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Gelsemium Low Doses Increases Bioenergetics and Neurite Outgrowth

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Abstract: Gelsemium sempervirens (GS) is a traditional medicinal plant, previously identified as a remedy for a variety of psychological and behavioral symptoms of anxiety and depression at ultra-low doses. Changes in neural plasticity have been shown to play a significant role in the onset and development of those mental illnesses. Mitochondria play an extremely important role in the central nervous system by being the main energy producer through oxidative phosphorylation and being involved in the regulation of cell survival and death, as well as synaptic plasticity. Neurite outgrowth is the differentiation process by which neurons establish synapses through the protrusion of neurons and their extension. Because the effects of GS dilutions on mitochondrial function and neuroplasticity remain elusive, we aimed to investigate whether a treatment with GS at low doses (centesimal dilutions, C) improved bioenergetic parameters such as ATP production, mitochondrial respiration, cellular glycolysis, and neurite outgrowth. Nerve growth factor (NGF), which is known as a promotor of cell growth and survival, was used as a positive control. Our results demonstrate that GS dilutions (3C and 5C) efficiently ameliorated the bioenergetics of SH-SY5Y neuroblastoma cells by increasing cellular ATP level and mitochondrial respiration as well as promoting cell survival. In addition, GS dilutions significantly improved neurite extension in 2D as well as 3D culture models after 3 days of treatment. 3C and 5C dilutions showed similar functional effects to those obtained with the positive control nerve growth factor (NGF). These findings indicate that GS dilutions modulate mitochondrial bioenergetic phenotype and improve neurite formation. The mitochondrial function-improving properties of GS dilutions may represent one possible pathway contributing to its neuroprotective effects.

Keywords: Gelsemium Dilutions, Mitochondria, Bioenergetics, Neurite Outgrowth

1. Introduction

Homeopathic Materia Medica suggested that Gelsemium sempervirens (GS) can be used as a remedy for neurological and behavioural symptoms, including general prostration, drowsiness, tiredness, mental apathy, lack of muscular coordination, anxiety, and depression [1]. GS itself was originally shown to have toxic effects, due to the high concentrations of alkaloids [2]. In contrast, there are no safety concerns for the use of ultra-low doses of GS [4]. Therefore, GS is currently only used in medicinal applications at homeopathic dilutions [3]. At low dilutions, GS has been shown to act on the emotional reactivity of mice by exerting anxiolytic-like effects [5, 6]. Basic evidence supported the existence of cellular effects of GS dilution 5C in the rat limbic system, in particular, regions that are known to pivotally modulate anxiety such as the hippocampus and amygdala [7]. The beneficial effect of GS dilution 5C in the

hippocampus and amygdala was mediated through the production of the neurosteroid allopregnanolone (3α, 5α-THP) [7], an endogenous steroid that is able to rescue neuronal cells from oxidative stress-induced death via bioenergetic improvement [8].

Mitochondria are the main energy producers of adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS) and this energy is required for almost all cellular processes, from cell survival and death, to the regulation of synaptic plasticity and intracellular calcium homeostasis [9-11]. Neurite outgrowth is an energy-consuming process where the neurons generate new projections as they grow in response to guidance cues. Neurotrophins, such as NGF, are one family of stimuli that modulate neurite growth [12]. Marzotto and colleagues [13] have shown that in SH-SY5Y cells, a human neuronal cell line, the GS dilution 2C modulated the expression of genes involved in neuronal functions such as G-protein coupled receptor signaling pathways known to play key roles in synaptic plasticity by strengthening or weakening synapses and/or shaping dendritic spines [14]. However, there is no evidence demonstrating that GS dilutions themselves can modulate mitochondrial function and/or neuroplasticity. To gain more insights into the cellular mechanisms underlying the mode of action of GS 3C and 5C dilutions, our first aim was to investigate its ability to modulate mitochondrial function via the determination of ATP levels, mitochondrial respiration and cellular glycolysis as well as cell survival. In addition, we evaluated the effect of GS dilutions on several parameters of neuroplasticity.

2. Methods

2.1. Gelsemium sempervirens Plant

GS plants, also known as Yellow Jessamine, were purchased from Herb's International Service SARL (France; Batch H140503595) in respect of the Good Agricultural and Collection Practices (GCAP) [15]. A double identification of GS plant was performed by the supplier and Boiron's quality control. The GS plant is not present in the International Union for Conservation of Nature (IUCN) lists as an endangered species [16]. In the present study, a GS batch sample used to manufacture the Mother Tincture was conserved at Boiron laboratories (Messimy, France).

2.2. Mother Tincture Composition

The main constituents of the mother tincture are alkaloids and coumarins with a total alkaloid content between 0,015-0,03% [17, 18]. In the mother tincture, Gelsemine is the main alkaloid but other such as gelsemicine, sempervirine, gelsedine can be detected [19-21]. HPLC characterization of Gelsemium sempervirens mother tincture by Patil P. et al. shows the presence of 9 majors active phytoconstituents among which the authors identified Gelsemine, Sempervirine and Scopoletin [22]. Scopoletin (β-methyl-6-esculetin) is the main coumarin in the roots and was previously described in the mother tincture along with esculetin [18]. A total up to 121 alkaloids were identified in Gelsemium Genus and are divided in 6 main classes: Sarpagine, Gelsedine, Koumine, Yohimbane, Humantenine and Gelsemine -types [2]. Several of those phytoconstituents identified in the mother tincture have previously been shown to have some biological activity. Intrathecal injection of Gelsemine in rats demonstrated antinociception in chronic pain through the glycine receptor (Gly-R) α3 subunit [23]. Moreover, Geslemine upregulated 3α, 5α THP production via Gly-R in rat spinal cord slices in a dose dependent manner and demonstrate an additive effect with glycine [24].

