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# A novel vitamin C preparation enhances neurite formation and fibroblast adhesion and reduces xenobiotic-induced T-cell hyperactivation

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**Background:**

Vitamin C (ascorbic acid, ascorbate) has been shown to enhance neurite outgrowth, promote fibroblast adhesion during wound healing, and reduce xenobiotic-induced leukocyte hyperactivity and inflammatory damage. In this study, a comparison was made between Ester-C® and PureWay-C™ on these various cellular activities.

**Material/Methods:**

PC12 cells were stimulated to form neurites with nerve growth factor, NIH 3T3 fibroblasts were seeded on fibronectin and H9 T-cells were stimulated to aggregate with the pyrethroid pesticide bifenthrin. The rate of neurite formation, fibroblast adhesion and T-cell homotypic aggregation was then measured in the absence and presence of various formulations of vitamin C including Ester-C® and PureWay-C™.

**Results:**

With PureWay-C™ treatment, 12% of PC12 cells extended neurites within one hour of treatment and 45% of the cells extended neurites by hour nine. With Ester-C®, 0% and 15% extended neurites at one and nine hours, respectively. NIH-3T3 fibroblast adhesion to fibronectin was enhanced by 4.7-fold with a 30 minute PureWay-C™ treatment while Ester-C® increased fibroblast adhesion by only 1.5 fold. Further, PureWay-C™ reduced pesticide-mediated T-cell homotypic aggregation by 83% within 30 minutes of treatment while the reduction seen with Ester-C® was only 33%.

**Conclusions:**

These data confirm the previous observations that vitamin C supplementation can promote neurite outgrowth, increase fibroblast adhesion and reduce xenobiotic induce immunocytes aggregation. More importantly, these data show that PureWay-C™ has a faster and greater beneficial effect on these parameters when compared to other vitamin C formulations.

**key words:**

**vitamin C • neurite outgrowth • fibroblast adhesion • homotypic aggregation • bifenthrin**

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## BACKGROUND

Vitamin C has been implicated as an important dietary component for the healthy physiological and metabolic activities which include; the development of healthy neurites [1]; prevention of neurodegenerative diseases [2,3]; wound healing *in vitro* [4], and *in vivo* [5]; and reduction of xenobiotic-induced leukocyte inflammatory hyperactivation [6,7]. The bioavailability of vitamin C in the diet has therefore been the focus of intense interest and research. When compared to other vitamin C formulations, Ester-C®, a calcium ascorbate preparation with small amounts of dehydroascorbate, calcium threonate, xylonate and lyxonate, has been shown to lead to the increased cellular uptake of vitamin C [8,9], to provide increased protection from vitamin C deficiency in rats [10], and to improve uptake and circulating levels of vitamin C in humans [11]. While these studies have shown that Ester-C® improves body weight and a variety of health parameters in rats [10], and increases white blood cells counts in humans [11], there are no published studies which indicate that Ester-C® has a more rapid or immediate beneficial effect on cell behavior or physiology in support of the conclusion that this form of vitamin C is responsible for the observed benefits.

Here, we present a new vitamin C formulation, PureWay-C™, which we compare with Ester-C® and other vitamin C formulations for rapid and immediate effects on cellular behaviors associated with beneficial physiology. For example, the PC12 neuronal cell line extends neurites when treated with nerve growth factor (NGF) [12], and vitamin C has been shown to enhance NGF-mediated neurite outgrowth in these cells [1]. In addition, NIH 3T3 fibroblast interaction with extracellular matrix proteins such as fibronectin, and subsequent migration to close wounds in cultured monolayers have been used to measure wound healing events [13–15]. Nutrient formulations containing vitamin C have been shown to enhance fibroblast adhesion to and interaction with the extracellular matrix C [16,17], in the context of wound healing models [16,17]. Further, leukocyte cell-cell adhesion is associated with xenobiotic induced hyperactivation and inflammatory damage [6,7,18], and vitamin C has been shown to prevent cigarette smoke-induced leukocyte aggregation and attachment to vascular endothelium [6,7]. Therefore, here, we examine the effect of PureWay-C™ on these cell behaviors known to be benefited by vitamin C supplementation and compared the efficiency of this formulation with Ester-C® and other vitamin C formulations. Further, the ability of PureWay-C™ to have a more rapid effect and display a more immediate benefit to the systems is used as an indication of a more rapid cellular internalization and enhanced bioavailability.

