

PolicosanolPlus® and Neuroprevin™ Enhance Neurite Regeneration and Prevent Neurite Degeneration: Dietary Supplements for Nerve Repair and Prevention, and Reduction of Neurodegenerative Disorders

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ABSTRACT

Objective

To determine if PolicosanolPlus® and Neuroprevin™ supplementation of neuronal cell culture system promotes neurite formation and prevents neurite degeneration.

Methods

The PC12 neuronal cells were seeded in wells of a twenty-four tissue cluster and immediately treated with nerve growth factor, Neuroprevin, PolicosanolPlus, the Neuroprevin ingredients, and PolicosanolPlus and Neuroprevin combined. Neurite formation was quantified at 200X magnification by visual inspection over a six-day period. Neurite outgrowth was determined as the percent of cells with neurites at least one cell diameter in length. Photomicrographs were also taken at 400X magnification.

Results

Ten percent of nerve growth factor treated expressed neurites at 9 hours. With the Neuroprevin ingredients, N1,

N2, and N3, and complete Neuroprevin (NT), 52%, 47%, 42%, and 55% of the cells, respectively, had neurites. Of cells treated with PolicosanolPlus (P) and NT +P, 45% and 57% expressed neurites. As the cell cultures aged, 7% of control cells maintained neurites, while 55% of the NT treated cells maintained neurites, and 17% of the NT and P treated cells maintained neurites.

Conclusion

Both Neuroprevin and PolicosanolPlus are dietary supplements that can enhance nerve regeneration, prevent neurodegeneration and reduce nerve damage upon stress or injury.

INTRODUCTION

The rat pheochromocytoma cell line (PC12) has long been used as a model of neuronal cell neurite outgrowth and neurite regeneration. As early as 1987, reviews of the literature demonstrated the wide use of these cells to model primary neuronal cell behavior.²⁰ The ability of nerve growth factor to stimulate neurite outgrowth and regeneration in primary cells was demonstrated in 1968,²² and in PC12 cells in 1976.²¹ Since 1974, neurite outgrowth in PC12 cells has been aggressively studied as a model of nerve regeneration and neurodegeneration. For example, NGF-treated PC12 cells have been used to identify neuronal plasticity factors involved in nerve repair of spinal cord.²³ Further, in studies using PC12 cells, a role was demonstrated for the MAPK pathway in neurite protection

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from hypoxia.²⁴ Moreover, NGF-treated PC12 cells have been used to investigate the mechanism of retrograde neurite degeneration in murine dying-back axonopathy, which serves as a model for human neurodegenerative diseases involving ubiquitination or proteasome abnormalities.²⁶ With regard to the nutritional status and starvation of neurons, PC12 cells have also been used to investigate agents that protect neurons from apoptosis and prevent the neurodegenerative events that occur in diseases such as Alzheimer's disease.²⁵ Therefore, the use of NGF-induced neurite outgrowth, regeneration and degeneration in PC12 cells is a widely accepted and excellent model to use when examining the potential benefits of dietary supplements on the promotion of nerve regeneration and the prevention of neurodegeneration.

PolicosanolPlus is a mixture of aliphatic alcohols, saturated fatty acids and polyunsaturated fatty acids (omega-3, omega-6 and omega-9) in their naturally occurring ratios, that have been extracted from natural waxes (available from sugar cane wax, rice bran wax, carnauba wax, candelilla wax, and/or beeswax). The mixture of aliphatic alcohols and fatty acids found in PolicosanolPlus indicate its principal use as a cholesterol-lowering natural supplement capable of reducing and/or preventing hypercholesterolemia diseases and cardiovascular diseases. However, dietary ingredients of this mixture have also been shown to have significant neurotrophic and neuroprotective properties, as well as many other clinical benefits.^{1,2,3,4,5,6,7,8,9,10,11,12,13,14,15} For example, hexacosanol enhances murine sciatic nerve regeneration after crush⁶ and promotes the survival of septal cholinergic neurons after fimbria-fornix transaction in rats.⁴ Further, the total mixture of fatty alcohols and fatty acids has been shown to protect neurons from microglia associated excitotoxicity.¹⁵

Neuroprevin is a previously undescribed proprietary blend of three dietary ingredients, the activity of which on neuronal cell cultures has not yet been reported. In this study, we tested the effect of PolicosanolPlus and Neuroprevin separately, and in combination in neuronal cell cultures to determine their potential use in the prevention of neuronal damage, the onset of neurodegenerative diseases, and the reduction of neuronal damage after injury.

