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Effects of blue-green algae extracts on the proliferation of human adult stem cells *in vitro*: A preliminary study

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

R. Douglas Shytle[1,2 ACDEF], Jun Tan[1,2 ACDEF], Jared Ehrhart[1,2 BCEF], Adam J. Smith[1,2 BCEF], Cyndy D. Sanberg[3 AE], Paul R. Sanberg[1,2 AE], Jerry Anderson[4 AE], Paula C. Bickford[1,5 ADEF]

[1] Center of Excellence for Aging and Brain Repair, Department of Neurosurgery, USF, Tampa, FL, U.S.A.
[2] Department of Psychiatry, USF, Tampa, FL, U.S.A.
[3] Natura Therapeutics, Inc. Tampa, FL, U.S.A.
[4] Simplexity Health, Klamath Falls, OR, U.S.A.
[5] James A. Haley Veterans' Hospital, Tampa, FL, U.S.A.

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Summary

Background: Adult stem cells are known to have a reduced restorative capacity as we age and are more vulnerable to oxidative stress resulting in a reduced ability of the body to heal itself. We have previously reported that a proprietary nutraceutical formulation, NT-020, promotes proliferation of human hematopoietic stem cells *in vitro* and protects stem cells from oxidative stress when given chronically to mice *in vivo*. Because previous reports suggest that the blue green algae, *Aphanizomenon flos-aquae* (AFA) can modulate immune function in animals, we sought to investigate the effects of AFA on human stem cells in cultures.

Material/Methods: Two AFA products were used for extraction: AFA whole (AFA-W) and AFA cellular concentrate (AFA-C). Water and ethanol extractions were performed to isolate active compounds for cell culture experiments.

For cell proliferation analysis, human bone marrow cells or human CD34+ cells were cultured in 96 well plates and treated for 72 hours with various extracts. An MTT assay was used to estimate cell proliferation.

Results: We report here that the addition of an ethanol extract of AFA-cellular concentrate further enhances the stem cell proliferative action of NT-020 when incubated with human adult bone marrow cells or human CD34+ hematopoietic progenitors in culture. Algae extracts alone had only moderate activity in these stem cell proliferation assays.

Conclusions: This preliminary study suggests that NT-020 plus the ethanol extract of AFA cellular concentrate may act to promote proliferation of human stem cell populations.

key words: **CD34+ • blue-green algae • stem cells • aging**

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Author's address: Paula C. Bickford, Center Excellence in Aging and Brain Repair, MDC78, Department of Neurosurgery and Brain Repair, University of South Florida College of Medicine, Tampa, FL, U.S.A., e-mail: pbickfor@health.usf.edu

BACKGROUND

Stem cells are found in many organs of the adult human including bone marrow, peripheral blood, umbilical cord blood, spleen, tooth pulp, adipose tissue and brain. These progenitor cells are being investigated for their potential use as transplanted tissues in the treatment of diseases such as cancer, neurodegenerative disease, diabetes, stroke, myocardial infarction, amyotrophic lateral sclerosis (ALS) and Parkinson's disease [1–7]. Little effort however is being directed toward enhancing the endogenous stem cells in the adult as an avenue to promote healing.

During aging, adult stem cells are known to have a reduced regenerative capacity [8,9] and are more susceptible to oxidative stress [10] resulting in a reduced ability of the body to heal itself. For example, neural stem cells, muscle satellite cells, and endothelial progenitors all show reduced proliferation in the aged and may play a role in pathology of age-associated diseases [11–14]. In cardiovascular disease, for example, there is a correlation between a reduction in peripheral blood endothelial progenitor cells and many risk factors for cardiovascular disease [15,16]. Neural stem cells also decrease in proliferative capacity with aging and some have postulated that declines in neurogenesis with aging are related to cognitive decline [17–19]. However, a growing body of literature now indicates that certain nutrients, vitamins, and flavonoids could have important roles in the proliferation and maintaining a of continuous replacement of stem cells required for healthy self-renewal of mature cells in the blood, brain, and other tissues.

