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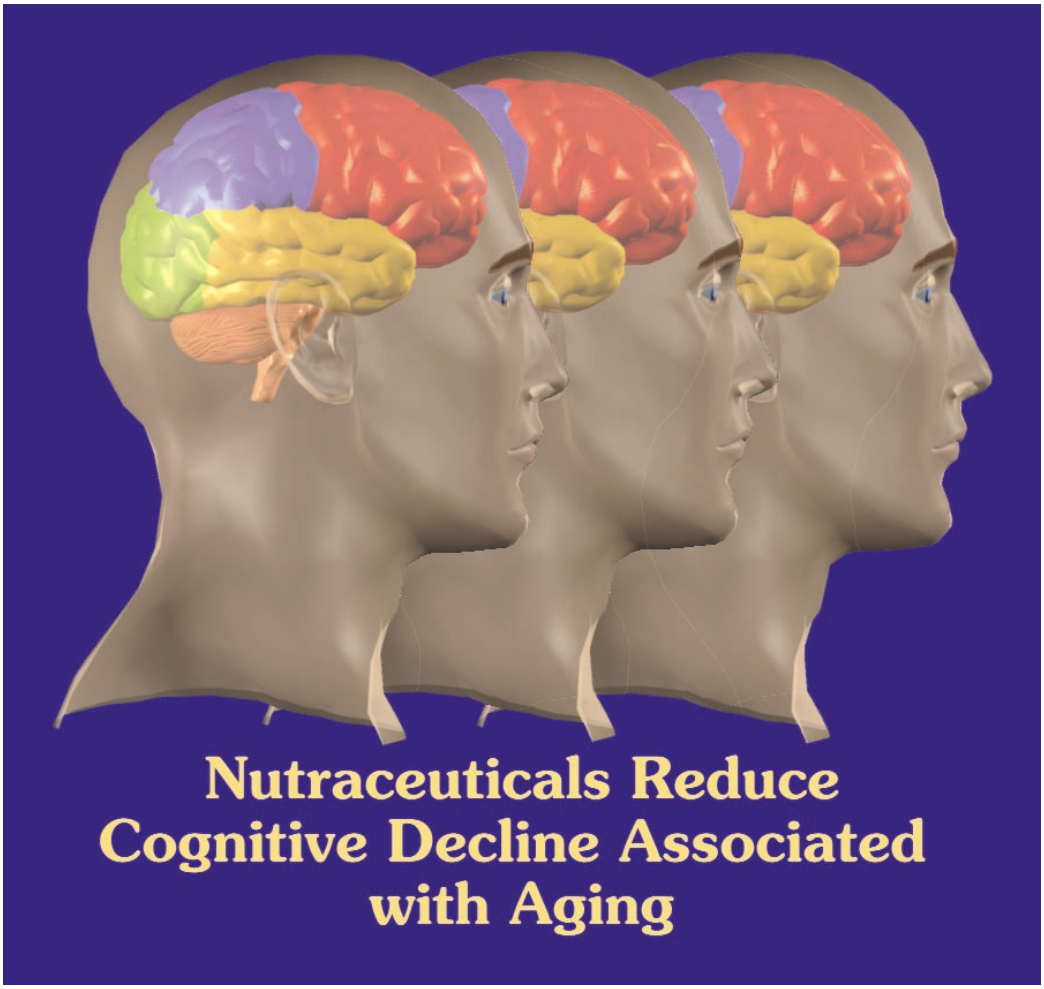

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Allen Montgomery, RPh

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CONFERENCE REPORTS

REVIEW ARTICLE

META-ANALYSIS STUDY

PILOT STUDY

ORIGINAL RESEARCH

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EDITORIAL COMMENTARY

Genetic Testing: Between Hope and Hype

Bernd Wollschlaeger, MD, FAAFP
Associate Editor
Journal of the American Nutraceutical Association

When I learned about my wife's pregnancy, we were both exhilarated and partially terrified by the news. Being Jewish and living in Israel at that time, we were both mandated to undergo genetic testing to screen for Tay-Sachs Disease, a fatal genetic lipid storage disorder in which harmful quantities of a fatty substance called *ganglioside* G_{M2} build up in tissues and nerve cells in the brain. The condition is caused by insufficient activity of an enzyme called *beta-hexosaminidase A* that catalyzes the biodegradation of acidic fatty materials known as *gangliosides.* Carriers of Tay-Sachs disease can be identified by a simple blood test that measures beta-hexosaminidase A activity. Both parents must carry the mutated gene in order to have an affected child. In these instances, there is a 25 percent chance with each pregnancy that the child will be affected with Tay-Sachs disease. The incidence of Tay-Sachs is particularly high among people of Eastern European and Askhenazi Jewish descent.

Affected children become blind, deaf and unable to swallow. Muscles begin to atrophy and paralysis sets in. Other neurological symptoms include dementia, seizures and an increased startle reflex to noise.

We both tested negative and our son is now a 19-year old healthy college freshman.

Needless to say, as a result of personal experience, I became a big fan of genetic testing and was looking forward to utilizing genetic testing and screening for a myriad of medical conditions. Seven years ago, scientists finally succeeded in decoding the entire human genome and I was convinced that the brave new world of personalized medicine was just around the corner. Ever since, dozens of genetic testing companies have entered the market place, with some of them promising to deliver solutions for those unfortunates among us with " bad" genes.

Some companies even went so far as to offer "nutrigenetic testing" with individualized vitamin supplementation tailored to a patient's DNA profile.

Having used some of those tests in my practice, my patients and I often asked ourselves how to interpret the results as well as how to translate them into medical treatments and life style recommendations.

These problems are further complicated by the fact that most doctors never received any education or training in Genomics, and the number of qualified genetic counselors is still very limited.

In addition, we tend to believe that ONE gene is responsible for ONE disease, often ignoring the fact that multiple genes on multiple chromosome locations may be responsible for medical conditions ranging from cancer to diabetes. Furthermore, the presence of a specific gene does not necessarily correlate to the development of a related disease.

For example, a test called BRACAnalysis, detects mutations in genes called BRCA1 and BRCA2. Women with a clinically significant mutation in one of those genes have a 35 to 84 percent probability of developing breast cancer by age 70, and a 10 to 50 percent probability of developing ovarian cancer, far higher than for women in general. But mutations in the genes account for less than 10 percent of all cases of breast cancer. And only 1 in about 400 women has the mutation. Women of Ashkenazi Jewish

descent have a ten-times higher risk of mutation, but that does not necessarily mean that they will get breast cancer. After the introduction of the test, many women who tested positive for the mutations(s) were faced with the tough choice of bilateral mastectomy or prophylactic chemotherapy. Furthermore, positive test results may jeopardize health and life insurance eligibility. We later learned to focus and limit intervention to women with positive BRCA1 or BRCA2 test and a relative with breast cancer, and to avoid aggressive therapy for EVERYONE who tested positive. Even though clinical science found high positive and negative predictive values for BRCA1 and BRCA2 positive women with pre-test risk between 4% and 40%, we still need to individualize the interventions and therapies.

Another company offers a test that looks for the variant of a gene TCF7L2, which is associated with Type 2 diabetes. We know that at least ELEVEN genes have been linked to the development of diabetes, but the company just tests for the presence of ONE gene. A recently published clinical study indicates that 80% of the people who tested positive for TCF7L2 didn't get Type 2 diabetes in old age and 40% of the people who had diabetes didn't carry the gene variant at all!

What does that mean? It means that at this point in time, the so-called predictive value of those tests and the proportion of patients with positive test results who have the disease, is still poor for many tests offered. Positive genetic screening results do not necessarily mean that the affected person will develop the disease. Physicians and other healthcare professionals need to be trained on how to perform and interpret the results. Patients should educate themselves about the rapidly increasing genetic testing options and opportunities, but should discuss the results with qualified professionals. Nutritional interventions based on genetic screening may be temping, but have yet to be substantiated by clinical science. Healthy and personalized nutrition should not be confused with fragmented mineral and vitamin supplementation that is allegedly tailored to an individual's genetic make-up.

I am still excited about the future of medical genomics and we will learn much more about their clinical application in the next few years. Meanwhile, I would be careful to jump to conclusions or to succumb to commercial pressures. Genomics may not be ready for prime time yet, but the time will come soon. I will keep you posted.

Confused About Diet and Health? Guidelines for the Clinical Practice: A 50-Year Research Journey

Proceedings Report from the American Nutraceutical Association's Fall 2007 CME Conference held in Memphis, Tennessee

T. Colin Campbell, PhD*
Professor Emeritus of Nutritional Biochemistry
Cornell University

For more than 40 years, T. Colin Campbell, Ph.D. has been at the forefront of nutrition research. His legacy, the *China Study*, is the most comprehensive study of health and nutrition ever conducted. Dr. Campbell is the Jacob Gould Schurman Professor Emeritus of Nutritional Biochemistry at Cornell University and Project Director of the China-Oxford-Cornell Diet and Health Project. The China Study was the culmination of a 20-year partnership of Cornell University, Oxford University and the Chinese Academy of Preventive Medicine.

His presentation at the ANA CME Conference touched on some of the highlights of the results from the *China Study* and other research conducted by Dr. Campbell during his career. The following report was prepared from his presentation by *JANA* Associate Editor Nadine Taylor, MS, RD.

Does Animal Protein Help or Hinder?

In the early stages of his career, Dr. Campbell began coordinating technical assistance in the Philippines for a nationwide project working with malnourished children. One aim of the project was to find out why so many Filipino children were developing liver cancer (typically an adult disease). Since liver cancer rates were highest in countries with the lowest protein intake, another goal of the project was to make sure the children were receiving plenty of protein. Surprisingly, the researchers on the Philippines project discovered that the children from the best-fed families who ate the *most* protein were most likely to develop liver cancer.

In 1968, a research paper from India was published showing the results of an experiment involving protein consumption and liver cancer in two groups of laboratory rats.[1] Both groups received aflatoxin, a fungus-produced toxin known to induce liver cancer in rats. Then one group was given a diet containing 20% protein, while the other was given 5% protein. All of the rats receiving 20% protein developed liver cancer, while none receiving 5% protein developed the disease. (Figure 1)

It seemed clear that while a 5% protein diet did not promote liver cancer, a 20% protein diet did. But at what level of protein did the cancer begin to grow? A study performed by one of Dr. Campbell's graduate students came up with an answer.[2] When cancer initiation is complete, tiny clusters of cancer-like cells called foci appear. By watching the foci develop, it is possible to study the effects of protein on cancer promotion without having to wait for the development of full tumors. In the study, it was discovered that foci growth could be increased and reversed simply by

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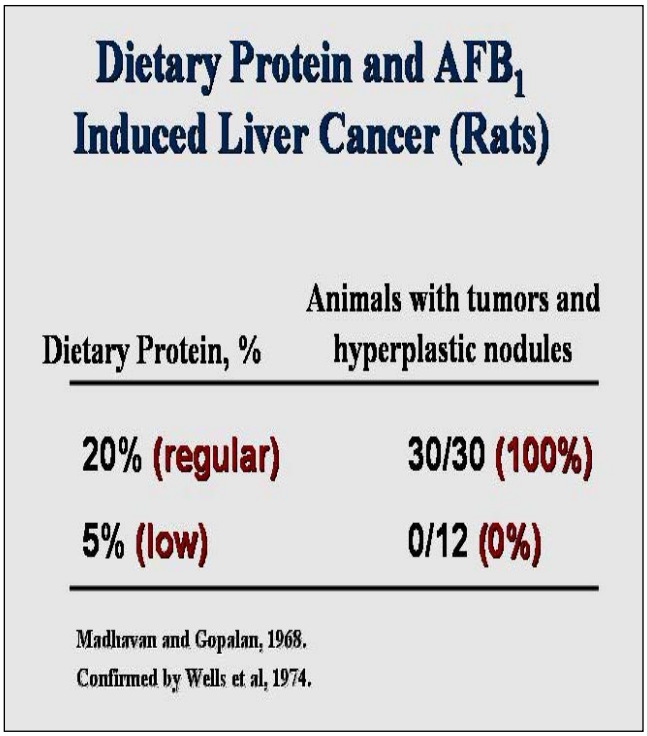
Figure 1.



