

Oxidative Stress of Neural, Hematopoietic, and Stem Cells: Protection by Natural Compounds

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ABSTRACT

During natural aging, adult stem cells are known to have a reduced restorative capacity and are more vulnerable to oxidative stress resulting in a reduced ability of the body to heal itself. We report here that the proprietary natural product formulation, NT020, previously found to promote proliferation of human hematopoietic stem cells, reduced oxidative stress-induced apoptosis of murine neurons and microglial cells *in vitro*. Furthermore, when taken orally for 2 weeks, cultured bone marrow stem cells from these mice exhibited a dose-related reduction of oxidative stress-induced apoptosis. This preclinical study demonstrates that NT020 can act to promote healing via an interaction with stem cell populations and forms the basis of conducting a clinical trial to determine if NT020 exhibits similar health promoting effects in humans when used as a dietary supplement.

INTRODUCTION

A STEM CELL is a special kind of cell that has a unique capacity to renew itself and to give rise to specialized cell types. These 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. Stem cell research is currently a popular subject in the media and science.¹ While stem cell therapies have been promised to offer important new treatments for a wide variety of illnesses, there are two primary obstacles to their ultimate success. First, stem cell therapies remain controversial due to the focus on fetal derived embryonic stem cells. Second, stem cell therapies may take many years to reach the medical market place because of the lengthy and costly regulatory requirements.

Fortunately, a growing body of evidence suggests that there are numerous stem cells continually being produced within the human body throughout the lifespan. These stem cells are located in many different tissues and can become or “differentiate” into virtually any cell type in the body. Internal or “endogenous” stem cells are crucial to the body’s ability to repair itself of degenerating tissues, or to replace cell populations, such as those that have been destroyed by injuries, diseases, disorders, or treatments such as chemotherapy. Healthy stem cells are vital for the body’s own natural regeneration and repair mechanisms to function.

During natural aging, adult stem cells are known to have a reduced restorative capacity² and are more vulnerable to oxidative stress³ resulting in a reduced ability of the body to heal

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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.⁴⁻⁶ 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.^{7,8} Neural stem cells also decline with aging⁶ and some have postulated that declines in neurogenesis with aging are related to cognitive decline.⁹⁻¹¹ However, a rapidly 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.¹²⁻²⁵ Thus, it appears 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*. Specifically, we showed for the first time that a proprietary combination of blueberry extract, green tea extract, carnosine, and vitamin D₃, identified as the dietary supplement formula, NT020, demonstrated synergistic activity in promoting proliferation of human hematopoietic stem cells in culture.²⁶

Because recent studies indicate that oxidative stress limits the capacity of stem cell self-renewal, we investigated if NT020 would reduce the effects of oxidative stress on the survival of murine cells *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

In vitro study

Reagents. All compounds were added to cell cultures as described in the results sections.

Sources of NT020 ingredients were as follows: blueberry (freeze dried powder, Van Drunen Farms, Momence, IL), green tea extract (Rexall Sundown, Boca Raton, FL), carnosine (Sigma, St. Louis, MO), and the active form

of vitamin D₃ (25-hydroxy-cholecalciferol, Sigma).

Cell cultures and lactate dehydrogenase assay. For analysis in the prevention of cell death, murine primary microglial and neuronal cells were prepared from cerebral cortices isolated from newborn BALB/c mice (1–2 days)²⁷ or from mouse embryo, between 15 and 17 d *in utero*²⁸ ($n = 8$). These cells were cultured in 96 well plates (5×10^4 /well) containing 200 μ L of complete minimum essential medium containing 5% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ML penicillin, 0.1 μ g/mL streptomycin, and 0.05 mM 2-ME). These cells were incubated for 24 hours with various extracts at a wide range of doses (8 ng/mL to 500 ng/mL) or molecular compounds (0.3125 μ M to 20 μ M).

After a 6 hour-incubation period, H₂O₂ (15 μ M) is added to all wells except appropriate controls and the plates are returned to the incubator for the remainder of the incubation period. Cell lysis buffer from the lactate dehydrogenase (LDH) kit (Promega, Madison, WI) was added to appropriate wells, 45 minutes prior to supernatant collection, in order to measure total LDH release (total cell death). After the total 24-hour incubation period, supernatants were collected and assayed using the LDH kit in strict accordance with the manufacturer's instruction.