It appears that certain nutrients, vitamins, and flavonoids could have important roles in maintaining the self-renewal of stem cells and stimulating the proliferation and differentiation of committed progenitors required for the continuous replacement of mature cells in the blood, brain, and other tissues. Furthermore, it may be possible to use certain natural products, either alone or synergistically, for the treatment of conditions where the stem cell replacement appears warranted. We recently investigated the ability of various natural compounds to stimulate the proliferation of human stem cells derived from bone marrow (CD34+) and progenitor cells from peripheral blood (CD133+) *in vitro* [20]. Specifically, we showed that a particular combination of blueberry extract, green tea extract, carnosine, and vitamin D3, a proprietary nutraceutical formulation known as NT-020, demonstrated synergistic activity in promoting proliferation of human hematopoietic stem cells in culture [21]. Moreover, in a follow-up study, we found that NT-020 reduced oxidative stress-induced apoptosis of murine neurons and microglial cells *in vitro* [21]. Cultured bone marrow stem cells from mice given NT-020 orally for 2 weeks exhibited a dose-related reduction of oxidative stress-induced cell death. These preclinical studies demonstrate that nutraceutical formulations, like NT-020, may act to promote healing via an interaction with stem cell populations [21].

Studies with NT-020 have shown that they promote migration of brain stem cells from the stem cell niche to the site of injury. In an animal model of stroke, NT-020 was shown in a controlled study to prevent damage from the ischemic insult by 70% when compared to control conditions. In these same animals it was shown that there was an increase in neural stem cell numbers in both the stem cell niche of the subventricular zone and at the site of injury in the striatum. This data demonstrated that NT-020 promotes the proliferation of stem cells in the niche and migration of these cells to damaged tissues [22].

An additional class of compounds has also been suggested to have activity in promoting stem cell function. Classified as cyanobacteria, blue-green algae (Aphanizomenon flos-aquae (AFA)) are abundant in phycocyanin, which gives it a blue pigmentation. The large amount of chlorophyll accounts for the vivid green color. Other additional carotenoids present contribute to the rich pigmentation of blue green algae. Studies have shown that these spirulina a related blue green algae promote adult neurogenesis following an inflammatory insult and in aging [23,24]. The blue green algae have also been shown to have effects on the function of immune cells including increases in the mononuclear cell fraction [18], which is the fraction that contains adult stem cells. In more extensive studies, a polysaccharide fraction referred to as "Immolina" isolated by ethanol extraction from the blue-green algae spirulina was found to potently and dose-dependently increase cytokines and the expression of genes encoding the members of a family of chemotactic cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from blood to tissues [25,26].

The purpose of the present study was to determine the effects of water or ethanol extracts of AFA on the proliferation of adult human stem cells when given alone or in combination with NT-020 *in vitro*.

MATERIAL AND METHODS

Reagents

All compounds were added to cell cultures as described in the results sections. Two AFA products were used for extraction: AFA whole (AFA-W) and a proprietary AFA cellular concentrate (AFA-C) that were kindly donated by Simplexity Health. The cell wall is removed in AFA cellular concentrate by means of a proprietary process (Simplexity Health) thereby concentrating the intracellular material. AFA is harvested and processed according to FDA's Good Manufacturing Practices and the regulations of the Oregon Department of Agriculture. AFA undergoes stringent quality assurance testing for the presence of any undesirable elements, including toxins, pesticides, heavy metals, and detrimental microorganisms.

Water extractions

Reagents were dissolved in distilled water, sonicated for 15 minutes and filter sterilized with a 45 µm filter prior to application to cell cultures.

Ethanol extractions

AFA preparations were also studied as ethanol extracts, reagents were dissolved in 70% ethanol, vortexed for 40 seconds and incubated at 65 degrees Celsius for 2 hours. Extracts were centrifuged at 1000 RPM for 2 minutes, the supernatant was collected. This process was repeated and then the supernatants combined and dried. The dried

