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In vitro effects of concentrated growth factors (CGF) on human SH-SY5Y neuronal cells

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Abstract. – OBJECTIVE: The aim of this study was to test the *in vitro* differentiation effects of concentrated growth factors (CGF), a platelet rich preparation, using SH-SY5Y cells, derived from human neuroblastoma.

MATERIALS AND METHODS: SH-SY5Y cells were cultured in presence of CGF or retinoic acid (RA). After 72 h of treatment, different parameters were investigated: cell proliferation by an automated cell counter; cell viability by thiazolyl blue tetrazolium bromide (MTT) assay; cell differentiation markers, i.e., neuronal nuclear antigen (NeuN), synaptophysin (SYP) and β3-tubulin, by immunocytochemistry and Western blotting techniques; release of nerve growth factor (NGF) and brain-derived growth factor (BDNF) by enzyme-linked immunosorbent assay (ELISA) and neurite outgrowth by a dedicated image software.

RESULTS: In presence of CGF, the cell proliferation rate and viability decreased, as expected for differentiated SH-SY5Y cells. On the contrary, the cellular differentiation markers increased their expression together with the release of growth factors. Moreover, the neurite outgrowth was improved.

CONCLUSIONS: The data suggest that CGF treatment positively affects the cell differentiation, regulating the expression of neuronal markers, the release of growth factors and the neurite length. Taken together these results seem to be promising in the development of new approaches for neural regeneration.

Key Words:
Concentrated Growth Factors (CGF), Neuronal differentiation, SH-SY5Y, Neural regeneration.

Introduction

Regenerative medicine finds important clinical applications influencing the daily practice. A promising innovative strategy is the use of platelet rich preparations for hard and soft tissues regeneration[1]. Platelet concentrates are blood derivatives[2,3], prepared from patient's own blood. They contain platelets, growth factors, and cytokines involved in the key processes of tissue regeneration, such as cell proliferation and differentiation, extracellular matrix synthesis, chemotaxis, and angiogenesis[4,5]. One of them is the concentrated growth factors (CGF), as reported by Kawase and Tanaka[1]. In its solid form, it is constituted by a tight fibrin network, a natural scaffold, rich in leukocytes, platelets, growth factors[6,7], and possessing great regenerative potentialities[8-13]. Autologous CGF is biocompatible, easy to obtain, safe and without any risk of transmitting infectious diseases.

Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are both released by platelets. In this context, the regeneration of the nervous system could be one of the main goals in this field. Recent data support the use of autologous platelet rich preparations to promote neurogenic differentiation[14], neural repair and regeneration[15-17]. Moreover, several growth factors present in platelets are reported to exert a regenerative but also antiapoptotic and neuroprotective effects on the nervous system[18-26]. These

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findings are supported by different authors. Zurita et al[27] have reported that platelet rich plasma scaffolds enriched with NGF, BDNF and retinoic acid (RA) and loaded with bone marrow stromal cells, enhanced differentiation into the neuronal phenotype. Moreover, autologous platelet-rich plasma gel could be used as a seeding matrix for Schwann cell-like cells to construct tissue-engineered nerves for peripheral nerve regeneration[28]. Finally, platelet rich preparations seem to be promising in the degenerative mouse model of Alzheimer's[17] and Parkinson's disease[29].

Based on these evidences, the aim of this study was to test the *in vitro* regenerative potentialities of CGF on SH-SY5Y cells, considering that no data are present in literature. The human neuroblastoma SH-SY5Y cells are dopaminergic neuronal cells commonly used as an *in vitro* model for neurotoxicity experiments[30], oxidative stress[31] and neurodegenerative diseases[32] which can be differentiated adding RA to the cell culture medium[33,34]. Differentiated cells reduce their proliferation rate[35] and synthetize neurotransmitters and neuropeptides[36]. So, cell proliferation and cell viability were evaluated. Moreover, three neuronal markers were monitored using both immunocytochemical and Western blotting techniques: neuronal nuclear antigen (NeuN), a marker of differentiated neuronal state; synaptophysin (SYP), a marker of synapse remodeling; β3-tubulin, a marker of axon growth and regeneration. The cumulative release of NGF and BDNF were evaluated using the enzyme-linked immunosorbent assay (ELISA). Finally, the neurite length was measured.

Our results are promising; in fact, the CGF treatment regulates the expression of the neuronal markers/growth factors improving the neurite outgrowth.