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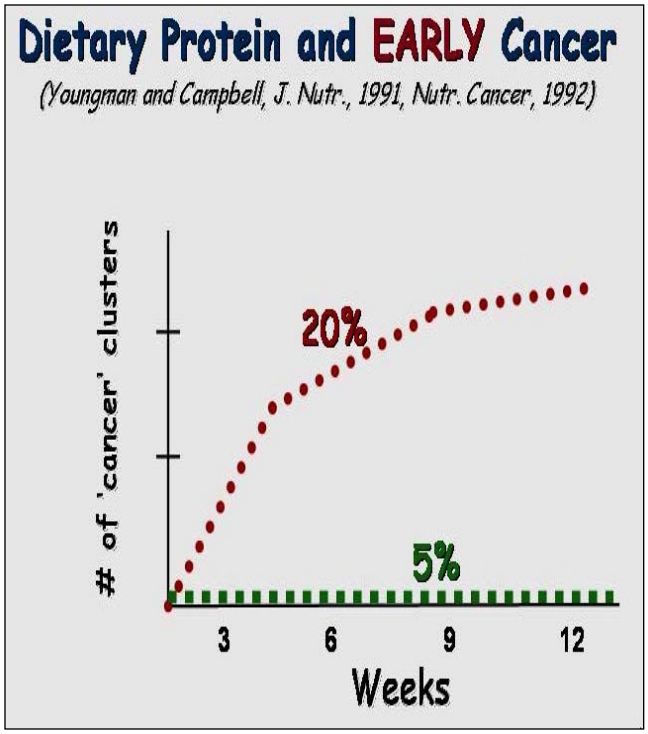


changing the amount of protein that was consumed. And foci did not develop until protein consumption reached about 10%. When the diet contained more than 10% protein, foci development increased dramatically. (Figure 2)

It's important to note that the protein used in the study was casein, the main protein found in cow's milk, and that soy and wheat protein do *not* increase pre-cancer development, even at 20% of the caloric intake.

THE CHINA STUDY

The Philippines research project eventually culminated in a 20-year partnership between Cornell University, Oxford University and the Chinese Academy of Preventive Medicine, the purpose of which was to survey diet, diseases and lifestyle factors in rural China and Taiwan. Known as "The China Study," it produced more than 8,000 statistically significant associations between various elements of diet and disease.

Researchers collected and analyzed mortality data for more than 50 diseases (including 7 different kinds of cancer) from 65 counties and 130 villages in rural mainland China. A total of 6,500 adults participated in the study. One hundred adults from each county (half of them male, half of them female; all 35-65 years of age) completed a diet and lifestyle questionnaire and provided a blood sample. Half of them produced a urine sample, as well. Blood (and/or urine??) samples were analyzed for dietary and nutritional factors.

Some of the massive dietary differences between people living in rural China and those living in Western societies included:

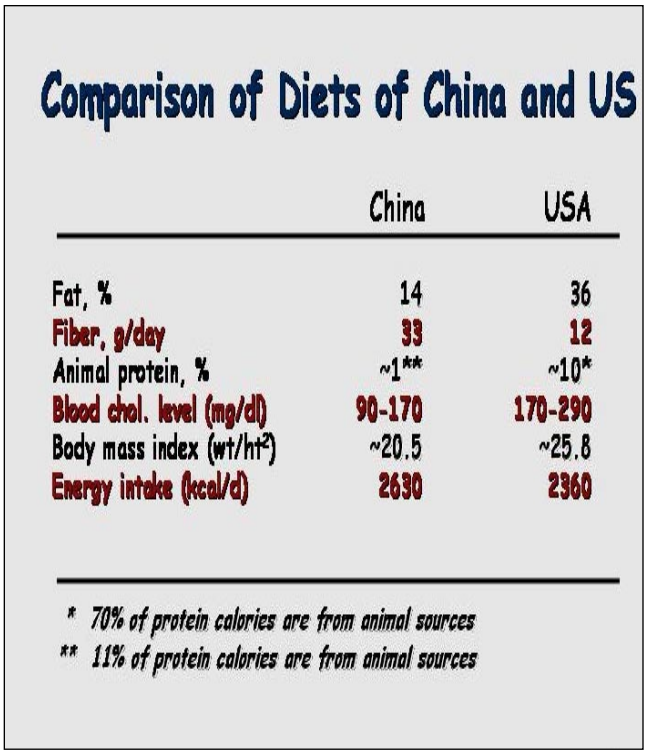
- Fat consumption in China was less than 50% of U.S. intake.
- Consumption of animal protein was about 10% of the U.S. intake.
- Fiber consumption was 3 times higher in China than U.S. intake. (Figure 3)

A few notable differences in health status between the two groups included:

- The average total cholesterol was 175 mg/dl in rural China versus 205 mg/dl in the U.S.
- Average body mass intake was 20.5 in rural China compared to 25.8 in the U.S., although calorie intake was 300 kcal/day higher in China.
- The death rate from coronary heart disease was 17 times higher in American men compared to rural Chinese men.
- The U.S. death rate from breast cancer was five times higher than the Chinese rate.
- During the years 1973-1975, *no one* in the Chinese provinces of Sichuan and Guizhou died of coronary heart disease before age 64.

In short, research has found that the people who had consumed the most animal-based foods were stricken with the greatest amounts of chronic disease. And those who had

Figure 3



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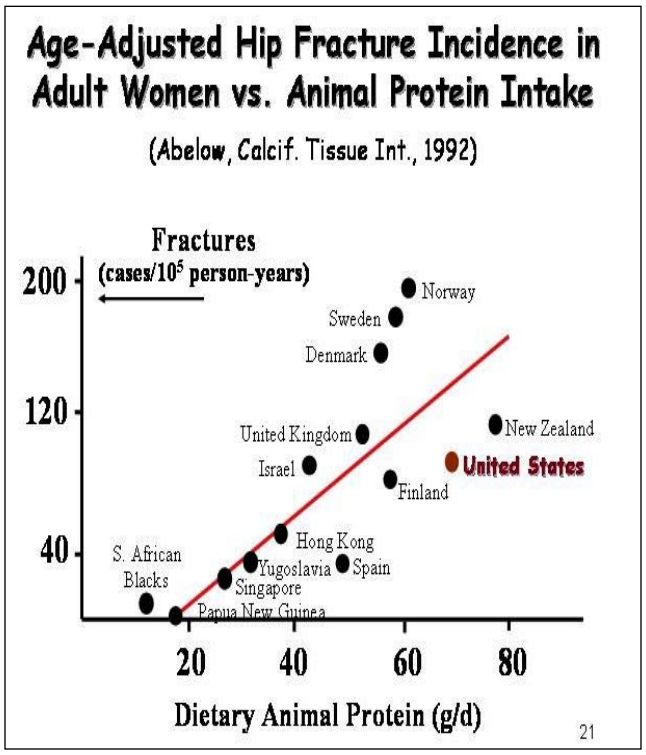


consumed the most plant-based foods were the healthiest and generally avoided chronic disease.

Expanding the Disease Horizon

Other studies have found similar results. One study involving the association between dietary animal protein and the incidence of hip fracture in women, found that the higher the protein intake, the more likely women were to fracture their hips. (Figure 4)

MILK CONSUMPTION

A study correlating food intake with the incidence of breast, ovarian and uterine cancer (using data taken from the Cancer Incidence in the Five Continents survey) found that meat and milk consumption were highly correlated with breast cancer incidence, while milk consumption was highly correlated with uterine cancer.[3]

FIGHTING DISEASE WITH A WHOLE FOOD, PLANT-BASED DIET

The results of the China Study and countless other studies of the effect of food on disease show that a whole food, plant-based diet can prevent, suspend or even cure a wide range of diseases, including cancer, osteoporosis, heart disease, macular degeneration, hypertension, depression and rheumatoid arthritis, just to name a few.

SAME Whole Food, Plant-Based Diet Prevents, Suspends and/or Cures All
(All supported by published peer-reviewed research)

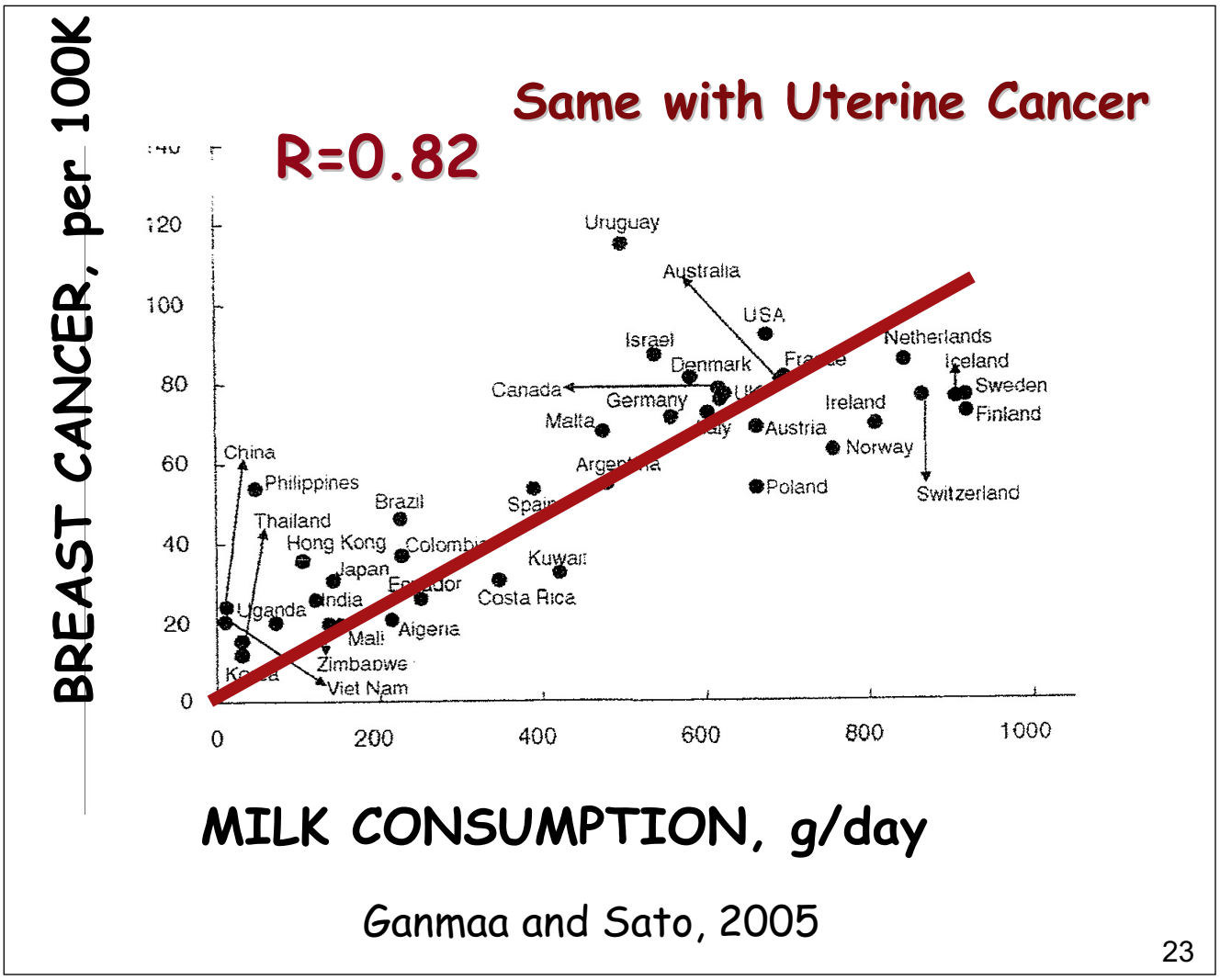
- Cancers
- Heart Diseases
- Multiple Sclerosis
- Kidney Stones
- Cataracts
- Osteoporosis
- Type I/II Diabetes
- Rheumatoid Arthritis
- Macular Degeneration
- Hypertension
- Acne
- Migraine
- Lupus
- Depression
- Alzheimer's Disease
- Colds and Flu

And Promotes Superior Physical Fitness

NUTRITION AND DISEASE: FOUR IMPORTANT PRINCIPLES

Nutrition involves the integrated effects of countless food constituents, which produce comprehensive health through infinitely complex mechanisms. To sum up the effect of nutrition on disease, consider these four principles:

Figure 5.