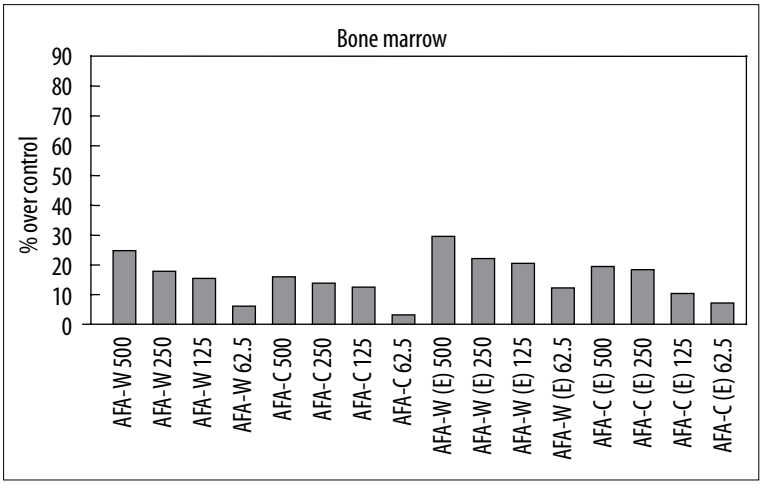

Figure 1. Bar graph showing the effects of whole AFA (AFA-W) or AFA cellular concentrate (AFA-C) either the water extract or ethanol extract (E) on the viability of bone marrow cells *in vitro*. Viability is measured using the MTT assay and data is presented as% over control with no addition of AFA. Extracts were added at concentrations varying from 62.5 ng/ml to 500 ng/ml as indicated below the figure. There is a concentration dependent effect of both AFA-W water and ethanol and AFA-C water and ethanol. Effects below 10% over control are not considered significant, thus not all concentrations showed significant effects.

extract was weighed and re-suspended in water, filter sterilized and used for cell culture experiments.

Cell cultures and MTT Assay

For cell proliferation analysis, human bone marrow cells or human CD34+ cells (AllCells, Inc.) were cultured in 96 well plates (5×10^4/well) containing 100 µL of complete medium (RPMI 1640 medium supplemented with 5% FCS). These cells were treated for 72 hours with various extracts at a wide range of doses (62.5 ng/mL to 500 ng/mL)with or without NT020 a proprietary formulation of blueberry, green tea, carnosine and Vitamin D3 previoulsy described [20]. This timing and dose range was found to be optimal in previous studies [20] and thus is the dose range we used for testing AFA in this study. Five hours before the end of the treatment, 20 µL of MTT solution (MTT kit, Sigma) was added to each well. These plates were then incubated in a CO2 incubator at 37°C for 5 hours and the cultured media removed with needle and syringe. 200 µL of DMSO was added to each well with pipetting up and down to dissolve crystals. These plates were put back into the 37°C incubator for 5 minutes, transferred to plate reader and measured absorbance at 550 nm. Data were represented as relative percentage mean proliferation, defined as O.D. reading number of each treated cells normalized to control cells (in the absence of treatment). This assay measures mitochondrial function and thus reflects cellular health and proliferation. Assays were run in duplicate for each individual experiment and the experiment was repeated at least 4 times to determine reproducibility.

Statistical analysis

Statistical significance of the data was assessed using a two-tailed student's t-test. The criterion for rejection of the null hypothesis was $P<0.05$.

Results

Effects of AFA alone on human bone marrow proliferation

As shown in Figure 1, when various AFA extracts were added to bone marrow cells in culture at doses between 62.5 and 500 ng/ml for 72 hours AFA-W and AFA-C both increased bone marrow proliferation as measured with MTT in a concentration dependent measure with the highest concentration of AFA-W having slightly greater efficacy (~25% over control) than AFA-C (<20% over control). Increases in proliferation below 10% over control were not considered significant, thus not all concentrations tested had effects above this threshold. There appeared to be little difference on measures of proliferation whether AFA was extracted from water or ethanol (E).

