fluorescence microphotographs of the positive staining for neuronal markers β-III-tubulin, MAP2 and tau, and the neuroblast marker DCX.

Figure 7. Immunocytochemical characterization of astrocyte cultures. Fluorescence microphotographs with a 20x and a 40x objective of GFAP staining of astrocyte culture.

Figure 8. Neurosphere conditioned medium increases astrocyte survival after an ischemic insult. The viability of astrocyte cultures in control and OGD conditions was assessed with Alamar Blue, a colorimetric dye that is reduced by cellular metabolism. The decrease in absorbance units at 570 nm after the OGD indicates that there are less

living cells after the ischemic insult. When the reoxygenation is done with neurosphere condition medium, the absorbance increases, meaning that the conditioned medium prevents astrocyte death induced by ischemia.

Figure 9. Neurosphere conditioned medium increases astrocyte survival after an ischemic insult. The viability of astrocyte cultures in control and OGD conditions was assessed by Flow Cytometry after staining the cells with propidium iodide and AnnexinV to detect necrotic and apoptotic cells. The reoxygenation in neurosphere condition medium reverted the proportion of live cells and apoptotic cells to the control levels. Moreover, the proportion of necrotic cells was significantly reduced.

Figure 10. Neurosphere conditioned medium increases neuronal survival after an ischemic insult. The viability of neuronal cultures in control and OGD conditions was assessed by Flow Cytometry after staining the cells with propidium iodide and AnnexinV to detect necrotic and apoptotic cells. The reoxygenation in neurosphere condition medium reverted the proportion of live cells, apoptotic cells and necrotic cells to control levels.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, the authors of the present invention have shown that, surprisingly, by adding conditioned media from neurospheres to a culture of neural cells which have suffered ischemic damage caused by oxygen and glucose deprivation (OGD) cell survival is increased.

Thus, in a first aspect, the present invention refers to a conditioned media from neurospheres or an extract thereof, hereinafter referred to as "conditioned media of the invention", for use as a medicament for the treatment of ischemic tissue damage. In a particular embodiment, said ischemic tissue damage is brain ischemia. In a more particular embodiment, said brain ischemia is focal brain ischemia.

As used herein, the term "conditioned media" refers to a growth medium that is further supplemented with soluble factors ("culture-derived growth factors") derived from neural stem cells, preferably human neural stem cells, cultured in the medium. In a particular case, said neural stem cells are adult neural stem cells, more particularly, subventricular zone (SVZ), olfactory bulb or cortex-derived adult stem cells.

Techniques for isolating conditioned medium from a cell culture are well known in the art. As will be appreciated, conditioned medium is preferably essentially cell-free. In this context, "essentially cell-free" refers to a conditioned medium that contains fewer than about 10%, preferably fewer than about 5%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001% than the number of cells per unit volume, as compared to the culture from which it was separated. As used herein, the term "conditioned media" also encompasses media conditioned by the growth of said neural stem cells that that has been treated by concentration, extraction, or other means for preserving, increasing the potency, improving the stability, removing impurities, etc. Thus, conditioned media includes extracts, for example, as defined below.

The term "extract" when used in reference to conditioned cell culture media refers to any subcomponent or fraction of the conditioned media, whether obtained by dialysis, fractionation, distillation, phase separation, gel filtration chromatography, affinity chromatography, hollow fiber filtration, precipitation, concentration, or the like.

In culture, neural stem cells grow in suspension forming a free-floating structure known as neurospheres. Thus, as used herein, the term "neurosphere" refers to a free-floating structure generated by neural stem cells *in vitro*. They are spherical clusters of cells comprised of a heterogeneous mix of neural stem cells, neural progenitors, differentiated cells and extracellular matrix proteins although many factors contribute to this composition to be variable. Neurospheres may be dissociate and subcultured repeatedly as disclosed in WO2004/013315.

As used herein, the term "stem cells" refers to cells that have the ability to self-replicate and give rise to specialized cells. Stem cells can be found at different stages of fetal development and are present in a wide range of adult tissues. Many of the terms used to distinguish stem cells are based on their origins and the cell types of their progeny. There are three basic types of stem cells. Totipotent stem cells, meaning their potential is total, have the capacity to give rise to every cell type of the body and to form an entire organism. Pluripotent stem cells, such as embryonic stem cells, are capable of generating virtually all cell types of the body but are unable to form a functioning organism. Multipotent stem cells can give rise only to a limited number of cell types. For example, adult stem cells, also called organ- or tissue-specific stem cells, are multipotent stem cells found in specialized organs and tissues after birth. Their

primary function is to replenish cells lost from normal turnover or disease in the specific organs and tissues in which they are found.

For the purposes of this disclosure, the terms "neural progenitor cell", "neural precursor cell" or "neural stem cell" are used interchangeably, and refers to a cell that can generate progeny that are either neuronal cells (such as neuronal precursors or mature neurons) or glial cells (such as glial precursors, mature astrocytes, or mature oligodendrocytes). Typically, the cells express some of the phenotypic markers that are characteristic of the neural lineage as described below. Typically, they do not produce progeny of other embryonic germ layers when cultured by themselves in vitro, unless dedifferentiated or reprogrammed in some fashion. Neural precursor cells give rise to all types of neural cells: neurons, astrocytes and oligodendrocytes. Neural precursor cells, as used herein, describes a cell that is capable of undergoing greater than 20-30 cell divisions while maintaining the potency to generate neurons, astrocytes and oligodendrocytes. Preferably, said cells are capable of undergoing greater than 40, more preferably greater than 50, most preferably unlimited such cell divisions.

Active cellular turnover does not occur in the adult nervous system as it does in renewing tissues such as blood or skin. Because of this observation, it had been a dogma that the adult brain and spinal cord were unable to regenerate new nerve cells. However, since the early 1990s, neural stem cells have been isolated from the adult brain as well as fetal brain tissues. Stem cells in the adult brain are found in the areas called the subventricular zone. Another location of brain stem cells occurs in the dentate gyrus of the hippocampus, a special structure of the cerebral cortex related to memory function. Stem cells isolated from these areas are able to divide and to give rise to nerve cells (neurons) and neuron-supporting cell types in culture.

Ischemia is the reduction or abolition of blood supply to a tissue. The associated deficiency of oxygen and nutrients leads to cell death in areas of the affected tissue. Ischemia can occur in many organs such as heart or brain. The damage induced by the lack of oxygenated blood in the brain occurs in two stages. First cellular metabolism is arrested due to lack of oxygen and some cells and tissue will die within minutes by necrosis in what is called the infarct core. Secondly a cascade of processes such as apoptosis is initiated and may last for hours or even days. The tissue damaged by the second cascade, called the ischemic penumbra, is non-functional but retains structural

integrity. The penumbral region can become part of the core region, and its death can be crucial and cause greater harm to the individual than the primary damage happening within the first minutes of ischemia. Once the ischemia is initiated, the necrotic cell death in the infarct core cannot be avoided, but preventing the apoptotic death in the penumbral region would imply the prevention of the major functional deficits.

The terms "stroke" or "focal brain ischemia" as used herein, refer to the condition that results from the blockage of a single artery that supplies blood to the brain or spinal cord, resulting in the death of cellular elements in the territory supplied by that artery. The terms "focal brain ischemia" and "stroke" can be used interchangeably.

In another particular embodiment of the invention, the neurospheres are primary neurospheres.

As used herein, the term "primary neurospheres" refers to neurospheres from a tissue culture started from cells, tissues, or organs taken directly from the donor organism. In a preferred embodiment, said primary neurospheres are derived from a tissue selected from the group consisting of subventricular zone, brain cortex, hippocampus, and olfactory bulb.

The term "subventricular zone" (SVZ) as used herein, refers to a paired brain structure situated throughout the lateral walls of the lateral ventricles. Along with the subgranular zone of dentate gyrus, subventricular zone serves as a source of neural stem cells in the process of adult neurogenesis. It harbors the largest population of proliferating cells in the adult brain of rodents, monkeys and humans. Neurons generated in SVZ travel to the olfactory bulb via the rostral migratory stream, which has until recently remained elusive in humans.

