

(19)



(11)

**EP 2 982 375 B1**

(12)

**EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:  
**25.04.2018 Bulletin 2018/17**

(51) Int Cl.:  
**A61K 35/60 (2006.01)**

(21) Application number: **15179955.8**

(22) Date of filing: **06.08.2015**

**(54) FACTORS EXTRACTED FROM FISH EMBRYOS AND USE OF MIXTURES THEREOF IN THE CONTROL OF STEM CELL MULTIPLICATION AND DIFFERENTIATION**

AUS FISCEMBRYONEN EXTRAHIERTE FAKTOREN UND VERWENDUNG VON MISCHUNGEN DAVON BEI DER KONTROLLE VON STAMMZELLVERMEHRUNG UND DIFFERENZIERUNG

FACTEURS EXTRAITS D'EMBRYONS DE POISSON ET UTILISATION DE MÉLANGES DE CEUX-CI DANS LE CONTRÔLE DE LA MULTIPLICATION ET DE LA DIFFÉRENCIATION DE CELLULES SOUCHES

(84) Designated Contracting States:  
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR**

(30) Priority: **07.08.2014 IT MI20141464**

(43) Date of publication of application:  
**10.02.2016 Bulletin 2016/06**

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(56) References cited:  
**EP-A1- 1 374 703 EP-A1- 1 570 853**

**WO-A1-98/11905 WO-A2-2007/020222**

- **ALESSANDRA CUCINA ET AL: "Zebrafish embryo proteins induce apoptosis in human colon cancer cells (Caco2)", APOPTOSIS ; AN INTERNATIONAL JOURNAL ON PROGRAMMED CELL DEATH, KLUWER ACADEMIC PUBLISHERS, BO, vol. 11, no. 9, 27 June 2006 (2006-06-27), pages 1617-1628, XP019390844, ISSN: 1573-675X, DOI: 10.1007/S10495-006-8895-4**
- **P M BIAVA ET AL: "A Systemic Approach to Cancer Treatment: Tumor Cell Reprogramming Focused on Endocrine-Related Cancers", CURRENT MEDICINAL CHEMISTRY, vol. 21, no. 9, 1 March 2014 (2014-03-01), pages 1072-1081, XP055181315, NL ISSN: 0929-8673**
- **D'ANSELMI F.: "Zebrafish stem cell differentiation stage factors suppress Bcl-xL release and enhance 5-Fu-mediated apoptosis in colon cancer cells", CURRENT PHARMACEUTICAL BIOTECHNOLOGY, vol. 12, 1 January 2011 (2011-01-01), pages 261-267, XP009183620,**
- **Pier M Biava ET AL: "Stem Cell Differentiation Stage Factors from Zebrafish Embryo: A Novel Strategy to Modulate the Fate of Normal and Pathological Human (Stem) Cells", Current Pharmaceutical Biotechnology, vol. 16, no. 9, 1 January 2015 (2015-01-01), pages 782-792, XP055382170,**

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**EP 2 982 375 B1**

**Description**

FIELD OF THE INVENTION

- 5 **[0001]** The present invention concerns extracts from zebrafish embryos and using mixtures thereof in the control of stem cell multiplication and differentiation.
- [0002]** The present invention originates in the epigenetic field and its applications in a nutritional, cosmetic and medical context.
- 10 **[0003]** Specifically, the present invention relates to cell growth and differentiation factors collected from zebrafish embryo at specific stages of stem cell differentiation.

BACKGROUND OF THE INVENTION

- 15 **[0004]** The considerable increase in average age exposes ever-increasing segments of the population to the risks of developing tumours and chronic degenerative diseases. These conditions constitute one of the most serious health problems for which an effective therapeutic response is not yet currently available, due to the multifactorial causation and the complexity of the pathogenetic mechanisms involved in their genesis and development.
- [0005]** Some progress has been made in recent times as a result of stem cell biology studies, from which it emerges that tumour malignancy could be, at least in part, attributable to the presence of altered stem cells (cancer stem-like cells). This finding has directed numerous lines of research on the reprogramming of cancer stem-like cells.
- 20 **[0006]** The use of certain growth factors in the cancer stem-like cell reprogramming field and stem cell transplant research has led to the hypothesis that stem cell growth and differentiation factors could also be useful for determining the fate of normal, as well as altered stem cells, regenerating and/or differentiating them.
- [0007]** The present invention originates precisely in this field of scientific research.
- 25 **[0008]** A general aim of the present invention is to identify and provide cell growth and differentiation factors that play a key role in stem cell regulation and differentiation and to find an application for these factors in the nutritional and/or medical context. Another aim of the invention is to identify and select biological factors that are produced by the stem cells of zebrafish species in specific stages of embryonic development and to find specific applications in the medicinal context, in particular, in the prevention and/or treatment of neurodegenerative diseases and/or tumoural forms.

30 SUMMARY OF THE INVENTION

- [0009]** The present invention originates in having identified specific differentiation stages of the stem cells of a zebrafish embryo in which specific stem cell growth and/or differentiation factors are produced, which find application in the medical, nutritional or cosmetics field.
- 35 **[0010]** According to certain aspects of the invention, an innovative method is provided for directing the fate of normal or pathological stem cells.
- [0011]** The present description also contains teachings aimed at improving the efficiency and the therapeutic results achieved with stem cell transplantation and identifies alternative routes to transplantation itself.
- 40 **[0012]** According to some aspects, the invention originates from having found that the biological and therapeutic effects consequent to stem cell transplantation are not so much attributable to transplantation of the stem cells themselves but to stem cell production of cell growth and/or differentiation factors.
- [0013]** The applicant has now identified specific cell growth and differentiation factors and the properties that make them applicable in slowing down cell and normal aging processes and in treating certain conditions such as neurodegenerative diseases and tumours.
- 45 **[0014]** It has, in particular, been found that a mixture of the cell growth and differentiation factors of selected stages have an effective cell regeneration and therefore find application in slowing down the body's aging process and have an anti-aging action. The factors extracted in three selected stages can then be combined with other factors extracted from further specific stages of stem cell differentiation, expanding the envisaged possibilities of use in the medical, nutritional, and cosmetic field for the factors extracted in the two selected stages of differentiation. According to a first aspect of the present invention, extracts of embryo stem cells, of zebrafish, in stages of embryonic stem cell differentiation or of embryonic development as claimed in claim 1, are therefore provided.
- 50 **[0015]** It was found that mixtures of cell growth and/or differentiation factors extracted in selected stages of embryonic development, in particular of zebrafish, play a part in the stem cell regeneration process and therefore find application in all the processes in which cell repair action and tissue regeneration is required, such as for example in the case of burns, necrotic tissue processes, in particular, of the epidermis, in fistulae, pressure sores or as antiaging agents for example.
- 55 **[0016]** In the course of experimenting on the stem cells of fish species, it was also observed that growth and/or

differentiation factors extracted in the differentiation stage of the embryonic stem cells at/or 24 hours after fertilisation intervene and exert an influence in the cell differentiation stage.

**[0017]** In particular, the combination or mixture of cell growth and/or differentiation factors according to claim 1 finds application in regulating the cell processes that are at the basis of aging and cell death. According to some embodiments, the combination of factors collected in these stages find application in preventing and/or treating degenerative diseases of the central nervous system, such as Alzheimer's or Parkinson's and/or of the cardio-circulatory system such as in the case of myocardial infarction.