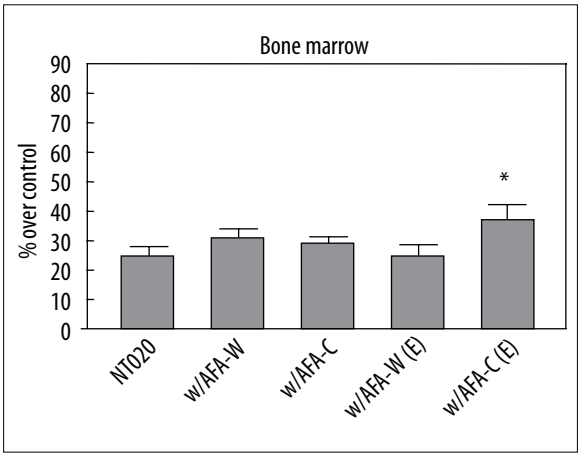

Figure 2. Bar graph showing the effects of addition of 500 ng/ml AFA-W and AFA-C both water and ethanol (E) extracts to NT-020 on the viability of bone marrow cells *in vitro*. Data are presented as described in figure 1. Only the AFA-C (E) showed additive effects with NT-020 ($P<0.05$).

Effects of AFA combined with NT020 on human bone marrow proliferation

As shown in Figure 2, when the 500 ng/ml dose of extracts were combined with NT020 (500 ng/ml), only the ethanol extract of AFA-C appeared to have additive effects on bone marrow cell proliferation as measured with the MTT assay.

Effects of AFA alone on human 34+ stem cell proliferation

As shown in Figure 3, when various extracts of AFA were incubated with CD34+ cells for 72 hours at doses ranging

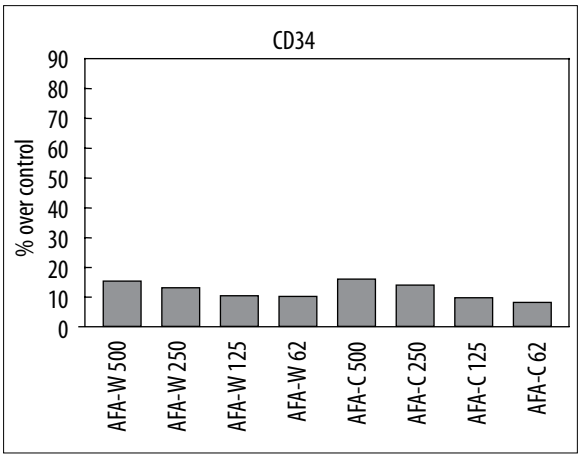

Figure 3. Bar graph showing the effects of AFA-W or AFA-C water extracts on viability of CD34+ cells *in vitro*. Data are shown as described in Figure 1. Doses of 250 ng/ml and 500 ng/ml showed small increases above baseline. Changes less than 10% over control are not considered significant.

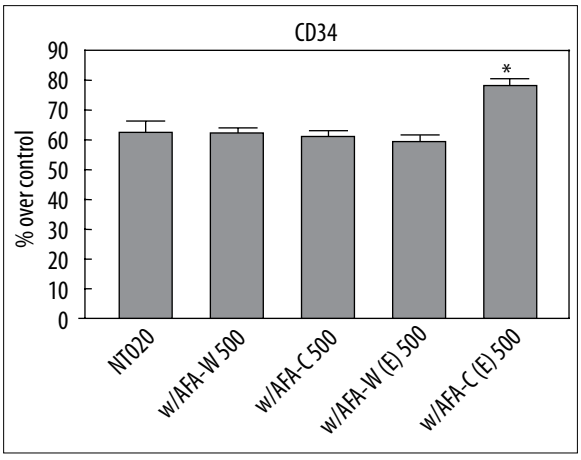

Figure 4. Bar graph showing effects of NT-020 with and without 500 ng/ml AFA-W and AFA-C either water or ethanol (E) extracts on CD34+ cell viability. Data are presented as in Figure 1. Only the AFA cellular concentrate ethanol extract shows additive effects with NT-020 on CD34+ cells *in vitro* ($P<0.05$).

from 62.5 ng/ml up to 500 ng/ml AFA-W and AFA-C (at doses from 250 ng/ml to 500 ng/ml) had effects to promote proliferation of the CD34+ cells above control conditions to a maximum of 14%. Doses lower than 250 ng/ml had no significant effects. Increases below 10% over control were not considered significant.