## MATERIAL AND METHODS

### Chemicals

Murine 7S nerve growth factor (NGF) and human serum fibronectin were purchased from Sigma Chemical Co., St. Louis, Mo. and dissolve in water. The NGF (100 µg/ml) was stored in aliquots at –80°C. The fibronectin (1 mg/ml) was stored at –20°C. Dimethylsulfoxide was purchased from Sigma Chemical Co., St. Louis, Mo. and bifenthrin was purchased from Chem. Services, West Chester, PA. Bifenthrin

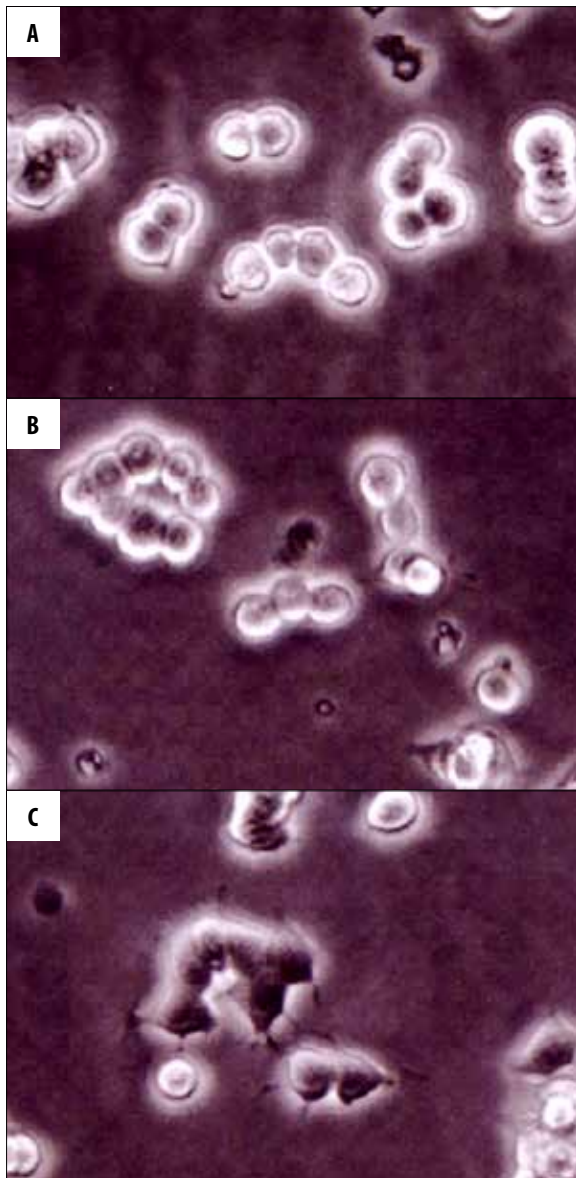
was brought to a stock concentration of 10<sup>–2</sup> M in DMSO. Phytohemagglutinin was purchased from Sigma Chemical Co. St. Louis, MO and suspended in RPMI 1640 and stored at –20°C. Formulations and certificates of analysis of ascorbic acid, calcium ascorbate, Ester-C® and PureWay-C™ were provided by Nature's Value, Coram, NY, from their respective suppliers, and were dissolved in sterile distilled water to a 50 mM ascorbic acid component concentration and passed through a 0.45 µm pore filter for sterilization. Stocks of 5.0 mM and 0.5 mM were also prepared by ten fold dilutions in sterile distilled water. The vitamin C formulations were dissolved at the same time and made fresh for each experiment. Prior to being dissolved, the ascorbic acid content of each formulation used in this study was determined as reducing equivalents by iodometric titration, and also by HPLC method. Analysis of the stability of vitamin C solutions showed retention of 90% vitamin C content for 24 hours when they were protected from light. Storage in a cool, dark place resulted in retention of activity for 7 days.