Principle I

Every biological event starts with genes (i.e. is genetic) but health/disease progresses through non-genetic mechanisms that are controlled by exogenous factors (i.e. nutrition).

Principle II

A nutrient functions through multiple mechanisms (rather than a single mechanism). For example, nutrient imbalances may contribute to cancer promotion through decreased NK cell activity, increased cell replication, increased oxidant activity, increased IGF-2 or altered energy activity.

Principle III

The same plant-based diet maintains health and prevents and/or reverses (cures) a broad range of degenerative diseases.

Principle IV

Nutrition is a "wholistic" concept, not a "reductionist" concept. Nutrition represents the combined activities of countless food substances, and the whole is greater than the sum of its parts. In short, nutrition is like a symphony.

REFERENCES

1. Madhavan TV, Gopalan C. The effect of dietary protein on carcinogenesis of aflatoxin. *Arch Pathol* 1968;85(2):133-37.
2. Dunaif GE, Campbell TC. Relative contribution of dietary protein level and aflatoxin B1 dose in generation of presumptive preneoplastic foci in rat liver. *J Natl Cancer Inst* 1987;78(2):365-69.
3. Ganmaa D, Sato A. The possible role of female sex hormones in milk from pregnant cows in the development of breast, ovarian and corpus uteri cancers. *Med Hypotheses* 2005;65(6):1028-37.

Pathophysiology, Diagnosis, Prevention and Treatment of Vascular Aging

Proceedings Report from the American Nutraceutical Association's Fall 2007 CME Conference held in Memphis, Tennessee

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Clinical Professor of Medicine, Vanderbilt University School of Medicine
Director, Hypertension Institute and Vascular Biology
Saint Thomas Hospital, Nashville, Tennessee

A summary of Dr. Houston's presentation at the ANA's Fall CME Conference prepared by *JANA* Associate Editor, Nadine Taylor, MS, RD.

INTRODUCTION

Sir William Osler once said, "A man is as old as his blood vessels," and indeed, even healthy aging in both men and women is an independent risk factor for coronary heart disease (CHD) and cardiovascular disease (CVD). Our blood vessels age just as surely as our bodies do, resulting in arterial stiffness, inflammation, oxidative stress, thrombosis, increased permeability, reduced angiogenesis, blood pressure that doesn't fall at night (impaired "circadian clock" genes), and vasoconstriction, among other problems.

Many factors contribute to vascular cell senescence, including DNA damage, the shortening of the telomeres, the tumor suppression pathway, insulin, the renin-angiotensin system, mitochondrial damage and a decrease in endothelial progenitor cells. A select few of these factors are discussed in this article.

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AGE-RELATED DAMAGE TO VASCULAR DNA

As the vascular cells age, the DNA repair system becomes imperfect; nucleotide bases are lost and cells become damaged. The aging vascular cell also develops problems with the telomere maintenance system. A telomere is a region of highly repetitive DNA at the end of a linear chromosome that functions as a disposable buffer. In a sense, the telomere is like a protective cap on the chromosome. Telomeres are crucial to the life of the cell, as they keep the ends of the chromosomes from accidentally becoming attached to each other. During replication, however, the extreme ends of the chromosomes are not duplicated completely, so a small portion of the telomere is "lopped off." In the process, every time a cell with linear genes divides, it loses a small piece of one of its strands of DNA. It is believed that this shortening of the telomeres is related to the aging process.

Besides aging, many other factors can contribute to the shortening of the telomeres and thus the aging of the vascular system, including inflammation, oxidative stress, CHD, hypertension, obesity, smoking, atherosclerosis, homocysteine, oxidized LDL, and a lack of arginine, among others.

Figure 1.

VASCULAR CELL SENESCENCE: TELOMERE MAINTENANCE SYSTEM

1. Telomere
   - Nonnucleosomal DNA / protein complex at end of chromosomes
   - Protective caps for chromosomes
   - Substrate for specialized replication mechanisms
2. Semi-conservative DNA replication leads to extreme chromosome ends that are not duplicated completely. This results in successive shortening of telomeres with each cell division.

Figure 1A.

VASCULAR CELL SENESCENCE: TELOMERE MAINTENANCE SYSTEM (continued)

3. Telomerase
   - Enzyme that repairs the telomere
   - Decreased activity with aging
   - Decreased activity with oxidative stress and inflammation
   - In VSMC and ED cells it is activated by mitogenic stimuli via PKC dependent path. Activity decreased with aging due to decreased TERT.

Circ Res 2007; 100:15-26

ANGIOTENSIN II AND VASCULAR AGING

An important cause of telomere shortening and vascular aging involves the angiotensin II pathway. Angiotensin II is a powerful blood vessel constrictor that triggers local damage to the arteries, endothelial dysfunction, vascular growth and blood clots. It also increases oxidative stress, produces pro-inflammatory cytokines and reduces NO bioavailability. All of these factors contribute to the aging of the vascular smooth muscle cells (VSMC) and the death of endothelial progenitor cells (EPC), the "stem cells" of the vascular system.

One way in which angiotensin II increases oxidative stress is by enhancing production of reactive oxygen species such as superoxide dismutase (SOD) anions. This leads to DNA damage and accelerated cellular aging. However, when oxidative stress is inhibited, for example, by the addition of catalase or SOD, damage to DNA is reduced, negative effects on the telomeres are interrupted and cellular aging is slowed.

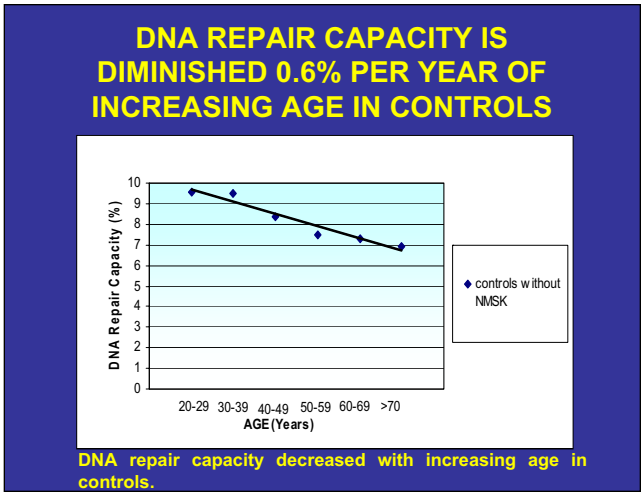
The Mitochondrial Damage Theory of Aging

One theory of aging holds that oxidative damage to the mitochondrial DNA (mtDNA) leads to mitochondrial dysfunction and the physiological decline associated with aging. Several factors make mitochondrial DNA susceptible to oxidative damage and rapid aging:

- MtDNA lacks protective histones.
- MtDNA is closer to the site of oxygen radical production and lipid peroxidation.
- Enzyme DNA repairing systems are lacking in the mitochondria.

For these and other reasons, DNA repair capacity decreases with age by 0.6% per year. (Figure 2)

Figure 2.



SLOWING THE AGING PROCESS

Caloric Restriction

It has been well established that in animals a caloric restriction of 30-50% can increase life expectancy (both average and maximum) by 15-60%, enhance mitochondrial function, decrease inflammation and reduce oxidative stress. But what about in humans?

In a 2007 study performed at Pennington Biomedical Research Group, 36 overweight subjects were assigned to one of three groups: calories restricted by 25%; calories restricted by 12.5% plus exercise sufficient to burn an additional 12.5%; or calories restricted by 100 kcal (control group).[1]

While no changes were seen in the control group, both the calorie restricted and calorie restricted-plus-exercise groups showed a significant decrease in DNA damage, plus an increase in mitochondrial DNA and mitochondrial biogenesis. The results suggest that caloric restriction improves mitochondrial function. Caloric restriction with exercise also reduces inflammation and lowers insulin levels. (Figures 3, 3A)

Interestingly, results that nearly equaled those achieved with caloric restriction have been seen in those who engage in three 12-hour fasts per week, fasting from 6 p.m. to 6 a.m. the following day, three days a week.

Simulated Caloric Restriction - The Sirtuins

Among the beneficial effects of caloric restriction is an increase in the copy number and expression of sirtuins (SIR-2, SIRT-1, SIRT-2, etc.). The sirtuins are involved in deacetylation and act as silent information regulators (thus the SIR part of their name). The sirtuins also play important roles in DNA repair, RDNA recombination, cell survival, energy metabolism and response to stressors. SIR-2, found in yeast, and SIRT-1, found in animals, have been shown to extend the life expectancy in animals by mimicking the effects of caloric restriction.

Since most people find caloric restriction a difficult and unpleasant way to achieve longevity, the idea of activating SIRT-1 to get the same results is intriguing. Tests to determine which substance was most effective at mimicking caloric restriction in this way found that the "winner" was resveratrol, activating both SIRT-1 and SIRT-3 activity by 10-fold. Other SIRT-1 activators include quercetin (35% as effective), IP6 rice bran oil (30% as effective) and genistein powder (40% as effective).

Resveratrol

Found in the skin of grapes and in red wine, resveratrol is believed to be a partial explanation for the French paradox - the fact that the French consume a high-fat diet yet develop less heart disease than Americans. Large amounts of resveratrol are found in the red wine they drink.

A recent study conducted at Harvard Medical School found that resveratrol shifted the physiology of middle-aged mice on a high-calorie diet to that of mice on a standard diet and significantly extended their life spans. Other benefits of resveratrol included increased insulin sensitivity, reduced insulin-like growth factor-1 (IGF-I) levels,

Figure 3.