**[0018]** The applicant has also found, according to an aspect of the invention, that combining extracts or cell growth and/or differentiation factors extracted from embryo stem cells in the mid-blastula-gastrula stage and tail bud stage with cell growth and/or differentiation factors extracted in the 5-somite, 20-somite stages of differentiation and in the pharyngula differentiation stage, a particularly intense combined differentiation and cell regulation action is obtained.

**[0019]** This combined action finds application in the medical field typically in preventing and treating conditions associated with an increased cell replication rate, as in the case of tumours or diseases of the skin in which the epithelial cells have a greater cell replication rate than normal, as in the case of psoriasis, eczema, erythematosus lupus, atopy.

**[0020]** According to some embodiments of the invention, the growth and differentiation factors used in the context of the invention are obtained from stem cells from zebrafish.

**[0021]** According to a fourth aspect, the present invention provides a composition comprising cell growth and/or differentiation factors extracted from zebrafish embryos according to one of the previously described aspects of the invention and an edible and/or pharmaceutically acceptable carrier.

**[0022]** According to some embodiments, the composition of the invention is a pharmaceutical composition in which the carrier is or comprises a pharmaceutically acceptable excipient.

**[0023]** According to other embodiments, the composition of the invention is a nutraceutical or dietetic product or a food for special purposes or a cosmetic composition.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0024]** Some of the characteristics and advantages of the present invention will be apparent from the accompanying drawings, wherein:

Figure 1 illustrates analysis of the proteins of the factors extracted from the stem cells of zebrafish in 5 stages of development and the mixture of the 5 stages;

Figure 2 illustrates a graph which reports the results of a proliferation assay (MTT) at 24 hours on human mesenchymal stem cells (hMSC) of individual growth factors (ZF1-ZF5) and of the mixture thereof (ZF6) at 4 concentrations;

Figure 3 shows a graph which reports the results of a proliferation assay (MTT) at 72 hours on human mesenchymal stem cells (hMSC) of individual growth factors (ZF1-ZF5) and of the mixture thereof (ZF6) at 4 concentrations;

Figure 4 shows bar graphs that illustrate the level of immunofluorescence with propidium iodide of the CA1 area of the hippocampus 24h after 1 h of serum deprivation in the presence or absence of the ABC mixture of factors of example 2,

Figure 5 illustrates the immunofluorescence with propidium iodide of the CA1 area of the hippocampus 24h after 1 h of treatment with NMDA (50  $\mu$ M) in the presence or absence of the ABC mixture.

Figure 6 illustrates the immunofluorescence with propidium iodide of the CA1 area of the hippocampus 24h after 1 h of treatment with NMDA (300  $\mu$ M) in the presence or absence of the ABC mixture.

Figure 7 illustrates the quantisation of the immunofluorescence with propidium iodide of the CA1 area of the hippocampus 24h after 1 h of serum deprivation in the presence or absence of the extracts A, B or mixture C.

Figure 8 illustrates the quantisation of the immunofluorescence with propidium iodide of the CA1 area of the hippocampus after 24h of treatment with NMDA (50  $\mu$ M; 1h) in the presence or absence of the extracts A, B or C mixture.

## DETAILED DESCRIPTION OF THE INVENTION

**[0025]** The applicant, studying the differentiation of embryonic stem cells of zebrafish in particular, has identified stem cell growth and differentiation factors that are useful for regenerating and/or differentiating stem cells.

**[0026]** In particular, the invention originates from:

A) having identified the most significant moments of the differentiation of various types of stem cells,

B) having analysed and selected the two early stages of stem cell differentiation in which essential factors for cell regeneration are produced,

C) having identified the specific percentage distribution of the protein fractions present in the two selected stages and the proteins produced in those specific early stages of cell differentiation, in particular at the time in which the

stem cells differentiate from totipotent to pluripotent;

D) having found specific applications in the medical field for the cell growth and differentiation factors identified by performing *in vitro* studies, for example by analysing the effects on the multiplication, proliferation, and differentiation of human mesenchymal stem cells collected from adipose tissue.

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**[0027]** According to some aspects of the invention, it was thus found that by providing extracts from zebrafish embryo, in particular containing stem cells in specific stages of embryonic development or stem cell differentiation, we are intervening in the regulation and/or differentiation of human cells.

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**[0028]** This approach revolutionises the conventional approach of using stem cells to intervene in some cell mechanisms deemed at the origin of diseases that originate from an uncontrolled cell growth.

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**[0029]** According to a first aspect of the present invention, an extract obtained by extraction from zebrafish embryos at selected stages of stem cell differentiation as claimed in claim 1, is provided. According to certain embodiments i) the extracts or ii) cell growth and/or differentiation factors extracted from zebrafish embryos in the mid-blastula-gastrula stage and in the tail bud and pharyngula stage have the following percentage distribution of protein fractions, referring to the total proteins having molecular weight of less than 100 Kdaltons

protein fraction of 14 kilodaltons: 14.6%

protein fraction of 20 kilodaltons: 4.2%

protein fraction of 25-30 kilodaltons: 28.4%

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protein fraction of 45 kilodaltons: 14.8%

protein fraction of 97 kilodaltons 38%.

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**[0030]** The preparation of the growth factors or embryonic extracts that contain them can be achieved following conventional techniques. In certain embodiments, preparation of the embryonic extracts of the invention comprises the following steps:

a) embryos are collected at a specific stage of embryonic development;

b) a solvent is added;

c) the mixture obtained in step b) is subjected to agitation, with a turbo-emulsifier for example;

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d) the mixture is optionally filtered, by 90 micrometre filters for example and

e) with 5-10 micrometre filters for example.

**[0031]** A stock solution is obtained from step c), which can be diluted with one or more conventional biocompatible solvents to obtain solutions with different degree of dilution depending on needs and uses.

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**[0032]** According to some embodiments, the solvent used in step b) is based on a mixture of glycerin and an aqueous solution of ethyl alcohol, for example 30% v/v, typically in a glycerol/alcohol ration of 85:15 v/v.

**[0033]** The weight ratio of said solvent and said embryos is preferably between 20:1 and 2:1.

**[0034]** The agitation of step c) is preferably carried out at room temperature, as also applies to the other operations, for a period of time typically ranging from 1 to 2 hours.

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**[0035]** According to some embodiments, step c) of mixing or agitation is performed with a turbo-emulsifier, emulsifying each one 1 to three times for 1-4 minutes.

**[0036]** According to another aspect, the present invention provides a mixture or combination of cell growth and/or differentiation factors extracted or collected from zebrafish embryos in the differentiation stage of embryonic mid-blastula-gastrula stem cells and in the tail bud stage with factors in the differentiation stage selected in the 5-somite, 20-somite stages and in the pharyngula differentiation stage, in particular in the early pharyngula stage.

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**[0037]** In certain embodiments, the mixture of extracts from zebrafish embryos or cell growth and/or differentiation factors contains 5% by weight of factors extracted in the mid-blastula-gastrula stage, 10% of factors extracted in the tail bud stage, 10% of factors extracted in the 5-somite stage, 25% of factors extracted in the 20-somite stage, 50% of factors extracted in the pharyngula stage.

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**[0038]** The mixture of factors according to this latter aspect of the invention finds application in the medical field in the treatment of conditions with abnormal cell replication rate as in the case of tumours or of certain affections of the skin such as in cases of psoriasis, eczema, erythematosus lupus, atopy.