Materials and Methods

Human Cell Line

Human neuroblastoma cell line SH-SY5Y, media and antibiotics were purchased from Sigma-Aldrich (Milan, Italy). The cells were cultured in "complete medium" constituted by a 1:1 mixture of Dulbecco Modified Eagle's Medium (DMEM) and Ham's F12 supplemented with 1.0 mM glutamine, 10% of heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin solution (antibiotics, 10 ml/L; 10.000 units penicillin and 10 mg streptomycin/ml), in a humidified atmosphere of 5% of CO_2 at 37°C. The culture medium was changed every two days and sub-cultures were used once they reached 80-90% confluence. Cells between third and six passage were used for *in vitro* experiments. During the experiments, "basal medium" has been also used constituted by a 1:1 mixture of Dulbecco Modified Eagle's Medium (DMEM) and Ham's F12 supplemented with 1.0 mM glutamine and penicillin/streptomycin solution (antibiotics).

CGF Production

The research was conducted according to the principles of the Declaration of Helsinki. For the experiments, the venous blood was collected with a 21-gauge needle from 3 healthy adult volunteers of Caucasian ethnicity consisting of 1 man and 2 women (age range: 39-54 years), who have expressed their informed consent for the re-use of human bio-specimens in scientific research. Inclusion criteria were: platelets, red blood cells and leukocytes levels within the normal range. Exclusion criteria were: systemic disorders, smoking, infections, non-steroidal anti-inflammatory drug use, hemoglobin level <11 g/dl for females and <13.5 g/dl for males.

Specific tubes (sterile Vacuette tubes, Greiner Bio-One, Kremsmünster, Austria) and a specific centrifuge (Medifuge MF200, Silfradent Srl, Forlì, Italy) were used to obtain CGF in solid form. After centrifugation, three distinct blood fractions were visible: 1) platelet poor plasma (PPP) at the top, 2) CGF in the middle and 3) red blood cells (RBC) at the bottom. Once obtained, CGF was removed from the tube under a laminar flow cabinet using sterile tweezers, rested upon a specific sterile trans-well inserts (ThinCert™ cell culture inserts, Greiner Bio-One, Kremsmünster, Austria) with a semi-permeable membrane at the bottom (pores of 0.4 μm), and finally inserted into the 6-well culture plates (one insert for each well).

Cell Treatments

For each volunteer, the experiments were performed in triplicate to ensure repeatability of the results. SH-SY5Y were seeded in six multi-well plates (Sarstedt, Nüembrecht, Germany) at a density of 10.000 cell/cm^2 and then subjected to 4 different treatments, for 72 h: 1) C: undifferentiated control – the cells were cultured in complete medium; 2) B+CGF: the cells were cultured in basal medium and in presence of CGF; 3) B+RA: differentiated control without growth factors – the cells were cultured in basal medium supple-

mented with 10 μM all-trans retinoic acid (RA, Sigma-Aldrich, Milan, Italy); 4) C+RA: differentiated control with growth factors – the cells were cultured in complete medium supplemented with RA. Different parameters have been investigated to evaluate the CGF differentiation properties: cell proliferation, cell viability, expression of neuronal specific markers (NeuN, SYP, β3-tubulin), release of NGF and BDNF and neurite outgrowth.

Cell Count and Cell Viability

Cell count was performed using an automated cell counter (Scepter™ 2.0 Cell Counter, Millipore, Billerica, MA, USA) and the data obtained were expressed as the percentage relative to the initial number of cells seeded (control, 100%).

Cell viability was evaluated using the thiazolyl blue tetrazolium bromide (MTT) colorimetric assay. At the end of the different treatment performed for 3 days, MTT (Sigma-Aldrich, Milan, Italy, 5 mg/ml) was added to each well for 4 h at 37°C. The culture medium was aspirated and the same quantity of dimethyl sulfoxide (DMSO – Sigma-Aldrich, Milan, Italy) was added to each well. The plate was put on the shaker for 15 min and after the supernatant was transferred to a 96-microplate well. Colorimetric changes were quantified at a wavelength of 540 nm. The data obtained were expressed as the percentage optical density relative to the control (C: undifferentiated control) of 100%.

