Scientific Research Publishing

Advances in Aging Research, 2019, 8, 155-164
https://www.scirp.org/journal/aar
ISSN Online: 2169-0502
ISSN Print: 2169-0499

Effect of 2LMISEN® on Long-Term Hippocampal Neurons Culture as a Screening Senescent Cells Model: p16^{INK4A} and Caspase 3 Quantification

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How to cite this paper: Lilli, N.L., Révy, D., Volteau, C., Robelet, S. and Lejeune, B. (2019) Effect of 2LMISEN® on Long-Term Hippocampal Neurons Culture as a Screening Senescent Cells Model: p16^{INK4A} and Caspase 3 Quantification. *Advances in Aging Research*, **8**, 155-164.
https://doi.org/10.4236/aar.2019.86012

Received: November 4, 2019
Accepted: November 24, 2019
Published: November 27, 2019

Abstract

The aim of the present study was to investigate the effect of one capsule of the micro-immunotherapy medicine (MIM) 2LMISEN® compared to vehicle, in a neuronal aging model. Senescence and apoptosis of hippocampal neurons were evaluated by measuring p16^{INK4a} and caspase 3 levels, respectively. The data presented is a single observation. Mice hippocampal neuron cultures were treated with MIM (11 mM) or vehicle (11 mM) from 22 days *in vitro* (DIV) until 27 DIV. After incubation, hippocampal neuron cultures were fixed at 15 (control condition), 22, 25 and 27 DIV and then incubated with primary antibodies p16^{INK4a}, MAP2 and Caspase 3. Quantification of p16^{INK4a} and Caspase 3-positive neurons was done using Developer software. We found that vehicle had no effect on p16^{INK4a} expression, whereas MIM was able to decrease p16^{INK4a} levels at 22, 25 and 27 DIV in a statistically significant manner. The MIM had no significative effect on Caspase 3 expression. Our preliminary results showed that the MIM capsule significantly reduced neuronal senescence and not apoptosis.

Keywords

Neurons, Senescence, Micro-Immunotherapy, Low Dose

1. Introduction

The concept of cellular senescence was first introduced by Hayflick and Moorhead showing that cells in culture could only undergo a limited number of divi-

sions [1]. During normal organism aging, cells undergo a wide range of structural and functional changes, including senescence and apoptosis [2]. Even though they have been proposed to contribute to the development of certain age-related diseases, senescence and apoptosis are critically important for the function of many tissues [3] [4]. Senescent cells undergo a durable growth arrest, associated with the expression of anti-proliferative molecules (such as $p16^{INK4a}$), activation of damage sensing signaling pathways and caspase-induced apoptosis [5] [6]. One of the causes of senescence is damage of the telomeres and the lack of the enzyme telomerase activity, leading to the "end-replication problem" [7]. Several studies have reported the link between telomere dysfunction and $p16^{INK4a}$ activation in p53-independent telomere-directed senescence [8] [9] [10].

The prototypical molecular changes occurring during senescence include altered morphology, expression of pro-inflammatory cytokines and growth factors. One of the characteristic features of aging mammals is that the function of the immune system decreases as a result of the decline in several components of the immune system (immune senescence), and a shifting to a chronic pro-inflammatory status (the so-called "inflammaging" effect) [11] [12].

Neuroprotective signaling pathways involving neurotrophic factors and cytokines can interfere and delay the effects of aging in experimental models of neurodegenerative disorders [6]. The regulation of cytokines and growth factors through micro-immunotherapy (MI) approach could regulate the neurodegenerative process, counteract immune senescence and delay age-related dysfunctions.

MI is an immunomodulation therapy which uses immune regulators, including cytokines, plant-derived total deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), and specific nucleic acids (SNA®) to readjust the immune response. The active substances, prepared in low doses (LD) and/or ultra-low doses (ULD), are used in sequential formulas developed to treat different acute and chronic diseases.

The aim of the present study was to investigate the effect of one capsule of the micro-immunotherapy medicine (MIM) 2LMISEN® in a neuronal senescence model.

2. Material and Methods

2.1. Hippocampal Neuron Culture

The experiments were performed in accordance to European guidelines for the care and use of laboratory animals (Directive 2010/63/UE) as described by Lilli *et al.* [13]. Briefly according to the decree 2013-118 of February 1, 2013 on the protection of animal used for scientific purposes, animal suffering has been minimized and the number of animals used in the experiments reduced. Animal sacrifice with the sole purpose of using their organs or their tissues is not considered as an experimental procedure (https://www.recherche-animale.org/sites/default/files/decret_2013-118, Pdf).

