Potential Role of the Neural Stem Cell Secretome in Modulating Neural Injury

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Abstract

Neural stem cells (NSCs) are present in well-defined neurogenic niches of the adult mammalian brain, where they maintain the ability to self-renew and differentiate into new functional neural cells throughout life. Curiously, emerging evidence suggests that NSCs may also induce neural protection through their paracrine activity. Additionally, three-dimensional (3D) culture systems, which better resemble the *in vivo* physiology, have already been proven to enhance the cell secretome profile. Thus, the aim of this project was to explore the therapeutic potential of NSC-derived secretome in different *in vitro* models of neural damage and test the best culture system for improving the therapeutic properties of NSC-released factors. Initially, different *in vitro* models of neurodegeneration were established with NSCs and N2a with 1-methyl-4-phenylpyridinium (MPP^+) and hydrogen peroxide (H_2O_2). Subsequently, for comparison of different culture NSC systems, a NSC spheroid culture model using Spinner flasks (3D system) was developed and validated with respect to spheroid formation and viability. However, the biochemical characterization of NSCs in 2D and the 3D culture systems, indicated that NSC spheroids present higher levels of neuronal differentiation markers when compared with the 2D system. Notably, 2D NSC-derived secretome was capable of rescuing cell viability in the models of neurodegeneration. Although not statistically significant, the assays demonstrated that 3D NSC-derived secretome also had an increased therapeutic value. Altogether, these results indicate that 2D is the better source of NSCs secretome, and more importantly, that NSC secretome present a therapeutic value which, in turn, can be improved in the future.

Keywords: Cell culture systems; Neurodegeneration; Neural stem cells; Secretome.

Introduction

Neural stem cells (NSCs) are a type of multipotent stem cell capable of differentiating into neurons, astrocytes and oligodendrocytes. Located in neurogenic niches, these cells can be activated by a myriad of both intrinsic and extrinsic factors, leaving the quiescent state to return to the cell cycle. . Intrinsic factors are considered part of the niche, including cells, matrix and all interactions *in situ* by proximity with the NSCs. Extrinsic factors, in turn, arise from other parts of the organism. This combined interplay is essential to keep stem cells in the potent state and to induce differentiation when required, being the key to understand neurogenesis and the role that

the cell secretome might play [1]. Their potential for differentiation was expected to be a target for therapy related with among others, neurodegenerative diseases, a type of disorders characterised by the selective loss of a subset of neural cells. The second most prevalent of which is Parkinson's Disease (PD) [2], which is characterized by the loss of dopaminergic neurons in the *Substantia Nigra pars compacta* [3]. PD is a very heterogenous disorder [4], and patients exhibit a range of symptoms, broadly categorized as motor, such as bradykinesia and tremor, cognitive and psychiatric, which also entails depression and apathy among others [5].

Considering the localized injury observed in the PD patients, regenerative medicine was considered a viable therapeutic method. However recent reviews showed that the therapeutic outcome of grafts depends on more than mere volume of dopaminergic neurons grafted, such as on the neural survival and integration levels [6]. Moreover, transplantation of stem cells entails the risk of teratoma formation, or uncontrolled growth of immature proliferative neural precursors. Even through selection of differentiated cells, it is possible for a few stem cells to evade detection and originate a teratoma, requiring more complex methods to ensure the safety of a graft [7]. The risk can also be decreased by increased maturation of the cells. However, more mature cells show decreased survivability in grafts [8], which would require that a delicate balance be achieved