**[0039]** The applicant has further, experimentally observed that the cell growth and differentiation factors, in particular in the mixtures of stages 1 and 2 with the 5-somite, 20-somite stages and in the pharyngula stage, determine a slowing down of cell proliferation and an increase in programmed cell death, or apoptosis, while the early stages have even highlighted a multiplicative effect.

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**[0040]** None of the stages and doses tested lead to death by necrosis or to toxicity for the cells. The extracts have thus proved harmless, and safe.

**[0041]** The experiments carried out have also demonstrated that when the information is sufficiently complete, unexpected results are obtained, both in protection against the insults that cause degeneration and tissue damage. This fact was confirmed by the experiments that related to the proliferation or, on the contrary, slowdown of the multiplication of mesenchymal stem cells, which demonstrate that they can therefore be directed both toward regeneration and toward differentiation, through the specific and selective use of the various protein networks identified in the individual differentiation stages of the embryo, of zebrafish, and above all, through the use of the mixtures thereof, or of the mixtures of multiple differentiation stages. The experimental data reported in the examples demonstrate that the various growth and differentiation factors studied have the ability to selectively and specifically regulate various genes that are the expression of cell staminality or differentiation.

**[0042]** Within the present description, the terminology extract(s) from zebrafish embryo(s) substantially has the same meaning of cell growth and/or differentiation factors. Typically, the cell growth and/or differentiation factors mentioned in the present description are extracts from zebrafish embryos.

**[0043]** The term factors should be intended as cell growth and/or differentiation factors.

**[0044]** The growth and differentiation factors studied constitute are epigenetically able to regulate stem cell gene expression, so as to direct them toward regeneration and/or toward differentiation. This finding is very important in both regenerative medicine, where tissues are to be regenerated, such as in the case of ulcers, wounds, etc., or tissues are to be regenerated tissues in the case of degenerative diseases.

**[0045]** This data supports the use of the factors related to the invention in stem cells transplantations, considering that, being small molecules having a low molecular weight, as demonstrated by the proteomics studies reported herein, they have a sublingual or percutaneous absorption.

**[0046]** A randomised, controlled clinical study of 179 cases of intermediate, advanced stage hepatocellular carcinoma, in which other treatments were no longer possible and therefore compassionately treated with these factors, sublingually, demonstrated regression in 20% and non-progression in 16% with a significant increase in the survival of patients treated. There is therefore a percutaneous absorption of these factors in psoriatic patients, in whom the unilateral treatment of lesions has resulted in an improvement or a disappearance of even contralateral lesions.

**[0047]** The cell differentiation factors or extracts from zebrafish embryo of the invention can consequently find application

a) as anti-aging agents

b) as tissue regeneration factors to treat scars, pressure ulcers, varicose ulcers, etc.

c) in conjunction with stem cell transplantations to improve the clinical effect of the transplantations

d) for preventing or treating various chronic degenerative diseases, such as neurodegenerative diseases, for example Parkinson's, Alzheimer's, multiple sclerosis, ALS, cardiovascular diseases, for example ischaemic cardiopathy, myocardial infarction, stroke and tumour. The extract of the invention finds application in treating stroke (Therapeutic Effects of hMAPC and hMSC Transplantation after Stroke in Mice, 2012 Silvia Mora-Lee et al. in PLoS ONE 7(8): e43683. doi:10.1371/journal.pone.0043683 editor: Brahim Nait-Oumesmar, Université Pierre et Marie Curie-Paris6, INSERM, CNRS, France).

e) in ocular conditions related to aging, such as exudative maculopathy According to certain aspects, the invention relates to extracts or the factors and the mixtures thereof according to any one of the previously described embodiments and compositions that contain them, for use in combination with stem cell transplantation or to replace stem cell transplantation.

**[0048]** According to certain aspects, the present invention therefore provides a composition comprising extracts or cell growth and/or differentiation factors extracted from zebrafish embryos according to any one of the previously described aspects and embodiments.

**[0049]** According to some embodiments, the composition of the invention is a pharmaceutical composition, a dietary supplement or a nutraceutical that can be introduced into the dietary regiment of an individual suffering from one or more of the previously described affections or conditions.

**[0050]** In some embodiments, the composition of the invention is a nutraceutical product comprising the mixtures of factors according to the previously described embodiments and an edible carrier. The nutraceutical product of the invention finds use in improving the nutritional conditions of the human body or performance status and quality of life.

**[0051]** In some embodiments, the composition of the invention can further comprise active substances, vitamins, nutritional substances, micronutrients, and/or minerals.

**[0052]** The term "carrier" as used herein, indicates a medium, excipient, diluent with which the combination of therapeutic or active substances is administered.

**[0053]** Any carrier and/or excipient suitable for the form of preparation desired for administration to humans is contemplated for use with the compounds described in the present invention.

**[0054]** For the purposes of the present application, the term "physiologically acceptable" or "edible" indicate edible

substances that are approved by the health authorities for use in pharmaceutical, nutritional or dietary applications.

**[0055]** A physiologically acceptable carrier can be a pharmaceutically acceptable carrier.

**[0056]** The compositions of the present invention comprise any composition produced by administering the extract or cell growth and differentiation factors of the present invention and a physiologically or pharmaceutically acceptable carrier. These compositions are suitable for dietary, nutritional, pharmaceutical, or dietetic use in mammals, in particular in human beings.

**[0057]** According to some embodiments, the composition of the invention is a food for special medical purposes.

**[0058]** According to another aspect, it is described a therapeutic treatment method of a disease selected among degenerative disease, in particular neurodegenerative disease, cardiovascular or tumour disease, comprising the administration of a therapeutically effective amount of an extract from zebrafish embryo in the mid-blastula-gastrula, tail bud and pharyngula, and 5-somite and 20-somite stages of cell differentiation.

**[0059]** The composition of the invention can take a wide variety of preparation forms, depending on the desired route of administration.

**[0060]** For example, for oral administration, the composition can be in solid form, as tablet, capsule, powder, granular, prolonged-release formulations of the active substances, for example. The compositions in solid form, in particular in granular or powder form, are preferred with respect to the other types of preparations.

**[0061]** The preparations in solid form can comprise one or more carriers such as starches, sugars, microcrystalline cellulose, and optionally diluents, granulating agents, lubricants, binders, disintegration agents, for example.

**[0062]** The tablets, pills, capsules, granules can also contain a binder such as tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; a sweetening agent such as sucrose, lactose or saccharin. If desired, the tablets can be coated by traditional techniques.

**[0063]** When the pharmaceutical form unit is a capsule, it can contain, in addition to the materials of the above-mentioned type, a liquid carrier such as an adipose oil.

**[0064]** In the case of preparations in liquid form for oral administration, such as for example in the case of suspensions, emulsions, solutions, a suitable carrier can be selected from water, glycols, oils, alcohol, and mixtures thereof.

**[0065]** Flavouring agents, preservatives, colouring agents and the like can also be present in the composition.

**[0066]** In some embodiments, the growth factors contained in the composition of the present invention can be combined or mixed as active substances in intimate mixture with a suitable edible carrier and/or excipient according to pharmaceutical and food industry or traditional nutritional techniques.

**[0067]** The compositions for pharmaceutical, cosmetic or nutritional use can be adequately presented in single pharmaceutical form and prepared by any well-known technique of the pharmaceutical or dietary art.