### Cells and culture condition

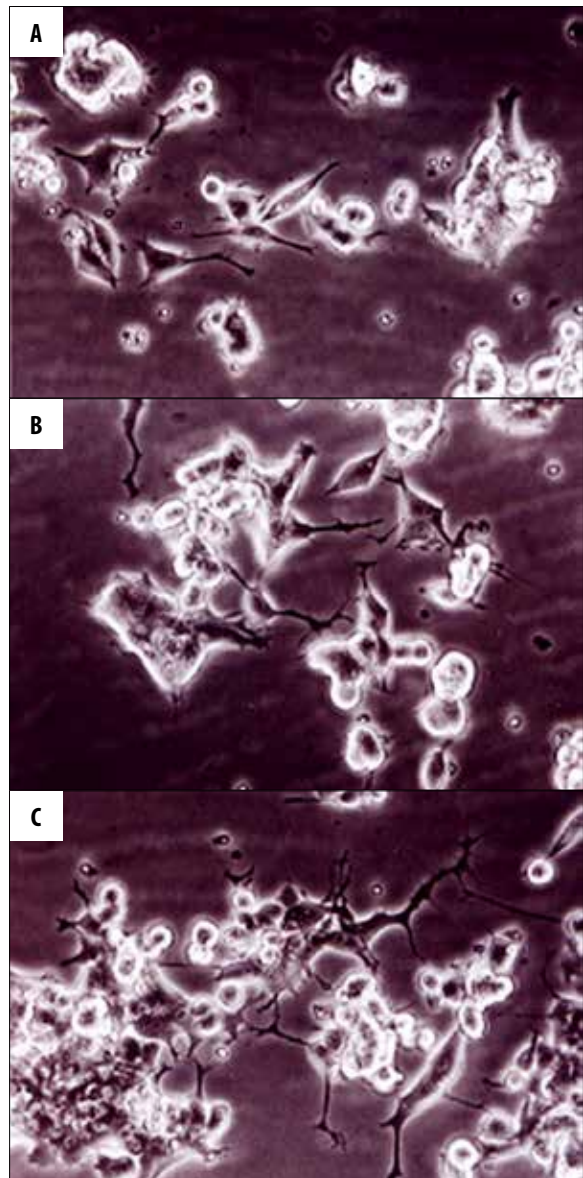
Rat PC12 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) containing 7.5% fetal bovine serum (FBS), 7.5% horse inactivated serum and 0.01% gentamycin and incubated in a CO<sub>2</sub> incubator at 37°C. For use in experiments, cells were collected by agitation when at approximately 70–80% confluence. NIH 3T3 Fibroblasts were culture in DMEM containing 10% FBS and 0.01% gentamycin and incubated in a CO<sub>2</sub> incubator at 37°C. For use in experiments the cells were rinsed with sterile serum-free DMEM and the incubated at room temperature for five minutes with trypsin, collected and immediately mixed with serum-containing growth medium to neutralize the trypsin, centrifuged at 1000 rpm and resuspended in growth medium. Human CD4+ H9 T-lymphocyte cell line was obtained from the NIH AIDS reagent program and the cells were cultured in RPMI 1640 containing 10% FBS and 0.01% gentamycin and incubated in a CO<sub>2</sub> incubator at 37°C. These cells grow in suspension and were simply collected by pipette. A hemocytometer was used to determine cell number for plating during experiments.

### Neurite outgrowth assays

PC12 cells were collected by agitation as described above and centrifuged at 1000 rpm. The old medium was discarded and the cells were resuspended in fresh medium at 60,000 cells/ml and treated with 100 ng/ml of nerve growth factor (NGF). In order to observe the effect of the vitamin C formulations over time, to each well of a 6 well tissue cluster plate, 3.0 ml of this cell suspension was added to triplicate wells which then were immediately treated with 3 µl of 50 mM ascorbic acid of the various vitamin C formulations as indicated. This resulted in a 50 µM test ascorbic acid concentration in the test wells and the plates were incubated for various times in a CO<sub>2</sub> incubator at 37°C. The percent of cells with neurites was then counted by visual inspection at 400× magnification and representative photographs were taken at 600× magnification at hours one and six. Cells were considered to be positive for neurite outgrowth if any evidence of process formation was observed on the cell. Cells that showed squaring were not counted as positive. A total



**Figure 1.** Morphology of PC12 cells one hour after treatment with nerve growth factor (NGF). Cells were treated with 100 ng/ml of NGF and either received no further treatment (A) or were treated with 50  $\mu$ M Ester-C® (B) or PureWay-C™ (C). The cells were then incubated for one hour and photographed at 600 $\times$  magnification.

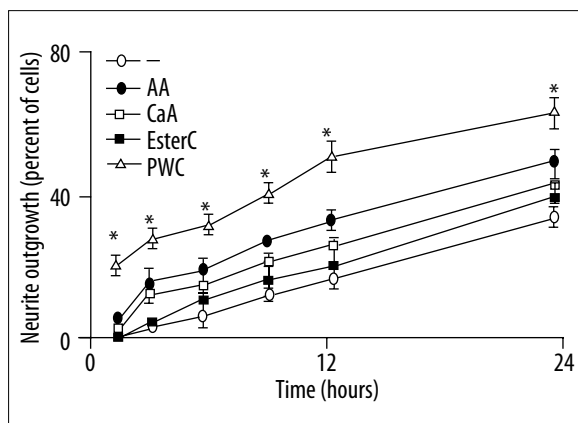


**Figure 2.** Morphology of PC12 cells six hours after treatment with nerve growth factor (NGF). Cells were treated with 100 ng/ml of NGF and either received no further treatment (A) or were treated with 50  $\mu$ M Ester-C® (B) or PureWay-C™ (C). The cells were then incubated for six hours and photographed at 600 $\times$  magnification.

of 100 cells was counted in each well, for a total of 300 cells per treatment. The experiment was conducted twice to give an n for each treatment of six and a total of 600 cells counted and the percent of positive cells was determined. In order to determine the dose effect of the vitamin C formulations, stocks of each formulation were prepared at 50 mM, 5 mM and 0.5 mM such that, subsequent to the seeding of 3.0 ml of 60,000 cells/ml in triplicate wells of the six-well tissue cluster, 3 ml of each stock could be added to achieve the test concentrations of 50  $\mu$ M, 5  $\mu$ M and 0.5  $\mu$ M ascorbic acid respectively using the same dilution factor and volumes of vehicle (water). For these experiments, the percent of cells expressing signs of neurites was determined at hours three and six at 400 $\times$  magnification.