The term "brain cortex" or "cerebral cortex" are used interchangeably and refer to the outer layer of gray matter of the cerebral hemispheres. The cerebral cortex is a structure within the brain that plays a key role in memory, attention, perceptual awareness, thought, language, and consciousness. In dead, preserved brains, the outermost layer of the cerebrum has a grey colour, hence the name 'grey matter'. Grey matter is formed by neurons and their unmyelinated fibers, whereas the white matter below the grey matter of the cortex is formed predominantly by myelinated axons interconnecting different regions of the central nervous system. The human cerebral

cortex is 2–4 mm thick. The surface of the cerebral cortex is folded in large mammals, wherein more than two-thirds of the cortical surface is buried in the grooves, called "sulci".

The term "hippocampus" as used herein, refers to a part of the forebrain, located in the medial temporal lobe. It belongs to the limbic system and plays major roles in short term memory and spatial navigation. Humans and other mammals have two hippocampi, one in each side of the brain.

The term "olfactory bulb" as used herein, refers to a structure of the vertebrate forebrain involved in olfaction. In most vertebrates, the olfactory bulb is the most rostral (forward) part of the brain. In humans, however, the olfactory bulb is on the inferior (bottom) side of the brain. Humans and other mammals have two olfactory bulbs, one in each side of the brain.

In another particular embodiment of the invention, said tissue is a mammal tissue. In another particular embodiment of the invention, said mammal is a rodent. In a more particular embodiment of the invention said rodent is mouse. In another particular embodiment of the invention, said mammal is a human.

The term "mammal" as used in this invention means any of various warm-blooded vertebrate animals of the class *Mammalia*, including humans, characterized by a covering of hair on the skin and, in the female, milk-producing mammary glands for nourishing the young.

As used herein, "rodent" is defined as any of various mammals of the order *Rodentia*, such as a mouse, rat, squirrel, or beaver, characterized by large incisors adapted for gnawing or nibbling.

In another embodiment of the invention, said cells present a normal karyotype.

The term "karyotype" as used herein, refers to the chromosome characteristics of an individual cell or cell line of a given species, as defined by both the number and morphology of the chromosomes. Typically, the karyotype is presented as a systematized array of prophase or metaphase (or otherwise condensed) chromosomes from a photomicrograph or computer-generated image. Alternatively, interphase chromosomes may be examined as histone-depleted DNA fibres released from interphase cell nuclei. It is considered a normal karyotype when the number of chromosomes is not altered compared to the number of chromosomes of the specie.

As used herein, the terms "treat", "treatment" and "treating" refer to the amelioration of one or more symptoms associated with a disorder that results from the administration of a therapeutically effective amount of the conditioned media of the invention or a pharmaceutical composition comprising same, to a subject in need of said treatment. Thus, "treatment" as used herein covers any treatment of a disorder, disease or condition of a mammal, particularly a human, and includes: (a) preventing the disease or condition from occurring in a subject which may be predisposed to the disease or condition but has not yet been diagnosed as having it; (b) inhibiting the disease or condition, i.e., arresting its development; or (c) relieving the disease or condition, i.e., causing regression of the disease or condition or amelioration of one or more symptoms of the disease or condition. The population of subjects treated by the method includes a subject suffering from the undesirable condition or disease, as well as subjects at risk for development of the condition or disease. Thus, one of skill in the art realizes that a treatment may improve the patient's condition, but may not be a complete cure of the disease. As used herein, the terms "disorder" and "disease" are used interchangeably to refer to an abnormal or pathological condition in a subject that impairs bodily functions and can be deadly.

The term "subject" refers to an animal, preferably a mammal including a non-primate (e.g. a cow, pig, horse, cat, dog, rat, or mouse) and a primate (e.g. a monkey or a human). In a preferred embodiment, the subject is a human..

Subjects that can be treated with the conditioned media of the present invention include, but are not limited to, subjects suffering from or at risk of developing conditions associated with hypoxia and/or ischemia that result in increased intracranial pressure and/or with cytotoxic edema of the central nervous system (CNS). Such conditions include, but are not limited to, trauma (e.g., traumatic brain or spinal cord injury (TBI or SCI), concussion) ischemic brain injury, hemorrhagic infarction, germinal matrix hemorrhage, stroke, atrial fibrillations, clotting disorders, pulmonary emboli, arterio-venous malformations, mass-occupying lesions (e.g., hematomas), etc. Still further, subjects at risk of developing such conditions can include subjects undergoing treatments that increase the risk of stroke, for example, surgery (vascular or neurological), treatment of myocardial infarction with thrombolytics, cerebral/endovascular treatments, stent placements, angiography, or individuals without

a medical condition who engage in sport activities that put them at risk for brain and spinal cord injury etc.

Subjects that may be treated with the conditioned media of the present invention include those that are suffering from or at risk of developing trauma (e.g., traumatic brain or spinal cord injury (TBI or SCI)), ischemic brain or spinal cord injury, primary and secondary neuronal injury, stroke, arteriovenous malformations (AVM), brain abscess, mass-occupying lesion, hemorrhagic infarction, or any other condition associated with cerebral hypoxia or cerebral ischemia resulting in cerebral edema and/or increased intracranial pressure, for example, but not limited to brain mass, brain edema, hematoma, end stage cerebral edema, encephalopathies, etc. Thus, the conditioned media can be a therapeutic treatment in which the therapeutic treatment includes prophylaxis or a prophylactic treatment. The conditioned media of the present invention is neuroprotective.

According to one embodiment, the conditioned medium of the invention can be concentrated at least 50 fold, at least 100- fold, at least 200 fold, or at least 1000-fold. Optionally, said concentrated conditioned medium is fractionated through a size exclusion resin or membrane. The concentrated conditioned medium of the invention is then optionally stabilized to protect degradation or loss of components.

While it is possible for the active agent, i.e. the conditioned media of the present invention, to be administered alone, it is preferable to present it as part of a pharmaceutical formulation or composition, comprising as active ingredient an effective amount of conditioned media according to the invention. The pharmaceutical formulation or composition in the context of the invention is intended to mean a combination of the active agent(s), together or separately, with a pharmaceutically acceptable carrier as well as other additives.

In a specific embodiment, the term “pharmaceutically acceptable” means approved by a regulatory agency of the Federal or a state government or listed in the US Pharmacopeia, or European Pharmacopeia, or other generally recognized pharmacopeia for use in animals and, more particularly, in humans.

The term “carrier” in the context of the present invention denotes any one of inert, non-toxic materials, which do not react with the conditioned medium of the invention and which can be added to formulations as diluents, carriers or to give form or

consistency to the formulation. The carrier may at times have the effect of the improving the delivery or penetration of the active ingredient to the target tissue, for improving the stability of the drug, for slowing clearance rates, for imparting slow release properties, for reducing undesired side effects etc. The carrier may also be a substance that stabilizes the formulation (e.g. a preservative), for providing the formulation with an edible flavour, etc. For examples of carriers, stabilizers and adjuvants, see E. W. Martin, REMINGTON'S PHARMACEUTICAL SCIENCES, MacK Pub Co (June, 1990).

The compositions of the present invention are administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The choice of carrier will be determined in part by the particular active ingredient, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable pharmaceutical compositions of the present invention.

Unlike cell therapies that inject stem cells at the point of injury, the process for treatment of injured nervous system cells, or cells prone to injury or neurodegenerative diseases does not require localized injection. Rather, it will be appreciated that since no living cells, which may die if used systemically, are being utilized, that a wide array of delivery systems may be used to ensure that the conditioned media of the invention, its fractions, concentrations, or distillations may be delivered systemically, via injection, intravenously, or otherwise. Optionally, the conditioned media of the invention may be delivered locally at the site of injury.

Pharmaceutical compositions or medicaments may be administered or coadministered by a wide variety of routes, including for example, orally, parenterally, intraperitoneally, intravenously, intraarterially, transdermally, sublingually, intramuscularly, rectally, transbuccally, intranasally, liposomally, via inhalation, vaginally, intraoccularly, via local delivery (for example by catheter or stent), subcutaneously, intraadiposally, intraarticularly, or intrathecally. The compositions may also be administered or coadministered in slow release dosage forms. Dosage forms known to those of skill in the art are suitable for delivery of the compositions of the invention.