Immunocytochemistry of Neuronal Specific Markers

Immunocytochemistry was performed to assess the effect of CGF on specific neuronal markers such as neuronal nuclear antigen (NeuN), synaptophysin (SYP), and β3-tubulin. At the end of treatments, both differentiated and undifferentiated cells were fixed in 4% paraformaldehyde (Immunofix, Bio-Optica, Milan, Italy) for 20 min at room temperature (RT). The UltraTek staining kit (ScyTek Laboratories, West Logan, UT, USA) was used to perform immunocytochemistry. Briefly, cells were washed 3 times with phosphate-buffered saline (PBS) and then incubated with Super Block solution for 5-10 min at 37°C, in order to block non-specific binding of antibody. The cells were subsequently incubated with anti-SYP (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:400), anti-NeuN (Chemicon, Temecula, CA, USA, 1:100) and anti-β3-tubulin (Abcam, Cambridge, UK, 1:100) for 2 h at room temperature. After that, SH-SY5Y were washed 4 times with PBS and incubated with UltraTek Ant-Polyvalent solution for 10 min at 37°C. After 4 washing with PBS, cells were incubated with Ultratek HRP for 10 min RT. The chromogen substrate diaminobenzidine (DAB, Amresco, Prodotti Gianni, Milan, Italy) was used to visualize the positive reaction. Digitally fixed images were acquired randomly and analyzed using a dedicated image software (Image Pro-Premier, Immagini & Computer, Milan, Italy) for the integrated optical density (IOD) normalized at 100 um^2 area[8,9].

Western Blotting Analysis

Immunoblotting was performed to assess the effect of CGF on specific neuronal markers such as neuronal nuclear antigen (NeuN), synaptophysin (SYP), and β3-tubulin. At the end of treatments, the cells were washed with phosphate-buffered saline (PBS), detached using the trypsin (0.025%)/ethylenediamine tetraacetic acid, 0.01% (EDTA) solution (Promocell, Heidelberg, Germany) and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and pellets were frozen at –80° until Western blotting analysis was performed. Briefly, pellets were washed 2 times with ice-cold PBS, and centrifuged at 10000 rpm for 2 min. At the end of centrifugation, the supernatant was removed and pellets were incubated with ice-cold lysis buffer, for 20 min, in agitation, for protein extraction. After samples preparation and protein quantification (Bio-Rad Protein assay, Bio-Rad Laboratories, Munich, Germany) protein extracts were separated in 10% sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE gel; Bio-Rad Laboratories, Munich, Germany) and then transferred onto a polyvinylidene difluoride (PVDF; Bio-Rad Laboratories, Munich, Germany) membrane. The membrane was incubated with the blocking solution, containing 5% of bovine serum albumin (BSA) in Tris-buffered saline (TBS; Sigma-Aldrich, Milan, Italy) and 0.1% Tween 20 (Sigma-Aldrich, Milan, Italy) at 4°C overnight. The day after, it was incubated for 1 h with antibodies: anti-SYP (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:2000), anti-NeuN (Chemicon, Temecula, CA, USA 1:1000), anti-β3-tubulin (Abcam, Cambridge, UK, 1:1000) and anti-β-actin antibody (Abcam, Cambridge, UK, 1:5000), used as loading control. Subsequently, the membrane was washed 2 times with TBS and 0.1% Tween 20 and developed using the chromogen substrate diaminobenzidine (DAB, Amresco, Prodotti Gianni, Milan, Italy) to visualize bands. Digitally fixed images were produced

and analyzed using a dedicated image software (Image Pro-Premier, Immagini & Computer, Milan, Italy) for the integrated optical density (IOD)[37] expressed as percentage of undifferentiated control (C).

Enzyme-Linked Immunosorbent Assay (ELISA)

Human nerve growth factor (NGF) ELISA kit (CUSABIO TECHNOLOGY LLC, Huston, TX, USA) and human brain-derived neurotrophic factor (BDNF) ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) were used to detect the levels of the growth factors in the cell supernatant. At the end of the different treatments, the conditioned medium was collected, centrifuged for 10 min at 1000 rpm, and stored at −80°C until the NGF and BDNF concentrations were measured using the specific ELISA kit, according to the manufacturer's instructions. The total quantity of the growth factors in the medium was evaluated.

Measure of Neurite Length

The neurite was identified as a cellular process that is greater than one cell body in length possessing a growth cone[37-39]. Digitally fixed images were produced randomly and analyzed using a dedicated image software (Image Pro-Premier, Immagini & Computer, Milan, Italy) to measure the neurite length.

Statistical Analysis

The data collected were analyzed by one-way ANOVA followed by the Bonferroni test. The level of significance was set at 5% ($p < 0.05$).