#### Fibroblast adhesion to fibronectin

The wells of a six-well tissue cluster plate were coated with fibronectin by first adding 1.5 ml of sterile water to each well and then adding 30  $\mu$ l of 1 mg/ml (30  $\mu$ g) fibronectin and the plates were then incubated at 37.5°C for one hour. The water was then removed from each well and 1.5 ml of medium was added to each well. Prior to cell seeding triplicate well received 3  $\mu$ l of the various 50 mM ascorbic acid formulations such that when the cells were added in a volume of 1.5 ml, the final test ascorbic acids concentrations were 50  $\mu$ M. The cells were suspended after collection at 4 $\times$ 10<sup>5</sup> cells/ml such that each well received a total of 6 $\times$ 10<sup>5</sup> cells. The plates were then incubated for 15 minutes in a in

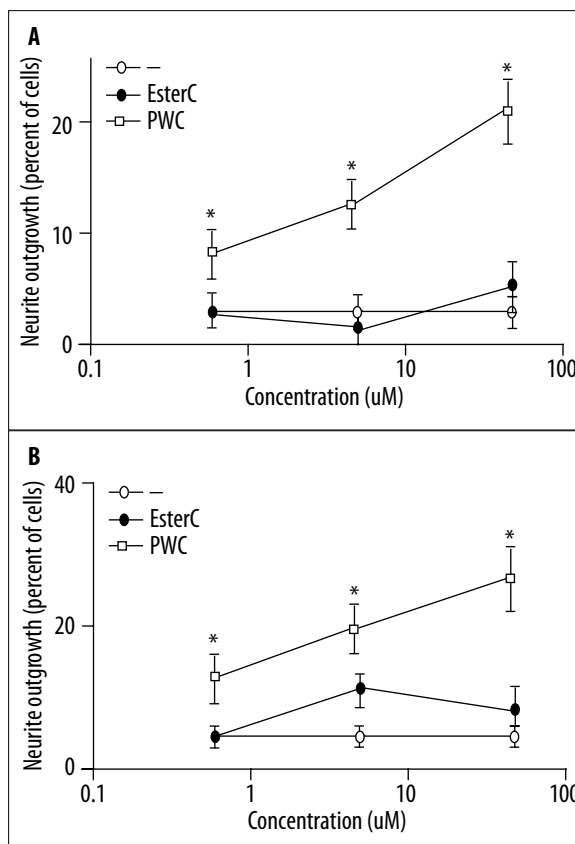


**Figure 3.** Vitamin C formulations enhance NGF-mediated neurite outgrowth. PC12 cells were treated with 100 ng/ml of NGF and incubated for a 24 hour period and then these cells either received not further treatment (—/open circles), or were treated with 0.5  $\mu$ M ascorbic acid (AA/closed circles), calcium ascorbate (CaA/open squares), Ester-C® (EsterC/closed squares) or PureWay-C™ (PWC/open triangle). After these treatments the cells were incubated over a 24 hour period during which the formation of neurites was assessed at hours 1, 3, 6, 9, 12 and 24 as described in the Material and Methods section. Error bars represent the standard error of the mean with an n of 6. An analysis of variance was performed and the asterisks indicate a significant difference at 95% confidence (Scheffe F test) between Ester-C® and PureWay-C™.

a CO<sub>2</sub> incubator at 37°C. Next the unattached cells were removed by aspiration and the attached cells were fixed and stained and counted at 400 $\times$  magnification. Three fields were counted in each well and this number was averages and multiplied by the number of times the field area could be divided into the entire well. This number is an extrapolation of the number of attached cells in each well and since  $6 \times 10^5$  cells were added to each well, the percent of cells which attached was determined. Triplicate wells were prepared in two separate experiments for an n of six and 18 fields counted per treatment. Representative fields were photographed at 600 $\times$  magnification.