Effects of AFA combined with NT020 on human CD34+ stem cell proliferation

As illustrated in Figure 4, when the 500 ng/ml dose of the various AFA extracts were combined with NT020 (500 ng/ml), only the ethanol extract of AFA-W appeared to have additive effects on human CD34+ stem cell proliferation as measured by MTT assay.

Discussion

The results of this preliminary pre-clinical study further support the concept that natural compounds have the ability to stimulate the proliferation of human stem cells in culture [20]. NT-020 increased stem cell proliferation up to 70% above baseline in human stem cell cultures, which supports our previous research with this formulation [20,21]. The water and ethanol extracts of AFA and AFA cellular concentrate alone produced only moderate effects on proliferation of bone marrow cell and effects only at the high doses on CD34+ cells *in vitro*. An ethanol extract of AFA cellular concentrate had additive effects on CD34+ cell proliferation when combined with NT-020.

As stated earlier, Aphanizomenon flos-aquae (AFA) contains numerous compounds which may be responsible for the various health benefits reported. For example, a polysaccharide fraction referred to as "Immolina" isolated by ethanol extraction from blue green algae was found to potently and dose-dependently increase cytokines and the expression of genes encoding the members of a family of chemotactic cytokines that stimulate leukocyte movement and regulate the migration of leukocytes from blood to tissues [25,26]. In another study, it was observed that consumption of AFA lead to an increase in immune surveillance [18]. Phycocyanin, a component of blue green algae including AFA, has been shown to have potent antioxidant activity, scavenge preoxynitirite, and to inhibit cyclooxygenase 2, and thus have potential to reduce inflammation [27–31]. In addition, Maitake beta-glucan (MBG) is a polysaccharide extract from the fruit body of the Grifola frondosa mushroom, which is being widely used to treat cancer in Asia. Recently, Lin et al., (2004) reported that MBG enhances mouse bone marrow cell hematopoiesis *in vitro* and protects bone marrow cells from doxorubicin (DOX) toxicity [32]. A follow-up study by this group found that MBG works to enhance stem cell proliferation by promoting granulocyte colony-stimulating factor (GM-CSF) production [33].

One caveat to remember for this study is that the method used to measure viable cells in this manuscript reflects the activity of certain enzymes in the mitochondrion and is thus only an indirect measure of cell number. It is possible that an effect to increase mitochondrial number per cell or mitochondrial function could produce similar results, however this would still reflect increased cellular function, which would be a desired outcome. Future studies should be performed to assess the effects of AFA using more specific assays of cell numbers to delineate effects on proliferation versus increased metabolic activity. Furthermore, it should be noted that many of the components thought to have activity for stem cell proliferation are polysaccharides that may have solubility in either the aqueous or ethanol extractions, thus extraction methods and conditions may influence the amount of active components. Further research could delineate the active molecules in this extract for additional validation and characterization. An additional caveat relates to the data we present here for bone marrow proliferation with NT-020 as the% increase over control in this manuscript is lower than that previously published [20]. There are several different possible reasons for this discrepancy. One possibility is that the vendor for the bone marrow cells in this paper was a different vendor from the previous study; a second possible reason is donor variability. Both of these

possibilities could result in variability in the numbers of various cell types found in bone marrow used in the two studies as bone marrow is a mixed cellular population that contains mature hematopoietic cells as well as a small number of stem cells including CD34+. The data presented in this study for the isolated peripheral blood derived CD34+ cells was similar to that previously published [20]. The CD34+ cell is a more defined population of hematopoietic progenitor cells and thus may more accurately reflect effects on stem cell function.

Conclusions

This study demonstrates for the first time that AFA-C ethanol extract when studied in combination with NT-020 had an additive effect on both human bone marrow cells and CD34+ stem cells *in vitro*. When examined alone the various AFA extracts have modest effects to promote the proliferation of human bone marrow or human CD34+ stem cells *in vitro*. This preliminary preclinical study suggests that NT-020 plus an ethanol extract of AFA cellular concentrate may promote the proliferation and health of human stem cell populations.

Competing interests

PRS and PCB are founders of and RDS and JT serve as consultants for Natura Therapeutics, Inc.

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