Results

Cell Count and Cell Viability

The cells treated both with CGF in basal medium (B+CGF) and RA in basal (B+RA – differentiated control without growth factor supplementation) or complete medium (C+RA) differentiated control) showed a decrease in proliferation rate and a consequent reduction of viability respect to complete medium alone (C – undifferentiated control) (Figure 1).

Expression of Neuronal Specific Markers

Immunocytochemistry for neuronal nuclear antigen (NeuN) showed that immunopositivity increased in SH-SY5Y treated with CGF in basal

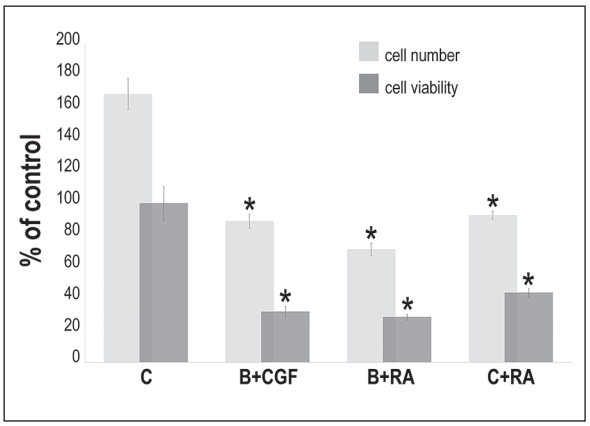

Figure 1. Evaluation of cell number and viability (MTT assay) on SH-SY5Y after 72 h of treatment in complete medium alone (C: undifferentiated control), basal medium supplemented with CGF (B+CGF), basal medium supplemented with retinoic acid (B+RA: differentiated control without growth factor supplementation), complete medium supplemented with retinoic acid (C+RA: differentiated control). Data represent the percentage of control (*$p < 0,05$ *vs.* C): the control for cell number evalutation was the total number of cell seeded, while for MTT assay was the treatment C.

medium (B+CGF: 2.46±0.22) respect to complete medium alone (C: 1.18±0.12 – undifferentiated control). The cells treated with RA in complete medium (C+RA: 3.5±0.39 – differentiated control) showed the highest positivity, while the cells treated with RA in basal medium (B+RA: 0.1±0.1 – differentiated control without growth factor supplementation) showed the lowest positivity respect to all the other conditions (Figure 2A). These results were confirmed also by Western blotting analysis (Figure 3A, D).

Immunocytochemistry for synaptophysin (SYP) showed that the highest value of immunopositivity was reached in SH-SY5Y cells treated with CGF in basal medium (B+CGF: 5.71±0.83) respect to complete medium alone (C: 2.11±0.24) and with RA both in complete (C+RA: 2.78±0.38) and in basal medium (B+RA: 1.83±0.14) (Figure 2B). These results were confirmed also by Western blotting analysis (Figure 3B, D).

Immunocytochemistry for the neuronal marker β3-tubulin showed that immunopositivity increased in SH-SY5Y cells treated with CGF in basal medium (B+CGF: 6.23±0.48) respect to complete medium alone (C: 2.91±0.14) and RA in basal medium (B+RA: 3.62±0.18). The cells treated with RA in complete medium (C+RA: 8.13±1.15) reached the highest value of immu-