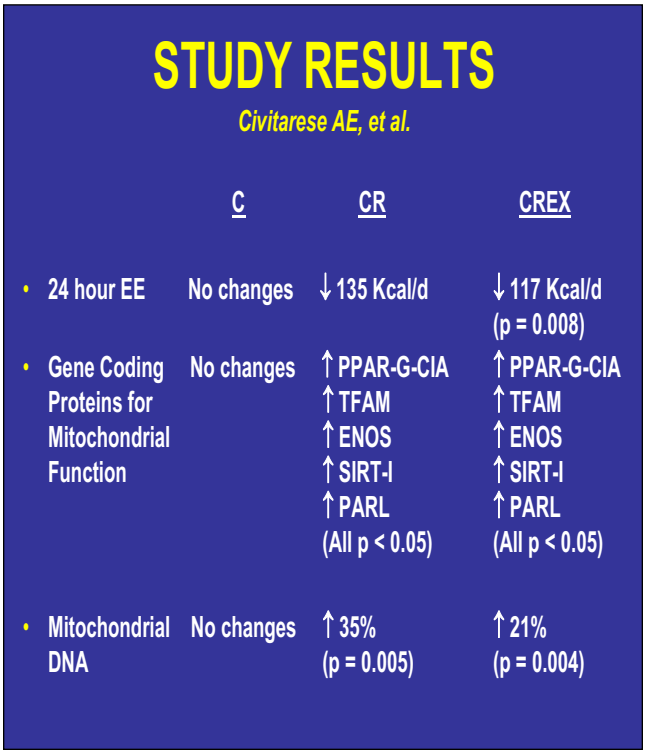
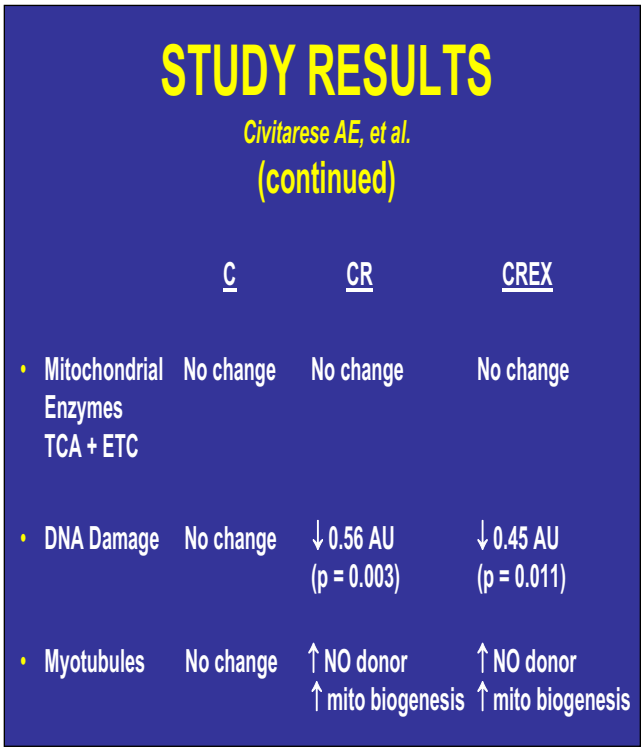


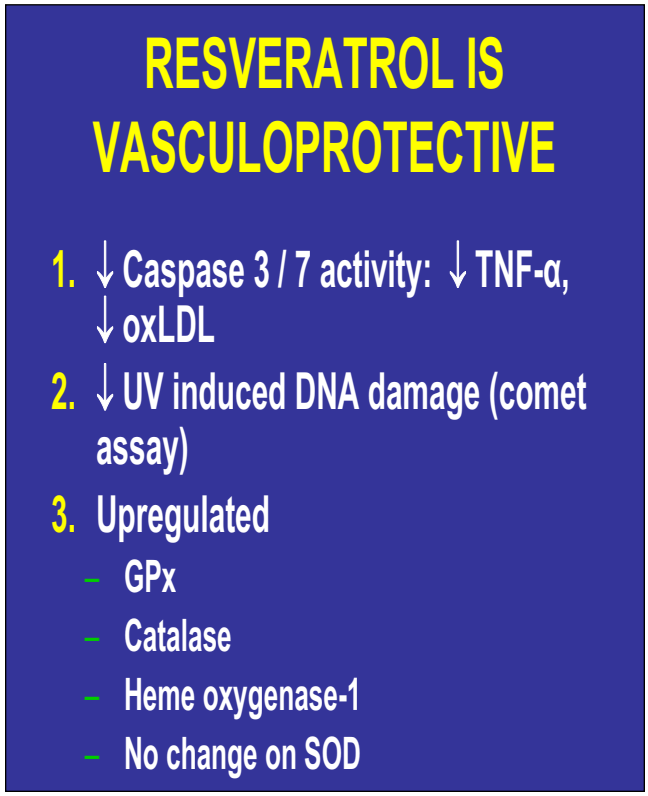
Figure 3A.



increased mitochondrial number, reduced inflammation and improved motor function.[2] The effective daily dose of resveratrol in animals is 22 mg/kg of body weight (the equivalent of 100 liters of red wine in humans!). The effective dose of resveratrol in humans is unknown, but is probably somewhere in the range of 200 - 400 mg/day.

Resveratrol has also been shown in numerous trials to be vasculoprotective, with beneficial effects including a decrease in endothelial cell death, platelet aggregation, vascular smooth muscle proliferation, endothelial activation, and monocyte adhesion, coupled with an increase in ROS scavenging and vascular oxidative stress resistance.

Figure 4.



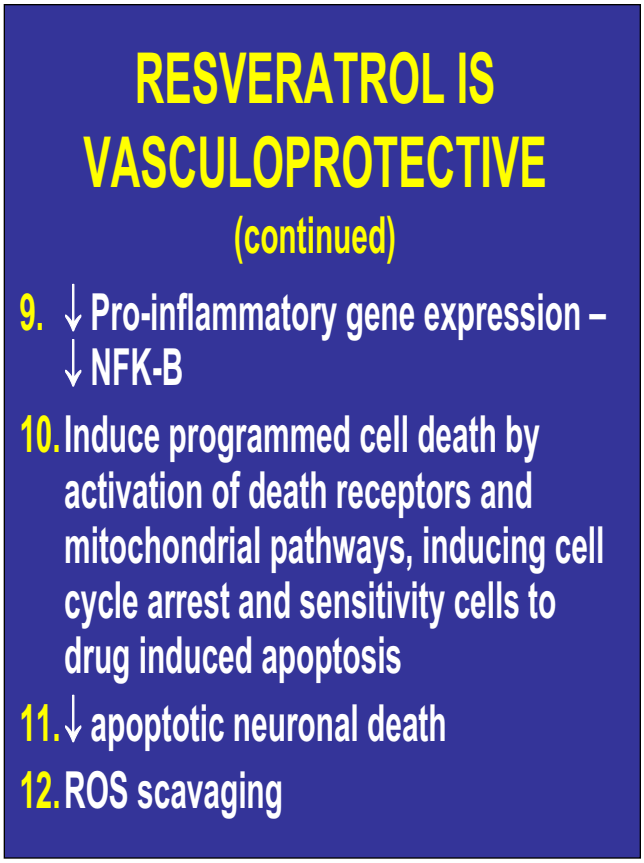
TREATMENT SUMMARY

To slow the aging process, protect mitochondrial DNA from damage, increase telomere length and protect the telomere from undue shortening, the daily guidelines found in figures 5-6A are recommended by Dr. Houston.

REFERENCES:

1. Civitarese AE, Carling S, Heilbronn LK, et al. Calorie restriction increases muscle mitochondrial biogenesis in healthy humans *PloS Medicine*, 2007;4(3):e76.
2. Baur JA, Pearson KJ, Price NL, et al. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006; 444(7117): 337-42.

Figure 4A.



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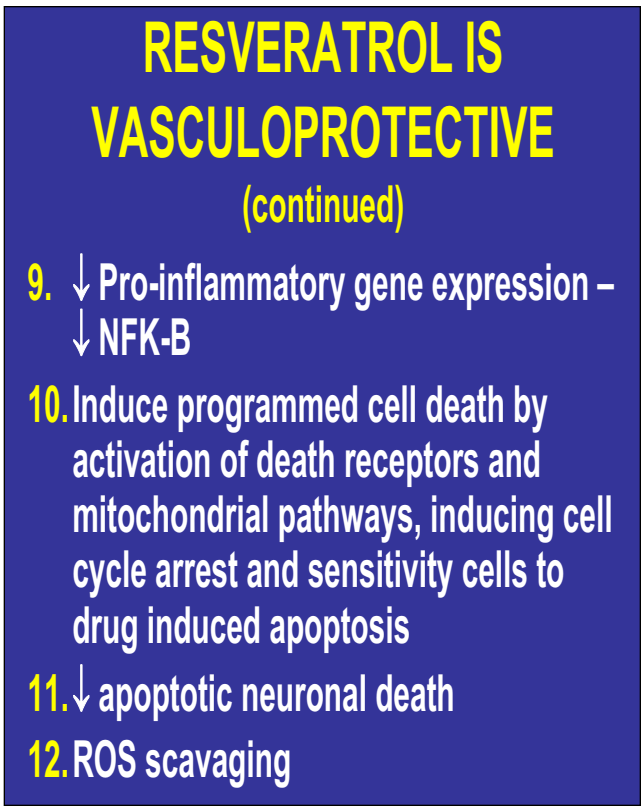

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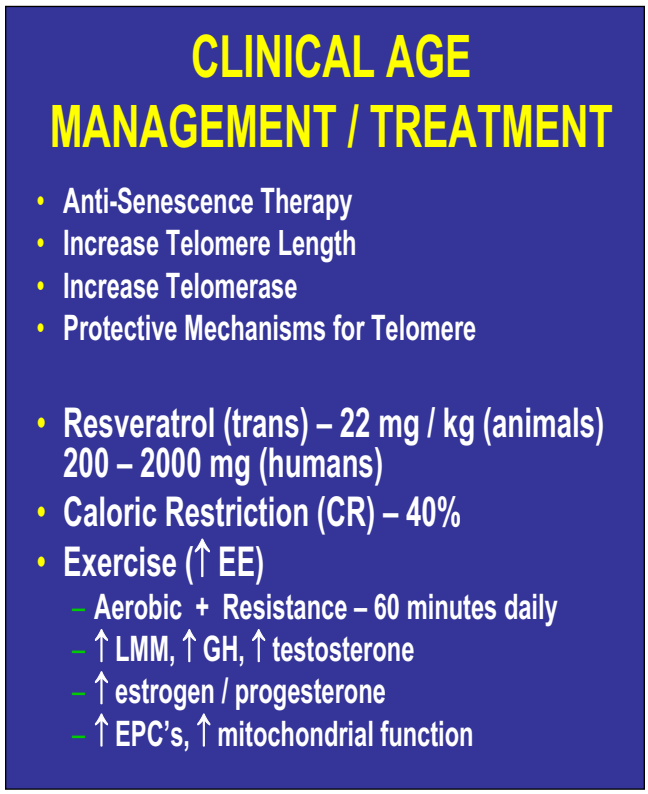

Figure 5A.

Figure 6.

Figure 6A.

Nutritional Support for Adult Stem Cells: Guidelines for the Clinical Practice

Proceedings Report from the American Nutraceutical Association's
Fall 2007 CME Conference held in Memphis, Tennessee

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Dr. Bickford has performed ground breaking work on the effects of fruits and vegetables that are high in antioxidants to reverse age-related deficits in learning and memory and in other biochemical and physiological markers. Her career has been dedicated to the study of aging and the role of oxidative stress. She was the President of the American Aging Association in 2002 and has served on its governing board for the past 15 years. The following report on her talk at the ANA Fall CME Conference was prepared by *JANA* Associate Editor Barry Fox, PhD.

WHAT ARE STEM CELLS?

Human stem cells (HSCs) are fundamental building blocks of the body that can divide a number of times and differentiate into many mature cell types for a variety of tissues, including bone marrow, brain, adipose and muscle. This plasticity, or pluripotency, allows stem cells to help serve as a repair system for the body.

Adult stem cells are undifferentiated cells residing among the differentiated cells found in tissues and organs, including fat, muscle, the brain and bone marrow. The stem cells reside in specific areas of the tissue and remain in a quiescent state undifferentiated until they are activated by disease or injury or required for normal tissue renewal. Then they migrate to the appropriate tissues and promote repair. Although the stem cells typically differentiate into the cell type of the surrounding tissue in which they reside – that is, stem cells in the bone marrow become blood cells, while stem cells in the brain become brain cells, etc. – however, under certain conditions it is possible for them to differentiate into other types of cells as well. For example, bone marrow stem cells can produce brain cells under specialized conditions.

Evidence that stem cells can migrate to the site of injury and differentiate is based on studies using Green Fluorescent Protein (GFP) mice. These mice are ideal for this type of study because their cells fluoresce green, allowing them to be traced inside the body. If cells from the bone marrow of GFP mice are transplanted into a control host and certain tissues in the host are then deliberately injured, you can easily track the GPF bone marrow cells as they move to the injury site, differentiate into the appropriate type of cell, and become incorporated into the surrounding tissue.

In one such study,[1] transplanted bone marrow cells migrated to the host pancreas and differentiated into islet cells.