Compositions are provided that contain therapeutically effective amounts of the conditioned media according to the invention. To prepare said compositions, conditioned media of the invention is mixed with a suitable pharmaceutically acceptable carrier. Upon mixing or addition of the compound(s), the resulting mixture may be a solution, suspension, emulsion, or the like. Liposomal suspensions may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known to those skilled in the art. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the compound in the selected carrier or vehicle. The effective concentration is sufficient for lessening or ameliorating at least one symptom of the disease, disorder, or condition treated and may be empirically determined.

The amount or concentration of active substance in those compositions or preparations is such that a suitable dosage in the range indicated is obtained. The compositions are preferably formulated in a unit dosage form. The term "unit dosage form" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

In addition, the active materials can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, or have another action. The compounds, i.e. the conditioned media of the present invention, may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients. The following examples of active ingredients to be combined with the conditioned medium of the invention are provided as merely illustrative and are not to be construed as limiting the scope of the invention: Erythropoietin, human chorionic Gonadotropin, Epoetin alfa, NTx-265, glutamate antagonists, calcium channel blockers, free radical scavengers, centrally acting catecholamines, selective serotonin reuptake inhibitors or any of the drugs described in Durukan and Tatlisumak, Pharmacology, Biochemistry and Behavior. 2007. 87, 179-197.

Where the compounds normally exhibit insufficient solubility, methods for solubilizing may be used. Such methods are known and include, but are not limited to,

using cosolvents such as dimethylsulfoxide (DMSO), using surfactants such as Tween.RTM., and dissolution in aqueous sodium bicarbonate. Derivatives of the compounds, such as salts or prodrugs may also be used in formulating effective pharmaceutical compositions.

The conditioned media of the present invention may be prepared with carriers that protect them against rapid elimination from the body, such as time-release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems. The active compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the subject treated. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery systems, and biodegradable, biocompatible polymers such as collagen, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid, and the like. Methods for preparation of such formulations are known to those skilled in the art.

Compounds of the invention may also be advantageously delivered in a nano crystal dispersion formulation. Preparation of such formulations is described, for example, in U.S. Pat. No. 5,145,684. The nano crystalline formulations typically afford greater bioavailability of drug compounds.

The conditioned media of the invention can be enclosed in multiple or single dose containers. The enclosed compounds and compositions can be provided in kits, for example, including component parts that can be assembled for use. For example, a therapeutic compound in lyophilized form and a suitable diluent may be provided as separated components for combination prior to use. A kit may include a conditioned media according to the present invention and a second therapeutic agent for co-administration. The conditioned media of the invention and second therapeutic agent may be provided as separate component parts. A kit may include a plurality of containers, each container holding one or more unit dose of the conditioned media of the invention. The containers are preferably adapted for the desired mode of administration, including, but not limited to tablets, gel capsules, sustained-release capsules, and the like for oral administration; depot products, pre-filled syringes,

ampoules, vials, and the like for parenteral administration; and patches, medipads, creams, and the like for topical administration.

As is well known in the art, a specific dose level of active compounds such as the conditioned media of the present invention for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy. The person responsible for administration will determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biology standards.

The conditioned media of the invention is administered in amounts which are sufficient to achieve the desired effect, in a preferred embodiment, a neuroprotective effect. As will be appreciated, the amount of the compound will depend on the severity of the disease, the intended therapeutic regiment and the desired therapeutic dose. An amount effective to achieve the desired effect is determined by considerations known in the art. Thus, it is appreciated that the effective amount or concentration depends on a variety of factors including the distribution profile of the compound within the body, a variety of pharmacological parameters such as half life in the body, on undesired side effects, if any, on factors such as age and gender of the subject to be treated, etc. The therapeutically effective amount or concentration may be determined empirically by testing the compounds in known *in vitro* and *in vivo* model systems for the treated disorder. The effective amount is typically tested in clinical studies having the aim of finding the effective dose range, the maximal tolerated dose and the optimal dose. The manner of conducting such clinical studies is well known to a person versed in the art of clinical development.

An amount may also at times be determined based on amounts shown to be effective in animals. It is well known that an amount of X mg/Kg administered to rats can be converted to an equivalent amount in another species (notably humans) by the use of one of possible conversions equations well known in the art. The concentration of active compound in the drug composition will depend on absorption, inactivation, and

excretion rates of the active compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

The conditioned media may be administered at once, or may be divided into a number of smaller doses to be administered at intervals of time. It is understood that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

If oral administration is desired, the conditioned media should be provided in a composition that protects it from the acidic environment of the stomach. For example, the composition can be formulated in an enteric coating that maintains its integrity in the stomach and releases the active compound in the intestine. The composition may also be formulated in combination with an antacid or other such ingredient. Oral compositions will generally include an inert diluent or an edible carrier and may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules, or troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as fatty oil. In addition, dosage unit forms can contain various other materials, which modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The compounds can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. Syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings, and flavors.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, or topical application can include any of the following components: a sterile diluent such as water for injection, saline solution, fixed oil, a naturally occurring vegetable oil such as sesame oil, coconut oil, peanut oil, cottonseed oil, and the like, or a synthetic fatty vehicle such as ethyl oleate, and the like, polyethylene glycol, glycerine, propylene glycol, or other synthetic solvent; antimicrobial agents such as benzyl alcohol and methyl parabens; antioxidants such as ascorbic acid and sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates, and phosphates; and agents for the adjustment of tonicity such as sodium chloride and dextrose. Parenteral preparations can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass, plastic, or other suitable material. Buffers, preservatives, antioxidants, and the like can be incorporated as required.

Where administered intravenously, suitable carriers include physiological saline, phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents such as glucose, polyethylene glycol, polypropyleneglycol, and mixtures thereof. Liposomal suspensions including tissue-targeted liposomes may also be suitable as pharmaceutically acceptable carriers. These may be prepared according to methods known for example, as described in U.S. Pat. No. 4,522,811.

The compounds of the invention can be administered intranasally. When given by this route, the appropriate dosage forms are a nasal spray or dry powder, as is known to those skilled in the art.

The compounds of the invention can be administered intrathecally. When given by this route the appropriate dosage form can be a parenteral dosage form as is known to those skilled in the art. The compounds of the invention can be administered topically. When given by this route, the appropriate dosage form is a cream, ointment, or patch. The compounds of the invention can be administered rectally by suppository as is known to those skilled in the art. The compounds of the invention can be administered by implants as is known to those skilled in the art. When administering a compound of the invention by implant, the therapeutically effective amount is the amount described above for depot administration.

Given a particular compound of the invention and a desired dosage form, one skilled in the art would know how to prepare and administer the appropriate dosage form.

The compounds of the invention can be used in combination, with each other or with other therapeutic agents or approaches used to treat or prevent the ischemic conditions that are the subject of this patent.

There is nothing novel about the route of administration or the dosage forms for administering the therapeutic compounds. Given a particular therapeutic compound, and a desired dosage form, one skilled in the art would know how to prepare the appropriate dosage form for the therapeutic compound.

It should be apparent to one skilled in the art that the exact dosage and frequency of administration will depend on the particular compounds of the invention administered, the particular condition being treated, the severity of the condition being treated, the age, weight, general physical condition of the particular patient, and other medication the individual may be taking as is well known to administering physicians who are skilled in this art.

As shown in the Example 1 accompanying the present invention, the inventors have shown that there is a significant increase in cell viability when cells, after an ischemic insult, are treated with the conditioned media of the invention. Inventors have demonstrated that the conditioned medium of the invention promotes cell survival after a ischemic insult and that the effects on cell viability after said insult can be reverted when reoxygenation is carried out in said conditioned medium (Table 5, Figure 10). Hence, the data herewith presented demonstrate that the conditioned medium of the invention can be used for the treatment of ischemic tissue damage and that said medium is capable, after ischemic damage, of restoring the levels of living, apoptotic and necrotic cells to control levels. Thus, in a particular embodiment of the invention, said conditioned media of the invention can be used as a medicament for the treatment of ischemic tissue damage, wherein said medium promotes cell survival. In a more particular embodiment, said cell is selected from the group consisting of a neuron and an astrocyte.