2.3. Chemicals and Reagents

Dulbecco's-modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin/streptomycin, Dimethylsulfoxid (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glutamax was purchased from Gibco Invitrogen (Waltham, MA, USA). B27 supplement was purchased from Gibco Invitrogen (Waltham, MA, USA). NGF was purchased from Lubio (Zürich, Switzerland). GS dilutions were prepared by Boiron laboratory (Messimy, France) based on the 1.1.10 method of the European Pharmacopoeia (Ph. Eur.) guidelines for the production of homeopathic remedies [25]. The Mother Tincture (MT) was produced by macerate for 10 days using dried G. sempervirens (L.) J.St.-Hil. plant roots were prepared with a 65% v/v ethanol solution. A ratio of 1g of plant for 9g of hydroalcoholic solution was used. Gelsemine is the major active principle of Gelsemium in the homeopathic monograph. The concentration of gelsemine estimated from the analyses of G. sempervirens mother tincture (batch number: M4090578, voucher number: 191138) was 6,49x10-4M (0,023%). All the centesimal (C) dilutions tested were prepared in sterile water (OTEC, Aguettant France) in order to avoid cell toxicity of alcohol. To obtain the first centesimal (1C) dilution, one volume of MT was diluted in 99 volumes of water and vigorously agitated using a mechanical shaker. The subsequent serial 100× dilutions, 3C and 5C GS dilutions were prepared using the same procedure with a final ethanol concentration largely below 0,0001% (v/v). The vehicle control was prepared according to the same procedure described above using only sterile water (OTEC). All GS dilutions as well as vehicle or control solutions were stored at 4°C before use.

2.4. Cell Culture

Human SH-SY5Y neuroblastoma cells (ATCC CRL-2266) were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FCS, 2 mM Glutamax and 1% (v/v) penicillin/streptomycin and incubated at 37°C in a humidified incubator chamber under an atmosphere of 7.5% CO_2. The cells were grown in 10 cm^2 dishes and split twice a week. When they reached ~80% confluence the cells were plated, 2 days prior treatment.

In the 2D cell culture method, collagen type I (Rat tail BD Bioscience) at 0.05 mg/ml was used to coat the cell plates. In

the 3D cell culture method, a BD PuraMatrix Peptide Hydrogel (BD Catalog #354250 packaged in one vial containing 1% solution (w/v) of purified synthetic peptide) was used. Cell plates were coated with 5mg/ml of PuraMatrix (0.5% diluted in sterile water, 50 µl for a 96-well plate) and gelation was induced by slowly and carefully adding medium to each well (100 µl for a 96-well plate). After 1 hour of incubation of the plates for a complete gelation, the medium was exchanged twice over a period of 1 hour to promote a physiological pH of the growth environment. Cells were seeded at a concentration of 5x103 cells/ well, as previously described [26].

2.5. Treatment Paradigm

In accordance with the treatment protocol and findings that were recently described [27], the effects of the GS dilutions 3C and 5C were investigated in this study. One day after plating, SH-SY5Y cells were treated in DMEM + 10% FCS either with DMEM alone (untreated control condition, CTRL) or NGF (positive control at a final concentration of 50 ng/mL), or vehicle control or the different dilutions of GS. Bioenergetic phenotype (OCR/ECAR profil) and effect on neurite outgrowth were investigated after treatment with CTRL or NGF (50 ng/ml), or 3C and 5C dilutions). Values were normalized to the untreated control group (CTRL).

2.6. MTT Assay

To assess cell viability, MTT reduction assays were performed in accordance with the protocol from Mensah-Nyagan laboratory [28] and confirmed preliminary MTT assay readout data generated by the Mensah-Nyagan laboratory. Briefly, SH-SY5Y cells were seeded at 5x103 cells/ well in a 96-well plate in replicates and allowed to attach. 48h after the plating, the treatments were initiated. After 24h treatments, cells were incubated with MTT (3-(4,5-dimethylthyazol-2-yl)-2,5-diphenyl-tetrazolium bromide) in DMEM for 3 hours. MTT is reduced to a violet formazan derivative by mitochondrial enzymatic activity. At the end of the reaction cells were dissolved in a MTT cell lysis buffer (DMSO). MTT absorbance was measured at 550 nm using the multi label plate reader Cytation3 (BioTek). MTT signal detected for the CTRL cells is arbitrary normalized to 100%.

2.7. ATP Levels

Total ATP content of SH-SY5Y cells was determined using a bioluminescence assay (ViaLighTM HT, Cambrex Bio Science, Walkersville, MD, USA) according to the manufacturer's instructions, as previously described [26, 29]. SH-SY5Y cells were seeded at $5x10^3$ cells/well into a white 96-well cell culture plate in 5 replicates [26, 29]. The bioluminescent method measures the generation of light from ATP and luciferin by luciferase. The emitted light was linearly correlated to the ATP concentration and was determined using the Cytation 3 cell imaging multi-mode reader [26, 29].