#### T-cell homotypic aggregation assays

H9 cells were collected and 3.0 ml of  $2 \times 10^5$  cells/ml ( $6 \times 10^5$  cells/well) were seeded in wells of a six-well tissue cluster and then treated with 10  $\mu$ g/ml of phytohemagglutinin and or  $10^{-5}$  M bifenthrin. The stock bifenthrin was  $10^{-2}$  M in DMSO so 3 ml of the stock was added to achieve  $10^{-5}$  M bifenthrin and a vehicle concentration of 0.1%. Therefore the control untreated cells for this experiment contained 1% DMSO. The PHA was diluted in RPMI 1640 and so did not require a vehicle control. Immediately after seeding the cells, triplicate wells were treated with the 50  $\mu$ M ascorbic acid from the various formulations and the cells were incubated in a CO<sub>2</sub> incubator at 37°C for 30 minutes. After treatment for 30 minutes the ability of vitamin C to inhibit homotypic aggregation was measured by counting aggregate size at 400 $\times$  magnification. The center field of each well was assessed by visual inspection and the number of cells in each aggregate was counted and divided by the



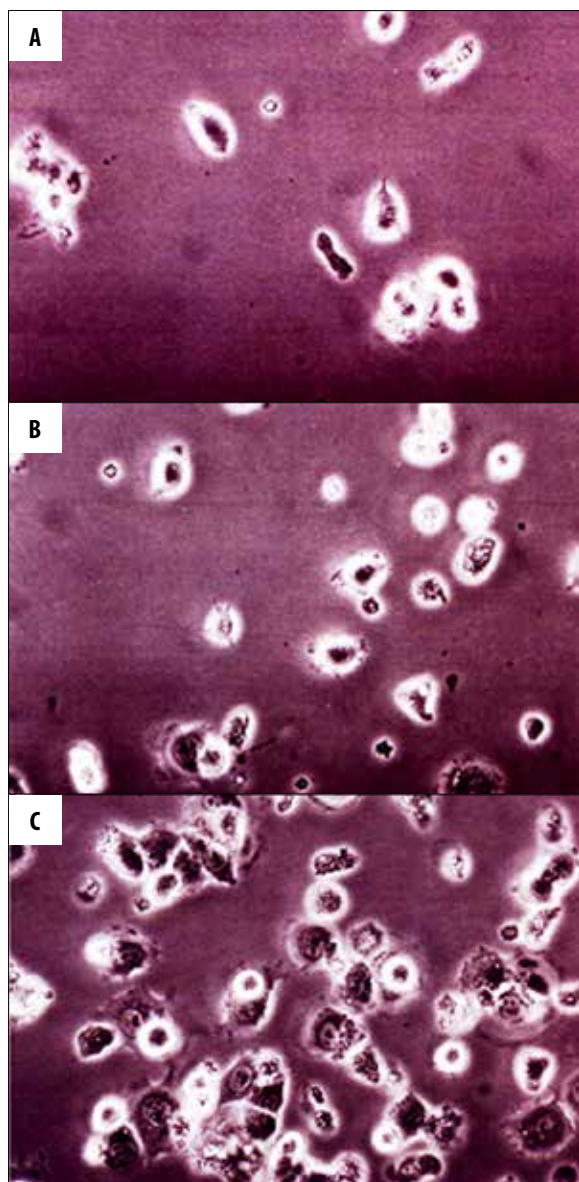
**Figure 4.** PureWay-C™ enhances NGF-mediate neurite outgrowth in a dose dependant manner. PC12 cells were treated with 100 ng/ml of NGF and subsequently untreated (—/open circles) or treated with the indicated concentrations of Ester-C® (EsterC/closed circles) or PureWay-C™ (PWC/open square). The cells not treated with a vitamin C formulation did not receive different doses of a treatment and so the line represents the same value and is presented as the zero treatment reference. Neurite outgrowth was then assessed at three (A) and six (B) hours as described in the Material and Methods section. Error bars represent the standard error of the mean with an n of 6. Asterisks indicate a significant difference at 95% confidence (Scheffe F test) between Ester-C® and PureWay-C™.

number of aggregates to derive the number of cells per aggregate. Since the treatments were done in triplicate, three fields were counted for each treatment. While the number of aggregates was noted, these data are not presented. The number of cells per aggregate is. The treatment with few cells per aggregate also had very few aggregates to count. The aggregate size was counted and representative photographs were taken at 400 $\times$  magnification.

#### RESULTS

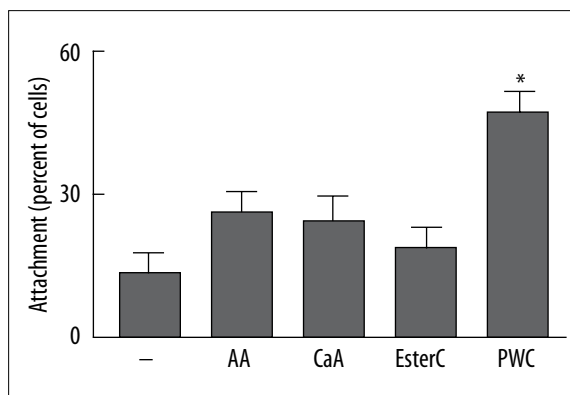
PC12 cells respond to nerve growth factor (NGF) treatment by extending neurites [reviewed in 12], and significant neurite outgrowth within three hours of NGF treatment of these cells has not been reported. Here we found a significant and observable neurite formation in 12% of the cells one hour after NGF treatment when the cells were concomitantly treated with PureWay-C™ (PWC), but not when