MATERIALS AND METHODS

Materials

The PolicosanolPlus extract (active material) was obtained from a blend of selected natural sugar cane waxes from continental United States sugar cane varieties, and produced by Innovation Laboratories, Inc. (Miami, Florida, Innovation lot 2002-01306-A). The compositions used of PolicosanolPlus and Neuroprevin were blended and formulated by Nature's Value, Inc. (Coram, New York) for this study. The 7S nerve growth factor was obtained from Sigma Chemical Company, Inc. (St. Louis, MO).

Cell Culture and Neurite Outgrowth Assays

PC12 cells were cultured in DMEM containing 7.5% heat inactivated fetal bovine serum and 7.5% heat inactivated equine serum, and incubated at 37.5°C in a 5% CO₂ water-jacketed incubator. For assay, the PC12 cells were harvested by agitation, centrifuged at 1000 X g, and pellets were re-suspended in 5 milliliters (ml) of culture medium. 10 µl of the cell suspension was loaded onto a hemacytometer. The cell numbers were counted, and the cells were then brought to 2 X 10⁴ cells/ml in culture medium containing a final concentration of 100 ng/ml 7S murine nerve growth factor (NGF). This cell suspension was added at a volume of 0.5 ml to each 16 mm diameter well of a 24 well tissue cluster, providing 1 X 10⁴/well. The cells were subsequently either treated with PolicosanolPlus, Neuroprevin, and/or the Neuroprevin ingredients, N1, N2, and N3. Since the PolicosanolPlus was dissolved in ethanol and the Neuroprevin ingredients were dissolved in water, cells also received the vehicle controls. All test concentrations were achieved by adding a volume of 10 µl of vehicle, or in the case of Neuroprevin and PolicosanolPlus, combined in 10 µl of ethanol and 10 ml of H₂O. After treatment, the cells were returned to the incubator and neurite outgrowth was assessed by visual inspection at 200 X magnification over a six-day period. The percent of cells expressing neurites was determined by counting three fields in duplicate well treatments. The number of cells with neurites at least one cell diameter in length was divided by the total number of cells counted and multiplied by 100 to give the percent of cells with neurites.

RESULTS

Nerve growth factor (NGF) treatment of PC12 cells stimulates a significant morphological change in PC12 cells (Figures 1A and 1B), and the cell processes that produce tyrosine hydroxylase and possess the characteristics of genuine neuritis.²² In this study, NGF treatment of PC12 cells resulted in 10% of cells with neurites at 9 hours (Figure 1B and 2). Co-treatment of the PC12 cells with the Neuroprevin ingredients and total Neuroprevin resulted in significant neurite outgrowth at 9 hours (Figures 1 and 2). Specifically, compared to 10% of cells expressing neurites in the presence of NGF alone, with the Neuroprevin ingredients, N1, N2, and N3, and complete Neuroprevin, 52%, 47%, 42% and 55% of the cells, respectively, had neurites (Figures 1 and 2). PolicosanolPlus also significantly promoted neurite outgrowth, with 45% of the cells extending neurites at 9 hours (Figures 2 and 3). When combined with Neuroprevin, PolicosanolPlus showed the greatest observed enhancement of neurite outgrowth with 57% of the cells expressing processes. Table 1 shows that PolicosanolPlus, Neuroprevin, the combination, and the Neuroprevin ingredients all enhance neurite outgrowth in a

Table 1. PolicosanolPlus® and Neuroprevin™ and its ingredients enhance neurite outgrowth in a dose dependent fashion.

| Percent Neurite Outgrowth | | | | | | | |
|---------------------------|----------------|----|----|----|----|----|--------|
| Ingredients: | | N1 | N2 | N3 | NT | P | NT + P |
| Dose(µg/ml) (N1) | (N2, N3 and P) | | | | | | |
| 0.0 | 0.0 | 10 | 10 | 10 | 10 | 10 | 10 |
| 10.0 | 0.2 | 29 | 16 | 12 | 31 | 22 | 26 |
| 50.0 | 1.0 | 47 | 32 | 28 | 51 | 25 | 49 |
| 100.0 | 2.0 | 52 | 47 | 42 | 55 | 45 | 57 |

PC12 cells were seeded in wells of a 24 well tissue cluster plate and treated with NGF. Immediately after seeding the PC12, cells were treated with varying doses of PolicosanolPlus (P), Neuroprevin (NT), and the three ingredients of Neuroprevin (N1, N2, N3). For the varying doses of NT and NT + P, the ingredients were added in combination at the indicated concentrations. The cells were then cultured for 9 hours and neurite outgrowth was assessed as described in the Materials and Methods. The data for no additions (0.0 µg/ml) and for the maximum dose are the same as those presented in Figure 3.