**[0068]** In some embodiments, the compositions or preparations of the invention can contain at least 0.0001% of each growth factor. The amount of growth factors present in these compositions is such that a prophylactically or therapeutically effective dosage will be obtained.

**[0069]** In some embodiments, the composition of the invention further comprises one or more additional components such as additives, fillers, stabilisers, emulsifiers, texturisers, film-forming agents, plasticisers, humectants and thickeners.

**[0070]** Various other materials can be present, such as coatings or to modify the physical form of the pharmaceutical unit. For example, tablets can be coated with shellac, sugar or both. To prevent disaggregation during transit through the upper part of the gastrointestinal tract, the composition can be a formulation with enteric coating.

**[0071]** A syrup or elixir can contain, in addition to the active substance, sucrose as a sweetening agent, suitable preservatives, a colourant and a flavouring agent such as cherry or orange flavour.

**[0072]** In some embodiments, the composition of the invention is a cosmetic composition comprising a cosmetically acceptable carrier.

**[0073]** Suitable cosmetic compositions are in the form of a cream, lotion, serum or emulsions of the oil/water type.

**[0074]** The cosmetic compositions of the invention find application in the treatment of skin aging, in the cosmetic treatment of wrinkles, skin blotches and dry and desquamating skin, for example.

**[0075]** According to certain embodiments, the pharmaceutical composition is for use in medicine in combination with stem cell transplantation or to replace stem cell transplantation.

**[0076]** In some embodiments, the formulation will contain quantities of growth factors that will depend on the severity of the associated symptoms, the condition, the further therapies underway, the individual's state of health and on the response to the combination of active substances. In some embodiments, the dose is in the range of 0.00001% by weight to about 10%, of 0.0001 to 5% of 0.001 to 1%, of 0.10 to 1% by weight of the formulation.

**[0077]** The present invention will now be described below with reference to the following examples, which are provided for illustrative purposes only and are not to be construed as limiting the present invention.

## EXAMPLES

EXAMPLE 1

5 **[0078]** Effect of cell growth and differentiation factors extracted in the mid-blastula-gastrula, tail bud, 5-somite, 20-somite stages and in the pharyngula stage from zebrafish embryo taken individually and in mixtures, on the proliferation and differentiation of mesenchymal stem cells (hMSC).

Materials and methods:

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**[0079]**

1. Cell model: adult human mesenchymal stem cells isolated from adipose tissue (hMSC) by means of the Lipogems system,
- 15 2. Determination of the protein concentration in the zebrafish (ZF) extracts and: assay with BCA,
- 3 Identification of the different individual proteins: Nano-LC-MS/MS (liquid chromatography combined with mass spectrometry) analysis
- 4 Study of cell proliferation: assay with MTT
- 20 5. Gene expression analysis: Relative quantitative RT-PCR

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**1. Adult human mesenchymal stem cells isolated from adipose tissue (hMSC) by means of Lipogems**

**[0080]** hMSC were isolated from human adipose tissue using Lipogems. This is a device for collecting stem cells from lipoaspirates using a mechanical approach instead of enzymatic digestion.

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**2. Assay with BCA (protein determination in the extracts)**

**[0081]** The protein content in the zebrafish extracts was determined using the BCA assay, which is a colorimetric assay for the quantification of protein. The protein content in each extract was assessed in quadruplicate using bovine serum albumin (BSA) as standard. The protein concentration in the extracts ranged from 1.5-2.7 mg/ml.

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**3. Nano-LC-MS/MS analysis**

**[0082]** The proteins were identified by means of LC-MS/MS analysis. The MS/MS analysis of peptides generated by in-gel digestion in gel was accomplished by a nano- LC-ESI-Q-TOF (QSTAR Elite, Applied Biosystem) apparatus. HPLC was accomplished on an LC Packings Ultimate 3000 nanoflow (Dionex, Sunnyvale, CA) system. Samples (5  $\mu$ L) were injected into a C18, 5  $\mu$ m, 100A, 300  $\mu$ m I.D. x 5 mm (LC Packing, Dionex) cartridge trap at 30  $\mu$ L/min for 5 min (2% ACN, 0.1% formic acid) to enrich and purify the peptides. The capillary separation of the peptides trapped was performed using a C18, 5  $\mu$ m, 300A, 75  $\mu$ m I. D. x 15 cm Vydac (Grace) column. The chromatographic conditions and mass parameters were adjusted as follows: buffer A (2% ACN, 0.1% formic acid) and buffer B (2% water, 0.1% formic acid in ACN) with the gradient of 8-50% buffer B in 35 min, 50-80% in 15 min, 80% for the next 15 min and 80-8% in 5 min, lastly the column was re-equilibrated at 8% B for 10 min. For each TOF MS (350-1300m/z) scan, the two ions with more intense double or triple charge were selected for the ionic scans of the potential product (110-1600 m/z). The ion spray voltage was 1.6 kV, the gas barrier was set to 15 and the potential sputtering was 80V. Mascot V1.6b20 (Matrix Science, London) software was used for the protein database search. Searches were performed using the NCBI database and the following standard parameters: zebrafish; tryptic digest with at most a scission failure; carbamidomethylation of cysteine, partial oxidation of methionine, and a mass tolerance of 100 ppm. For MS/MS data, the searches were performed with the following additional criteria: maximum tolerance of 0.3 Da for MS/MS data and the search for 2+ and 3+ peptide charge.

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**[0083]** Chemical compounds and reagents.

**[0084]** The general chemical compounds were obtained from Sigma-Aldrich (Poole, UK). All the chemical and biochemical compounds used were of analytical grade.

**4. Assay with MTT (cell proliferation)**

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**[0085]** The proliferation of human mesenchymal stem cells after treatment with zebrafish extracts was determined using the assay with MTT. In metabolically active cells, MTT is converted into formazan, which can be spectrophotometrically evaluated. The amount of formazan is proportional to the number of cells in the well.

Optimisation

[0086] Initially, preliminary studies were performed to determine optimal cell density, as well as the time for the metabolism of MTT. The following experimental conditions were selected on the basis of these studies:

Number of cells: 4000 or 5000 seeded cells per cm<sup>2</sup> in a 48-well plate.  
 Treatment: the cells were treated with extracts of zebrafish in the following stages:

- stage 1: mid-blastula-gastrula stage
- stage 2: tail bud stage
- stage 3: 5-somite stage
- stage 4: 20-somite stage
- stage 5: early (typically around 24 hours after fertilisation) differentiation stage of the pharyngula stage
- stage 6: mixture of the stages 1-5 ;

each stage at four different concentrations: 10, 1, 0.1, 0.01 µg/ml and incubated for 24 or 72 h respectively.  
 MTT metabolism time: 3h  
 Statistics: The data is presented as mean absorbance compared with respect to the control (untreated cells).

Results

[0087] The results on the fractions having different molecular weight for the different stages of differentiation are indicated in Figure 1.

Table 1 below, indicates the individual different proteins identified by LC-MS/Ms analysis.