In other aspect, the present invention refers to the use of a conditioned medium of the invention for preventing, treating, or ameliorating one or more symptoms

associated with a tissue ischemic condition in a subject suffering from said disorders or diseases.

In other aspect, the present invention provides methods of preventing, treating, or ameliorating one or more symptoms associated with a tissue ischemic condition, in a subject suffering from said disorders or diseases, which comprises administering to said subject in need of such treatment of a prophylactically or therapeutically effective amount of a conditioned medium of the invention. In a particular embodiment, said ischemic tissue damage is brain ischemia. More particularly, said brain ischemia is stroke.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used, is intended to be in the nature of words of description rather than of limitation. Obviously, many modifications and variations of the present invention are possible in light of the above teaching. It is therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described hereinafter.

The following examples are provided as merely illustrative and are not to be construed as limiting the scope of the invention.

EXAMPLE

Neurosphere culture medium promotes cell survival

I. Materials and Methods

Neural Stem Cell Neurosphere Primary Culture

Primary cultures of neural stem cells were obtained from adult mouse subventricular zone (SVZ) or from neonatal mouse cortex.

Animals were sacrificed by asphyxiation, and the brain tissue was dissected under a stereomicroscope inside a horizontal laminar flow. National guidelines for animal welfare and experimental conduct were followed.

For adult neurosphere culture, female mouse adult brains were cut in slices at the SVZ level and the SVZ was dissected and digested. For neonatal neurosphere culture, the brains were dissected to obtain the cortex, the meninges were removed and the

tissue was digested. Both digestions were done with papain (20 units/ml, Sigma), DNase I (2000 units/ml, Roche), 1mM L-cysteine (Sigma) and 0,5 mM EDTA (Sigma) in Earl's Balance Salt Solution (EBSS, Sigma) in a 2 ml total volume at 37°C and agitation at 800 rpm for 30 minutes. Then the tissue was dissociated with 10 passes with a micropipette blue tip and the cell suspension was placed in a tube, on top of 2 ml of a 10 mg/ml BSA (Sigma), 10 mg/ml ovomucoid (Sigma) solution in EBSS and centrifuged for 5 minutes at 1000 rpm. The cell pellet was resuspended in 5 ml of neurosphere proliferation medium (DMEM:F12 (Sigma), B27 (Gibco-Invitrogen), N2 (Gibco-Invitrogen), 20 µg/ml insulin (Sigma), 2 µg/ml heparin (Sigma), 20 ng/ml bFGF (Peprotech), 20 ng/ml EGF (Peprotech) and Penicillin/Streptomycin (Sigma), and seeded on a T-25 flask (BD). The cells were incubated at 37°C in an atmosphere with 5% CO_2, 5% O_2 and 90% N_2. After two or three days, the first small neurospheres start to show. The medium was changed twice a week and the neurospheres were passed by mechanical dissociation to single cells once a week.

Neuronal Culture

Primary cultures of neurons were obtained from neonatal mouse hippocampus. Animals were sacrificed by asphyxiation, and the brain tissue was dissected under a stereomicroscope inside a horizontal laminar flow. First, the cortex was dissected, then the meninges were removed and the hippocampus was dissected and cut into 1mm pieces before digestion. The hippocampi pieces were digested in a final volume of 5 ml Hanks' Balance Salt Solution (Sigma) with 0.125 mg/ml trypsin (Sigma) and 200 units/ml DnaseI (Roche) for 10 minutes at 37°C. Then, the tissue was mechanically dissociated with10 passes with a micropipette blue tip and the digestion was stopped by adding trypsin inhibitor (Sigma) to a final concentration of 0,27 mg/ml. The cell suspension was centrifuged for 10 minutes at 700 rpm and the pellet was resuspended in Neurobasal-A medium (Gibco-Invitrogen) supplemented with B27 (Gibco-Invitrogen), Glutamax-I (Gibco-Invitrogen) and Penicillin/Streptomycin (Sigma). The cells were seeded on 0,1 mg/ml poliornithine (Sigma) coated dishes or coverslips at a density of 50.000 cells/cm^2. The cells were incubated at 37°C in an atmosphere with 5% CO_2, 5% O_2 and 90% N_2. The medium was changed twice a week.

Astrocyte culture

Primary cultures of astrocytes were obtained from neonatal mouse cortex. Animals were sacrificed by asphyxiation, and the brain tissue was dissected under a stereomicroscope inside a horizontal laminar flow. The cortex was dissected, the meninges were removed and the tissue was mechanically dissociated by passing through a fire polished pasteur pipette. The cell suspension was then filtered through a 40 micron cellstrainer (BD) and centrifuged for 5 minutes at 900 rpm. The cell pellet was then resuspended in DMEM High Glucose (Sigma), 10% Foetal Bovine Serum (FBS, Sigma), 2mM Glutamine (Sigma), and Penicillin/Streptomycin (Sigma) and seeded on 0.1 mg/ml poliornithine (Sigma) coated T-75 flask (BD). The cells were incubated at 37°C in an atmosphere with 5% CO_2 and 95% air. The medium was changed every two days. After one week in culture, the astrocyte monolayer was confluent and the flasks were agitated over night at 260 rpm to detach the microglial cells and the oligodendrocyte progenitor cells. The astrocytes that remain attached were then trypsinized (0,25% trypsin-EDTA solution, Sigma) and seeded on 0,1 mg/ml poliornithine (Sigma) coated 96-well culture dishes (BD) at a density of 10.000 cells per well.

Culture characterization

Neurosphere characterization by immunocytochemistry

As neurospheres grow in suspension, they were pelleted by centrifugation and seeded on 0,1 mg/ml poliornithine (Sigma) coated glass coverslips. Two hours after seeding, the neurospheres were attached to the coverslips and were fixed in 4 % formaldehyde for 20 minutes at room temperature, washed three times in phosphate buffer solution and stained for the neural stem cell marker nestin with a polyclonal anti-nestin antibody (Abcam) 1:1000 in blocking solution (phosphate buffer saline, 0,1% triton x-100 (Sigma), 5% donkey serum (Sigma)) over night at 4°C. The primary antibody was washed and the cells were incubated for 1 hour at room temperature in a Donkey anti-Rabbit-Cy2 (Jackson Immuno) 1:1000. Nuclei were stained with bisbenzimide (Sigma).

For characterizing the neural differentiation potential of the neurospheres, they were seeded on 0,1 mg/ml poliornithine (Sigma) coated glass coverslips and incubated

for 1 week in neurosphere differentiation medium (DMEM:F12 (Sigma), B27 (Gibco-Invitrogen), N2 (Gibco-Invitrogen), 2 µg/ml heparin (Sigma), 1% FBS (Sigma) and Penicillin/Streptomycin (Sigma)). During this week, the neurospheres flattened against the coverslip and differentiated into neurons, astrocytes and oligodendrocytes, identified by immunostaining for specific markers.

Target	Antigen	Manufacturer	Dilution
neural progenitor	nestin	Abcam	1:1000
neuron	Beta-III-tubulin	Abcam	1:1000
neuroblast	doublecortin	Abcam	1:350
neuron	MAP2a+b	Sigma	1:500
neuron	tau	Abcam	1:100
astrocyte	GFAP	Sigma	1:2000
oligodendrocyte	RIP	DSHB	1:1000
oligodendrocyte	MBP	Covance	1:1000
oligodendrocyte	CNPase	Abcam	1:1000

Table 1: The immunocytochemical characterization of the neurosphere, the astrocyte and the neuronal cultures was done using the antibodies listed above.