**Figure 5.** NIH 3T3 fibroblast adhesion to fibronectin. NIH 3T3 fibroblasts were seeded into fibronectin coated wells of a tissue cluster plate in the absence of a vitamin C formulation (A) or in the presence of 50 µM Ester-C® (B) or PureWay-C™ (C). The cells were incubated for 15 minutes after which the unattached cells were removed and the attached cells were observed as described in the Material and Methods section. Cells were photographed at 600× magnification.

they were treated with Ester-C® with an observed percent of cells with neurites at 0% (Figure 1). The ability of PWC to more rapidly stimulate neurite outgrowth compared to Ester-C® was also evident by cell morphology at six hours post NGF treatment with 31% of cell extending neurites, compared to only 8% with Ester-C® treatment (Figure 2). PureWay-C™ also enhance NGF-induced neurite outgrowth to a greater extent than ascorbic acid and calcium ascorbate (Figure 3). While all vitamin C formulations did enhance NGF stimulated neurite outgrowth, at all time points measured, including 24 hours post NGF treatment, PWC treated cells had a significantly greater number of the popula-



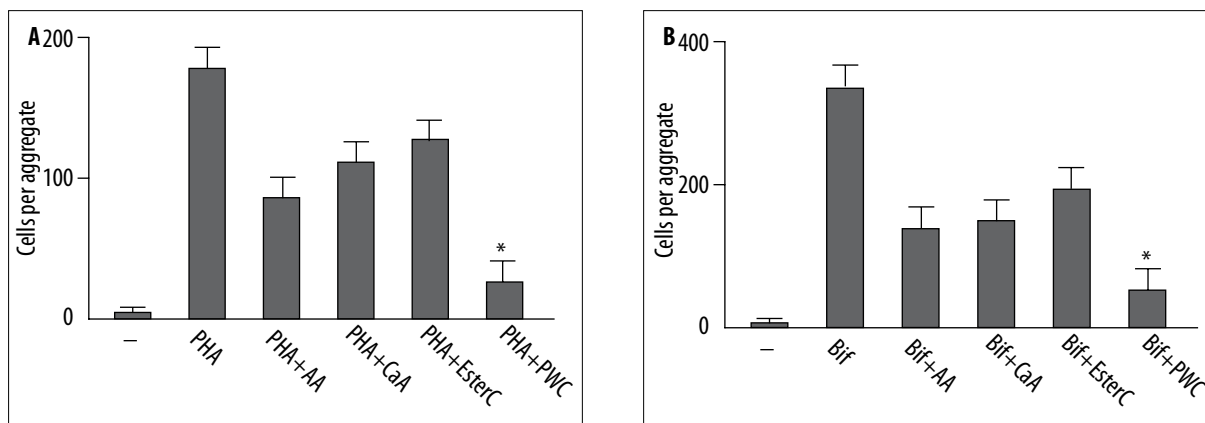
**Figure 6.** Vitamin C enhances fibroblast adhesion to a fibronectin substrate. NIH 3T3 fibroblasts were seeded in the fibronectin coated wells of a tissue cluster plate in the absence of a vitamin C formulation (–) or in the presence of 50 µM ascorbic acid (AA), calcium ascorbate (CaA), Ester-C® (EsterC) or PureWay-C™ (PWC). The cells were then incubated for 15 minutes and the percent of attached cells was determined as described in the Material and Methods section. The error bars represent the standard error of the mean with an n of 18. The data for Ester-C® and PureWay-C™ were compared by a Student's-t test and the asterisk indicates a significant difference between Ester-C® and PureWay-C™ at 95% confidence.

tion extending neurites with an 8-fold increase compared to Ester-C® at 3 hours and over a 2-fold increase compared to Ester-C® at 12 hours (Figure 3).