The largely unfulfilled regenerative potential of stem cell grafts leads to the exploration of the *paracrine hypothesis*, according to which stem cells can ameliorate the symptoms of chronic inflammation not only through cell replacement, but also through the secretion of growth factors and other components of their secretome [9]. In particular, grafts of SVZ-derived NSCs in PD mice models ameliorated motor symptoms but resulted in reduced effective regeneration of tissue [10]. Notably, the administration of culture media conditioned by the presence of NSCs induced a significant recovery of motor function and increased tyrosine hydroxylase expression when compared with simple NSC grafts [11]. The secretome bypasses the difficulties associated with grafts and can be enhanced using different cell culture methods. One such method pertains to growing cells as 3D aggregates, instead of a monolayer, which is not a good simulacrum for physiological tissue [12]. Cellular aggregates, on the other hand display a gradient of nutrients, growth factors and oxygen along their axis of the structure. This gradient results in a very heterogeneous population of cells, in varying states of differentiation. Furthermore, cells cultured as spheroids exhibit a behaviour similar to that of *in vivo* cells, making them a more suitable model for drug testing and disease modelling [13].

Aims

Based on possibility to strategically modulate NSC secretome and, therefore, better respond to neural damage, we aimed to:

1. Evaluate the potentially protective effect of NSCs secretome on *in vitro* models of Parkinsonism and neural oxidative stress;
2. Perform a 2D and 3D cell culture comparison to offer the best protective effect of the NSC secretome.

3. Perform a 2D and 3D cell culture comparison to provide a homogenous NSC population for molecular biology studies and future manipulation of the secretome.

Material and Methods

2D Cell culture

In 2D culture system, NSCs and N2a cells were cultured in a monolayer and passaged after reaching 80% confluency. The medium used was Euromed-N (EuroClone S.p.A., Pavia, Italy) supplemented with 1% of both N-2 supplement (Gibco, Thermo Fisher Scientific, Inc., USA) and Penicillin-Streptomycin (P/S, Gibco, Thermo Fisher Scientific, Inc.), and 0.2% of Epidermal Growth factor (EGF, PeproTech EC, UK) and Fibroblast Growth factor (FGF, Gibco, Thermo Fisher Scientific, Inc.) for NSCs and a culture medium composed of equal parts Opti-MEM (Gibco, Thermo Fisher Scientific, Inc.),) and a mix of D-MEM (Gibco, Thermo Fisher Scientific, Inc.) with 10% Fetal Bovine Serum (FBS, Gibco, Thermo Fisher Scientific, Inc.), 2% P/S and 2% Glutamine (Gibco, Thermo Fisher Scientific, Inc.) for N2a cells. Cells were then seeded at a concentration of 50.000 cells/cm^2 in tissue culture-treated flasks (Falcon, Corning Inc., NY, USA) and maintained at 37ºC in a humidified atmosphere of 5% CO_2.

Viability assay

Cell viability was evaluated using the Guava Easycyte 5HT flow cytometer (Luminex Corp., Austin, Texas, USA), according to the manufacturer's specifications using cells seed into 12-well culture plates. All cells were collected from each individual well and resuspended in PBS. 40 μL of cell suspension was added to 110 μL of Guava Viacount Reagent (Luminex Corp. Austin, Texas, USA). After five minutes of incubation the sample was analysed by the cytometer. Guava Suite Software (Luminex Corp. Austin, Texas, USA) was used to analyse the results.

3D cell culture

After propagation in 2D, cells were cultured as 3D aggregates in Spinner Flasks under two different conditions. In Condition 1, 1.000.000 cells/mL were seeded in culture medium with 5% of fetal bovine serum (FBS), whereas in Condition 2 we used 500.000 cells/mL and no FBS.

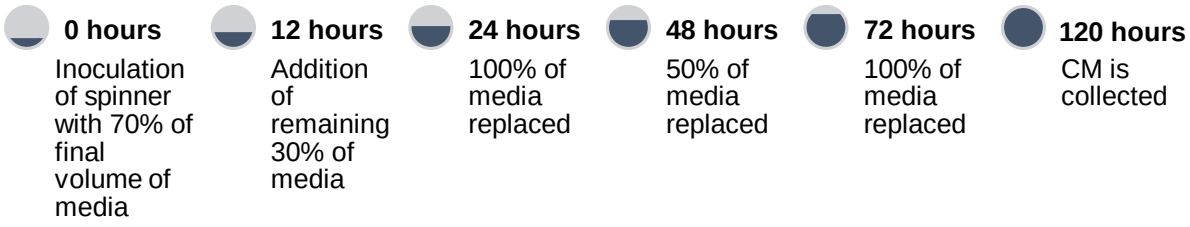

Figure 1 – Schematic representation of the protocol for NSC 3D-culture system using spinner flasks. The culture medium was fully replaced at 24 and 72 hours post inoculation and

half of it was replaced at 48 hours post inoculation. The culture was left undisturbed for the last 48 hours.