2.8. Determination of Oxygen Consumption Rate (Mitochondrial Respiration) and Extracellular Acidification Rate (Glycolysis)

The Seahorse Bioscience XF24 Analyser (North Billerica, MA, USA) was used to perform a simultaneous real-time measurement of oxygen consumption rate (OCR, respiration) and extracellular acidification rate (ECAR, glycolysis). XF24 cell culture microplates (Seahorse Bioscience) were coated with 0.1% gelatine and SH-SY5Y cells were plated at a density of 2.5x104 cells / well in 100 µl of the medium containing 10% FCS, 1 g/l glucose and 4 mM pyruvate and treated with CTRL or NGF or 3C and 5C dilutions After 24 h of treatment, all the cells were washed with PBS and incubated with 500 µl of assay medium (DMEM, without $NaHCO_3$, without phenol red, with 1g/l glucose, 4 mM pyruvate, and 1% L-glutamine, pH 7.4) at 37°C in a CO_2-free incubator for 1 hour.

The OCR and ECAR values from the basal respiration state were recorded simultaneously. Data were extracted from the Seahorse XF-24 software, and the bioenergetic profiles using the OCR and ECAR values were calculated according to the guidelines of the company [26].

2.9. Neurite Outgrowth

For the 2D or 3D cell culture, SH-SY5Y neuroblastoma cells were cultured in coated 96 well plates (black with clear bottom). The following day, cell differentiation was initiated by adding neurobasal medium containing 1% fetal bovine serum and 10 µM retinoic acid (RA) for 3 days. Then, cells were treated either with CTRL, NGF (50 ng/ml) or with 3C and 5C dilutions. After 3 days of treatment, cells were fixed with 2% paraformaldehyde. All media were exchanged every 2 days to ensure the availability of growth factors and GS dilutions components in the culture [30].

2.10. Immunostaining, Microscopy and Analysis (Software)

The protocol was used with 2D or 3D surface cultures of cells in plates. For 96-well black microplates with a clear bottom, it was possible to directly image the samples without transferring the gel to a glass slide. Immunolabeling of neurites was performed using an anti βIII-tubulin (R&D Systems, Biotechne, Minneapolis, MN, USA) and Alexa Fluor 488-conjugated secondary antibody (Thermofisher scientific, Waltham, MA, USA) [29]. DraQ5 (Biostatus, Shepshed, Leicestershire, UK) or DAPI (Thermofisher scientific, Waltham, MA, USA) were used for the nucleus staining.

Images were obtained in a blinded manner using an inverted confocal microscope (Leica Microsystems TCS SPE DMI4000, 10x objective) connected to an external light source for enhanced fluorescence imaging (Leica EL6000). Axially, all the cells were entirely present within the confocal volume for the pinhole settings. One layer was taken for the 2D culture method. To visualize the whole 3D network, z-stacks were generated (3-4 layers). Maximum intensity projections were then generated for subsequent 2D image

analysis using ImageJ (Neurophology plugin) software to investigate parameters of neuroplasticity such as Neurite count, Total neurite length, attachment point (Number of branching points), endpoint (Number of contact points) [30].

Data are given as the mean ± SEM. Values were normalized to the untreated control group (= 100%). Statistical analyses were performed using the Graph Pad Prism software version 5.02 (GraphPad-Prism, San Diego, CA, USA). One-way ANOVA followed by Dunnett's multiple comparison tests versus the control group were used for statistical comparisons of more than two groups. Student unpaired t-test was used for statistical comparisons of two groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. GS dilutions Increased ATP Levels and Cell Viability

We first investigated the effects of the GS dilutions 3C and 5C on ATP production and cell survival in human neuroblastoma cells (SH-SY5Y) after 24h of treatment (Figure 1). The GS dilutions 3C and 5C significantly increased ATP levels compared to untreated control cells (Figure 1a) (3C: +7%; 5C: +8%) as well as to vehicle treated cells (3C: +10%; 5C: +11%). GS dilutions 3C and 5C raised the ATP level at the same range as the positive control NGF (+6% vs CTRL). No effect of treatment with the vehicle solution compared to the untreated control condition was observed.

We next assessed whether treatment with GS dilutions was able to improve cell survival in SH-SY5Y cells (Figure 1b). After 24h of treatment, the dilutions 3C and 5C significantly increased the viability of the SH-SY5Y cells when compared to untreated control cells (3C: +23%; 5C: +40%) as well as to vehicle treated cells (3C: +26%; 5C: +43%).

Because no effect of vehicle treatment was observed in both assays, we compared the effects of the GS dilutions to the untreated control condition in the following experiments.