It might be thought that the presence of this "stem cell repair system" would prevent the development of many of the ailments associated with aging. Unfortunately, this does not appear to be the case. Indicators of aging, such as markers of oxidative stress and inflammation, increase with the passing years. A reduction in the ability of body tissues to regenerate, and an increase in degenerative processes are

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also apparent. These changes affect the body and the mind in challenging ways. (Figure 2)

- Concomitantly, as aging occurs the number of adult stem cells decreases and the cells lose their ability to help maintain optimal health (figures 3A, 3B). Some of the changes include:
- The reduced proliferation of bone marrow stem cells. Bone marrow cells transplanted from aged hosts are not as successful as those from younger hosts.[2,3]
- In addition, a reduced proliferation of brain progenitors is observed.[4]
- More over, there is a reduced proliferative capacity of muscle stem cells (satellite cells), which may underlie some aspects of reduced muscle repair in the elderly and those with sarcopenia.[5]

A number of factors may contribute to the age-related decrease in stem cell regenerative capacity, including programmed senescence and various environmental influences, including elevated glucose levels and exposure to alcohol.

The decline in the proliferation and abilities of adult stem cells to differentiate, which leaves the body increasingly exposed to injury and other health-related issues, raises an intriguing question: Is aging a stem cell disease?

This question gives rise to a second question: Can nutritional supplementation increase the proliferation of stem cells in the aged? If so, which supplements, either individual or in combination, have beneficial effects?

It is known that nutrition plays a role in regulating oxidative stress and inflammation. For example, adding spirulina, apple and spinach to the diet of test animals improved norepinephrine levels and reduced pro-inflammatory substances, allowing for an improvement in behavioral functioning (Figure 4).

Figure 1. Stem cells have been used in transplants for 30 years.

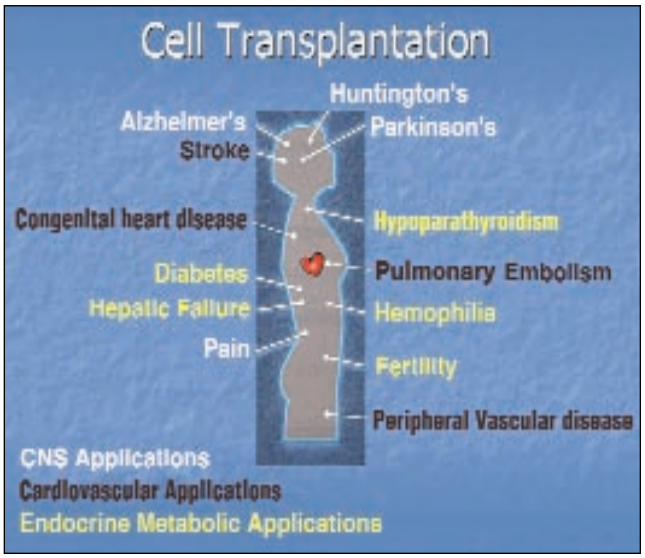


Figure 2.

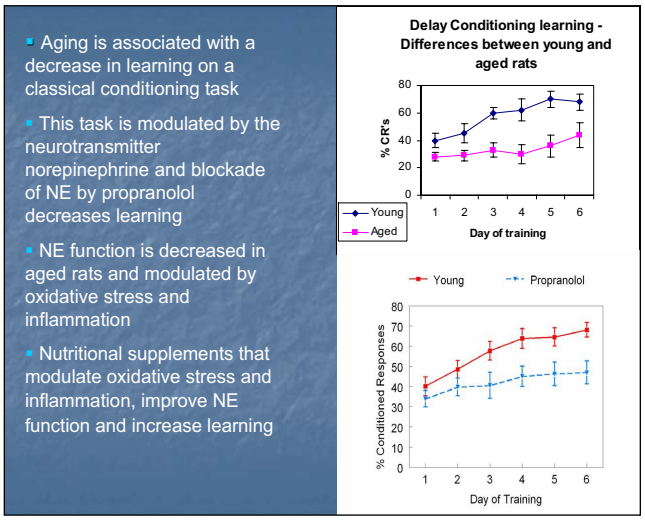


Figure 3A.

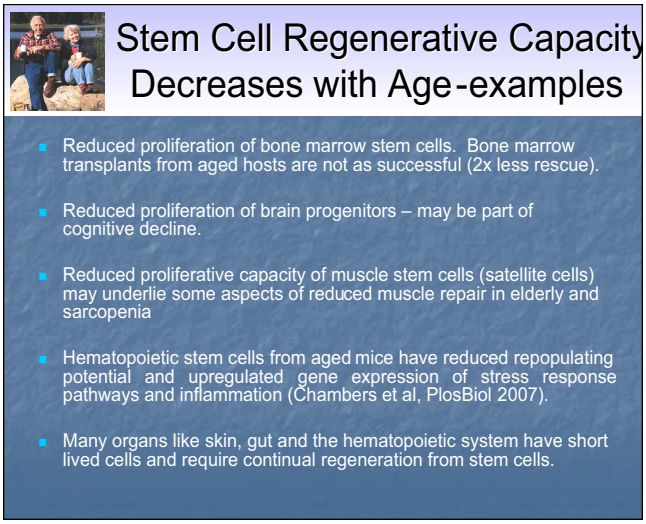
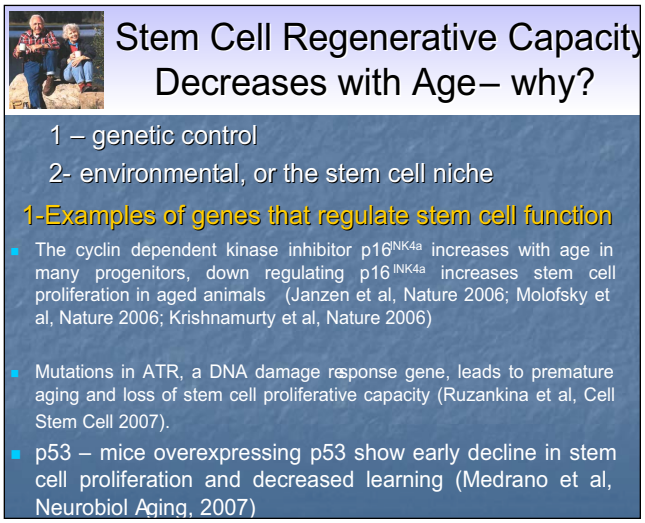


Figure 3B.



Studies suggest that spirulina may be helpful in suppressing inflammation and neurogenesis in the adult brain. Spirulina's beneficial effects can by correlated by noting that inflammation is detrimental to the development of neurons in the adult brain. Moreover, markers of inflammation tend to increase with age. Rao, Hattiangady, Shetty.[11] demonstrated in rats the number of newly-generated brain cells decreases with age. This is tracked by measuring the decline in the number of doublecortin+ cells, a marker of newly-generated neurons. However, supplementing the diet with spirulina can reduce markers in inflammation. (Figure 5.)

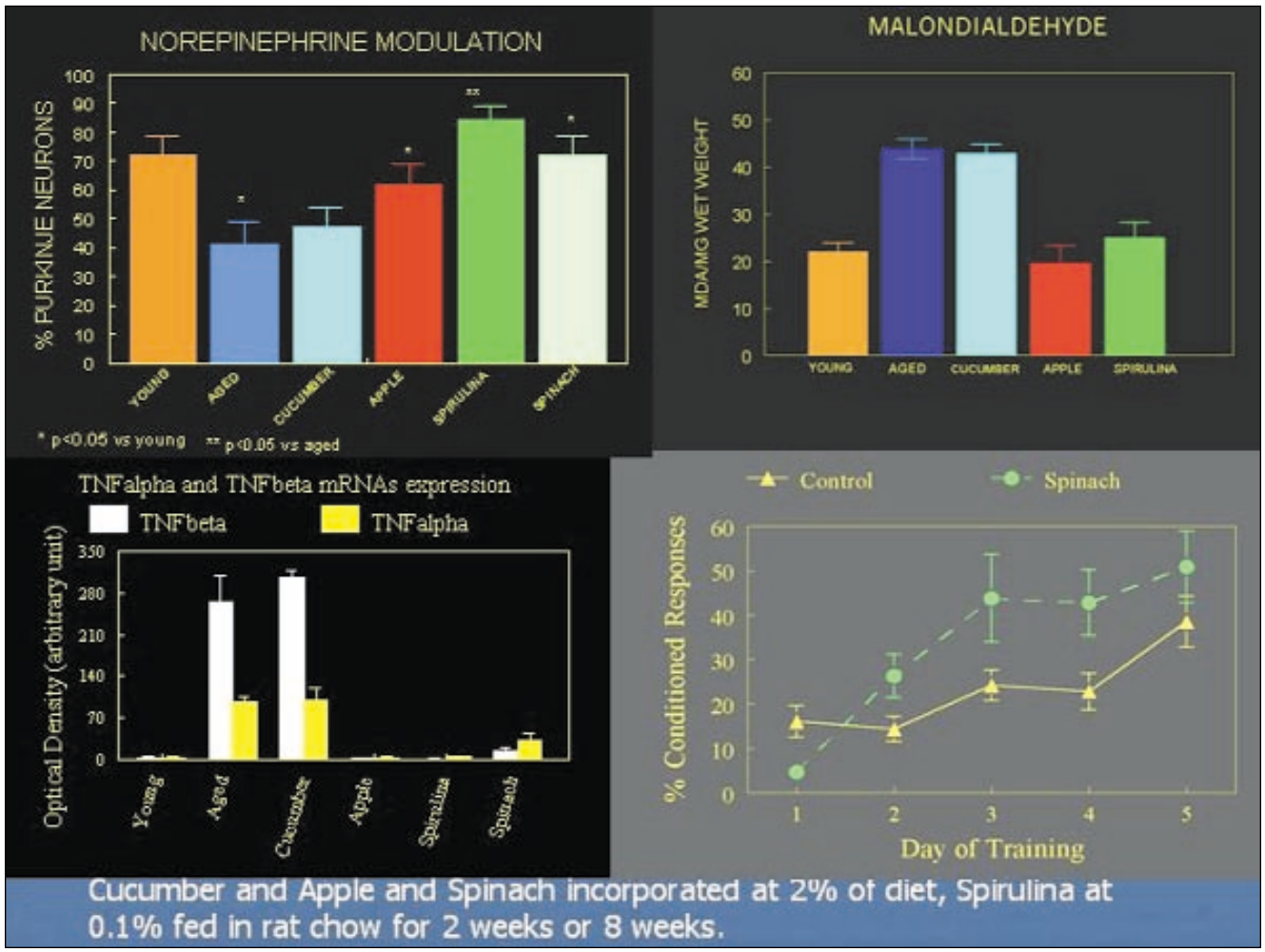
Blueberry has also proven to be beneficial. Researchers have experimented with the transplantation of neural tissue to cells that were damaged or destroyed by neurodegenerative disease or brain injury. Unfortunately, transplanted tissues have poor survival prospects, especially if the recipients are aged. A 2005 study found that fetal hippocampal transplants to middle-aged recipient animals had reduced growth and compromised tissue organization, compared to similar transplants in young animals.[6] But when the middle-aged animals were fed a diet supplemented with 2% blueberry extract, transplant growth and cellular organization was comparable to that seen with the young recipients. (Figure 6.) Blueberry may exert its beneficial effects by increasing neural stem cell proliferation, as more stem cells are observed in the transplants grown in blueberry-fed aged animals. The effect is at least in part mediated by increasing stem cell growth.

A NUTRITIONAL APPROACH TO IMPROVING STEM CELL FUNCTION

Numerous natural compounds have been found to promote healing, but it was not known whether they exerted their beneficial effects by stimulating stem cells. In a 2006 study, Dr. Bickford and her colleagues examined the effect of selected natural compounds on the proliferation of human bone marrow and CD34(+) and CD133(+) cells.[7] They found that blueberry, green tea, catechin, carnosine and vitamin D(3) can promote healing by interacting with stem cell populations.(Figure 7).