Cell aggregate measurement

Samples of NSC aggregates were extracted throughout the aforementioned protocol to measure the size and stability of the NSC spheroids. To account for different dimensions across width and length, three measures offset by a 60° angle were taken in each aggregate, and the average was then considered. The size of the NSC aggregates was evaluated by Image Analysis using the ImageJ Software (public domain).

Haemotoxylin-eosin staining

The aggregates were sectioned into 10 μm sections which were stained for 20 minutes in Harris Hematoxilin (HH S32, Sigma-Aldrich), washed with flowing tap water and then further stained for 2 min in Eosin stain (Sigma-Aldrich) prior to another round of washing using distilled water.

Protein quantification

Protein quantification in both NSC culture systems led us to assure that 2D- and 3D-associated NSC secretome derived from the same number of cells and therefore could be compared. This quantification was performed using the Bradford Protein Assay. Firstly, a calibration curve was prepared by successive dilutions of Bovine Serum Albumin (BSA) in water. Then, 200 μL of Bradford reagent (Bio-Rad Laboratories, USA) were added to each sample and properly homogenised. After 5 min, the absorbance was measured in a microplate reader (BMG Labtech, Ortenberg, Germany). Samples from 2D-cultured NSCs were used to generate the following calibration curve:

$$Cell\ Concentration = \frac{\text{Absorbance } - 0{,}3593}{0{,}0009}$$

This expression was then applied to the absorbance measured in samples from 3D cultured NSC, resulting in ~ 4.5 thousand cells per mL. After obtaining this result, it became possible to culture cells in 2D in an equivalent manner to the 3D and obtain two comparable sources of CM.

Secretome concentration

To avoid cell clogging, the collected CM was centrifuged at 500 *g* for 5 min to remove cells and aggregates. The Amicon® Ultra-Centrifugal Filter Unit (Merck) with a cut-off of 3 KDa was used, allowing the concentration of 15 mL of culture medium per centrifugal cycle. To operate the concentrator, the CM was pipetted to the sample reservoir and the concentrator was placed in

the centrifuge, taking care to place the filters aligned with the radius of the device. The concentrators were then centrifuged at 2 700 *g* for 90 min, after which the filtrate was removed, and more CM was supplied. The concentrated medium was then removed from the sample reservoir and frozen at -80°C.

Total RNA extraction

Cells were collected, homogenized in 500 μL Trizol (Thermo Fisher Scientific, Inc.) and stored at -80°C until processing. To extract RNA, 100 μL of chloroform were added to the sample. After 15 min of centrifugation at 12 000 *g*, the aqueous phase was isolated, and the RNA was precipitated by the addition of 250 μL of isopropanol. After another round of centrifugation, the supernatant was removed, and the pellet was washed with 75% ethanol. The sample was resuspended in water and finally quantified in a QuBit fluorometer (Thermo Fisher Scientific, Inc.).