For this reason, no authorization was required for this project. Prior to the administration of drugs, animals used to prepare mesencephalic cell cultures were naive. After receipt at the animal house at the latest the day before the sacrifice, pregnant mice were euthanized with CO_2, according to the authorized procedures. The nervous tissues of interest were then recovered from the embryos and cultured. Central nervous system (CNS) cultures of mice embryos were not subject to any authorization.

As described by Harrison [14], mice hippocampal neurons were cultured. Briefly pregnant female mice (Swiss mice; Janvier) of 15 days gestation were euthanized with CO_2. The foetuses were removed from the uterus and placed in ice-cold medium of Leibovitz 15 (L15; PanBiotech, Germany) containing 2% of Penicillin-Streptomycin (PS; PanBiotech, Germany) and 1% of bovine serum albumin (BSA; PanBiotech, Germany). The hippocampi were dissociated by trypsinisation for 20 min at 37˚C (Trypsin EDTA 1X; PanBiotech, Germany). The reaction was stopped by the addition of Dulbecco's modified Eagle's medium (DMEM; PanBiotech, Germany) containing DNAase I grade II (0.1 mg/ml; Roche Diagnostic, France) and 10% of foetal calf serum (FCS; Invitrogen, USA). Cells were then mechanically dissociated by 3 passages through a 10 ml pipette and then centrifuged at 180x g for 10 min at 4˚C on a layer of BSA (3.5%) in L15 medium. The supernatant was discarded and the cells of pellet were re-suspended in a culture medium consisting of Neurobasal (Invitrogen, USA) supplemented with B27 (2%; Invitrogen, USA), L-glutamine (2 mM; PanBiotech, Germany), 2% of PS solution and 10 ng/ml of brain-derived neurotrophic factor (BDNF; PanBiotech, Germany). Viable cells were counted in a Neubauer cytometer using the trypan blue exclusion test. The cells were seeded at a density of 20.000 cells/well in 96 well-plates (pre-coated with poly-D-lysine; Greiner, Austria) and were cultured at 37˚C in a humidified air (95%)/CO_2 (5%) atmosphere. Half of the medium was changed every 2 days with fresh medium. After 12 days of culture, astrocytes are present in the culture and release growth factor allowing neurons differentiation. Hippocampal neuron cultures (6 wells par condition) were treated with MIM (2LMISEN®; 11 mM) or vehicle (11 mM) from 22 days *in vitro* (DIV) until 27 DIV and senescence was evaluated at 22, 25 and 27 DIV (**Figure 1**).

2.2. Quantification of $p16^{INK4a}$ and Caspase 3

Cells were fixed at 15 (control condition), 22, 25 and 27 DIV by a solution of paraformaldehyde 4% (Sigma-Aldrich, USA) for 25 min at room temperature and washed two times with phosphate buffered saline (PBS, PanBiotech, Germany). Cells were then permeabilized and non-specific sites were blocked with a solution of PBS containing 0.1% of saponin (Sigma Aldrich, USA) and 1% of FCS for 15 min at room temperature. Cells were then incubated with primary antibodies $p16^{INK4a}$ produced in mouse (Thermo Scientific, USA), MAP2 produced in chicken (Abcam, France) and Caspase 3 produced in rabbit (Abcam, France). These antibodies were revealed with Alexa Fluor 488 goat anti-mouse

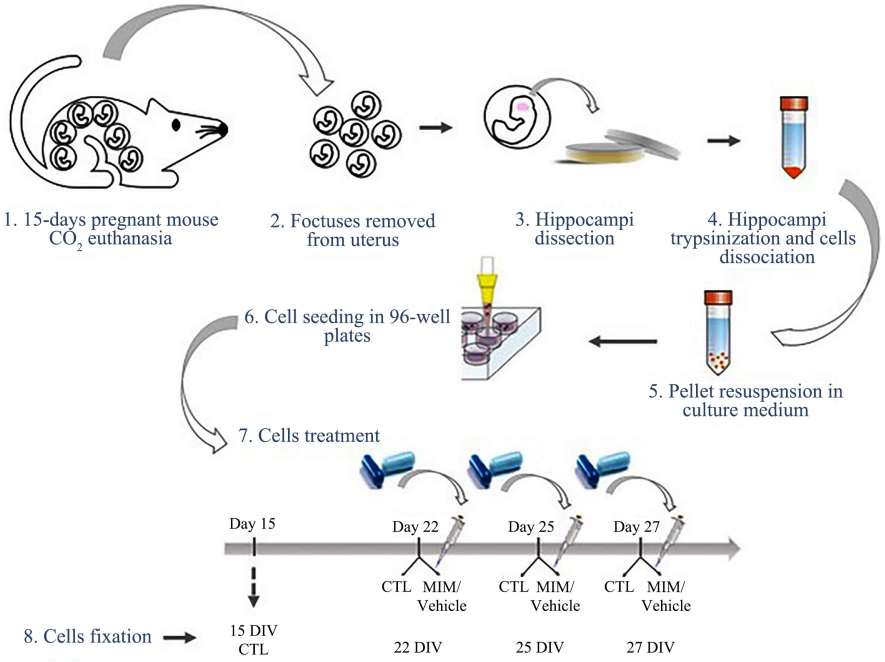

Figure 1. Experimental schema of the *in vitro* study.