In vivo study

Oral treatment of NT020 in mice. All experimental techniques and procedures were approved by the Institutional Animal Care and Use Committee.

BALB/c mice, 3 months of age, were purchased from Jackson Labs (Bar Harbor, ME) for use in all *in vivo* experiments. All animals were housed under normal conditions ($20 \pm ^\circ\text{C}$, relative humidity of $50 \pm \%$, and a 12-hour light-dark cycle) and provided a normal food diet *ad libitum*. To further test of the effects of NT020 *in vivo*, mice were treated with NT020 at two different doses. The "low" dose of the proprietary formulation, 13.5 mg/kg per day, was based on the human daily dose derived from our initial *in vitro* study.²⁶ Because mice have a considerably higher metabolic rate than humans, we decided to use a dose 10-fold

AU1 → higher for the “high” dose condition in this *in vivo* study. Therefore, mice received 13.5 mg/kg per day (low dose, $n = 5$) or 135.0 mg/kg per day (high dose, $n = 5$) NT020, or water ($n =$) for 14 days by oral gavage. On day 15, the bone marrow was isolated and cultured (blind to treatment condition) as described below and challenged with varying doses of H_2O_2 (15 μM 500 μM).

QU1 → *Cell isolation and tissue collection.* Animals were anesthetized with Nembutal (500 μl), and bone marrow cells were collected in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 3% FBS. Bone marrow was obtained by gently flushing the femur with IMDM. The marrow was then mechanically dissociated, cells counted, and plated in 24 well plates (5×10^6 /well).

Statistical analysis

All data were normally distributed; therefore, in instances of single mean comparisons,

Levene’s test for equality of variances followed by *t* test for independent samples was used to assess significance. In instances of multiple mean comparisons, analysis of variance (ANOVA) was used, followed by *post hoc* comparison using Bonferonni’s method. α Levels were set at 0.05 for all analyses. The statistical package for the social sciences release 10.0.5 (SPSS Inc., Chicago, IL) was used for all data analysis.

RESULTS

Natural products reduce oxidative stress of primary cultured murine cells in vitro

Blueberry (BB), green tea (GT), carnosine (Ca), vitamin D₃ (D₃), and their combination (NT020) were found to reduce oxidative stress of murine cells in culture (Fig. 1). Cell viability was determined by LDH assay and displayed as the average percent of cell death for each treatment group, over total cell death \pm stan-