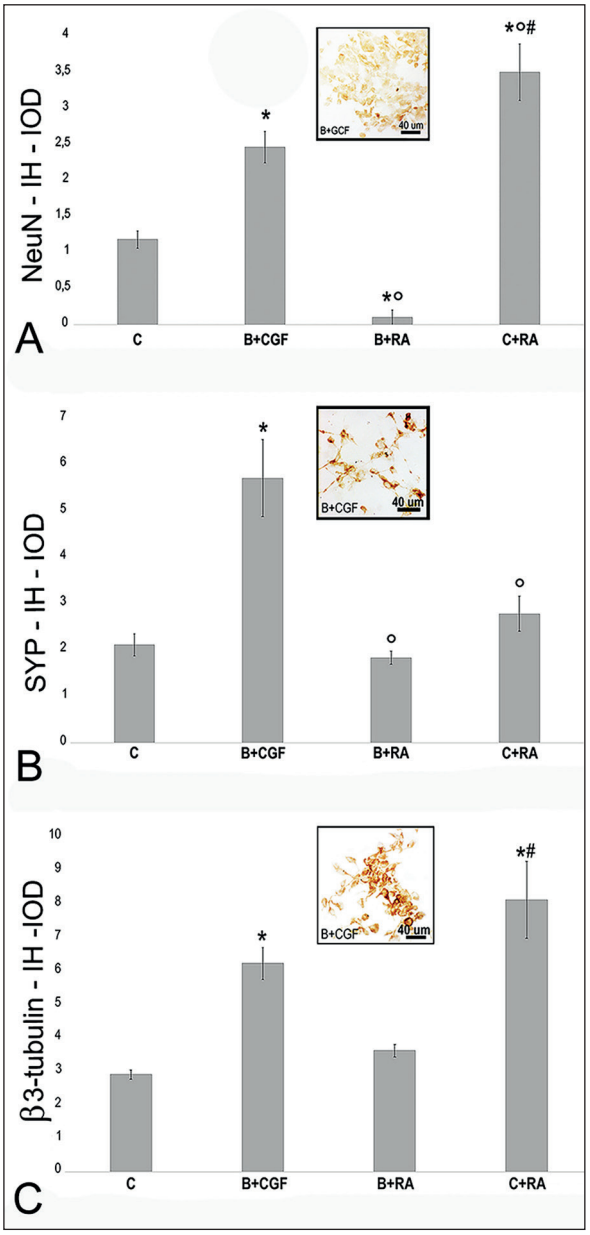

Figure 2. Immunocytochemical evaluation of neuronal markers on SH-SY5Y after 72 h of treatment in complete medium alone (C: undifferentiated control), basal medium supplemented with CGF (B+CGF), basal medium supplemented with retinoic acid (B+RA: differentiated control without growth factor supplementation), complete medium supplemented with retinoic acid (C+RA: differentiated control). **A,** Neuronal nuclear antigen (NeuN); **B,** Synaptophysin (SYP); **C,** β3-tubulin. Data are reported as integrated optical density (IOD) ± SE. *$p < 0,05$ *vs.* C; °$p < 0,05$ *vs.* B+CGF; #$p < 0,05$ vs. B+RA. Inset: example of immunostaining micrograph - magnification: 200×; bar = 40 µm.

nopositivity respect to all the other treatments (Figure 2C). These results were confirmed also by Western blotting analysis (Figure 3C, D).

Cumulative Release of NGF and BDNF

The NGF detected in the culture medium increased, in the same way, in SH-SY5Y treated with CGF in basal medium (B+CGF: 1006.90±224.25), RA in basal medium (B+RA: 1200.87±187.23)