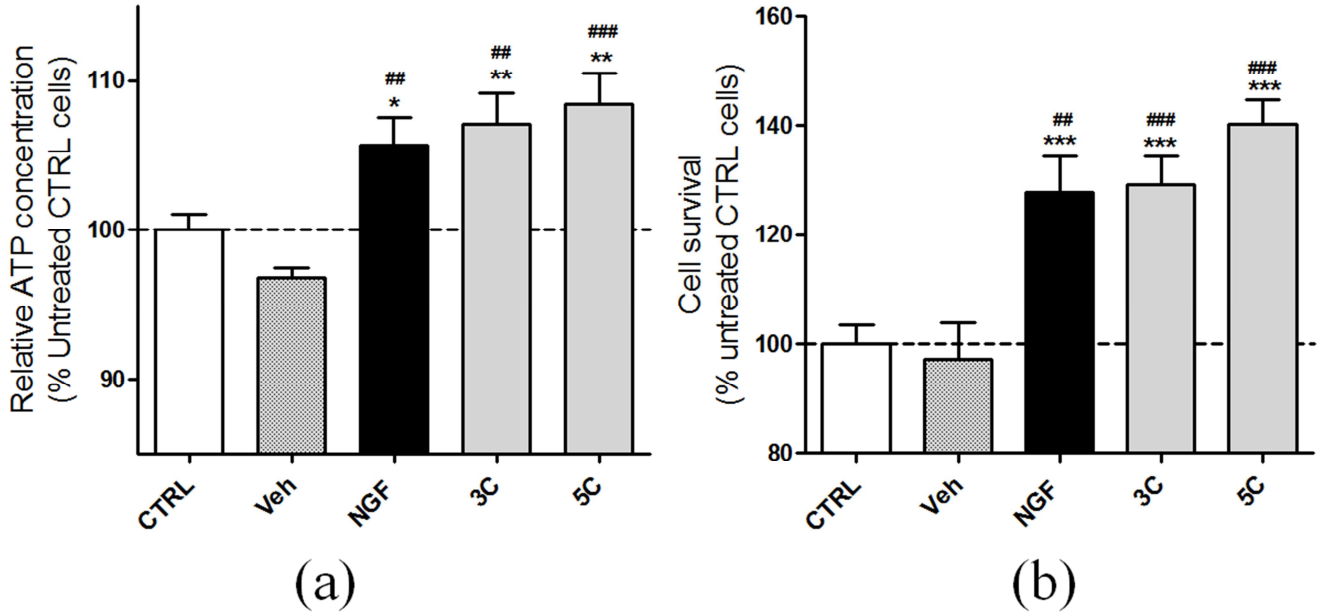

Figure 1. *Effect of GS dilutions on ATP levels and cell viability after 24h treatment. 3C and 5C increased both parameters: (a) the ATP production and (b) the cell survival. Vehicle (Veh) treatment had no effect on ATP levels compared to CTRL cells. Values represent the mean± SEM (n = 13-18 replicates) of five independent experiments and were normalized to the untreated control group (CTRL, 100%, Table 1). One-way ANOVA ((a, b): P<0.0001) and post hoc Dunnett's multiple comparison test versus untreated control cells, *P<0.05, **P<0.01, ***P<0.001. One-way ANOVA and post hoc Dunnett's multiple comparison test versus vehicle treated cells, ##P<0.01, ###P<0.001.*

Table 1. *The data are presented as mean (M), standard deviation (±SD) corresponding to the number of cells (N) (Figure 1). Values are normalized on 100% of the control group (untreated CTRL).*

	CTRL M±SD (N)	Veh M±SD (N)	NGF M±SD (N)	3CM±SD (N)	5CM±SD (N)	p (One way)
ATP assay	100±7.584 (48)	94.72±3.331 (24)	105.7±9.041 (24)	107.1±8.637 (17)	108.4±8.175 (15)	< 0.0001
MTT assay	100±19.33 (31)	94.43±23.05 (12)	127.8±23.06 (12)	129.2±17.77 (12)	140.4±14.51 (11)	< 0.0001

3.2. GS Dilutions Activated the Metabolic State of the Human Neuroblastoma Cells

ATP molecules are mainly produced via mitochondrial oxidative phosphorylation (OXPHOS) and cellular glycolysis. Therefore, we evaluated the efficiency of 3C and 5C, and the positive control NGF, to modulate one or both pathways. Seahorse Bioscience XF24 Analyzer was used to simultaneously monitor in real-time the OCR, an indicator of mitochondrial respiration and the ECAR, an indicator of glycolysis (Figure 2). Treatment with 3C and 5C induced a strong and significant improvement in basal

OCR compared to CTRL-treated cells, with +45% and +41% of increase respectively, while NGF induced a +58% increase compared to the control (Figure 2a). The 3C dilution had a similar effect to NGF (+52%) in significantly ameliorating glycolysis compared to CTRL, while 5C was more efficient, inducing an increase up to 85% (Figure 2b). The bioenergetic phenotype of the cells (Figure 2c), represented by OCR versus ECAR of the basal respiration, revealed that treatment with 3C and 5C were particularly efficient in improving both parameters, switching the cells to a metabolically more active state, with a comparable effect between NGF and 3C.