IgG (Molecular probe, USA), Alexa Fluor 568 goat anti-rabbit IgG (Molecular probe, USA) and Alexa Fluor 633 goat anti-chicken (Molecular probe, USA). Nuclei of neurons were labeled by a fluorescent marker (Hoechst solution, Sigma-Aldrich, USA). Quantification of $p16^{INK4a}$ and Caspase 3-positive neurons was done using Developer software (GE Healthcare, France). For each well of culture, 20 pictures (20x magnification) per well were taken in the same conditions using InCell Analyzer™ 2200 (GE Healthcare, France).

2.3. Investigational Product

The MIM is notified to the Belgian Federal Agency for Medicines and Health Products under notification number 1507CH47F1. As reported by Lilli *et al.* [13], MI medicines for oromucosal administration are composed of lactose-saccharose pillules impregnated with LD and/or ULD of ethanolic preparations of immune mediators and nucleic acids. Active substances are obtained through a "serial kinetic process" reproduced a defined number of times, consisting of a 1/100 dilution process followed by vertical shaking. Centesimal Hahnemannian (CH) dilutions are used to express medicines composition indicating the number of times by which the two proceedings are carried out for each active substance. The composition of the MIM tested capsule is as follow: recombinant human (rh) interleukin-2 (IL-2; 10CH); rh epidermal growth factor (EGF; 10CH); dehydroepiandrosterone (DHEA; 10CH); dimethylsulfoxide (DMSO; 10CH); ribonucleic acid (RNA; 10CH); specific nucleic acid® (SNA) targeting human leukocyte antigen (HLA) class I (SNA-HLA I; 10CH); SNA® HLA class II (SNA-HLA II; 10CH); SNA® targeting human telomerase reverse transcriptase (TERT) (SNA-MISEN; 16CH). Vehicle used as control consists of

lactose-saccharose pillules impregnated with the vehicle solution without any active substance.

The pillules contained in one capsule of MIM or vehicle control (380 mg) were dissolved in cell culture media (100 ml). Indeed, the tested lactose-saccharose concentration was 11 mM.

2.4. Statistical Analysis

Statistical analyses and graphs have been performed using SAS software 9.4 (SAS Institute, Cary NC). The data were represented by box plot with scatter diagram (of 6 data per condition, 1 culture). The comparison between groups was performed using a one-way analysis of variance (ANOVA) following by Dunnett's test to compare control 15 DIV vs controls at 22, 25 and 27 DIV and Tukey test to compare vehicle vs MIM at 22, 25 and 27 DIV. The level of significance is set at $p \leq 0.05$.

3. Results

3.1. Effect of MIM on $p16^{INK4a}$ Expression

We showed that an increase of $p16^{INK4a}$ immunostaining appeared at 22 DIV in hippocampal neurons culture and remained at 25 DIV and 27 DIV (**Figure 2**). We found that vehicle had no effect on $p16^{INK4a}$ expression, whereas MIM was able to decrease $p16^{INK4a}$ levels at 22 DIV, 25 DIV and 27 DIV in a statistical significant manner (control 15 DIV vs controls 22, 25, 27 DIV [$p \leq 0.0001$]; control vs MIM 22 DIV [$p = 0.0004$], 25 DIV [$p = 0.0004$] and 27 DIV [$p = 0.0272$]; vehicle vs MIM 22 DIV [$p = 0.0017$], 25 DIV [$p = 0.0037$], 27 DIV [$p = 0.1333$]). Representative pictures of hippocampal neurons staining with primary antibodies $p16^{INK4a}$ are reported in **Figure 4**.

3.2. Effect of MIM on Caspase 3 Expression

We showed that a statistically significant increase of caspase 3 immunostaining appeared at 22 DIV in culture (**Figure 3**). We found that neither vehicle nor MIM had effect on caspase 3 level at any time of culture (control 15 DIV vs control 22 DIV [$p = 0.0098$]; control vs MIM 22 DIV [$p = 0.8077$], 25 DIV [$p = 0.8655$] and 27 DIV [$p = 0.7868$]; vehicle vs MIM 22 DIV [$p = 0.7156$], 25 DIV [$p = 0.6677$], 27 DIV [$p = 0.9886$]). Representative pictures of hippocampal neurons staining with primary antibodies caspase 3 are reported in **Figure 4**.