The following antibodies were used: anti- beta-III-tubulin (rabbit polyclonal anti- TUJ1 antibody, Abcam, 1:1000), anti- doublecortin (rabbit polyclonal anti- DCX antibody, Abcam, 1:350), anti- MAP2a+b (mouse monoclonal antibody, Sigma, 1:500) and anti-tau (rabbit polyclonal anti-tau antibody, Abcam, 1:100) as neuroblast or neuronal markers, anti- GFAP (mouse monoclonal anti- glial fibrillary acidic protein (GFAP, Sigma) 1:2000) as astrocyte marker and anti- myelin basic protein (mouse monoclonal anti- MBP (SMI 99) antibody, Covance, 1:1000) and anti- CNPase (mouse monoclonal anti-RIP antibody (Developmental Studies Hybridoma Bank, DSHB) 1:1000, and mouse monoclonal [11-5B] to CNPase antibody, Abcam, 1:1000)) as

oligodendrocyte markers. Donkey anti-Rabbit-Cy2 (Jackson Immuno) 1:1000 and Donkey anti-Mouse-Cy3 (Jackson Immuno) 1:1000 were used as secondary antibodies. Nuclei were stained with bisbenzimide (Sigma).

Neuronal characterization by immunocytochemistry

The cells were processed for immunocytochemistry as described above and stained for the neuronal markers cited above.

Astrocyte characterization by immunocytochemistry

The cells were processed for immunocytochemistry as described above and stained for the astrocyte marker GFAP.

Ischemic insult

Prior to the oxygen and glucose deprivation (OGD), the cells were washed once in phosphate buffer saline. The cells were then incubated in Earl's balance salt solution (EBSS) with or without glucose for the control and the OGD cells, respectively. The control cells were incubated under normal conditions (37°C in an atmosphere with 5% CO_2, 5% O_2 and 90 % N_2) whereas the OGD cells were incubated in an hypoxic chamber at 37°C in an atmosphere with 5 % CO_2, 0,5 % O_2 and 94,5 % N_2.

The OGD lasted from 10 minutes to 2 hours (time started counting when the hypoxic chamber oxygen concentration reached the set point), after which the EBSS was removed and either neuronal culture medium or neurosphere conditioned medium was added to the cells, that were incubated again under normal conditions.

Neurosphere conditioned medium

When the neurosphere culture density was high enough (when there were between 5 and 10 neurospheres per field using a microscope with a 10 x objective), the neurospheres were collected by centrifugation and washed 3 times in phosphate buffer saline. These washes allow eliminating the rest of the proteins of the proliferation medium that can be attached to the cell surface. After the last centrifugation, the neurospheres were resuspended in neuronal cell culture medium (Neurobasal-A medium (Gibco-Invitrogen) supplemented with B27 (Gibco-Invitrogen), Glutamax-I (Gibco-

Invitrogen) and Penicillin/Streptomycin (Sigma)), seeded on a new uncoated T-25 flask (BD) in a final volume of 5 ml and incubated under normal conditions for 6 hours. After this time, the medium was collected and centrifuged. The supernatant was filtered through a 0,45 micron syringe filter and frozen at -20°C. The neurospheres were resuspended in 5 ml of neurosphere proliferation medium and returned to their original flask.

Neuroprotection assay

Colorimetric viability assay:

To quantify the viability of the astrocyte cultures after the OGD, Alamar Blue (AbD Serotec) was used as an indicator of cell metabolic activity. Eighteen hours after reoxygenation, Alamar Blue was added to the cells and to control wells without cells, only with neuronal cell culture medium. At 24 hours after reoxygenation (6 hours after adding Alamar Blue), the absorbance at 570 nm and 600 nm was measured with an iMark microplate absorbance reader (Bio-Rad). Each well of the 96-well culture plates was considered as a biological replicate. Each experiment was done with 16 wells per situation and experiments were repeated at least twice. Data were exported and analyzed with a two tailed Student t test (Excel) and are expressed as mean ± SEM.

Flow Cytometry:

Cell viability was also assessed by flow cytometry. At 24 hours after reoxygenation, cells were harvested by adding 50 µl trypsin-EDTA solution (Sigma) and stained with 5 µg/ml propidium iodide (PI, Sigma) and 1:40 annexinV-DY634 (Immunostep). Without inactivating the trypsin and centrifugating the cells, 150 µl of 1,3x Binding Buffer (Immunostep), PI and annexinV were added to the cell suspension and incubated for 15 minutes at room temperature. Then, the cells were analyzed using a Hypercyt (Intellicyt) high throughput screening connected to a Cyan ADP (Beckman-Coulter) flow cytometer. Cells positive for PI were considered necrotic, cells positive for annexinV and negative for PI were considered early apoptotic and cells negative for both were considered to be alive. Each well of the 96-well culture plates was considered as a biological replicate. Each experiment was done with 16 wells per situation and

experiments were repeated at least twice. The data were exported and two tailed Student t test statistical analysis was carried out using Excel (Microsoft).

II. Results

Cell culture immunocytochemical characterization

Neurosphere characterizarion:

Nestin is an intermediate filament protein expressed in dividing cells during the early stages of development of the central nervous system (CNS) and in the neural progenitors of the adult CNS. When differentiation takes place, nestin synthesis decreases and GFAP and neurofilament proteins start to be expressed in differentiating astrocytes and neurons, respectively.

Both the neonatal and adult neurospheres were positive for nestin staining, confirming that the cells forming the neurosphere are neural progenitors (Figures 1 and 2).

As neural progenitors, neurospheres can differentiate into the three neural lineages: neurons, oligodendrocytes and astrocytes. β-III-tubulin is a microtubule protein expressed exclusively in neurons, DCX is transiently expressed in proliferating progenitor cells and newly generated neuroblasts and MAP2 is a microtubule associated protein that is present mainly in the neuronal bodies and the dendrites of mature neurons. These three proteins were identified in the differentiated neurospheres and confirmed that they can give rise to neurons (Figure 3).

In the CNS, the oligodendrocytes form the myelin sheath surrounding the axons of neurons. This myelin is composed of lipids and proteins, among which is the myelin basic protein (MBP). CNPase is an enzyme that has been found to be present only in myelinating cells, in the inner and outer loops of myelin. For confirming the neurosphere ability to differentiate into oligodendrocytes, immunocytochemistry against MBP and CNPase was done. Both proteins were found to be present in differentiated neurospheres, and the morphology of the cells that were positive for the staining was that of oligodendrocytes (Figure 4).

GFAP is the cell specific intermediate filament protein in astrocytes. The presence of GFAP positive cells after neurosphere differentiation confirmed their ability to give rise to astrocytes (Figure 5).

Neuronal culture characterization:

Four neuronal markers were used for the immunocytochemical characterization of the hippocampal neuronal cultures. One of them, DCX, is a marker for immature neurons or neuroblasts, and MAP2 is a marker of mature neurons and is located more specifically in neuronal bodies and dendrites (Figure 6).

Astrocyte culture charaterization:

The astrocyte marker GFAP was used for the immunocytochemical characterization of the astrocyte cultures (Figure 7). After the over night agitation step, almost a 100% of the cells in the culture were positive for GFAP staining. No microglia was found in the cultures when immunocytochemistry against the microglial markers CD11b and tomato lectin was done (data not shown).

Neurosphere conditioned medium promotes astrocyte survival after OGD

To test the neuroprotective effect of the neurosphere conditioned medium, astrocyte cultures with 3 days *in vitro* (DIV) and around 90% confluence were subjected to OGD for 20 minutes or 1 hour.

Each experiment was carried out using two parallel 96-well culture plates, one for the control (see Methods above) and one for the OGD. Reoxygenation was done in neuronal culture medium for half of the wells in the control plate and the OGD plate, and in neurosphere conditioned medium for the rest of the wells of both plates.

	Abs_{570nm}-Abs_{600nm}
Control	0.113 ± 0.011
Control OGD	0.045 ± 0.006
Conditioned Control	0.116 ± 0.005
Conditioned OGD	0.071 ± 0.004

Table 2. Absorbance data for testing the viability of astrocyte cultures after OGD and reoxygenation with control and neurosphere conditioned medium. Data are expressed as mean and SEM. (n = 16 for each treatment).

Cell viability was tested 24 hours after reoxygenation with two different methods, a colorimetric indicator and flow cytometry.

Alamar Blue, a soluble dye that undergoes colorimetric change in response to cellular metabolic reduction, was used. Alamar Blue was added 18 hours after reoxygenation and the absorbance at 570 nm and at 600 nm was measured with a microplate absorbance reader 6 hours later. The result data are shown in table 2.