**Table 1**  
**List of proteins identified using the nano LC-ESI-1-TOF in the zebrafish embryo in the mid-blastula-gastrula phase**

Access	Protein name	Score	Molecular weight	Calculated pl	Sequence coverage
gi 166795887	precursor of vitellogenin 1	1108	150308	8,68	19
gi 94733730	Vitellogenin 1	1039	149825	8,74	21
gi 94733733	New protein similar to vitellogenin 1 (vg1)	913	149828	8,92	19
gi 94733734	New protein similar to vitellogenin 1 (vg1)	835	150550	8,83	16
gi 145337918	Vtg1 protein	780	116965	9,07	18
gi 94733731	New protein similar to vitellogenin 1 (vg1)	762	149911	8,84	19
gi 94732723	New protein similar to vitellogenin 1 (vg1)	745	147826	8,73	17
gi 159155252*	Protein Zgc:136383	720	124413	8,78	17
gi 68448530	Vitellogenin 5	559	149609	8,77	13
gi 92097636	Zgc:136383	402	28924	9,33	36
gi 63100501	Protein Vtg1	345	36580	9,23	28
gi 57864789	Vitellogenin 7	341	24490	8,37	40
gi 57864783	Vitellogenin 4	334	31304	9,48	27
gi 113678458	precursor of vitellogenin 2 isoform 1	323	181208	8,70	11
gi 125857991	Protein Zgc:136383	171	149328	8,93	9
gi 152093112*	Type 1 procollagen alpha chain 2	169	147826	9,35	4
gi 57864779	Vitellogenin 2	122	69906	7,84	8
gi 11118642	precursor of vitellogenin 3	117	140477	6,92	2
gi 303227889	Vitellogenin 6	73	151677	8,84	4

Continued

Access	Protein name	Score	Molecular weight	Calculated pI	Sequence coverage
gi 13242157*	Protein ZP2 of the pellucid membrane of the egg	71	48194	6,04	5
gi 6644111*	nucleoside diphosphate kinase-Z1	69	17397	7,77	14
gi 18859071*	nucleoside diphosphate kinase 3	69	19558	7,68	7
gi 126632622*	New prot. cont. a galactose binding lectin domain	67	19245	9,33	13
gi 66773080*	Mitochondrial ATP-synthase beta-like subunit	66	55080	5,25	4
gi 38541767*	Ppia protein	60	19745	9,30	13
gi 1865782	Protein H5C7D	58	71473	5,18	2
gi 28279108	Heat shock protein B	58	71382	5,32	4
gi 41152402*	Histone H2B 3	49	13940	10,11	11
gi 41393113*	Collagen, type I, alpha 1b precursor	46	137815	5,39	4
gi 94732492*	RAS homologue gene family, member F	46	24035	9,00	6
gi 47778620*	tryptophan hydroxylase D2	45	55686	6,56	1
gi 68448517*	Glycoprotein of the pellucid zone 3, precursor 2	44	47365	4,92	2
gi 326677766*	ENVISAGED: RIMS binding protein 2-like	41	138659	5,86	0
gi 112419298	Protein Vtg3	40	60622	6,32	2
gi 54400406*	Glutaredoxin 3	39	36541	5,18	11
gi 41152400*	peptidylprolyl isomerase a-like, a-like	37	17763	8,26	7

The ionic score is  $-10 \cdot \log(P)$ , wherein P is the possibility that the pairing observed is a causal event.  
Individual ionic scores  $>36$  indicate identity or extensive homology ( $p < 0.05$ ).  
Individual protein scores are derived from ionic scores non-probabilistic basis for ranking protein hits.

### 30 Results of the cell proliferation tests

[0088] Cell proliferation after 24h and 72h of incubation with zebrafish extracts, in different independent experiments.

[0089] The data is presented as normalised absorbance (treated cell abs/ untreated cell abs) and as the average of 3 independent experiments.

[0090] The results of the assay with MTT after 24 h of treatment with zebrafish extract are illustrated in Fig. 2.

[0091] From the data, the following can be concluded:

- After 24 h of treatment, there was a slight reduction in proliferation;
- Extract 6 (mix of all the stages) had the most pronounced effect, and the effect was more evident with the minimal concentration (i.e. 0.01  $\mu\text{g/ml}$ ).

[0092] The results of the assay with MTT after 72h of treatment with zebrafish extract are illustrated in Fig. 3.

[0093] After 72 h of treatment, we can conclude that:

- Stages 4, 5 and 6 inhibit the proliferation of hMSCs;
- The effect of the extracts was dose-dependent (for all stages, 10  $\mu\text{g/ml}$  had the more pronounced effect).

[0094] Comparison of the results with MTT after 24 and 72 h, assay with MTT after 72 h of treatment with zebrafish.

[0095] Comparing the 24 and 72 h, highlights that the early stages (i.e. 1, 2 and 3) and the late stages influence proliferation in a different way, with stages 4, 5 and 6 being the most effective (i.e. less proliferation):

### 4. Relative quantitative RT-PCR (gene expression)

[0096] In order to study the effect of embryonic zebrafish extracts on differentiation, gene expression was analysed after incubation with the extracts. Since the assay with MTT indicated that the more pronounced effect was observed after treatment with the ZF6 extract (a mixture of 5 stages), gene expression was studied after treatment with this extract in concentrations ranging from 0.01 -10  $\mu\text{g/ml}$ .

[0097] The studied genes are markers for:

## EP 2 982 375 B1

- stem cell potential (OCT3/4 and KLF4)
- cardiogenesis (NKX2.5 and MEF2C)
- vasculogenesis (VEGF)
- neurogenesis (NEUROG1)
- 5 - myogenesis (MyoD).

**[0098]** Gene expression was analysed using relative quantitative RT-PCR after 24, 72 hours, and 7 days of treatment. Gene expression was normalised to a domestic gene (HK) (GAPDH) and compared against the untreated cells.

**[0099]** Fundamental principle.

10 **[0100]** On the basis of the result of the assay with MTT, ZF6 was used to treat the cells before analysing gene expression.

**[0101]** The following concentrations were used:

- 10 µg/ml in the 24 and 72 h experiments;
- -0.01 -0.01 µg/ml in the 7-day experiments.

15 **[0102]** In the first experiments, low concentrations of ZF6 (0.01, 0.1 µg/ml, 1µg/ml) were used to study gene expression after 7 days of treatment. An additional concentration (10µg/ml) was added in the 24 and 72 h experiments.

**[0103]** The following experiments were then carried out:

- 20 - hMSCs treated with ZF6 at 0.01 and 0.1 µg/ml and RNA extraction after 7 days (n= 2);
- hMSCs treated with ZF6 at 1µg/ml and RNA extraction after 7 days (n= 1);
- hMSCs treated with ZF6 at 10, 1, 0.01 and 0.1µg/ml and RNA and protein extraction after 24 h (n= 1);
- hMSCstreated with ZF6 to 10, 1, 0.01 and 0.1 µg/ml and RNA and protein extraction after 72 h (n= 1).

### 25 Results

**[0104]** The results after 7 days of treatment with ZF6 have highlighted:

- 30 - Treatment with ZF6 0.01 µg/ml induces a weak negative modulation (change in times < 1) of OCT3/4, the expressions of KLF4 VEGF (blue bars) as well as a positive modulation of the expressions of NKX2.5 iMEF2C;
- Treatment with ZF6 0.1 µg/ml appears to induce a positive modulation of the expression of OCT3/4, NKX2.5 and MEF2C and a negative modulation of the expression of VEGF, while the expression of KLF4 is similar to the control. Analysis of gene expression after 24 h of treatment with ZF6 (10µg/ml).