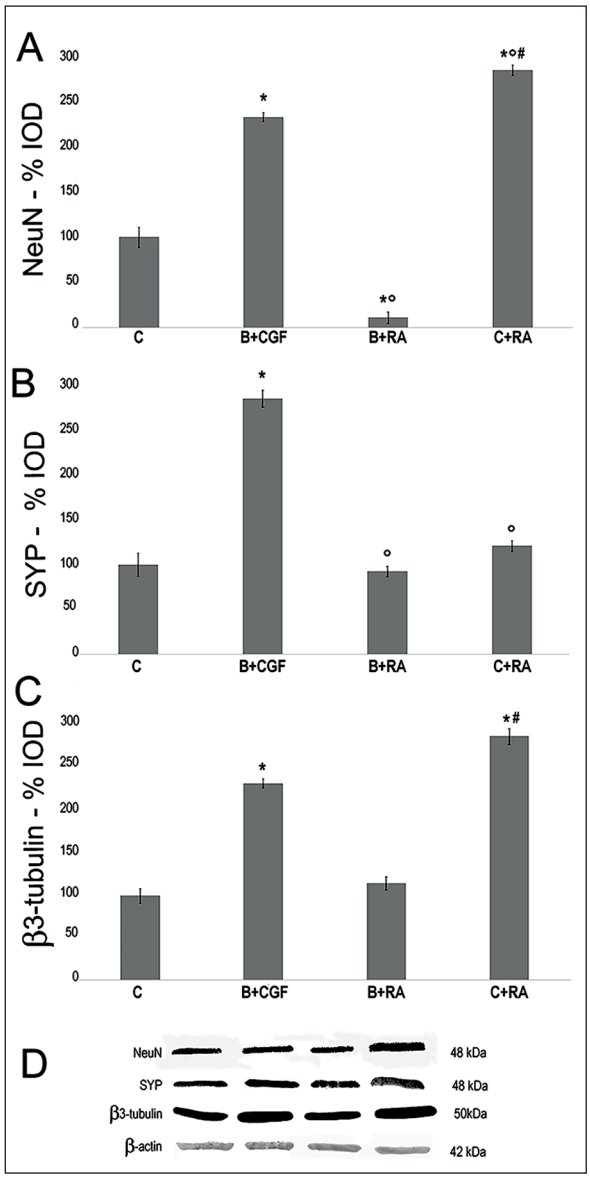

Figure 3. Immunoblotting evaluation of neuronal markers on SH-SY5Y after 72 h of treatment in complete medium alone (C: undifferentiated control), basal medium supplemented with CGF (B+CGF), basal medium supplemented with retinoic acid (B+RA: differentiated control without growth factor supplementation), complete medium supplemented with retinoic acid (C+RA: differentiated control). **A,** Neuronal nuclear antigen (NeuN); **B,** Synaptophysin (SYP); **C,** β3-tubulin; **D,** Western blotting bands. Data are reported as percentage of integrated optical density (IOD) respect to control (C) ± SE. *$p < 0,05$ *vs.* C; °$p < 0,05$ *vs.* B+CGF; #$p < 0,05$ *vs.* B+RA.

and in compleate medium (C+RA: 868.39±71.87) respect to the cells in complete medium alone (C: 426.48±81.64) (Figure 4A).

The BDNF detected in the culture medium increased, in the same way, in SH-SY5Y treated with CGF in basal medium (B+CGF: 2885.67±468.87), RA in basal medium (B+RA: 3522.00±791.47) and RA in compleate medium (C+RA: 2394.00±214.93) respect to the cells in complete medium alone (C: 132.00±14.80) (Figure 4B).

Neurite Length

The neurite length was increased in all treatments (B+CGF: 104.8±8.07; B+RA: 102.07±8.8; B+RA: 122.91±9.98) compared with complete medium alone (C: 60.37±5.7). So, CGF in basal medium was a treatment with an effect comparable with the RA inducing differentiation (Figure 5).

Discussion

The potential use of platelet rich preparations is today not completely explored and supported by scientific experiments. Nevertheless, the growth factor treatment is one of the most promising therapy for neurodegenerative diseases, promoting neurorestoration and neuroprotection[40].

This work underlines the neural regenerative potential of a specific platelet rich preparation, the CGF in its solid form. Previous studies[41-47] on stem cells indicated the CGF treatment to promote proliferation, activation and differentiation of stem cells. In our experiments, we observed a decrease in cell proliferation rate, comparable to the RA treatment, which was indicative of the differentiation ability of CGF. Moreover, three different neuronal differentiation markers were considered: Neuronal nuclear antigen (NeuN), synaptophysin (SYP) and β3-tubulin. NeuN is a neuronal mature phenotype marker[48] and has recently been used also in several studies on neural regeneration[49-51]. SYP plays a key regulatory role in the release of neurotransmitters and its up-regulation indicates a remodeling of synapses to maintain/recover the neural circuits[52-54]. β3-tubulin protein is expressed most prominently in axon-like cell extensions[55] and increases when PC12 cells differentiate into neurons[51] and during axon growth and regeneration[56].

Our results showed an increase of these three markers after CGF stimulation. In particular, the value of β3-tubulin under CGF stimulation (B+CGF) is higher than the differentiated control (C+RA), while the other two markers of differentiation increased respect to the undifferentiated control (C), but did not reach the positive control value (C+RA). So, CGF seems to especially promote the axon-like cell extensions. Another interesting data are related to the cells cultured in basal medium plus RA (B+RA), in fact, only SYP increased while NeuN decreased and β3-tubulin was unaffected compared with complete medium alone (C). These data suggest that the lack of a controlled release of growth