The cells that suffered the ischemic insult showed a viability of 40% with reference to the control cells, whereas the cells that suffered the OGD but were reoxygenated in the neurosphere conditioned medium, showed a viability of 61% with reference to the conditioned control cells (Figure 8).

This significant increase in cell viability shows that neurosphere conditioned medium promotes astrocyte survival. The statistical significance of the changes in viability was assessed with a Student t test. The statistical significance is indicated by the p values shown in table 3.

	p value
Control vs Control OGD	0.001
Control OGD vs Conditioned OGD	0.0003

Table 3. Viability changes analyzed with Alamar Blue after OGD and with neurosphere conditioned medium reoxygenation.

For flow cytometry, the cells were detached from the plate with trypsin and stained with propidium iodide and AnnexinV to detect necrotic cells (positive for propidium ioide) and apoptotic cells (positive for annexinV). The cells negative for both markers were considered as living cells. Each experiment was done with 16 biological replicates and a minimum of 4.000 cells were analyzed from each well.

	% cells		
	live	apoptotic	necrotic
Control	62 ± 6	7 ± 1	31 ± 6
Control OGD	46 ± 4	16 ± 2	38 ± 3
Conditioned Control	57 ± 5	13 ± 2	30 ± 5
Conditioned OGD	69 ± 4	9 ± 1	21 ± 3

Table 4. Flow cytometry viability analysis of the astrocyte cultures subjected to 1 hour OGD.

	p value		
	live	apoptotic	necrotic
Control vs Control OGD	0,04	0,003	0,3
Control OGD vs Conditioned OGD	0,0002	0,0143	0,0006

Table 5. Viability changes observed with flow cytometry after OGD and with neurosphere conditioned medium reoxygenation.

The OGD caused a significant decrease in the proportion of living cells and an increase in apoptosis, whereas no change was observed in terms of necrosis in reference to the control (Tables 4-5, Figure 9).

The percentage of living cells increased dramatically when reoxygenation was performed with the neurosphere conditioned medium, whereas the proportion of apoptotic and necrotic cells was reduced to control levels or less, showing that astrocytes subjected to an ischemic insult were protected by the neurosphere conditioned medium (Tables 4-5, Figure 9).

Neurosphere conditioned medium promotes neuronal survival after OGD

To test the neuroprotective effect of the neurosphere conditioned medium, neuronal cultures with 3 DIV were subjected to OGD for 1 hour.

Each experiment was carried out using two parallel 96-well culture plates, one for the control and one for the OGD. Reoxygenation was done in neuronal culture

medium for half of the wells in the control plate and the OGD plate, and in neurosphere conditioned medium for the rest of the wells of both plates.

Cell viability was tested by flow cytometry as described above.

	% cells		
	live	apoptotic	necrotic
Control	84 ± 2	7 ± 1	9 ± 1
Control OGD	76 ± 5	13 ± 3	11 ± 2
Conditioned Control	89 ± 2	4 ± 1	7 ± 2
Conditioned OGD	86 ± 2	7 ± 2	7 ± 1

Table 6. Flow cytometry viability analysis of the neuronal cultures subjected to 1 hour OGD.

The OGD induced an 8% decrease in the number of living cells, as well as an increase in the number of apoptotic and necrotic cells. This effect of the ischemic insult was reverted when the reoxygenation was done in neurosphere conditioned medium (Table 6, Figure 10). The neuroprotective effect of the neurosphere conditioned medium restores the levels of living, apoptotic and necrotic cells to control ones.

CLAIMS

1. A conditioned media from neurospheres or an extract thereof for use as a medicament for the treatment of ischemic tissue damage.

2. Conditioned media according to claim 1, wherein said ischemic tissue damage is brain ischemia.

3. Conditioned media according to claim 2, wherein said brain ischemia is stroke.

4. Conditioned media according to any one of claims 1 to 3, wherein said neurospheres are primary neurospheres.

5. Conditioned media according to claim 4, wherein said primary neurospheres are derived from a tissue selected from the group consisting of subventricular zone brain cortex, hippocampus, and olfactory bulb.

6. Conditioned media according to claim 5, wherein said tissue is a mammal tissue.

7. Conditioned media according to claim 6, wherein said mammal is a rodent.

8. Conditioned media according to claim 7, wherein said rodent is a mouse.

9. Conditioned media according to claim 6, wherein said mammal is a human.

10. Conditioned media according to any one of claims 1 to 9, wherein said cells present a normal karyotype.

11. Conditioned media according to any one of claims 1 to 10, wherein said conditioned media promotes cell survival.

12. Conditioned media according to claim 11, wherein said cell is selected from the group consisting of a neuron and an astrocyte.