35 **[0105]** We can conclude that after 24 h:

- Treatment with ZF6 induced a positive modulation of the expression of VEGF and MEF2C;
- Treatment with ZF6 induced a negative modulation of the expression of KLF4 and NKX2.5.
- The expression of OCT3/4 (marker of stem cell potential) was not affected.

40 Analysis of gene expression after 72 h of treatment with ZF6 (10µg/ml).

**[0106]** We can conclude that after 72 h:

- 45 - Treatment with ZF6 induced a positive modulation of the expression of VEGF and MEF2C;
- Treatment with ZF6 induced a negative modulation of the expression of NKX2.5.
- The expression of OCT3/4 and KLF4 was not affected.
- The expression of NEUROG and MyoD was not detected in the cells treated or in the controls.

50 **[0107]** In conclusion, it has been demonstrated that the factors (in particular those present in ZF1+ZF2, ZF5 and ZF6) have led to a slowing down of cell proliferation and to an increase in programmed cell death, or apoptosis, while the early stages also highlighted a multiplicative effect. None of the stages and doses tested led to death by necrosis or to toxicity for the cells. The extracts thus proved harmless, and safe.

### 55 EXAMPLE 2

**[0108]** Study of the effects produced by factors collected in 3 different stages of differentiation [A=ZF1+ZF2 (mid-blastula-gastrula + tail bud), B=ZF1+ZF2+ZF5: (mid-blastula-gastrula + tail bud + early pharyngula stage), C=ZF6 (a

mixture of mid-blastula-gastrula + tail bud + 5-somite + 20-somite + early pharyngula stage) in preventing the neurodegeneration of hippocampus cells.

**[0109]** Organotypical hippocampal slices were prepared as previously described (Gardoni et al., 2002) using 7-8-day rats; all pharmacological treatments were carried out in organotypical slices on the 14th day in culture (*DIV14*).

**[0110]** To test the effect of the zebrafish extract, organotypical slices were exposed to NMDA 50  $\mu$ M or to NMDA 300  $\mu$ M in serum free medium, in the presence or absence of the extracts. In some experiments, the slices were incubated for one hour in serum-free medium in the presence or absence of the extracts. For each type of treatment, the neuroprotective activity of both the mixture of the extracts (A+B+C) and the individual extracts (A or B or C), was evaluated.

**[0111]** After one hour, the slices were washed with serum-free medium and then incubated with their medium in the presence or absence of the zebrafish extract for 24 hours.

**[0112]** Fluorescence was used, in combination with the use of propidium iodide (PI) (5 mg/ml) as previously described (Pellegrini-Giampietro et al. 1999), to evaluate the cell damage induced by the different treatments. Quantitative analysis of cell mortality was performed in the CA1 area of the hippocampus using the maximum cell damage obtained by exposing the organotypical slices to treatment with NMDA, as term of comparison.

**[0113]** The images were acquired with a Zeiss Axiovert 200M (10x lens) epifluorescence microscope, and CoolSnap CCD camera. For the quantitative analysis, the images were acquired with the same settings and exposure times. The average intensity of fluorescence was determined after tracing the area corresponding to the CA1 area and the mortality analysed as a function of the fluorescence intensity and of the area expressed as pixel<sup>2</sup>.

## Results.

**[0114]** A first series of experiments was carried out in order to establish the optimal experimental conditions for the evaluation of a possible action of the neuroprotective type of the zebrafish extracts.

**[0115]** For this purpose, we verified that treatment for 1 h with NMDA 50  $\mu$ M and 300  $\mu$ M induced a significant mortality in our experimental conditions. Organotypical hippocampal slices (*DIV14*) were treated for one hour with NMDA 50  $\mu$ M, or with NMDA 300  $\mu$ M, so as to have a condition that determines certain mortality, and staining with PI was carried out 24h thereafter. After fixation, the CA1 was acquired and the mortality analysed as described in the materials and methods. Treatment with NMDA 50  $\mu$ M determines a 47% increase in mortality (NMDA 50  $\mu$ M vs CTRL) while NMDA 300  $\mu$ M determines a 139% increase (NMDA 300  $\mu$ M vs CTRL).

**[0116]** The neuroprotective properties of the extracts were then evaluated following exposure of the organotypical hippocampal slices to three different toxic stimuli, of different intensity (deprivation serum, NMDA 50  $\mu$ M, NMDA, 300  $\mu$ M; 1h). In a first series of experiments, the possible neuroprotective effect of the mixture of C extracts was analysed. The mixture was contemporaneously added (1:100 dilution) to treatment with NMDA or to simple serum deprivation and the analyses were carried out after 24 h.

**[0117]** As shown in Figure 4, treatment with the ABC mixture determines a significant reduction in neuronal mortality ( $-31.6 \pm 6.2\%$ ,  $*p=0.005$ ) induced by 1h of serum deprivation.

**[0118]** Treatment with NMDA (50  $\mu$ M) caused a significant increase in mortality in the CA1 area ( $** p=0.002$ , NMDA 50  $\mu$ M vs CTRL); cotreatment with the ABC mixture determines a significant reduction in the mortality induced by NMDA (50  $\mu$ M) (Figure 5;  $*p=0.01$ , NMDA 50  $\mu$ M +ABC vs NMDA 50  $\mu$ M).

**[0119]** In a last series of experiments, the capacity of the ABC mixture to induce neuroprotection even after treatment of hippocampal slices with even higher concentrations of NMDA (300  $\mu$ M). Even under these experimental conditions, cotreatment with the ABC mixture determines a significant reduction in the mortality induced by NMDA (300  $\mu$ M) (Figure 6;  $* p=0.0002$ , NMDA 300  $\mu$ M vs control;  $** p=0.009$  NMDA 300  $\mu$ M + ABC vs NMDA 300  $\mu$ M).

**[0120]** The possible neuroprotective properties of the individually administered extracts (A, B or C) was subsequently evaluated. In this case also, the neuronal mortality was evaluated, through the assay with propidium iodide, following exposure of the organotypical hippocampal slices to serum deprivation or to NMDA, in the presence of the various zebrafish extracts. As shown in Figures 7 and 8, none of the individual extracts has shown a significant neuroprotective capacity, either following serum deprivation (Figure 7) or following treatment with NMDA (50  $\mu$ M; Figure 8).

## Discussion and conclusions

**[0121]** In this study we have assessed the neuroprotective properties of zebrafish extracts, both administered as a mixture of the three extracts and as individual extracts (A, B, C), following exposure of organotypical hippocampal slices to various types of neurotoxic insults.

**[0122]** The best results in terms of neuroprotection with regard to each type of toxic insult analysed were obtained using the A+B+C mixture. Extract A also showed a certain neuroprotective action, which can be defined at the limit of significance, especially with regard to cell death induced by serum deprivation.

**[0123]** The A+B+C mixture of extracts has, on the other hand, demonstrated a significant neuroprotective activity with

regard to the neuronal death induced by 1 h of serum deprivation or induced by treatment with NMDA (both 50  $\mu$ M and 300  $\mu$ M). This means that a certain redundancy of information must be used to obtain the best response.

5 **Claims**

1. An extract of fish embryos in selected stages of stem cell differentiation, **characterised in that** it is a mixture of extracts from zebrafish embryos in the mid-blastula-gastrula stage, tail bud stage, 5-somite stage, 20-somite stage and pharyngula stage.  
10
2. The extract according to claim 1 for use as medicament.
3. The extract according to claim 1 for use in preventing or treating neurodegenerative diseases.
- 15 4. A pharmaceutical composition comprising an extract according to claim 1 and a pharmaceutically acceptable carrier.