qRT-PCR assay

The expression of several genes was investigated in NSCs by qRT-PCR. Firstly, the synthesis of the complementary DNA (cDNA) was achieved using the NZY First-Strand cDNA Synthesis Kit (NZYTech Lda., Lisbon, Portugal). According to the specifications, 1 μg of RNA was diluted in 12.5 μL of RNase free water. A 10% solution of DNase diluted in Buffer 10X was prepared and 1.5 μl was added to the sample, which underwent a cycle of 20 min at 37°C and another of 10 min at 75°C to remove any remaining DNA contamination. To synthesize the cDNA, 3 μL of random hexamers and dNTP's was added and the sample placed for 5 min in the thermocycler at 65°C. Finally, the reverse transcriptase and Reaction Buffer mix were added. The thermocycler was programmed thusly: 10 min at 25°C, 50 min at 50°C and 5 min at 85°C. The sample was then kept at -20°C until the next step. Each sample was diluted 1:1 in RNAse-free water and 2 μL of the sample were added to 5 μL of Sybr Green (bio-92020; Bioline, London, Uk), 0.4 μL of each primer and 0.2 μL of water. The analysis was performed in an Applied Biosystems® QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following cycle: 2 min at 50°C, 10 min at 95°C, followed by 30 steps of 95°C for 15 sec and 60°C for 1 min. To calibrate, the levels of gene expression were normalized to the housekeeping gene HPRT1.

Table 1 – List of primers used for qRT-PCR.

GENE	PRIMER FWD (5'- 3')	PRIMER REV (5'- 3')
SOX2	AGGGTTCTTGCTGGGTTTTGATTCT	CGGTCTTGCCAGTACTTGCTCTCA
NESTIN	CTCAGATCCTGGAAGGTGGG	GCAGAGTCCTGTATGTAGCCA
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
HPRT1	GGTGAAAAGGACCTCTCGAAGTG	ATAGTCAAGGGCATATCCAACAACA

NSC-derived CM-based assays

To assess and compare the impact of NSC secretome on different target cells and injury models, a series of controls were established, namely a control without CM exposure, and a control with the same volume of non-conditioned concentrated medium (fresh medium, FM). These different control conditions allow to evaluate the effect of two types of NSC-CMs, the 2D- and the 3D-derived CMs, each at both 5x and 10x concentration. The models were exposed to the toxic for 24 hours and then incubated with the medium for another 24 hours, after which cell viability was evaluated with the Guava Viacount assay.

Statistical analysis

All results are presented as a mean ± Standard Error of the Mean (SEM). The SEM is calculated as follows:

$$SEM = \frac{\text{Standard Deviation}}{\sqrt{\text{n}}}$$

The statistical significance was determined by t-test, comparing a data set to the indicated control. A *p*-value lower than 0.05 was considered statistically significant, with additional emphasis placed on threshold indicated by the following subtitles: * for $p < 0.05$, ** for $p < 0.005$ and *** for $p < 0.001$.

Results and Discussion

After testing their aggregation capabilities, NSCs were cultured as aggregates in Spinner Flasks as described [14]. Initially, two different conditions were set up for a specific Spinner and controlled for five days, with regular sampling of the aggregates size. By the end of the protocol, it became apparent that the Condition 1 resulted in aggregates disproportionally large, with diameters of 702 µm (± 29 µm, n=9) (Figure 1.C). Moreover, after staining with haematoxylin-eosin, condition 2 did not exhibit the presence of necrotic centres (Figure 1.A, 1.B), whereas the larger aggregates found in condition 1 exhibit necrotic centres. Thus condition 1 was deemed unfit for CM collection, as cell death normally results in increased release of cytokines that can negatively impact bordering cells [15]. Therefore, the experimental condition 2 was selected as the best 3D culture system condition to maintain NSCs and collect NSC secretome for future experiments. This secretome was compared with secretome derived from 2D grown cells in two concentrations (5X and 10X) [14], by supplementing the medium of NSCs which were subjected to injury through the addition of MPP^+ or H_2O_2.Two different concentrations of CMs were tested for each type of CMs, 5X and 10X [14].

The results presented in Figure 1.B represent the effect of CM *vs* the effect of FM, in each specific injury context. Interestingly, in undifferentiated NSCs, the addition of CM was beneficial across both toxic conditions. However, higher concentrations of CM did not correlate with higher levels

of live cell count, even showing a decreased effect. Moreover, in toxic conditions, 3D-derived CM appears to have a very mild improvement when compared with the 2D- derived CM.