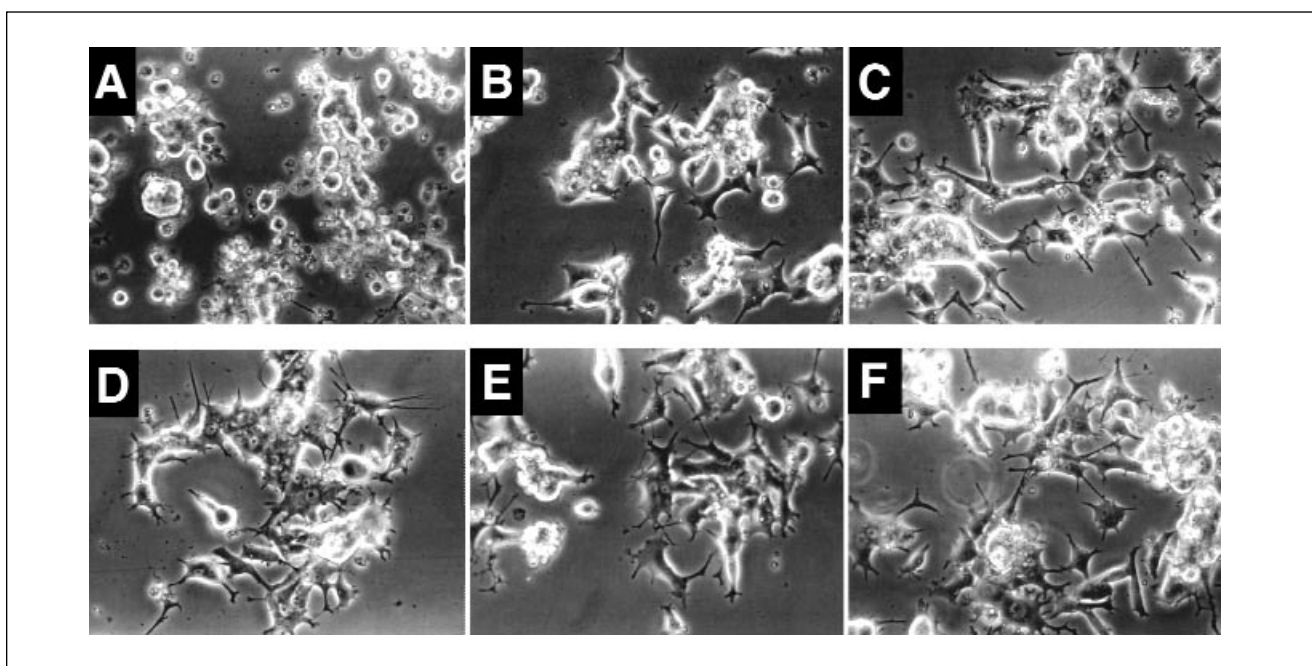


Figure 1. Neurite outgrowth in PC12 cells treated with Neuroprevin and its ingredients. PC12 cells that are not treated with NGF do not become neurons and therefore do not express neurites (A). With NGF treatment, the PC12 cells extend neurites (B). Concomittant treatment of the cells with NGF and the Neuroprevin ingredients: 1 (C), 2 (D), and 3 (E) results in enhanced neurite formation. The total Neuroprevin ingredient (F) also enhances neurite outgrowth. The cells were cultured for nine hours with the indicated treatments and then photographed at 400 X magnification.