Dr. Bickford and her colleagues have formulated a combination of these nutraceuticals called NT-020. A 2007

Figure 4.



study conducted by Dr. Bickford and colleagues found that NT-020 protected bone marrow stem cells.[8] (See Figure 8) In their published report, the researchers noted that NT-020 "reduced oxidative stress-induced cell death 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 cell death. This preclinical study demonstrates that NT-020 can act to promote healing via an interaction with stem cell populations"

Dr. Bickford and her colleagues found in 2005 that feeding laboratory animals diets enriched with blueberry, spinach or spirulina reduced ischemia/reperfusion-induced apoptosis and cerebral infarction following ligation and reperfusion of the right middle cerebral artery.[9] (Figure 9)

A later study conducted by Dr. Bickford and colleagues found that the specific nutraceutical combination that comprises NT-020 had a direct, positive effect on behavioral deficits following a stroke[10] (Figure 10). One other aspect of this effect is that there was a significant increase in the numbers of neuronal stem cells found in the region of stroke damage. This indicates that the NT-020 might be acting by increasing the recruitment and function of neural precursors following brain injury. Furthermore, there is a synergistic effect of combining these ingredients together as the dose needed to achieve the protection from stroke is 100 times lower in the combined NT-020 compared to individual ingredients given by themselves.

These and other studies suggest that specific nutraceuticals, and the nutraceutical combination NT-020, can help protect the brain against injury by interacting with stem cells, among other actions.

Dr. Bickford summed up her presentation by reviewing the following key points:

- Aging is associated with decreased stem cell proliferative capacity; this is modulated by both intrinsic (genetic) and extrinsic (inflammation/oxidative stress/ other modulators) factors.
- Nutritional supplements offer one approach to increasing stem cell proliferation in older subjects, and ameliorating neurodegenerative disease.
- Many nutritional approaches can lead to improvements of neurodegenerative disease and may increase stem cell proliferation in animal models.
- combination of blueberry, green tea, carnosine and vitamin D3 can increase human stem cell proliferation. This formulation can also improve recovery from stroke in an animal model via an interaction with stem cell proliferation. Synergistic effects of the combination lower the doses of individual compounds needed to produce the positive effects.

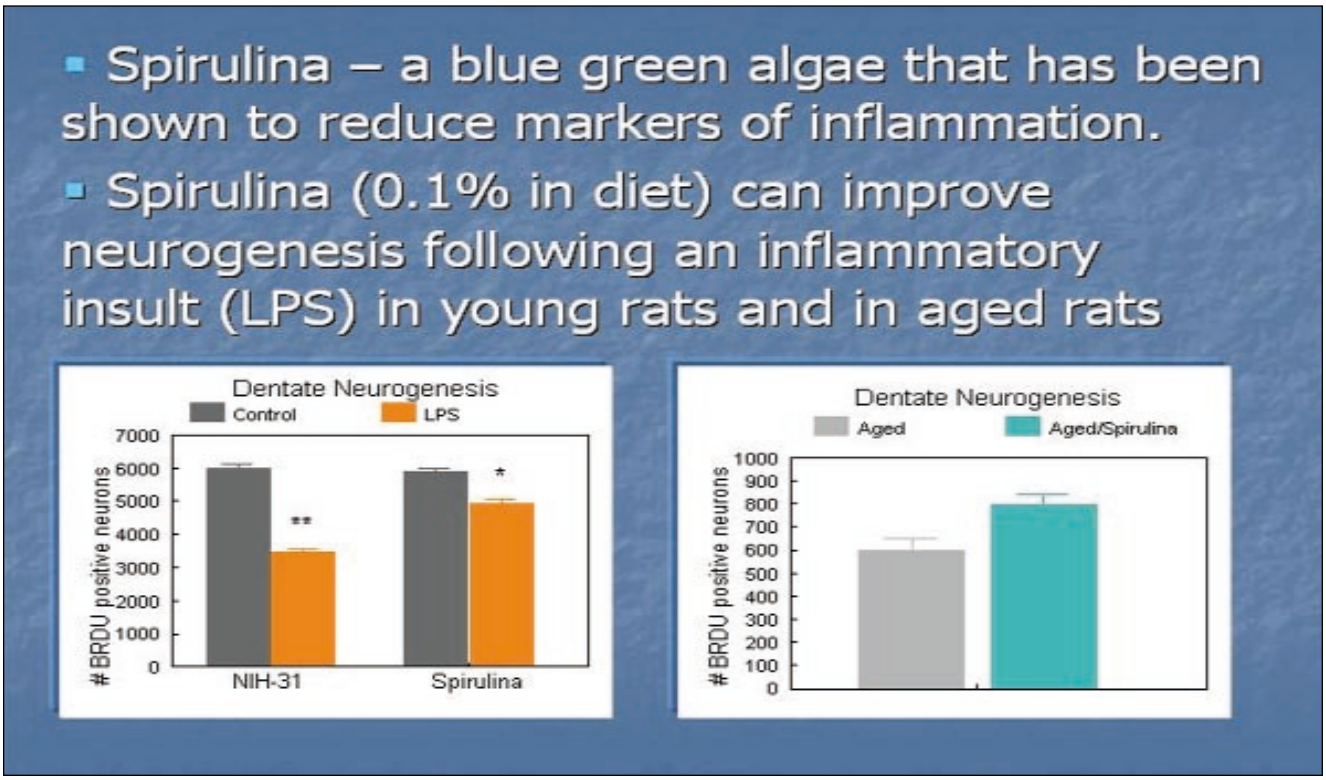
DISCLOSURE

Dr. Bickford has received Grant/Research support from the National Institute of Health (NIH) and Veteran Affairs. She is also a consultant for Saneron CCEL Therapeutics, Inc., and is the Co-Founder of Natura Therapeutics, Inc.

REFERENCES

1. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. *J Clin Invest.* 2003. March 15; 111(6): 843–850.
2. Chambers, SM, Goodell, MA. .Hematopoietic stem cell aging: wrinkles in stem cell potential. *Stem Cell.* 2007; *Rev., 3,* 201-211.
3. Chambers, SM, Shaw, CA. Gatza, C, Fisk, CJ, Donehower, LA, Goodell, M A. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS.Biol.* 2007;5:e201.
4. Shetty, AK, Hattiangady, B, Shetty, GA. Stem/progenitor cell proliferation factors FGF-2, IGF-1, and VEGF exhibit early decline during the course of aging in the hippocampus: role of astrocytes. *Glia.* 2005;51:173-186.
5. Conboy, IM, Conboy, MJ, Wagers, AJ, Girma, ER, Weissman, IL, Rando, TA. Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature.* 2005;433:760-764.
6. Willis L, Bickford P, Zaman V, Moore A, Granholm AC. Blueberry extract enhances survival of intraocular hippocampal transplants. *Cell Transplant.* 2005;14(4):213-23.
7. Bickford PC, Tan J, Shytle RD, Sanberg CD, El-Badri N, Sanberg PR. Nutraceuticals synergistically promote proliferation of human stem cells. *Stem Cells Dev* . 2006.15(1):118-23.
8. Shytle RD, Ehrhart J, Tan J, Vila J, Cole M, Sanberg CD, Sanberg PR, Bickford PC. Oxidative stress of neural, hematopoietic, and stem cells: protection by natural compounds. *Rejuvenation Res.* 2007. Jun;10(2):173-8.
9. Wang Y, Chang CF, Chou J, Chen HL, Deng X, Harvey BK, Cadet JL, Bickford PC. Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. *Exp Neurol.* 2005. May;193(1):75-84.
10. Yasuhara T, Hara K, Make M, Bickford PC, Borlongon CV. Dietary supplementation exerts neuroprotective effects in ischemic stroke model. *Cell Transplantation*. 2007;16:351-352.
11. Rao, MS, Hattiangady, B, Shetty, AK. The window and mechanisms of major age-related decline in the production of new neurons within the dentate gyrus of the hippocampus. *Aging Cell.*2006;5:545-558.

Figure 5. Villa et al. 2005, 2006



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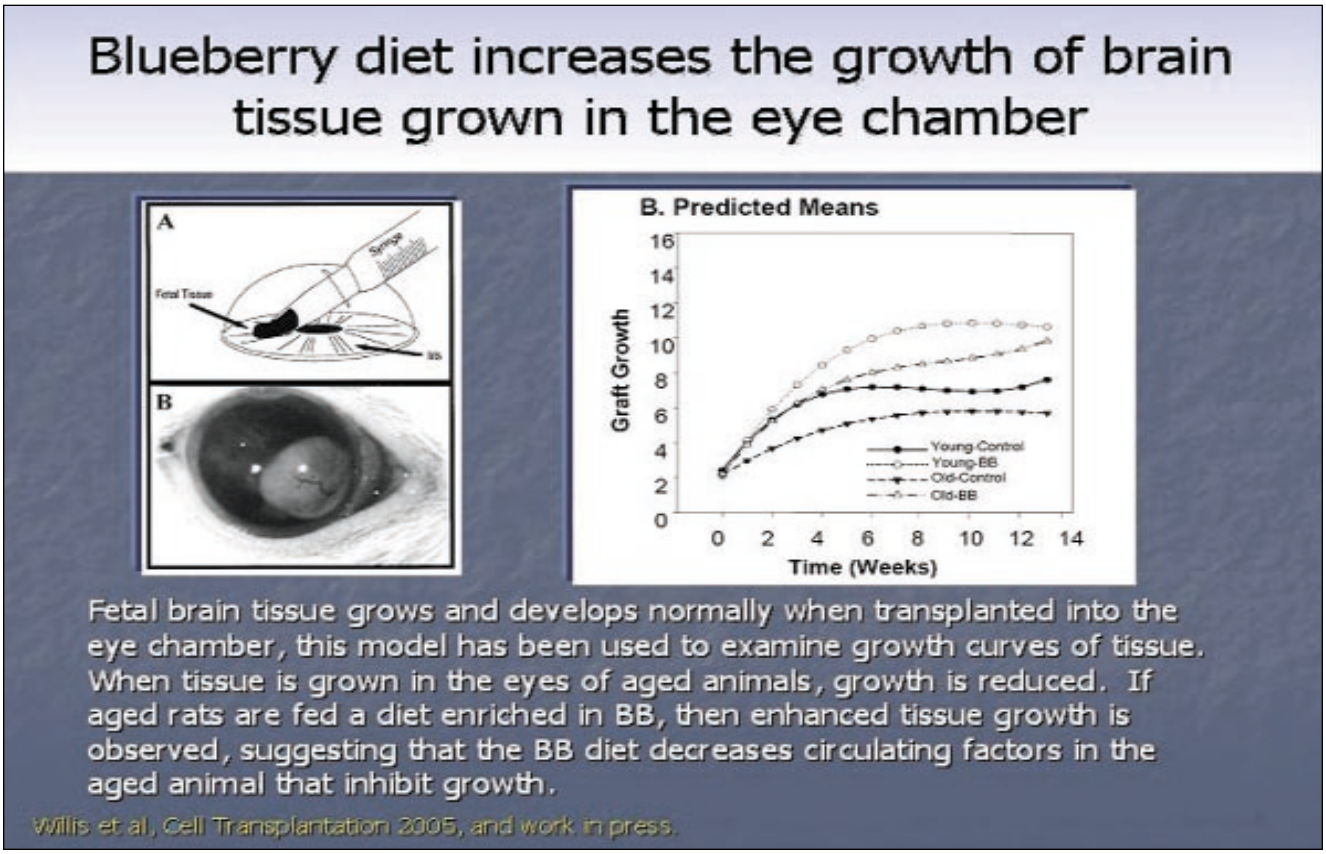