**Patentansprüche**

- 20 1. Extrakt aus Fischembryonen in ausgewählten Stadien der Stammzelldifferenzierung, **dadurch gekennzeichnet, dass** es sich um ein Gemisch von Extrakten aus Zebrafischembryonen in der Mittel-Blastula-Gastrula-Stufe, der Tail-BuD-Stufe, 5-Sommit-Stufe, der 20-Sommit-Stufe und der Pharyngula-Stufe handelt.
2. Extrakt nach Anspruch 1 zur Verwendung als Arzneimittel.  
25
3. Extrakt nach Anspruch 1 zur Verwendung bei der Vorbeugung oder Behandlung von neurodegenerativen Erkrankungen.
4. Pharmazeutische Zusammensetzung, die einen Extrakt nach Anspruch 1 und einen pharmazeutisch annehmbaren Träger aufweist.  
30

**Revendications**

- 35 1. Extrait d'embryons de poisson dans des stades choisis de la différenciation des cellules souches, **caractérisé en ce qu'**il s'agit d'un mélange d'extraits d'embryons de poisson zèbre dans le stade mid-blastula-gastrula, le stade du bourgeon caudal, le stade 5 somites, le stade 20 somites et le stade pharyngula.
2. Extrait selon la revendication 1 pour une utilisation en tant que médicament.  
40
3. Extrait selon la revendication 1 pour une utilisation dans la prévention ou le traitement de maladies neurodégénératives.
4. Composition pharmaceutique comprenant un extrait selon la revendication 1 et un support pharmaceutiquement acceptable.  
45

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Analysis of zebrafish extract proteins:  
5 stages of development (ZF1-ZF5) and a mix of the 5 stages (ZF6)

### SDS page of the Embryonic Zebrafish extracts

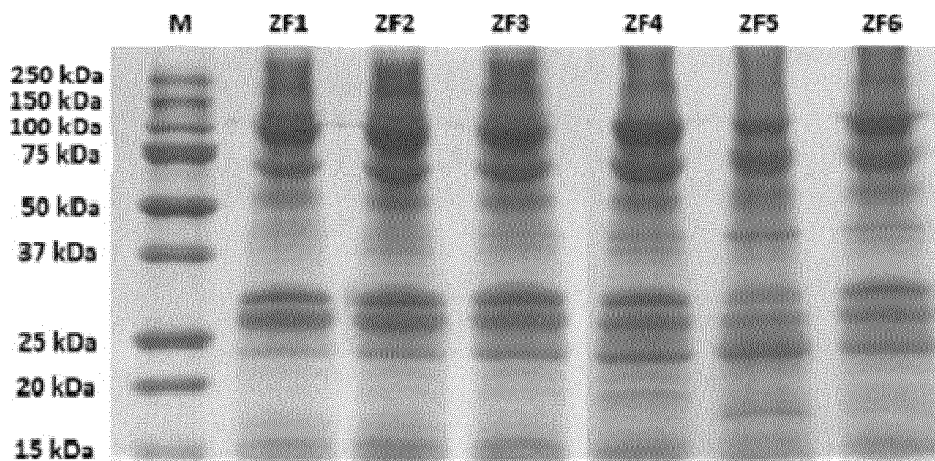


Figure 1

Proliferation assay:  
 Zebrafish extracts: 6 stages (ZF1-ZF6) at 4 concentrations,  
 After 24 hours of treatment

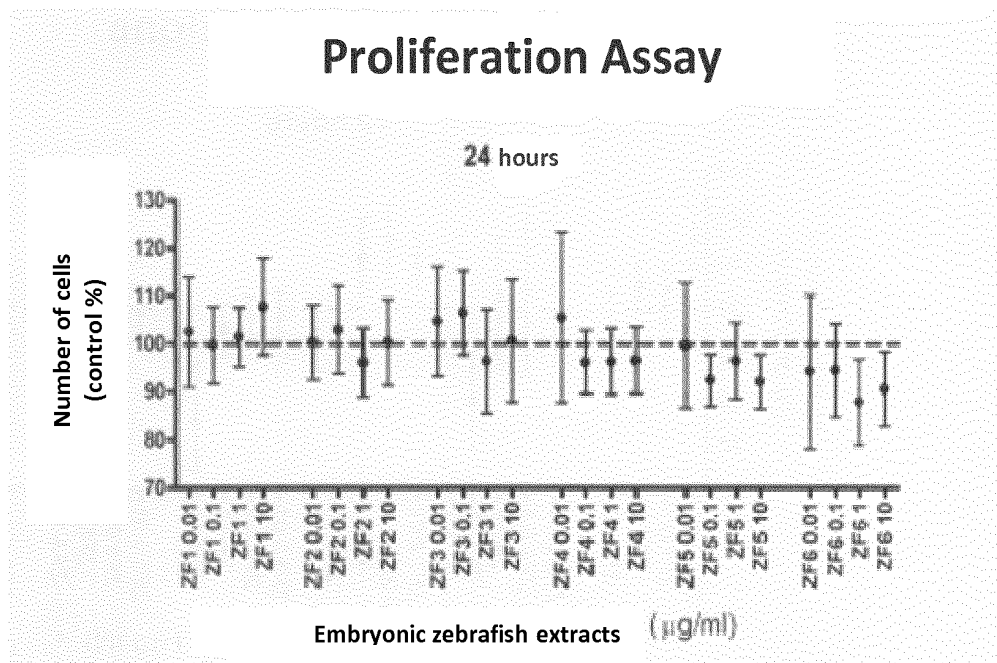


Figure 2

Proliferation assay:  
Zebrafish extracts: 6 stages (ZF1-ZF6) at 4 concentrations,  
After 72 hours of treatment