dose dependent manner. N1 was tested at 0, 10, 50, and 100 µg/ml, and the observed neurite formation at these concentrations after 9 hours of treatment were, 10%, 29%, 47% , and 52%, respectively (Table 1). N2, N3, and PolicosanolPlus were all tested at 0, 0.2, 1.0, and 2.0 µg/ml, and of these, PolicosanolPlus showed the greatest increase from 10% to 22% with a 0.2 µg/ml treatment (Table 1), with each ingredient increasing the percent of cells with neurites to over 40% with a 2.0 µg/ml treatment (Table 1). The strongest result was seen with the combination of PolicosanolPlus and Neuroprevin, with 57% of the cells

expressing neurites within 9 hours (Table 1). Furthermore, NGF treatment alone resulted in 20% of cells with neurites at 12 hours, and a peak of 61% at 48 hours (Figure 4). As nutrients were consumed and the cell cultures aged, by 96 hours post treatment only approximately 40% of the cells retained neurites, and by 120 hours, 12% of the cells retained neurites, which dropped to 7% by hour 144 (Figure 4). In contrast, with Neuroprevin, the maximum neurite outgrowth was 67% by 24 hours, and dropped by only 12% by hour 144, at which time 55% of the cells maintained neurites (Figure 4). Treatment with PolicosanolPlus also

extended the survival of the neurites as the cultures aged, with over 30% of the cells retaining neurite at hour 120, and more than double the number expressing neurite at 144 hours compared to the 7% control treatment in the absence of PolicosanolPlus (Figure 4). However, with regard to the maintenance of neurites, the Neuroprevin was significantly more effective than the combination of PolicosanolPlus and Neuroprevin (Figure 4).

DISCUSSION

Scientific literature is filled with studies which demonstrate that neuronal damage associated with injury, stroke, and neurodegenerative diseases can be reduced and possibly repaired with a wide range of nutritional supplements. For example, dietary supplement with grapes ameliorates ischemia-induced cerebral neuronal death in gerbils.¹⁶ Further, dietary supplementation with the herb youkongan can reduce ischemia-induced damage in rodents.¹⁷ Dietary supplement with creatine in both mice and rats protects against traumatic brain injury,¹⁸ and antioxidant supplementation has been shown to reduce the damage associated with neurodegenerative diseases in human case reports.¹⁹

In this study, we report the potential neuroprotective activity of two dietary supplements, PolicosanolPlus and Neuroprevin. Both of these natural supplements, either

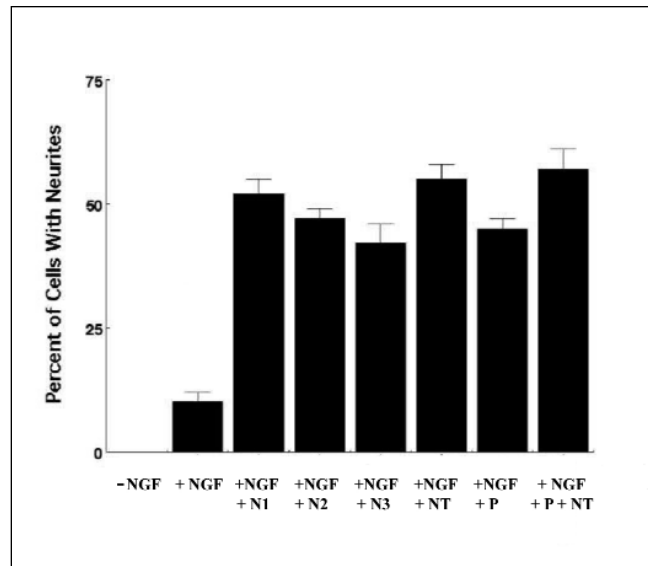


Figure 2. Percent of PC12 cells with neurites after treatment with PolicosanolPlus® and Neuroprevin™. PC12 cells were cultured for nine hours in the absence (-) or presence of NGF (+NGF), or NGF and the Neuroprevin ingredients (N1, N2, N3), the total Neuroprevin mixture (NT), PolicosanolPlus (P), or PolicosanolPlus and Neuroprevin (NT + P). After the nine hour culture period the cells were assessed for neurite outgrowth as described in the materials and methods section.