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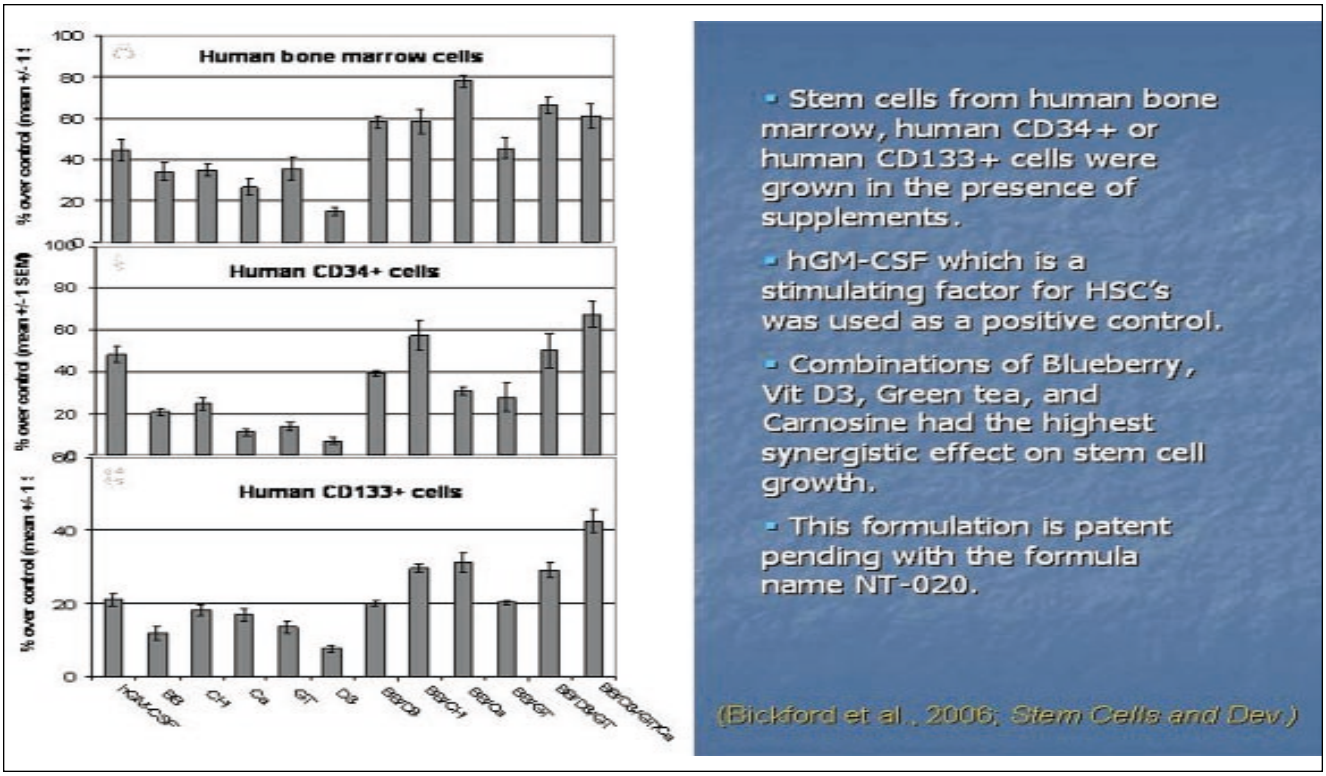

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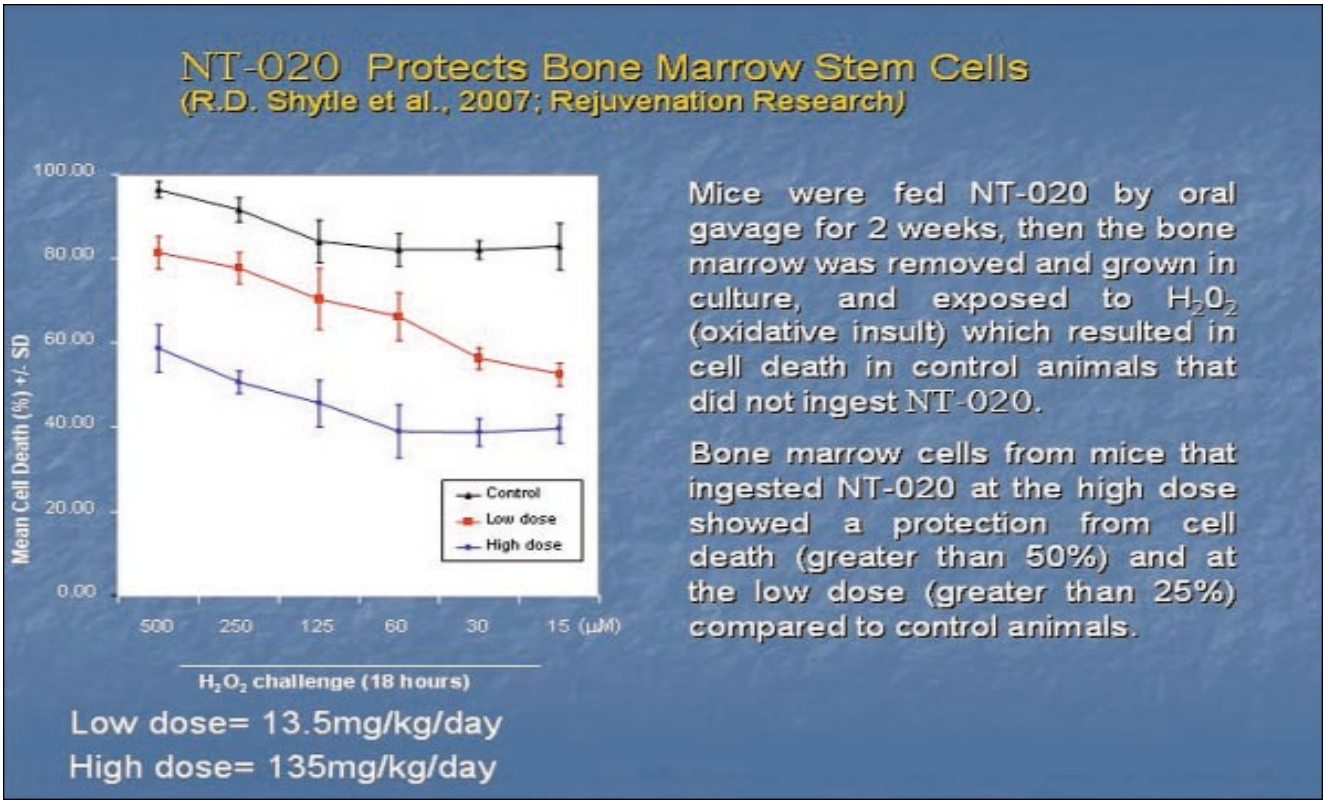

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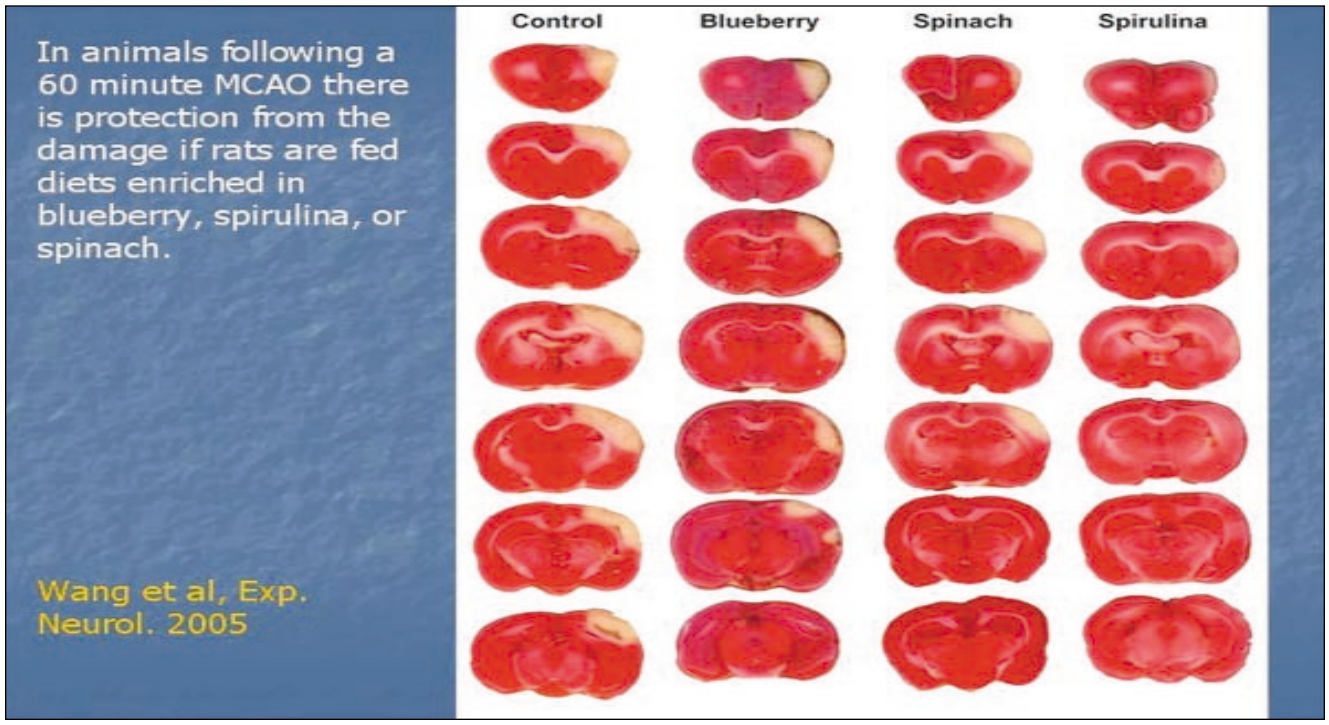

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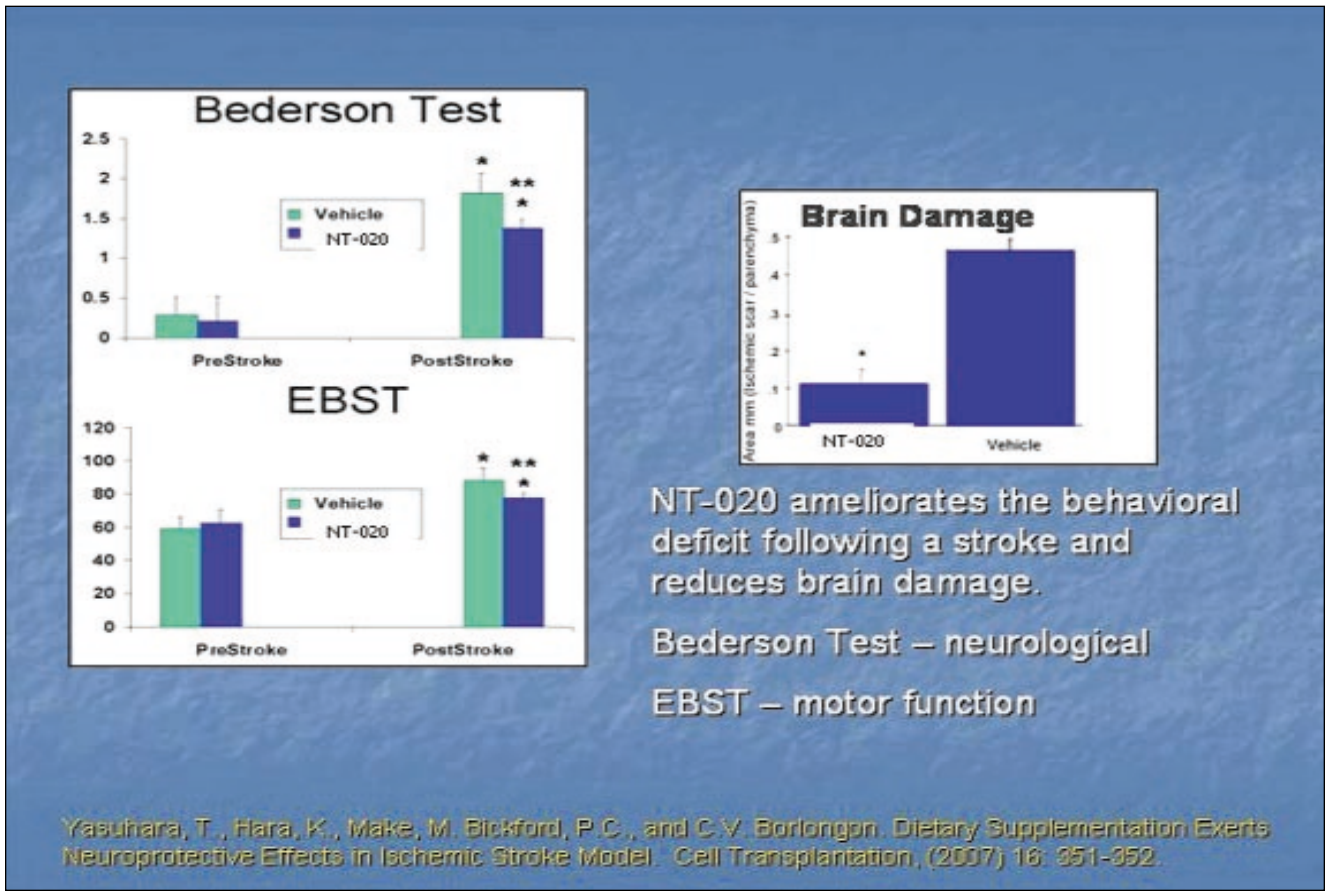

REVIEW ARTICLE

Retarding Cognitive Decline with Science-based Nutraceuticals

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ABSTRACT

A review of five natural ingredients that combine with potential synergy to address key multiple factors common to normal and accelerated cognitive decline. A combination of well- studied pleotropic ingredients has been shown to provide broad spectrum antioxidant, anti-plaque, anti-excitotoxicity and anti-inflammatory neuroprotective effects, while enhancing neurotransmitter function, cerebral energy metabolism, calcium homeostasis and mitochondrial function.