4. Discussion

Aging is associated with replicative senescence and increased $p16^{INK4a}$ levels in most mammalian tissues [15] [16]. Using an *in vitro* model of neuronal senescence, this study demonstrated that the combination of LD of the components contained in the tested MIM capsule significantly reduced $p16^{INK4a}$ levels in a long-term hippocampal neurons culture.

MI employs LD and ULD of specific substances to regulate the immune sys-

tem and protect cells, including neurons, from aging. MIM uses IL-2 at 10 CH to promote neuronal viability and functions and decrease age-related neuronal loss and dysfunctions. IL-2 promotes the survival and neurite extension brain neurons in culture and increases the survival of hippocampal neurons in rat [17] [18]. In fact, the expression of IL-2, in humans and rodents, decreases with age and is associated with immune senescence features and age-related decrease in immunologic function [19].

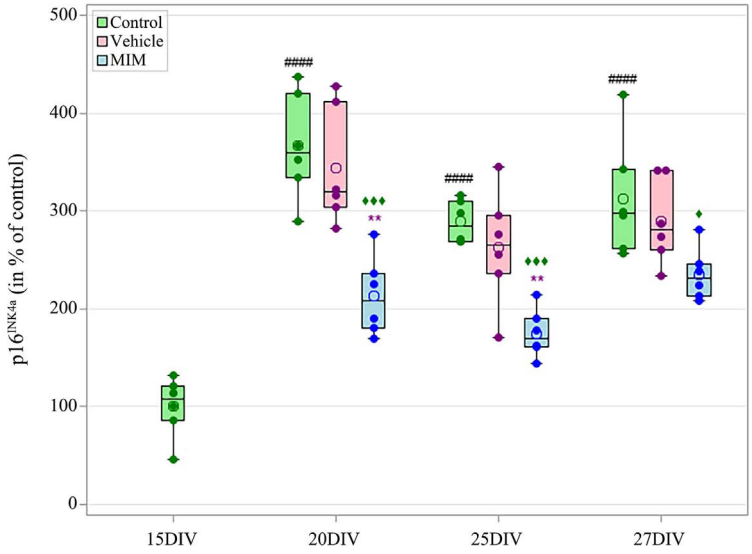

Figure 2. Effect of MIM on $p16^{INK4a}$ protein content in hippocampal neurons over time. Data are expressed in percentage of control at 15 DIV (####p ≤ 0.0001 Control at 15 DIV vs controls at other times of cultivation; one-way ANOVA followed by Dunnett test; ♦p ≤ 0.05, ♦♦♦p ≤ 0.001 MIM vs control; one-way ANOVA followed by Tukey test; **p ≤ 0.01 MIM vs vehicle; one-way ANOVA followed by Tukey test).

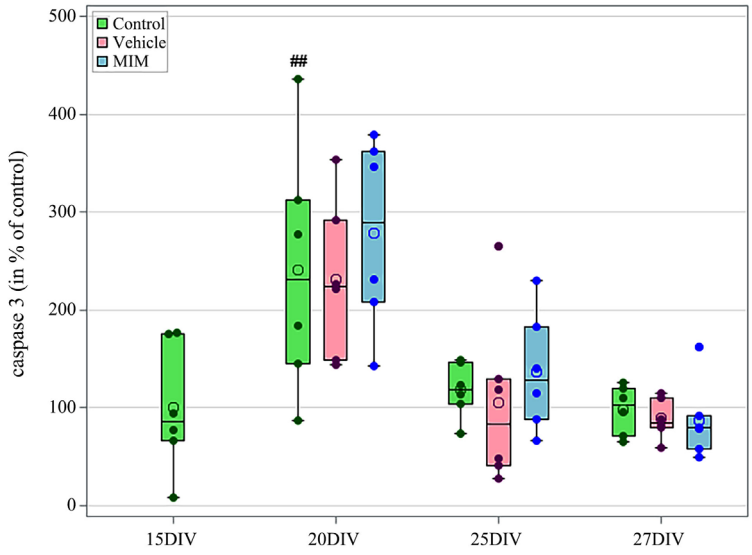

Figure 3. Effect of MIM on caspase 3 protein content in hippocampal neurons over time. Data are expressed in percentage of control at 15 DIV (##p ≤ 0.01; Control at 15 DIV vs controls at other times of cultivation; one-way ANOVA followed by Dunnett test).