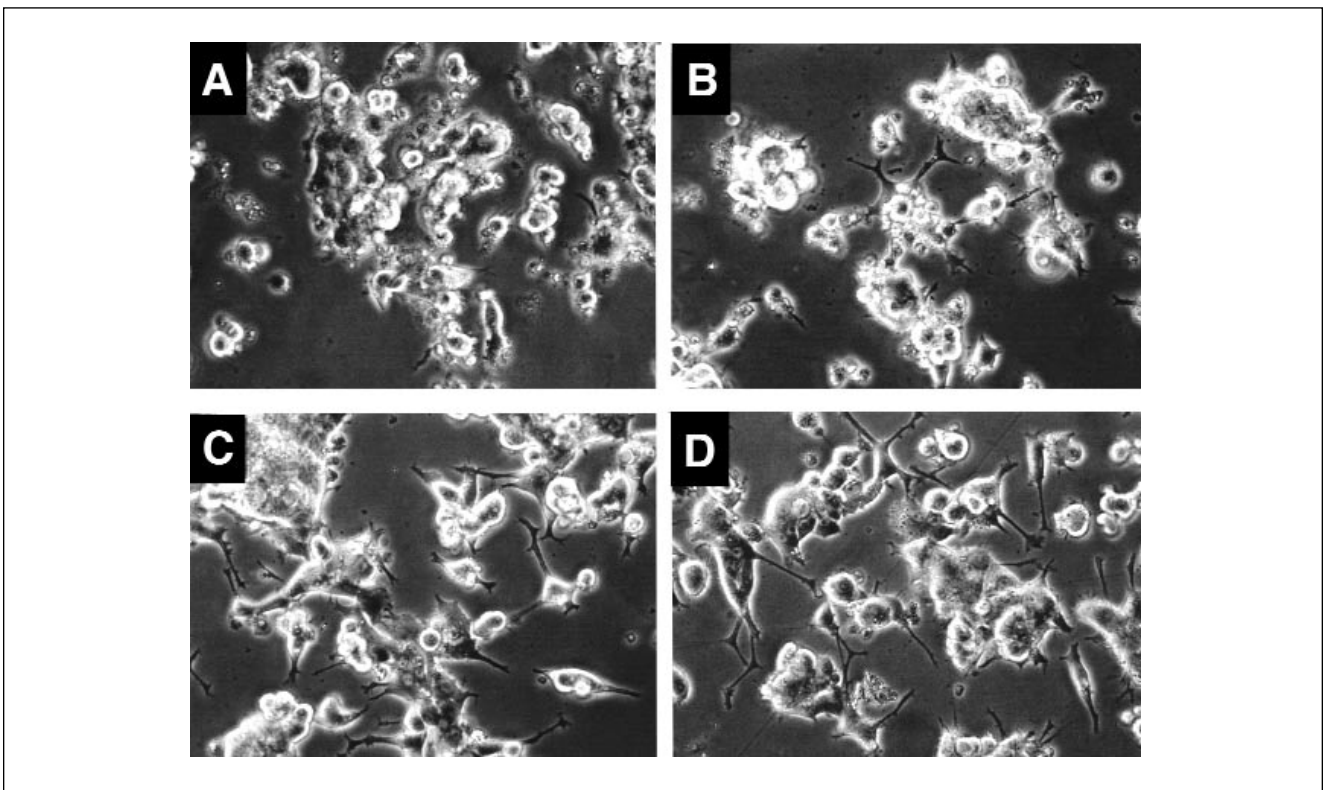


Figure 3. Neurite outgrowth in PC12 cells treated with PolicosanolPlus and Neuroprevin. PC12 cells were either untreated (A), or treated with NGF (B), or NGF and PolicosanolPlus (C), or PolicosanolPlus and Neuroprevin (D), and incubated for nine hours prior to being photographed at 400 X Magnification.