Table 2. *The data are presented as mean (M), standard deviation (±SD) corresponding to the number of cells (N) (Figure 2). Values are normalized on 100% of the control group (untreated CTRL).*

	CTRL M±SD (N)	NGF M±SD (N)	3C M±SD (N)	5C M±SD (N)	p (One way)
OCR	100±60.94 (31)	207.5±78.36 (31)	197.9±51.16 (39)	164.9±63.48 (28)	0.0031
ECAR	100±44.82 (24)	151.6±46.81 (16)	151.9±71.22 (32)	185.4±82.51 (32)	0.0001

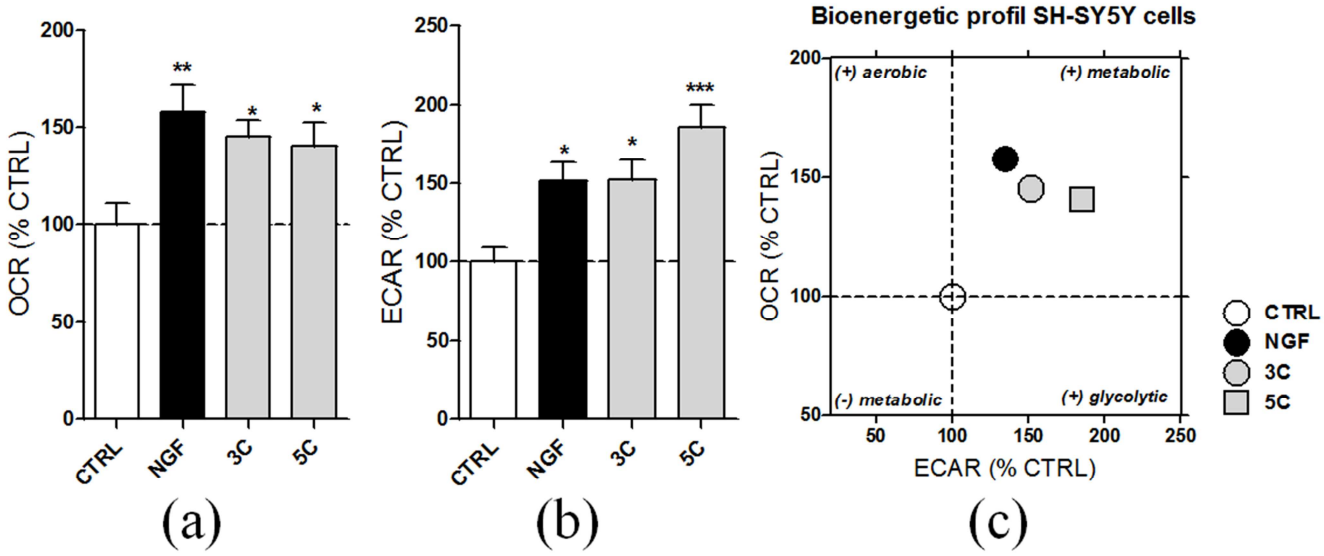

Figure 2. *GS dilutions positively regulates bioenergetic activity in SH-SY5Y neuroblastoma cells. (a) Oxygen consumption rate (OCR) and (b) the extracellular acidification rate (ECAR) were measured simultaneously in SH-SY5Y cells after treatment (24h) with the GS dilutions 3C and 5C as well as the positive control NGF and compared to the untreated control cells after normalization using a XF24 Analyser (Seahorse Bioscience). Values represent the mean± SEM (n = 19-39 replicates) of four independent experiments (Table 2). (c) Bioenergetic phenotype (OCR versus ECAR) of SH-SY5Y cells revealed an increased metabolic activity after treatment with 3C and 5C. Values represent the mean of each group (mean of the ECAR in abscissa/ mean of the OCR in ordinate) and were normalized to the untreated control group (CTRL, 100%). One-way ANOVA ((a): P=0.0031, (b) P=0.0001) and post hoc Dunnett's multiple comparison test versus untreated control cells *P<0.05, **P<0.01, ***P<0.001. OCR, Oxygen Consumption Rate (mitochondrial respiration); ECAR, Extracellular Acidification Rate (Glycolysis).*

3.3. GS Increased the Neurite Extension in 2D and 3D Surface Culture

To investigate the effect of GS dilutions on neurite outgrowth, 3C and 5C dilutions were tested on differentiated SH-SY5Y cells after 3 days of treatment. NGF (50 ng/ml) was again used as positive control because of its action as a promotor of survival and neuritic growth.

After 3 days of treatment, 3C and 5C were able to improve the neurite outgrowth when compared to the untreated control cells, with a higher effect than the positive control NGF itself (Figures 3 and 4).

In fact, after 3 days of treatment with GS dilutions 3C and 5C, significantly increased neurite count (about +94.1% and +133.2% of increase respectively), total neurite length (about +173.3% and +214.4% of increase respectively), attachment point (up to 300% of increase) as well as the number of endpoints (up to 330% of increase) was observed compared to untreated control cells (Figures 4). NGF significantly ameliorated neurite outgrowth, with a +73.5% increase in neurite count, +130% increase in total neurite length, up to 176.9% increase in attachment points, as well as 187.7% increase in the number of endpoints compared to untreated control cells (Figure 4).

Based on the above results, we confirmed the beneficial effect of the GS dilutions on neurite extension using the 3D cell culture method to obtain a 3D view of the neuroplasticity. Figure 5 displays a 3D view of the enhanced neurite length into the 3D-matrix after a treatment with 3C and 5C dilutions compared to the untreated control cells. We observed that GS dilution treatment is able to ameliorate the neurite outgrowth by enhancing the formation of neurite extension (Figure 5).