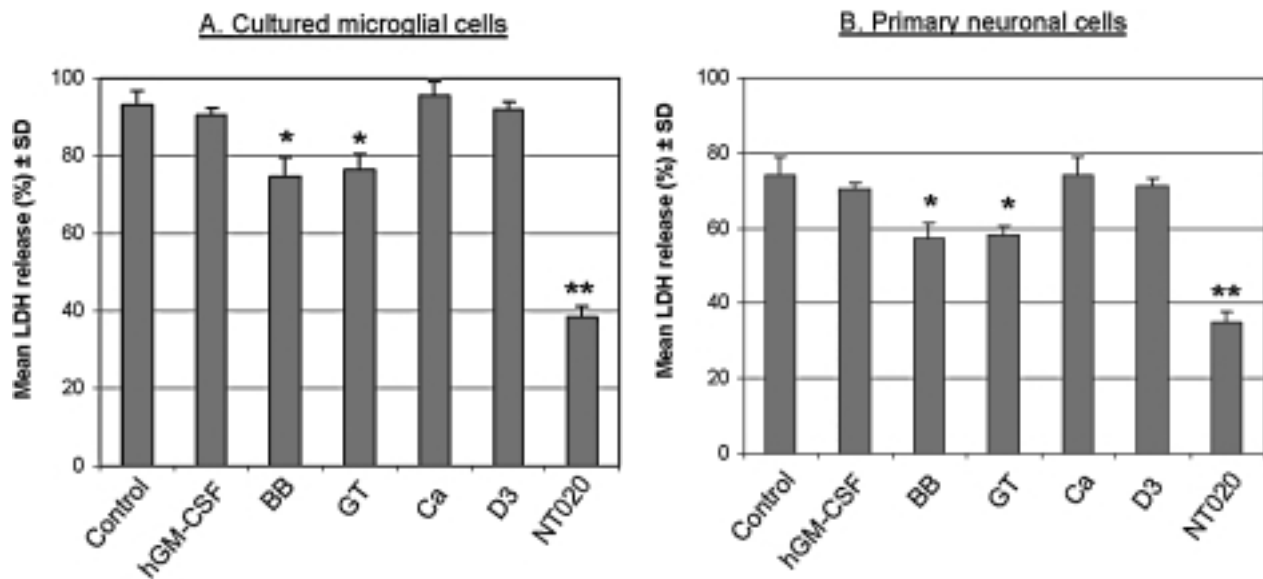


FIG. 1. In order to investigate NutraStem™ and its individual ingredients in an *in vitro* model of oxidative stress, we decided to utilize a hydrogen peroxide (H_2O_2) model in murine primary cultured microglial cells (A) and murine primary neuronal cells (B). Cells were cultured in 96 well plates (5×10^4 /well) using complete medium (minimum essential medium containing 5% FBS, 2 mM glutamine, 100 U/ML penicillin, 0.1 μg /mL streptomycin, and 0.05 mM 2-ME). Cells were then treated with either natural extracts (BB and GT) or natural compounds (Ca and Vitamin D₃), and their combination (NutraStem™). After 6 hours H_2O_2 was added and allowed to incubate for the remainder of the 24 hour period. After treatment supernatants were collected and assayed for LDH release as described in materials and methods. Data were represented as the percentage of cell death (LDH release) over total cell death (max. LDH release). For A and B, one-way ANOVA followed by *post-hoc* comparisons (Bonferonni corrected) revealed significant differences between BB or GT and control ($*P < 0.01$) as indicated. In addition, this analysis also revealed an even greater significant difference between NT020 and control ($*P < 0.005$).

dard deviation (SD; cell lysis, maximum LDH release). Controls represent cells cultured in the same condition (H_2O_2 insult) without any extract or compound added. Results showed a significant decrease in LDH release for the natural extracts of BB and GT, but not for the compounds of Ca or vitamin D_3 , when used in individual treatments (* denotes significance). When these extracts and compounds are used in a combination we observed a synergistic effect resulting in even greater decrease in the LDH released.

Effects of low and high dose oral NT020 treatment on oxidative stress in mice

Bone marrow isolated from BALB/c mice gavaged with a low or high dose of NT020 was able to reduce oxidative stress from H_2O_2 administered in culture.

Results from this *in vivo* study showed that there was a dose-dependent decrease in LDH release for each treatment group, and that this decrease corresponds to the decreasing concentrations of H_2O_2 administered. Cell viability was displayed as the average percent of cell death for

each H_2O_2 concentration administered per gavage group, over total cell death \pm SD (cell lysis, maximum LDH release). The low-dose treatment displayed a significant decrease in LDH release, for many of the H_2O_2 concentrations administered compared to controls.

Additionally, H_2O_2 administration to cells from the high-dose group resulted in greater decrease in LDH release, showing significance from both control and low-dose groups.

DISCUSSION

In this study, we have demonstrated for the first time that the proprietary natural product formulation, NT020, designed to promote the proliferation of human stem cells, also reduces oxidative stress, a well-known physiologic process associated with aging and one that reduces the capacity for stem cell self-renewal. A major strength of this study was that NT020 not only reduced oxidative stress *in vitro*, but also dose dependently promoted the viability of bone marrow cells and increased the resistance of these cells to oxidative insults *in vivo* when given orally to mice for 2 weeks. These findings suggest that NT020 should have the capacity to increase the viability of stem cell populations and reduce oxidative stress in humans when given orally as a dietary supplement.

When tested individually, the compounds that compose NT020 were effective in decreasing cell death within *in vitro* microglial and neuronal cultures.