When we performed the same experiments but using N2a cells as target injured cells (Figure 1.C), an additional control condition was added. Incubation with FM allowed us to evaluate the effects of concentrated growth factors present in the culture medium on viability of recipient cells. In accordance with the results obtained in NSCs, the higher concentration (10X) of both CM and FM induced a negligible or negative impact in the survival levels of target cells in all conditions. In the N2a models, the effect of the CMs on injury was less visible and presented higher variability, particularly with respect to the MPP^+ model. Nonetheless, the H_2O_2-induced injury of N2a suggests a trend similar to what was previously observed in NSC target cells in both injury models, namely a tendency to increase in viability when exposed to the various CMs. Once again, the 3D-derived CM did not appear to exhibit stronger therapeutic properties when compared with the 2D-derived NSC secretome.

Thus, our results indicate that NSC-derived CM is beneficial, displaying increased live cell count in the majority of injury conditions, when compared with the corresponding controls. In the case of NSC target cells, we observed a very consistent and robust effect of NSC-derived CMs in both injury models, indicating that this model of target cells is accurate and reproducible. In contrast, the use of N2a cell line as target cells did not show the same robustness, with a lot of variability in MPP^+-induced injury model.

To analyse the cell population that conditioned the 3D-derived secretome, we compared the cell fate of 2D- and 3D-secretome producing cells. In fact, whereas the stemness and multipotency of 2D cultured NSCs have been well described [16], the characterization of these new 3D-generated NSC spheres was not. To that end, mRNA was extracted from both 2D- and 3D-derived NSCs, on the same time point of secretome collection, and qRT-PCR was performed to assess proliferation, differentiation markers and mitochondrial activity.

Our results revealed profound changes between the 2D and 3D NSC populations (Figure 1.D), with 3D-derived NSCs showing high levels of cellular differentiation. In fact, Sox2, a key hallmark of undifferentiation [17], was significantly reduced in 3D-derived NSCs. Nestin, a marker of undifferentiated NSCs was shown to be similarly expressed in 2D and 3D conditions. Indeed, since Nestin is also a marker for neural progenitor cells [18], it is not surprising that its expression may transiently co-exist with the presence of other differentiation markers.

In addition, since differentiation of stem cells has been associated with an increase of oxidative phosphorylation, we also assessed the expression levels of SOD2, the major scavenger of mitochondrial reactive oxygen species (ROS), respectively [19]. Our data validated the idea that cells present in 3D-derived NSCs are indeed under differentiation process, as undifferentiated NSC are known to depend mostly on glycolysis [19].