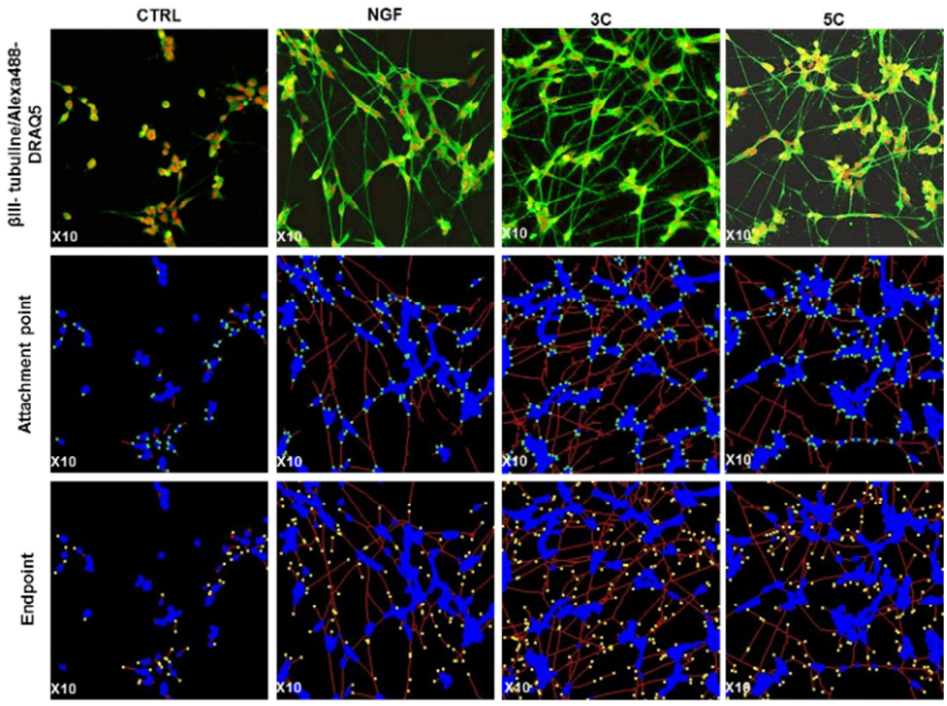

Figure 3. *The GS dilutions 3C and 5C improved the neurite outgrowth of neuroblastoma cells after 3 days of treatment in a 2D cell culture. Pictures were taken using a confocal microscope (x10). Pictures in the upper panel (S1 Figure) display neurite extension between the cells (βIII- tubuline/Alexa488, green) and DraQ5 (nucleus, red). Quantification of the neurite outgrowth parameters such as the attachment points (middle panels) and the endpoint numbers (lower panels), after NGF or GS treatment are shown in the middle and lower panel (Blue: soma, red: neurite, green point: attachment point, yellow point: endpoint). CTRL: untreated control cells.*

Original images (confocal microscope, x10, immunostaining (IMS) with βtubuline/Alexa488 (green) and DraQ5 (red) of a 2D cell culture was used for analysis using ImageJ software (Figure 3).

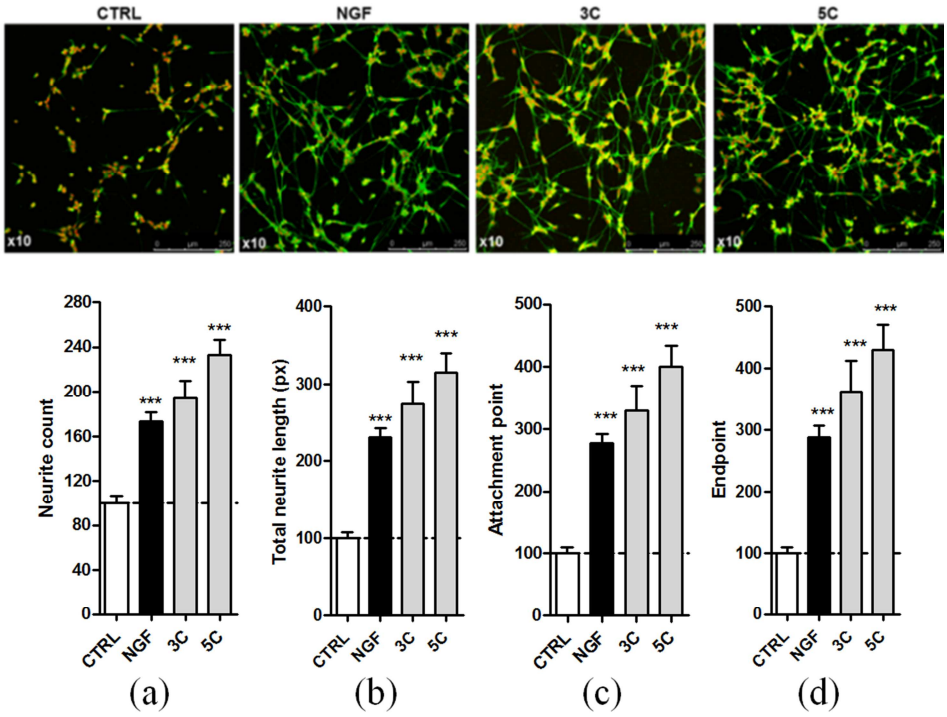

Figure 4. *GS dilutions 3C and 5C increased the neurite outgrowth of neuroblastoma cells after 3 days of treatment in a 2D cell culture. Quantification of Figure 3 using NeurophologyJ (Table 3). 3C and 5C significantly increased: (a) number of neurites (neurite count), (b) total neurite length, (c) number of attachment points and (d) number of endpoint. The effect of GS dilutions was similar than the positive control NGF when compared to the untreated cells. Values represent the mean± SEM of three independent experiments and were normalized to 100% of untreated control cells (CTRL). One way ANOVA (a-d: P <0.0001)) and post hoc Dunnett's multiple comparisons versus untreated control cells ***P<0.001.*