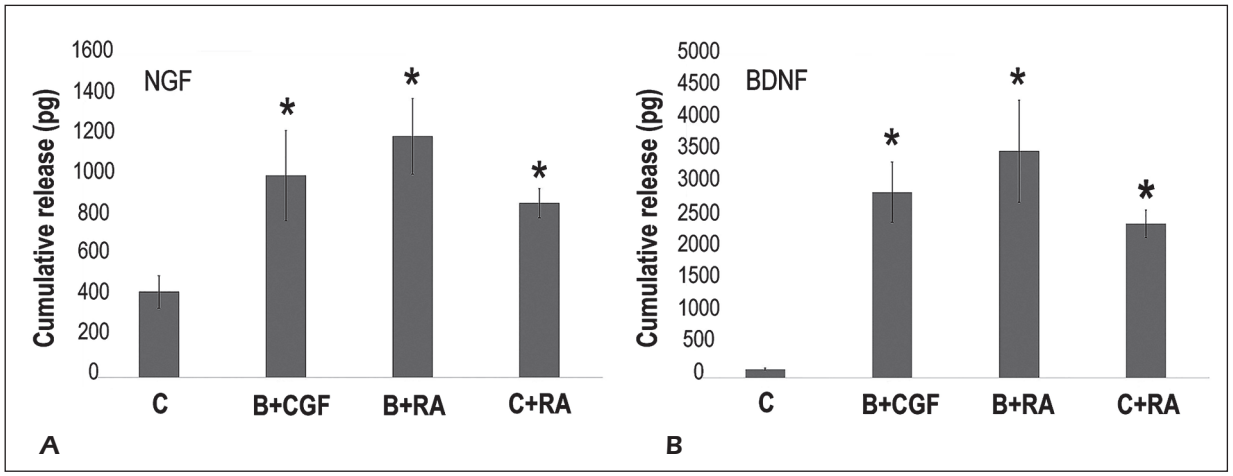

Figure 4. Quantification by ELISA test of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) released (cumulative release) after 72 h of treatment in complete medium alone, which represent the control (C), basal medium supplemented with CGF (CGF), basal medium supplemented with retinoic acid (B+RA), complete medium supplemented with retinoic acid (C+RA). Data are reported in picograms (pg) ± SE. *$p < 0,05$ *vs.* C.

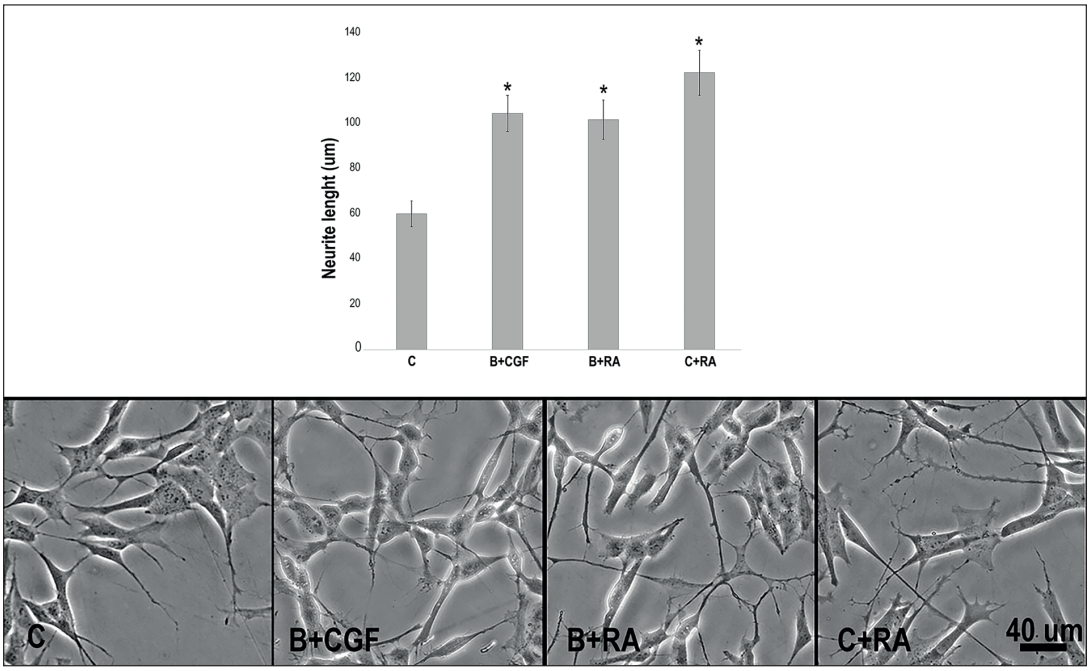

Figure 5. Neurite lenght of SH-SY5Y after 72 h of treatment in complete medium alone, which represent the control (C), basal medium supplemented with CGF (B+CGF), basal medium supplemented with retinoic acid (B+RA), complete medium supplemented with retinoic acid (C+RA). Data are reported in micrometers (μm) ± SE. **p* < 0,05 *vs.* C. Magnification: 200×; bar = 40 μm.

factors, as in CGF treatment, determines an impairment in differentiation process and indicates a precise direction of neuronal remodeling under the CGF stimulation.