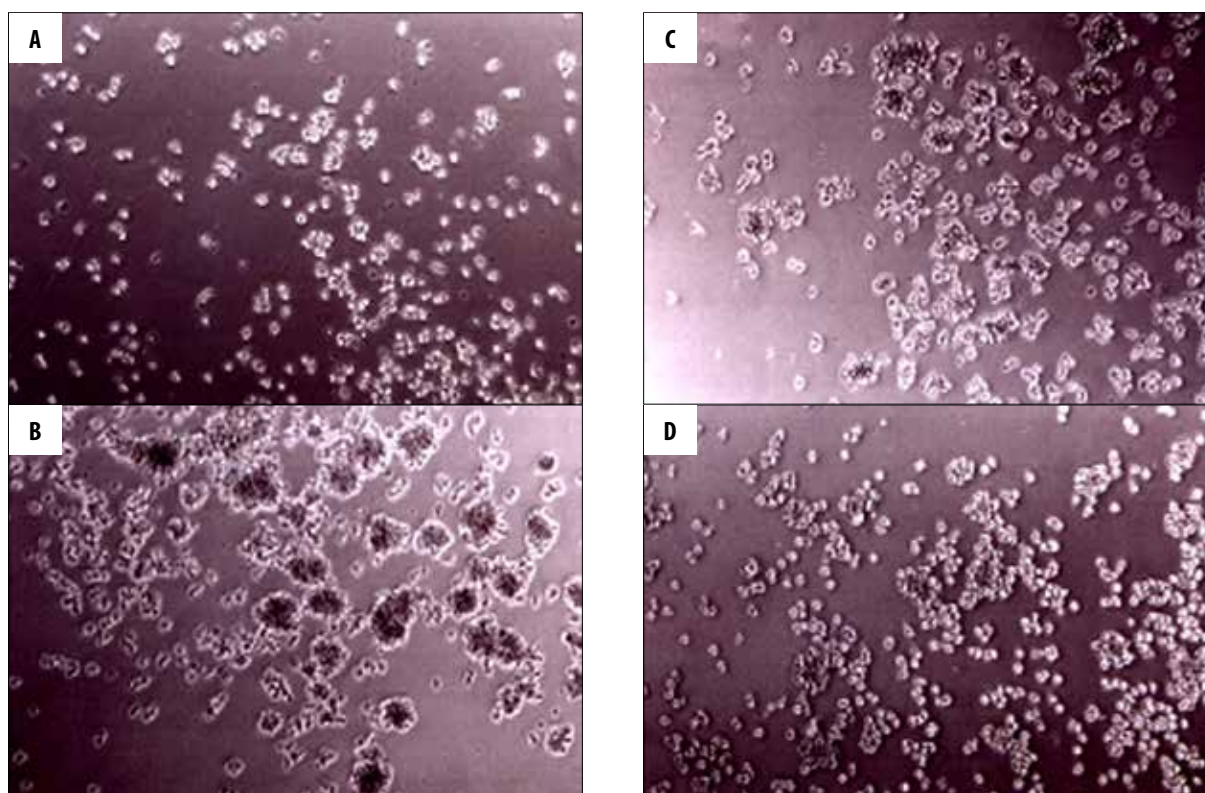
PureWay-C™ enhanced NGF-mediated neurite outgrowth was dose dependant with 8% of the cells extending neurites three hours after NGF and a 0.5 µM PWC treatment (Figure 4A). This 8% increased to 22% three hours post treatment when PWC was added at 0.5 µM (Figure 3A). Ester-C® did not increase NGF-mediated neurite outgrowth at all doses tested three hours post NGF treatment (Figure 4A). However, at six hours post-treatment, 50 µM Ester-C® did stimulate over a two-fold increase in the percent of cells with neurites (Figure 4B) but this compares to an 6-fold increase with PWC. Indeed PWC enhanced NGF-mediated neurite outgrowth by three-fold six hours after treatment with 0.5 µM, a concentration at which Ester-C® failed to show any effect (Figure 4B).

PureWay-C™ enhanced fibroblast adhesion to fibronectin by over three-fold compared to Ester-C® (Figure 5,6). In addition to adhesion, fibroblast spreading on fibronectin is an important next step to migration and wound healing behavior. Figure 5 shows not only improved adhesion with PWC, but also a significantly greater spreading as judged by cell morphology. Ascorbic acid and calcium ascorbate also increased fibroblast adhesion to fibronectin by nearly two-fold compared to untreated cells, but this again was only nearly half of the enhanced adhesion to fibronectin when the fibroblasts were treated with PWC (Figure 6).

T-cell homotypic aggregation can be activated by phytohemagglutinin (PHA) within 30 minutes of treatment (Figure 7A). All of the various vitamin C formulations are able to reduce PHA-induced aggregation, with ascorbic acid



**Figure 7.** Vitamin C formulations reduce phytohemagglutinin (PHA) and bifenthrin (Bif) induced T-cell aggregation. Human H9 CD4+ T-cells were either untreated (–) or treated with phytohemagglutinin (A) or bifenthrin (B) and then either given no further treatments (PHA) (A) or (Bif) (B) or treated further with 50  $\mu$ M ascorbic acid (AA), calcium ascorbate (CaA), Ester-C® (EsterC) or PureWay-C™ (PWC). The cells were then incubated for 30 minutes and the number of cells per aggregate was determined as described in the Material and Methods section. The error bars represent the standard error of the mean with an n of 6. The data for Ester-C® and PureWay-C™ were compared by a Student's-t test and the asterisk indicates a significant difference between Ester-C® and PureWay-C™ at 95% confidence for both PHA (A) and bifenthrin (B).