5

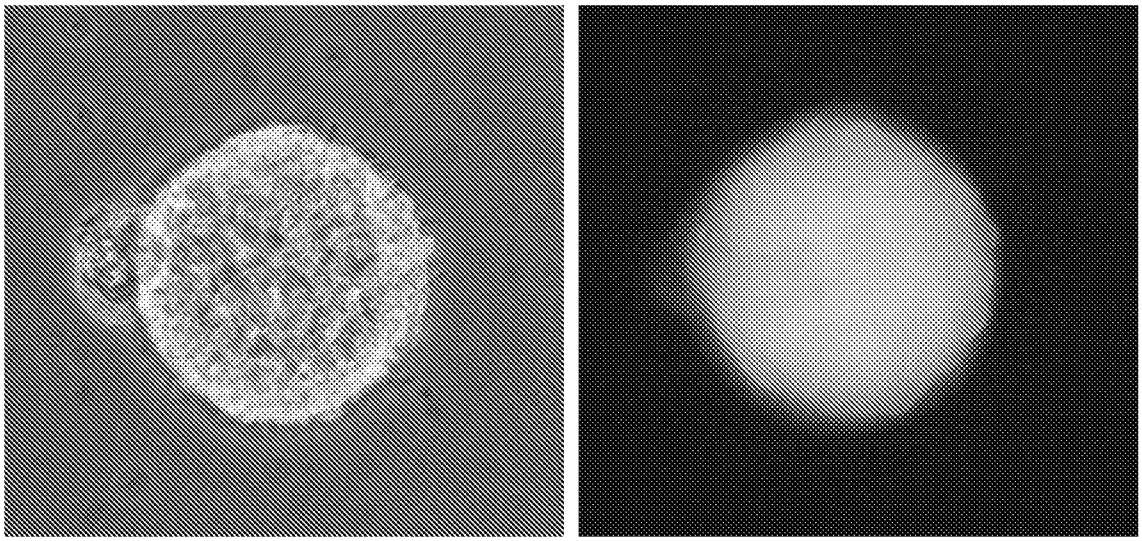


FIGURE 1

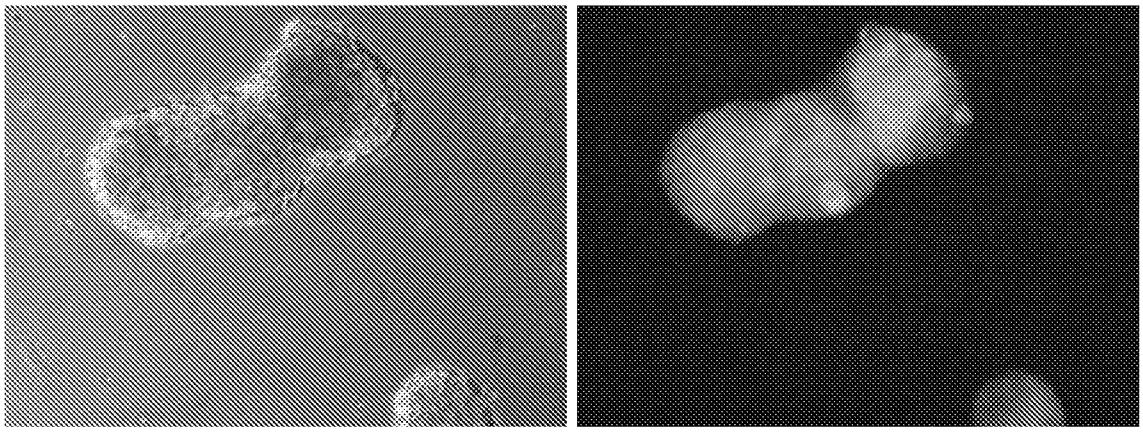


FIGURE 2

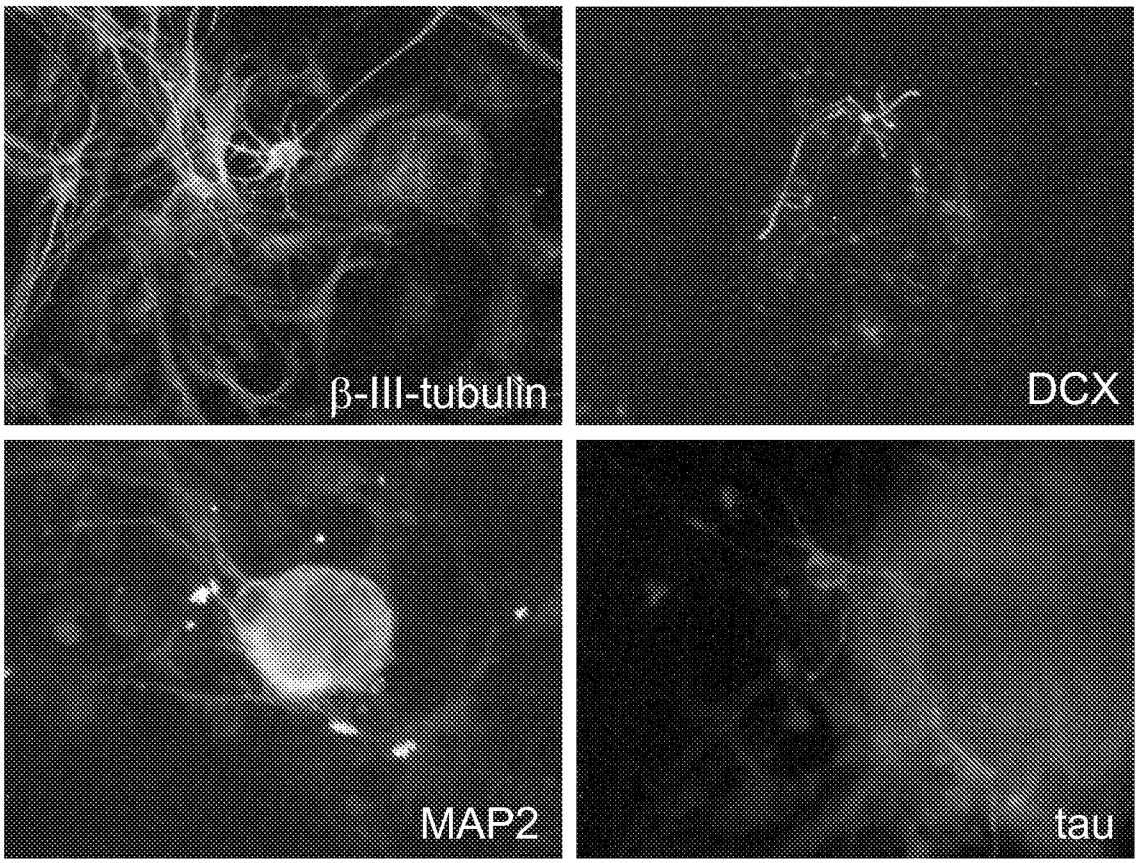


FIGURE 3

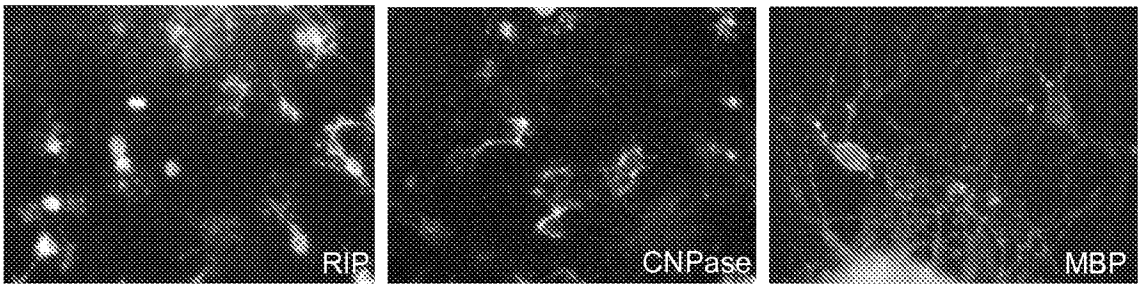


FIGURE 4

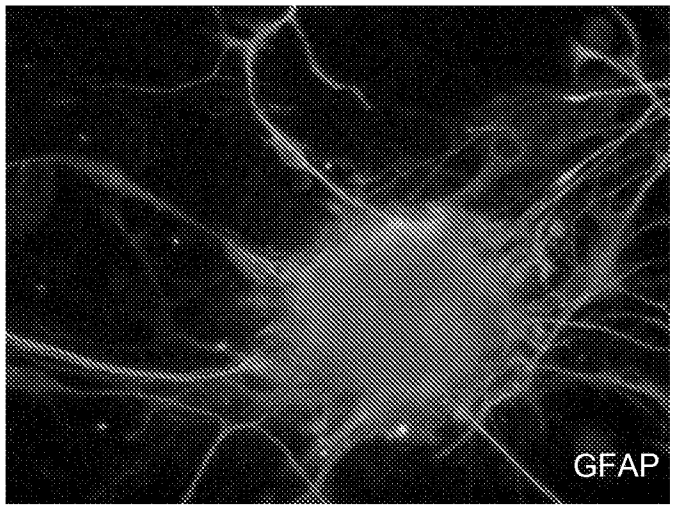


FIGURE 5

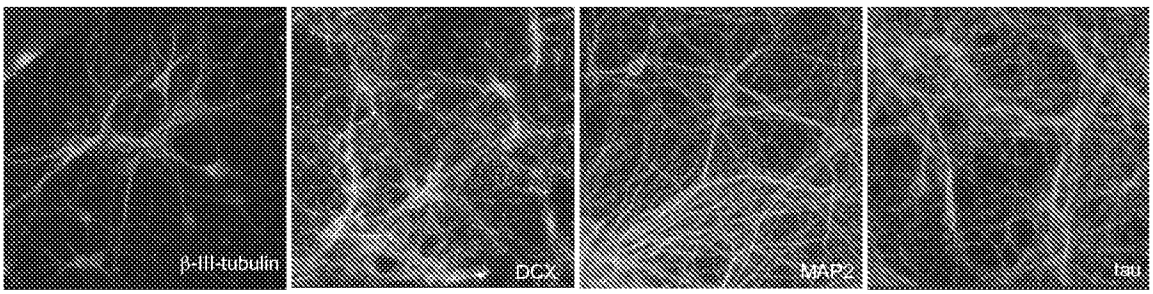


FIGURE 6

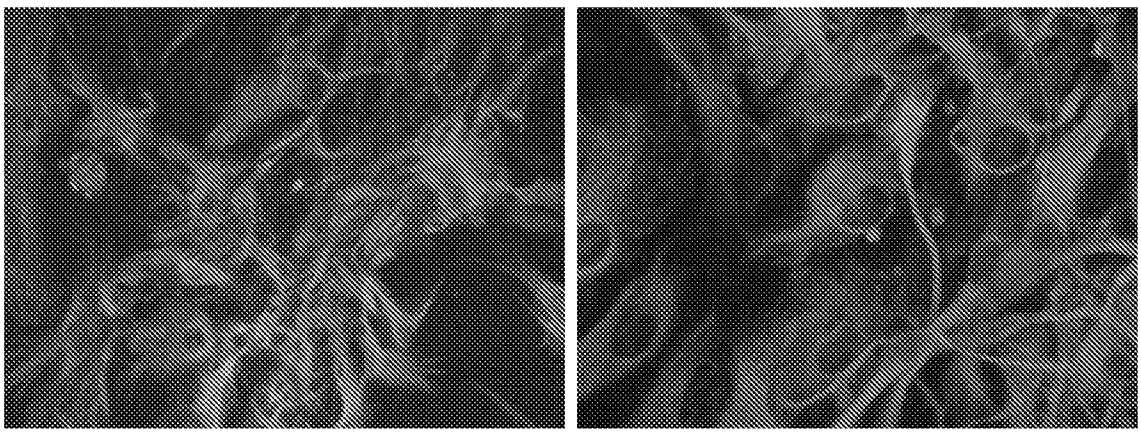