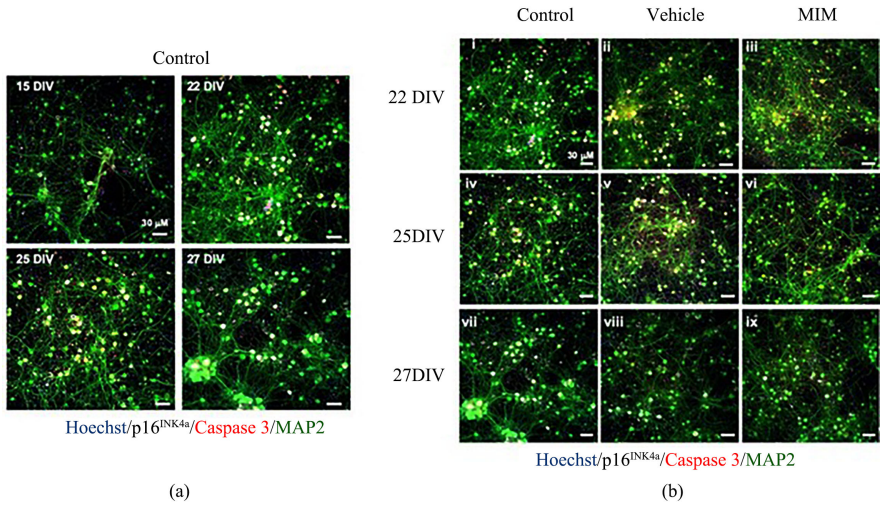

Figure 4. Representative pictures of hippocampal neurons. Images taken using InCell AnalyzerTM 2200 with 20x magnification. (a) Controls at 15 DIV, 22 DIV, 25 DIV and 27 DIV; (b) Control (i), vehicle (ii) and MIM (iii) at 22 DIV; Control (iv), vehicle (v) and MIM (vi) at 25 DIV; Control (vii), vehicle (viii) and MIM (ix) at 27 DIV. Blue: Hoechst, Grey: $p16^{INK4a}$, Red: Caspase 3, Green: MAP2. Scale bar 30 μm.

Specific EGF receptors (EGFR) have been identified in a variety of nerve cells, including the subventricular zone, hippocampus, and cerebellum. The distribution of EGFR expression in the two primary areas of adult neurogenesis suggests that the EGFR could also play a pivotal role in age-related neuronal survival and regeneration [20] [21] [22]. EGF is also expressed in various regions of the CNS, enhancing neurite outgrowth and survival [23]. MIM uses EGF at 10 CH to induce pro-survival and trophic signals.

DHEA is a neurosteroid which has multiple actions in the CNS, such as neuronal differentiation during development [24] and neurogenesis [25]. DHEA has also several neuroprotector effects, protecting hippocampal cells from oxidative stress [26]. Interestingly, DHEA is able to promote neurogenesis and neuronal survival in human neural stem cell cultures in an EGF and leukemia inhibitory factor-dependent manner [27]. MIM uses DHEA at 10 CH to exert neuroprotective effects.

HLA class I molecules are expressed by neurons during development and early adulthood in brain regions, including hippocampus [28] [29]. A coordinated up-regulation of HLA class I pathway expression with hippocampal aging was showed in rats [30]. Several studies also clearly showed that HLA class II up-regulation at the protein level is a prominent immunophenotype of normal brain aging [31] [32]. MIM uses SNA® targeting HLA class II proteins at 10 CH to regulate their expression and limit overexpression.

TERT is active in the adult rat cerebellum at a higher level than in young rats [33] and promotes tissue regeneration by delaying the entrance of cells into senescence. Moreover, being constitutively expressed in the hippocampus and the olfactory bulbs, TERT is also important for regulating normal brain functions

[34]. MIM uses SNA° targeting TERT at 16 CH to regulate the expression of TERT during aging retarding telomere shortening.

Our results showed that the MIM capsule significantly reduced neuronal senescence and not apoptosis.

5. Conclusion

The preliminary results of this study allowed to investigate the effect of a single capsule of the MIM sequence in an *in vitro* model of neuronal senescence. Only one experiment has been performed. Further molecular and mechanistic analyses are required to demonstrate the functioning of the whole MIM sequence *in vitro* and *in vivo*.

Acknowledgements

We would like to thanks Mrs Anne Naedts for having prepared the vehicle used in the study, Mr. Cédric Wolf and Mr. Michel Gangolf for having sent medicine and vehicle capsules to Syncrosome laboratory. We are grateful to Dr Ilaria Floris for reviewing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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