**Figure 8.** PureWay-C™ reduces bifenthrin induced T-cell aggregation. Human H9 CD4+ T-cells were either untreated (A) or treated with bifenthrin (B). Bifenthrin treated cells were also exposed to 50  $\mu$ M Ester-C® (C) or PureWay-C™ (D). The cells were incubated for 30 minutes and assessed for aggregate size and photographed at 400 $\times$  magnification.

and PWC showing the greatest reductions of 2.3-fold and 8.5-fold respectively (Figure 7A). Ester-C® showed the least effect on PHA-induced aggregation with only a 13% reduction at 30 minutes (Figure 7A).

The pesticide, bifenthrin, also stimulates T-cell homotypic aggregation within 30 minutes of treatment (Figure 7B, 8).

Untreated T-cells show little aggregation and when aggregates do form, they contain 10 or fewer cells (Figure 7B). Bifenthrin increases the number of aggregates and moreover increases the aggregate size by 30-fold (Figure 7B). Ester-C® does decrease the number of cells in the bifenthrin induced aggregates on average by 34% (Figure 7B, 8). PureWay-C™ decreases the size of the bifenthrin induced aggregates by

6-fold (600%) (Figure 7B, 8). Indeed, all of the vitamin C formulations significantly inhibited the size of the bifenthrin induced aggregates within 30 minutes (Figure 7B, 8), with PWC having at least double the effect of the next best vitamin C formulation (Figure 7B, 8).

## DISCUSSION

Nerve growth factor stimulates significant neurite formation in neuronal cells between hours 3 and 6 post-treatment [19]. Vitamin C treatment of nerve growth factor-treated neuronal cells has been shown to enhance neurite formation within a 24 hour treatment period [1]. Here, we confirm that vitamin C enhances nerve growth factor-mediated neurite outgrowth and further demonstrate that the vitamin C formulation, PureWay-C™, is able to enhance neurite outgrowth within one hour of treatment of neuronal cells and maintain a measurable difference compared to Ester-C®, ascorbic acid and calcium ascorbate over a 24 hour period. Nerve growth factor-mediated neurite outgrowth involves the activation of the MAPK cascade [20–23]. While the role of vitamin C has been primarily ascribed to that of antioxidant, coenzyme and inducer of the collagen pathway [reviewed in 24], Vitamin C can also activate the MAPK cascade [1,25] and in this way synergize with the signaling activity of nerve growth factor. In this regard the rapid effect of PureWay-C™ suggests that it is more efficiently capable of effecting the intracellular activity of treated neurons when compared to Ester-C® and the other vitamin C formulations tested here.

Neurite outgrowth is inhibited by xenobiotics such as pyrethroid pesticides [19,26] and nutritional supplements can protect neurons from pyrethroid toxicity [27]. Pyrethroids can also stimulate the hyperactivation of the immune system [18] and here we show that supplementation with PureWay-C™ can ameliorate pyrethroid-activated CD4+ T-cell homotypic aggregation *in vitro*. Homotypic aggregation involves the leukocyte integrin-mediated adhesion mechanisms associated with inflammation [reviewed in 28]. Xenobiotic insults, such as those associated with pesticides cigarette smoke, diesel exhaust, aryl hydrocarbons, sulfites and heavy metals have been shown to be proinflammatory through the activation of leukocyte aggregation and adhesion to vascular endothelium. [6,7,18,29–32] Further, Vitamin C has been shown to reduce cigarette smoke associated leukocyte cell-to-cell adhesion and the associated inflammatory damage [6,7]. Our data confirm the observation that vitamin C can reduce xenobiotic induced leukocyte adhesion mechanisms and that the PureWay-C™ formulation of vitamin C demonstrates the greatest and fastest amelioration of pyrethroid pesticide induced aggregation.

Fibroblast adhesion to the extracellular matrix component, fibronectin, and subsequent spreading and migration are important events in the wound healing process [33] and vitamin C has been shown to promote fibroblast migration and wound healing [4,5,34–36]. Our data confirm that vitamin C may have benefits to wound healing by promoting fibroblast adhesion and spreading on a fibronectin substrate. Indeed, PureWay-C™ promoted enhanced fibroblast adhesion to and spreading on fibronectin to a greater extent than any of the other vitamin C formulations tested.

## CONCLUSIONS

Vitamin C has been implicated in protecting the nervous system from neurodegenerative diseases, the promotion of wound healing and protection from xenobiotic-induced inflammatory damage. Here we present a new vitamin C formulation, PureWay-C™ which is more active and effective *in vitro* than ascorbic acid, calcium ascorbate and Ester-C® with regard to stimulation of neurite outgrowth, fibroblast wound healing activities and the protection of the immune system from xenobiotic induced inflammatory mechanisms. The ability of PureWay-C™ to quickly affect cultured cell behavior is an indication of its rapid cellular uptake and improved activity.

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