We also explored the production of NGF and BDNF, suggesting a positive correlation between the two growth factors. NGF and BDNF actions are dependent on binding to transmembrane receptor systems, the tropomyosin receptor tyrosine kinase (TrK) family and the p75 neurotrophin receptor[57]. NGF receptors are present most predominantly in nociceptive sensory neurons and α-motor neurons[58,59], and NGF stimulates the survival and maturation of developing neurons in the peripheral nervous system and protects them from degeneration[60,61]; BDNF exerts neuroprotective and growth-promoting effects[62,63] on a variety of neuronal populations after injury. Our results indicate that the release of both NGF and BDNF increased significantly under CGF treatment compared with the undifferentiated control (C).

It has been well documented as the neural regenerative process becomes in an orchestrated expression of growth factors; these active molecules change their expression in a spatio-temporal way. For example, Nakamura and Bregman[64] described how each neurotrophic factor underwent different temporal changes in lesioned

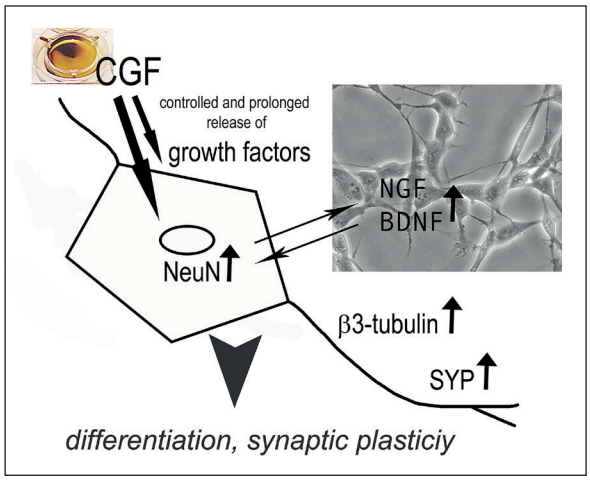

Figure 6. Concentrated growth factors (CGF) releases high quantity of growth factors[8], among which nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which induce an intracellular signal trasduction pathways mediated by specific receptors[48] in SH-SY5Y cells (micrograph: 200× magnification). Neuronal activity needs molecular mediators and neurotrophic factors are prime candidates, even if not the only ones, in fact, other non-neurotrophic factors can contribute to neuronal survival and differentiation[38,52,56]. The release of growth factors by CGF is modulated in a temporal way[8] promoting the expression of key molecules involved in neuronal differentiation and plasticity, such as neuronal nuclear antigen (NeuN), synaptophysin (SYP), β3-tubulin and the synthesis of neurotrophic factors by cells itself, promoting an autocrine stimulating mechanism.

spinal cord. Moreover, different neuronal populations are differently responsive to neurotrophins[62]. Thus, the development of a useful delivery system able to protect the growth factors from proteolytic degradation and, in the same time, their spatio-temporal release, would greatly improve their clinical efficacy[40] and could avoid side effects from high concentrations of growth factors.

In this context, the CGF responds perfectly to this request delivering platelets with a controlled release of growth factors and cytokines during the time. In addition, it has been reported that the co-delivery of multiple growth factors improved their clinical efficacy[40]. Accordingly, CGF releases different active molecules from platelets that exert multiple stimulations on neuronal cells. In fact, not only NGF and BDNF can contribute to neural regeneration but also other growth factors are promising (i.e., basic fibroblast growth factor – bFGF)[40,61,65]. A recent study[40] supports this consideration showing that the *in vitro* co-delivery of bFGF and NGF in PC12 and SH-SY5Y cells gives better neuroprotective results than the use of NGF or bFGF alone. Moreover, also hormones (e.g., thyroxine, estradiol, progesterone, growth hormone) and ions present in plasma can contribute to this process[66,67], also influencing the growth factor release by platelets and resident cells.

Finally, CGF treatment (B+CGF) enhances neurite outgrowth with results comparable with the cells treated with RA both in basal (B+RA) and complete (C+RA) medium. These data indicate that RA is able to induce neurite outgrowth also in absence of growth factors in cell culture medium promoting the release of growth factors by the cells without supporting a proper neuronal maturation (low expression of NeuN and β3-tubulin). In this context, CGF exerts additional effects supported by an orchestrated release of growth factors which promote the neural regeneration process (Figure 6).

Conclusions

Our data suggest that treatment with CGF positively affects cell differentiation and neuronal phenotype regulating the expression of the neuronal markers and improving the neurite outgrowth. Taken together these results seem to be promising in the development of new approaches for neural regeneration.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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