Table 3. *The data are presented as mean (M), standard deviation (±SD) corresponding to the number of cells (N) (Figure 4). Values are normalized on 100% of the control group (untreated CTRL).*

2D-COLLAGEN	CTRL M±SD (N=1733)	NGF M±SD (N=4688)	3C M±SD (N=4066)	5C M±SD (N=3518)	P (One way)
Neurite count	100±40.3	173.5±55.23	194.2±97	233.2±83.17	< 0.0001
Total neurite lenght	100±50.86	230±84.86	273.3±183.1	314.4±150.4	< 0.0001
Attachement point	100±58.66	276.8±104.8	329.6±251.8	400.3±205.2	< 0.0001
Endoint point	100±51.78	287.7±128.8	362.3±307	430.1±241.2	< 0.0001

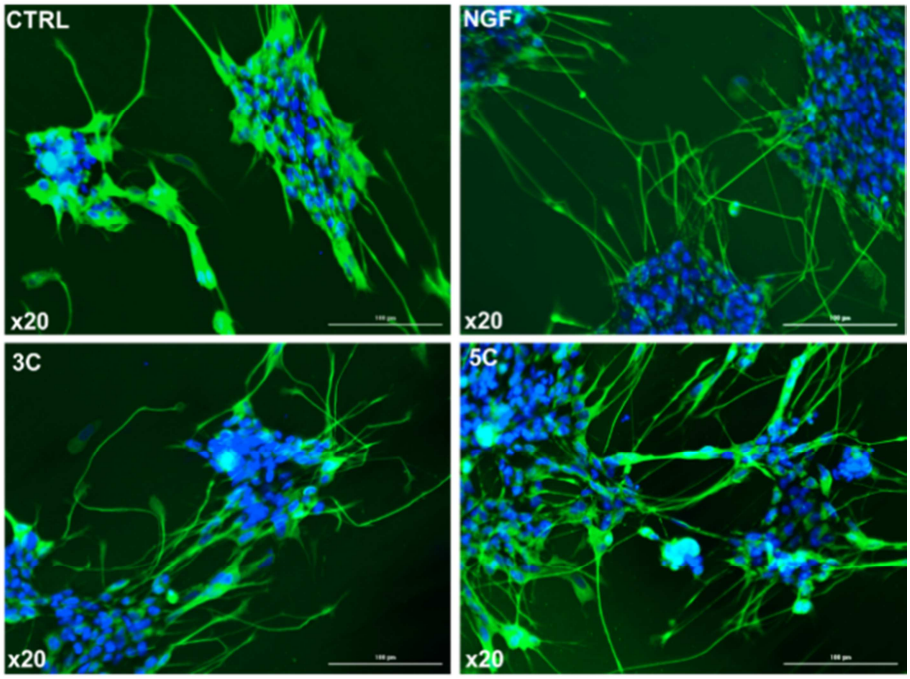

Figure 5. *GS dilutions 3C and 5C induced neurite extension in a 3D-matrix by increasing the neurite outgrowth of neuroblastoma cells after 3 days of treatment. Pictures were obtained by merging 3–4 layers of cells (z-stack projection) on 3D-matrix using the multi-label plate reader Cytation3 (x20). Pictures display neurite extension between the cells (Immunostaining (IMS) with βIII- tubuline/Alexa488 (green) and DAPI (blue)). CTRL: untreated control cells.*

4. Discussion

In the present study, we showed that the GS dilutions 3C and 5C were able to improve mitochondrial bioenergetic metabolism, as well as to initiate the neurite outgrowth in SH-SY5Y cells by: (1) ameliorating cellular ATP levels; (2) promoting cell survival; (3) stimulating mitochondrial respiration and cellular glycolysis; and (4) inducing neurite extension in 2D, as well as 3D cell culture models.