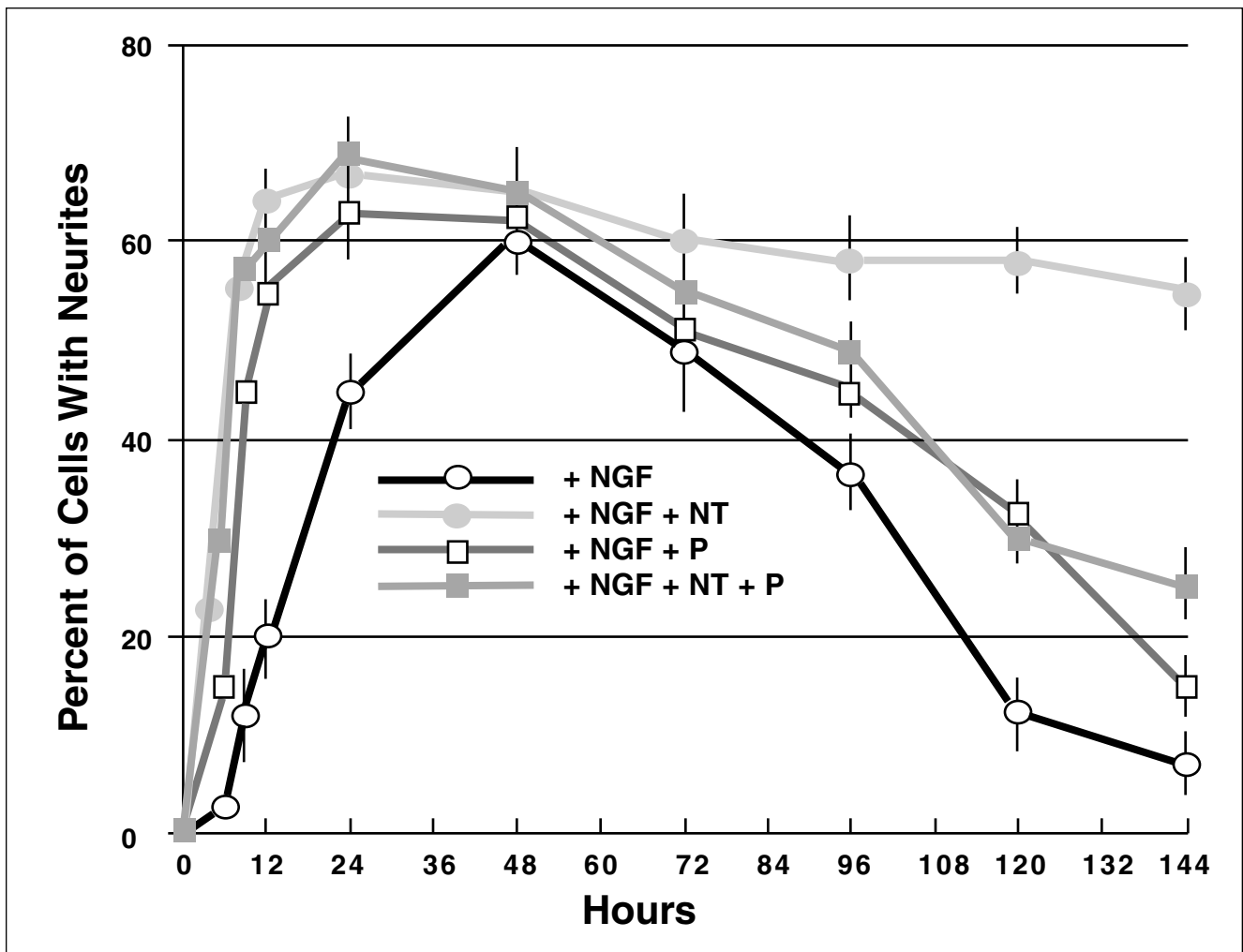


Figure 4. Percent of PC12 cells retaining neurites with PolicosanolPlus® and Neuroprevin™ treatment. Cells were treated with NGF (open circles), NGF + Neuroprevin (NT) (closed circles), NGF + PolicosanolPlus (P) (open squares), and NGF + Neuroprevin + PolicosanolPlus (closed squares) and assessed for neurites and various times over a 6 day period (144 hours).

used separately or combined, showed significant neurite regenerating potential. Moreover, PolicosanolPlus and Neuroprevin both showed the ability to reduce age related neurodegenerative events. It is interesting to note that the combination of PolicosanolPlus and Neuroprevin showed the greatest stimulus for neurite formation, while Neuroprevin did better alone compared to combination with PolicosanolPlus with regard to the maintenance of neurites, although PolicosanolPlus did enhance neurite maintenance. These activities are unique and suggest that PolicosanolPlus and Neuroprevin may be powerful daily dietary supplements that will reduce the risk of neuronal injury and delay the onset of neurodegenerative diseases.

CONCLUSION

Neuronal damage and neurodegenerative diseases are debilitating to the individual. While stem-cell therapy pro-

vides a promise for repair of some forms of neuronal injury, these solutions remain obscured and do not address the complications of the central nervous system networks. PolicosanolPlus and Neuroprevin, separately and combined, show the ability to enhance nerve formation and then promote the maintenance of these nerves. In this regard, PolicosanolPlus and Neuroprevin are indicated dietary supplements that can help not only those with existing injury, but can serve as a prophylactic against the consequences of unexpected injury, stroke, or the onset of neurodegenerative diseases when these supplements are used as part of a daily dietary routine.

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