### Proliferation Assay

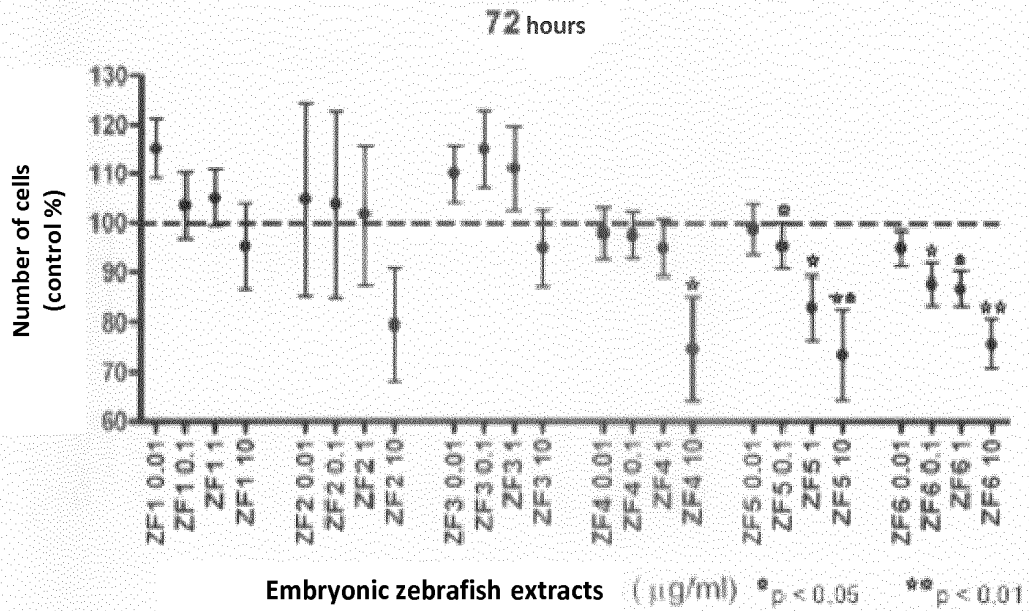


Figure 3

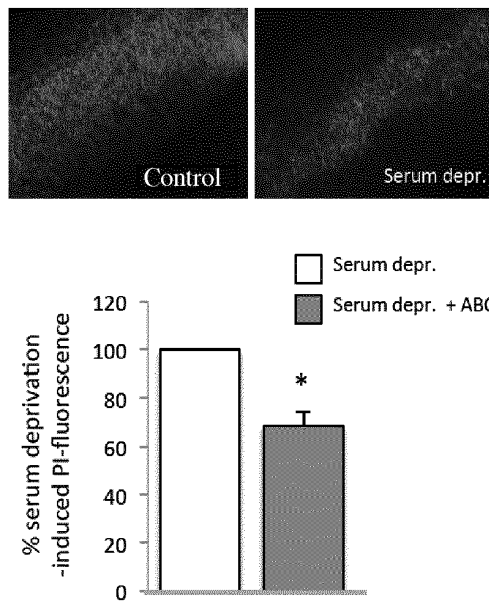


Figure 4

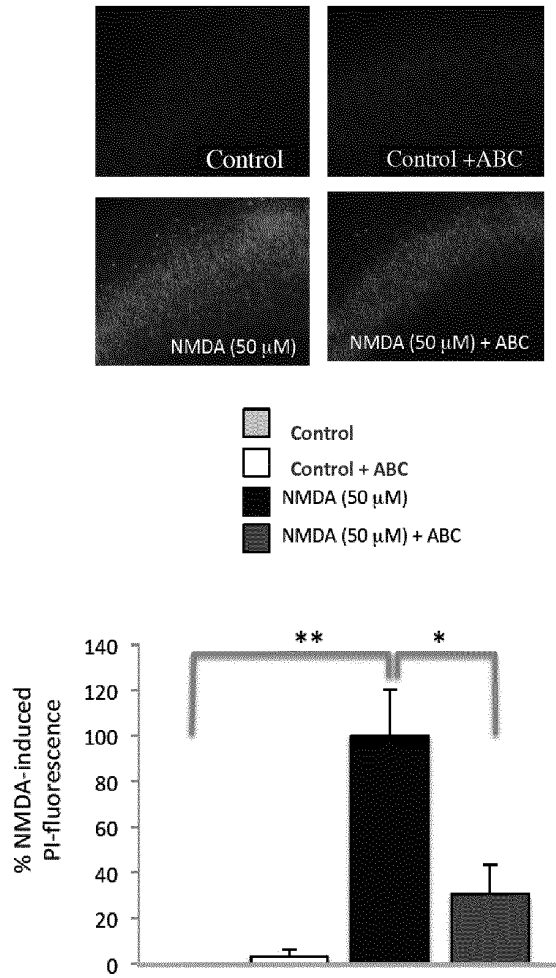


Figure 5

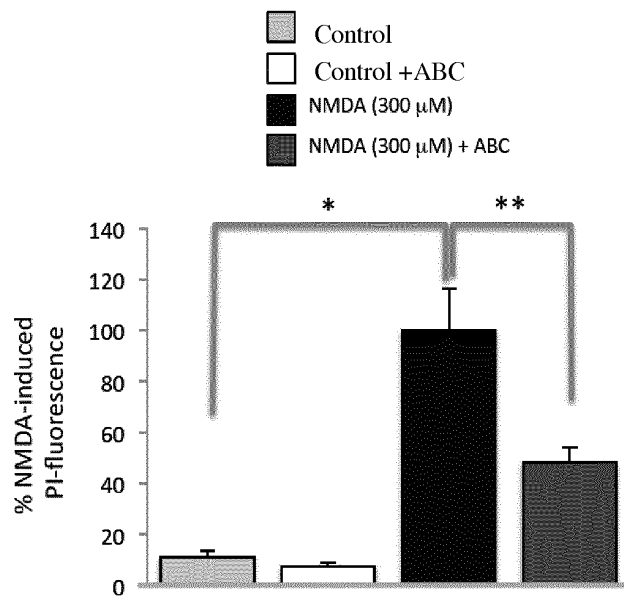
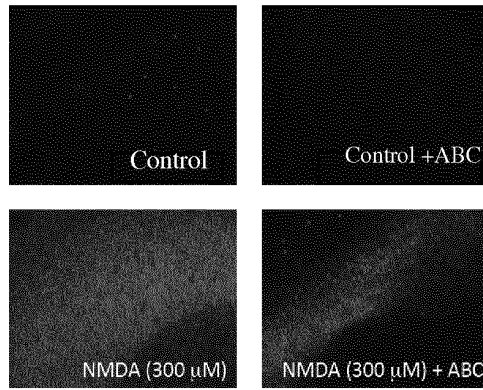


Figure 6

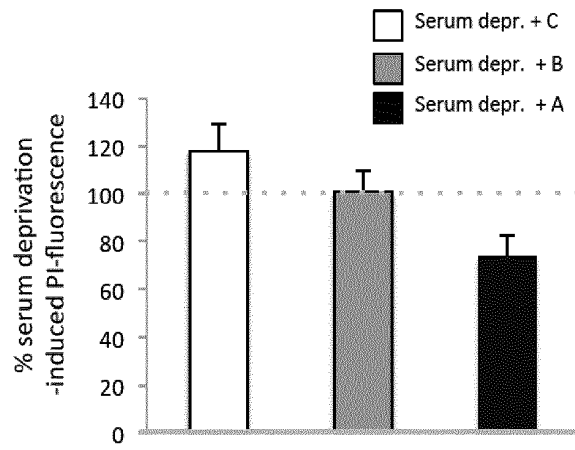


Figure 7

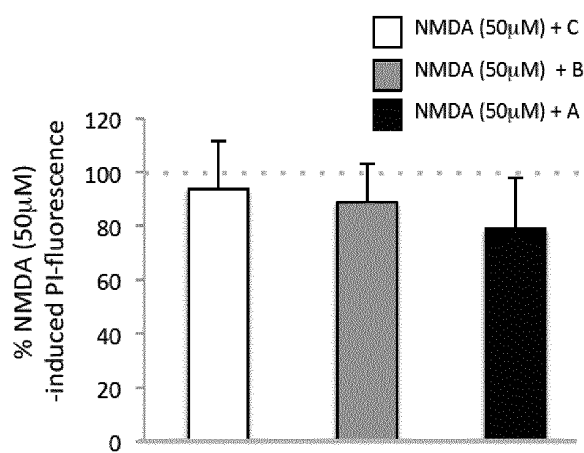


Figure 8

**REFERENCES CITED IN THE DESCRIPTION**

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**Non-patent literature cited in the description**

- Therapeutic Effects of hMAPC and hMSC Transplantation after Stroke in Mice. **SILVIA MORA-LEE et al.** PLoS ONE. Université Pierre et Marie Curie-Paris6, 2012, vol. 7, e43683 **[0047]**