In detail, GS 3C and 5C dilutions significantly increased ATP levels, OCR as well as ECAR, switching the SH-SY5Y cells to a more metabolically active state. We highlighted a similar efficacy of the GS dilutions in comparison to the positive control (NGF) in modulating OXPHOS-derived energy production and neurite outgrowth. In a recent study, we previously demonstrated that NGF plays a crucial role in the stimulation of neurite outgrowth in SH-SY5Y cells by increasing the neurite count and length as well as the attachment point and endpoint parameters [30]. In line with this study, here we showed that, after 3 days of incubation, 3C and 5C dilutions induced a significant increase of the neurite extension in neuroblastoma cells. These effects were comparable with those of the positive control NGF. GS dilution-induced neurite outgrowth was confirmed in 3D-matrix of SH-SY5Y cultures. In accordance with the beneficial effect of GS dilutions reported in many studies as a treatment for several neuroses including anxiety and depression [2, 22, 31], we propose that the efficacy of 3C and 5C is mediated by their ability to boost mitochondrial activity, particularly mitochondrial bioenergetics. This is supported by the fact that mitochondrial function is a critical mechanisms involved in neurite outgrowth [32]. Indeed, the brain requires a considerable amount of energy in order to activate, sustain, and consolidate neuronal functions and plasticity [11]. In particular, nerve cells exhibit high energy demands due to their postmitotic polarization state [11]. In neurons, energy in the form of ATP produced by OXPHOS is directed into the development of the synapses [10]. Apart from the production of energy, other pathways could be implicated in the effect of GS dilutions because mitochondria are the key modulators of brain cell survival and death by controlling redox equilibrium (which can in turn affect neuronal plasticity) and generating reactive oxygen species (ROS) [11]. Furthermore, the regulation of mitochondrial dynamics plays an important role in neurite outgrowth via the mechanisms of extension, regeneration, and branching that require a continuous supply of energy [32-34].

Additional investigations are required to characterize the

specific mechanisms of action or cell signalling triggered by the GS dilutions 3C and 5C for the modulation of cellular bioenergetics and cell survival, as well as the stimulation of neuroplasticity. A possible mechanisms of action would rely on the ability of GS dilution (5C) to induce the production of the neurosteroid allopregnanolone, as was already shown in the rat hippocampus and amygdala [7]. These effects might be mediated by the GS compound Gelsemine [7, 24, 35]. Gelsemine stimulated strongly the upregulation of 3α, 5αTHP biosynthesis at 1x10-8M in rat spinal cord slices [24]. Interestingly, allopregnanolone was also shown to rescue neuronal cells from oxidative stress-induced death via bioenergetic improvement [8]. In addition, a recent study showed that another compound of GS, Koumine, an alkaloid, exerted cytoprotective effects against oxidative stress-induced apoptosis in a porcine intestinal epithelial cell line by suppressing the reactive oxygen species production, inhibiting the caspase-3 activity and influencing the expression of Bax and Bcl-2, key regulators of mitochondrial function [36]. These findings suggest that Gelsemine and Koumine might act on pathways involved in the regulation of reactive oxygen species generation or the redox equilibrium, probably through the action of a single component or synergistic interaction with other unidentified constituents of GS dilutions. Other components might act through additive or synergic effects. Demangeat et al. postulate that at low dilutions (below 3-4C), the presence of the source of molecules in the dilution would ensure a specific molecular action at the level of receptors, in a manner analogous to classical pharmacology [37]. At higher dilutions, above 3-4C, the molecules of the substance source, are included within nanostructures (NS) and/or nanoparticules (NP) with a wide variety of sizes from 3nm up to hundreds nanometers [38-42]. The NS grow, with dilution, so according to their size, NP may induce various biological properties such as cross easily cell and nuclear barrier and might be responsible for some results observed here. NP would act not by direct pharmacological effects on receptors, but as biological signals (stressors) that stimulate the organism's allostatic biological stress response network.

5. Conclusion

The present study suggests that GS dilutions markedly promote neurite outgrowth via the stimulation of mitochondrial bioenergetics and the cell survival. The beneficial effect of GS dilutions through modulation of mitochondrial function leads to new working hypotheses on the anxiolytic and anti-depressant action of this plant as well as an appreciation for GS dilutions as a potential agent for neuroprotection.

Declaration

Ethics Approval and Consent to Participate

Plant: GS as a traditional medicinal plant is no longer used because of its toxicity [3]. It is currently used in homeopathic dilutions only [3]. GS plants were purchased from Herb's International Service SARL (France; Batch H140503595) in respect of the Good Agricultural and Collection Practices (GCAP) [15]. In the present paper, GS dilutions were prepared by Boiron laboratory (Messimy, France) based on the 1.1.10 method of the European Pharmacopoeia (Ph. Eur.) guidelines for the production of homeopathic remedies [25].

Source: It is not present in the International Union for Conservation of Nature (IUCN) lists as an endangered species [16].

Animals: not applicable.

Humans: There were no humans participating in this study and therefore there are no ethical issues that should be addressed. The biological materials that were used are anonymized and are excluded from the Human Research Act (HRA). Origin of the cells: Human SH-SY5Y neuroblastoma cells (ATCC CRL-2266, Virginia, USA, ATCC company).

Consent for Publication

Not applicable.

Availability of Data and Materials

All relevant data are within the manuscript and its supporting information files.

Competing Interest

AE received an investigator research grant from Boiron Laboratory, France. NB and PT are employed by Boiron Laboratory, France. IL and AG declare that they have no competing interests.

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Author Contributions

Conceptualization, A. E. and I. L.; Formal analysis, I. L.; Funding acquisition, A. E.; Investigation, I. L.; Methodology, I. L.; Project administration, A. E.; Resources, N. B., P. T. and A. E.; Supervision, A. E. and I. L.; Writing original draft, I. L., A. G., N. B., P. T. and A. E. All authors have read and agreed to the published version of the manuscript.

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