Additionally, when examined in combination the additive and synergistic effects showed a much greater decrease (>50%) in oxidative induced cell death. This effect was also observed with *in vivo* administration of a low or high dose of the NT020 combination. Results showed a significantly large decrease in cell death for the high dose (>50%) for several concentrations of H_2O_2 , while the low dose also resulted in a significant decrease in cell death (>25%) below controls. These treatments have displayed significant protection, for various cell types, from the oxidative damage and cell death induced from the exposure to H_2O_2 . An interesting aspect of this result is that NT020 is increasing the resistance of these bone marrow cells to an oxidative insult even af-

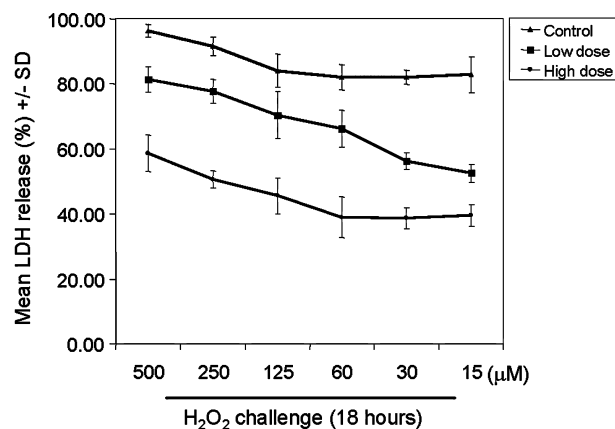


FIG. 2. In a further test of the effects of NutraStem™ *in vivo*, mice received 13.5 mg/kg/day (low dose) or 135.0 mg/kg/day (high dose) NutraStem™, or water (n=5/group) for 14 days by oral gavage. On day 15, the bone marrow was isolated and cultured as described above and treated with varying doses of H_2O_2 . As can be observed in Figure 2, Nutra Stem™ treatment significantly attenuated the cell death from the oxidative insult. Data were represented as the percentage of cell death (LDH release) over total cell death (max. LDH release) for each dose of H_2O_2 given. An ANOVA followed by *post hoc* comparisons (Bonferroni corrected) revealed significant differences between the low and high dose *versus* control with H_2O_2 challenge at each concentration ($P < 0.05$).

ter NT020 is no longer present, as it was not added to the cultures after the cells were removed for the mice. This suggests that NT020 is producing changes at the cellular level that increase the viability of these cells and make them more resistant to an oxidative insult. Further work will be necessary to determine the mechanism of this effect. Also, further studies into the reactive oxygen scavenging abilities of NT020 in more aged animals may provide insight into its ability to limit oxidative damage, which is known to increase with age. Further refinement in dosing may also be achieved through investigations into the length of time that protection, from oxidative insult, is present after NT020 treatment.

While millions of dollars are currently being spent to study stem cell function, very little research has been devoted to determining which natural nutrients from food and plants can be used to optimize production or to maintain cellular health of existing endogenous stem cell populations. Clearly, research into this area holds huge potential for developing safe and effective nutraceutical formulations with unique and untapped therapeutic possibilities. Moreover, because the ingredients come from nature, they are often safer than pharmaceutical or costly invasive cell therapies. As a result, they often require less preclinical toxicology studies and can enter clinical trials very rapidly. It is our hope that NT020 will serve as a proof of concept for this therapeutic and preventative approach to health.

In conclusion, we have demonstrated that NT020, which we previously found to promote proliferation of human hematopoietic stem cells *in vitro*, reduced oxidative stress-induced apoptosis of murine neurons and microglia in culture. Furthermore, when taken orally for two weeks, cultured bone marrow stem cells from these mice exhibited a dose-related reduction of oxidative stress-induced apoptosis. These findings form the basis of conducting a clinical trial to determine if NT020 exhibits similar health promoting effects in humans.

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SHYTLE

AU1

Indicate quantity.

AU2

Provide email address.

QU1

Second 2 OK? or inferior?

QU2

Placement of Figure 2 OK? No callout in text for Figure 2.