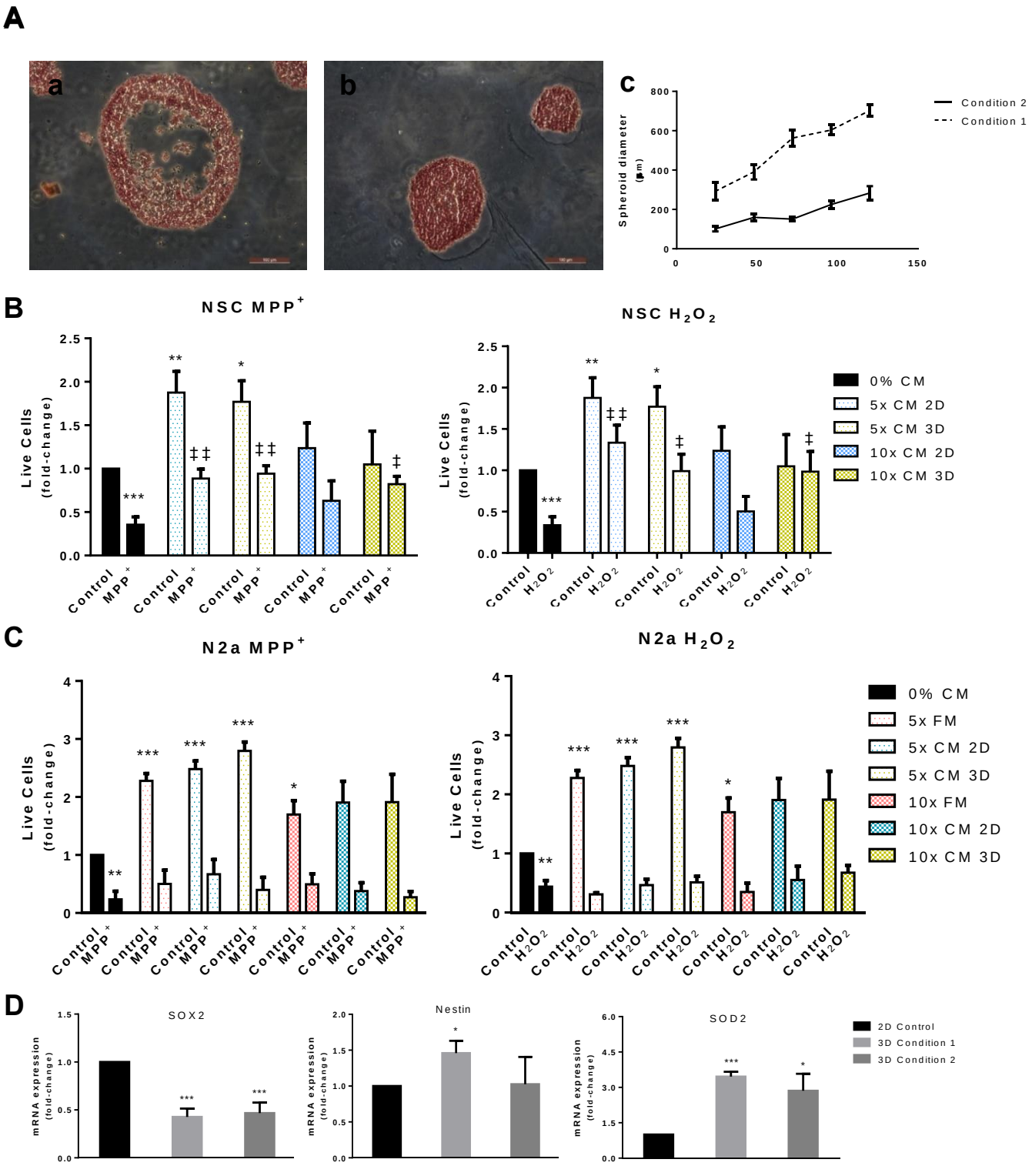

Figure 1 – (A) Analysis of aggregates: (a, b) Haematoxylin-eosin staining of aggregates in condition 1 and 2 respectively (scale bar, 100 µm), (c) size of aggregates along the protocol. (B, C) Live cell counts of injury models supplemented with secretome in NSC and N2a cultures respectively. (D) Results of qRT-PCR of samples from 2D and 3D cultured NSC's.

Conclusions

After testing both types of CMs, it became apparent that there was little improvement when 3D sourced CM was compared with 2D sourced CM. Moreover, the higher concentration of CM did not correlate with increased CM-protection on target injured cells. However, considering the lack of a thorough optimization step in the spinner protocol, it stands to reason that 3D culture resulted in the production of cytokines that induce cellular death. Furthermore, different CM concentrations should be also attempted, as the points tested appear to be outside of the linear portion of the dose-response curve.

On the other hand, qRT-PCR assays showed that cells in 3D aggregates are no longer a homogeneously stem population, being instead in several stages of differentiation. While this was to be expected, it raised the question that the populations responsible for the production of secretome are fundamentally different and not comparable. Despite the presence of numerous avenues left to explore in order to improve and continue the project, the results that were achieved can support further experiments and optimizations in an effort to harness the clinical potential of NSC-sourced secretome.

1. References

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