FIGURE 7

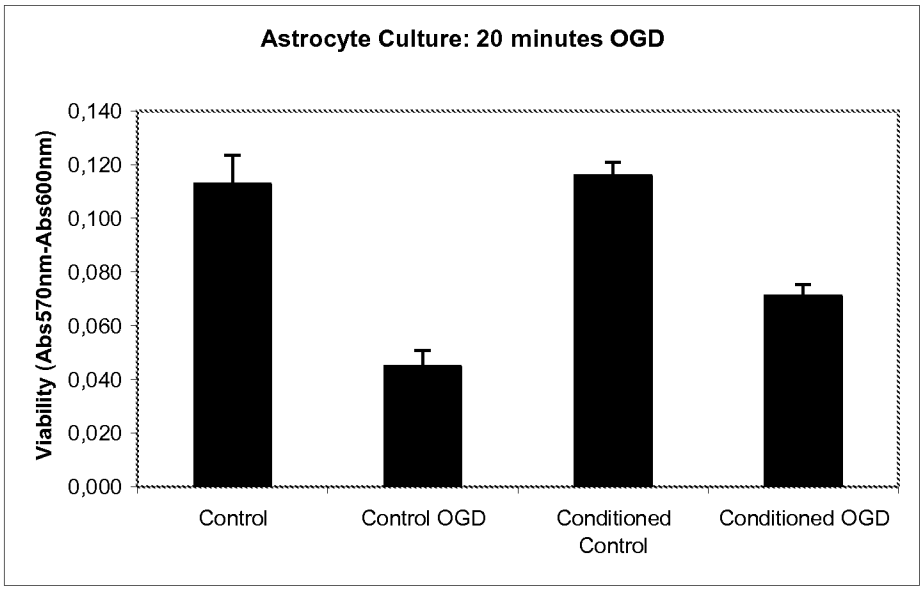


FIGURE 8

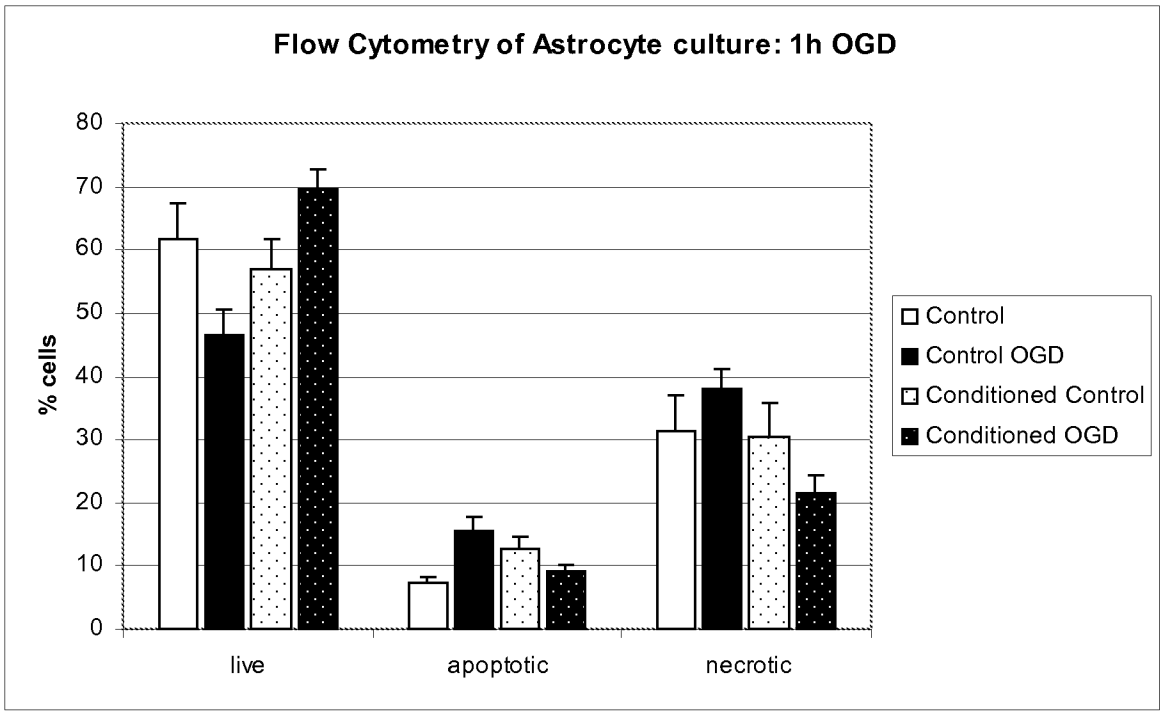


FIGURE 9

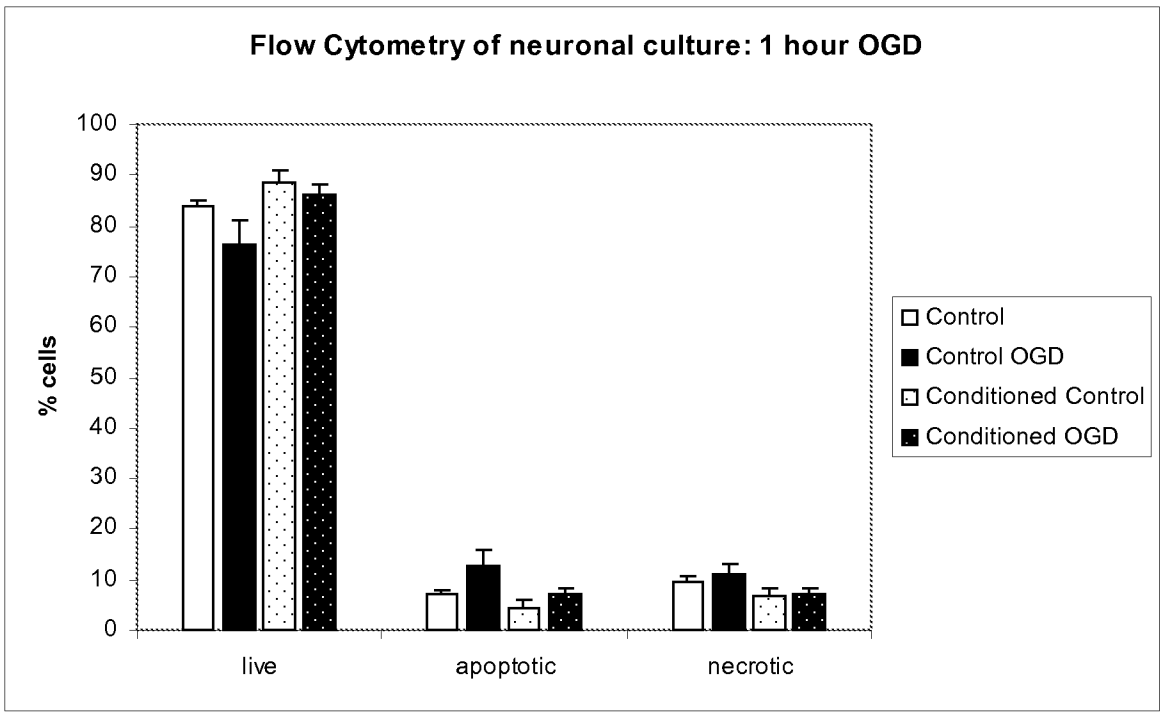

Flow Cytometry of neuronal culture: 1 hour OGD
% cells
100
90
80
70
60
50
40
30
20
10
0
live
apoptotic
necrotic
Control
Control OGD
Conditioned Control
Conditioned OGD

FIGURE 10