INTRODUCTION

"Cognitive vitality is essential to quality of life and survival in old age. With normal aging, cognitive changes such as slowed speed of processing are common, but cognitive decline is clearly not inevitable. Various therapeutics, including cognitive enhancers and protective agents such as antioxidants and anti-inflammatories, may eventually prove useful as adjuncts for the prevention and treatment of cognitive decline with aging." *Mayo Clinic, 2002*

Cognitive decline is a relative term. Decline rate can be age-associated, i.e., normal, or accelerated, i.e., abnormal. There are *states* of cognitive function, aka *cognitive status*, ranging from normal and low normal, e.g., AAMI, or age-associated memory impairment,[1] to the borderline transitional state of MCI, or mild cognitive impairment,[2] to Alzheimer's disease (AD). Each state has standardized memory and cognitive test score metrics allowing for clinical assessment based on percentile, or standard deviations (SD) from the general population and/or age-matched norms.

AAMI is indicated by a memory score at or below 1SD (i.e., 17th percentile) of a young, high functioning group or population, e.g., a 20-30 year old. AAMI may apply to 50% or more of the general population over age 50-60. Statistically, AAMI is three times more likely to progress to dementia. MCI is indicated by a memory score at or below 1.5SD below age-matched norms, and progresses to AD at the rate of 10-15% per year.[2] Incidence of full blown AD approaches 50% by age 80-90. Alzheimer's is now assumed to be a 20-40 year process until its obvious manifestation and clinical diagnosis. AD has been identified via PET scans in late 20 and early 30 year old brains.

The author's patented, (US Pat 5,911,581; 6,435,878) computerized and web-enabled cognitive tests of brain processing speed, clinically validated by Stanford and used by medical schools such as Scripps and UC Irvine, were administered to nearly 100,000 individuals and revealed a "normal" decline in processing speed (aka brain power) by up to 50% by age 50. A score between the 17th percentile and 33rd percentile is typically considered normal, albeit "low normal."

The bottom line is that the brain appears to be declining faster than the body. This is a gradual process and it may be important if not mandatory, in today's stressful and neurotoxic environment, to intervene and address this

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decline before it accelerates and converts from a normal to an impaired status.

One purpose of this review is to put forth a comprehensive theory, including common mechanisms of action, of the multiple causes and contributory factors to normal age associated cognitive decline, accelerated decline including low normal states, and transitional and abnormal states of impairment. A second purpose is to put forth an ideal formulation of the fewest number of natural ingredients that can optimally address the multiple mechanisms and factors underlying normal age-associated states of cognitive decline, accelerated rates and stages of decline, and abnormal states of cognitive impairment, e.g., AD.

Multifactorial Nature of Cognitive Decline

Many (if not most) states of cognitive decline from healthy normal to diseased (e.g., AD) share common underlying causative or contributory factors, principally: decreased brain blood flow (CBF), cerebral circulation (blood volume, or CBV), cerebral metabolism (CMR) and oxygen utilization (CMR02); [3,4,5,6,7] increased oxidative stress, especially within the mitochondria;[8] deficits in calcium [Ca2+] regulation,[9] cholinergic,[10] and mitochondrial function;[8,11] inflammation (immunity dysfunction); and glutamate-induced NMDA receptor over-activation (excitotoxicity) and subsequent calcium [Ca2+] mobilization and ultimate cellular overload.[9]

Many of these factors are often locked into self-reinforcing feedback loops such as amyloid-beta or Abeta=>oxidative stress=>Abeta, etc.,[12] Abeta=> decreased blood flow=> increased Abeta=> reduced blood flow=>increased oxidative stress,[10] and others.

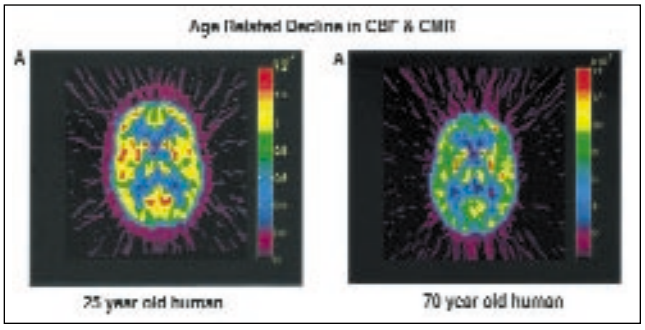
Micro array gene chip analysis of age-related genes in cognitive function has revealed downregulation of transcriptional regulators expressed by mid-life in the absence of observable cognitive deficits.[13] These transcriptional events were found to predict later cognitive impairment, therefore, changes in gene expression at the transcriptional level may significantly precede and predict later cognitive impairment. This suggests the necessity to address a number of the above referenced causal or contributory factors, especially reduced CBF, CBV, CMR, cholinergic and increased oxidative stress factors that are believed to begin relatively early in the process of accelerated brain aging, cognitive decline and ultimate impairment.

Reduced Brain Metabolism, Blood Flow and Volume, or Cerebral Circulation

General brain aging and cognitive decline, as well as states of impairment, are accompanied by reduction in cerebral vascular blood flow (CBF) and metabolism (CMR).[3,4] Regional cerebral blood flow (CBF) and cerebral blood volume (CBV, aka cerebral circulation), and cerebral oxygen utilization (CMRO2) decline approximately 5% per decade,[7] possibly exceeding that rate in accelerated brain aging. In addition, reduced brain blood flow and cerebral circulation directly impact brain metabolism and energy dynamics. Impaired brain energy production may drive AD pathogenesis leading to Abeta overproduction,[14] a process representing one of the earliest pathogenic events in AD and possibly akin to the plaque accumulation process in atherosclerosis.

Cerebral blood flow and volume (circulation), or CBF&V, can be compromised by a number of factors, including age, reduced capillary elasticity and vasodilatory capacity, cholinergic deficits, vascular lesions and plaques, oxidative stress, inflammation, stress (cortisol) and others, thereby reducing oxygen and glucose supply to the brain, as well as impairing glucose and oxygen uptake and utilization.[5] This leads to further oxidative stress-induced energy deficits, vascular insults and blood flow decline resulting in an eventual neurodegenerative cascade effect.[14] Reduced blood flow can also promote the vascular and neuronal deposition of Abeta plaques, which increase oxidative stress and in turn, render neurons significantly more susceptible to the damaging effects of reduced blood flow.[10] Reduced brain blood flow can also compromise cholinergic function, a leading theory of AD, and cholinergic dysfunction can further compromise blood flow.[10]

The following PET scans show the dramatic age-related difference in cerebral blood flow and metabolism.



Reference: Bentourkia M., et al., (2000) Comparison of regional cerebral blood flow and glucose metabolism in the normal brain: effect of aging. *Journal of the Neurological Sciences.*181;19-28

CHOLINERGIC DYSFUNCTION

The prevailing conventional wisdom regarding the cause of AD is the "cholinergic dysfunction" theory. (10) In recognition of the key role of impaired cholinergic function in cognitive impairment, the first four FDA approved drugs for Alzheimer's, i.e., tacrine, donepezil, rivastigmine and galantamine, were based on improving levels of acetylcholine (ACh) by inhibiting the enzyme acetylcholinesterase (AChE). AChE breaks down and thus reduces the synaptic levels of acetylcholine. These pharmaceutical agents along with a most promising herbal agent,

huperzine A, are collectively known as acetylcholinesterase inhibitors (AChEIs) and are used to slow down the rate of cognitive decline.

Cholinergic deficits may be one of the earliest contributors to mild forms of cognitive decline, such as AAMI. In fact, the acetylcholinesterase inhibitor, donepezil (Aricept), was successfully used to retard memory decline in airline pilots with AAMI.[15] Cholinergic enhancement may also help normal individuals as evidenced by use of the plant based cholinesterase inhibitor, huperzine A, to improve learning and memory in university students.[16]

Cholinergic deficits may promote the vascular and neuronal deposition of amyloid beta (Abeta) plaques, which in turn contribute to further chronic hypoperfusion.[10] Lower ACh levels have been linked to decreased cerebral vascular blood flow as well as an increase in inflammation, including microglial activation of pro-inflammatory cytokines, now believed to contribute to both the formation and/or support of Abeta plaques, a hallmark of AD pathology. ACh levels and cholinergic deficits also coincide with the level of impairment or cognitive status (e.g., MCI, AD).

CEREBRAL ENERGY METABOLISM

Cerebral glucose metabolism (CMR) is reduced in preclinical AD, suggesting that impaired energy production may be an early pathologic event in AD, as well as in the earlier stages of accelerated and even normal age-associated cognitive decline. CMR may be one of the earliest indicators, if not causative trigger factors, in the initial stages of accelerated decline. The question is, does decreased CBF&V reduce CMR, or vice versa?

Research has shown that experimentally induced impairment of energy production in the brain increases Beta-secretase [beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1)], a key rate-limiting enzyme for the production of beta-amyloid (Abeta) peptide, which is directly involved in the pathogenesis of Alzheimer's disease. Therefore, a reduction in cerebral glucose metabolism may drive AD pathogenesis by elevating BACE1 levels and activity, which in turn leads to Abeta overproduction and neuro-degeneration.[14] Cerebral energy deficits have also been shown to lead to an increase in both reactive oxygen and nitrite radicals, such as the highly neuro-toxic peroxynitrites, and have been widely implicated in oxidative neuronal apoptosis. Lipid peroxidation and peroxynitrites inflict significant damage to cellular proteins, lipids and nucleic acids. Thus, cerebral energy enhancers such as vinpocetine and acetyl-l-carnitine combined with antioxidants specific to hydroxyl radicals, peroxynitrites and lipid peroxidation (e.g., vinpocetine, acetyl-l-carnitine, alpha-lipoic acid, Rhodiola, ginkgo and huperzine A) might contribute significantly to prevent and retard the accelerated rate of cognitive decline.

OXIDATIVE STRESS

Oxidative stress may represent one of the earliest and yet most pervasive and ubiquitous contributing factors to neuro-cognitive decline and impairment. One reason is because a multitude of factors contribute to oxidative stress, including reduced brain blood flow, reduced oxygen and glucose metabolism, mitochondrial dysfunction, hypercortisolemia ("stress"), brain plaques (e.g., Abeta, AGEs, calcium, cholesterol, et al.) and inflammation.[10,12]

Natural agents that address oxidative stress by conferring neuroprotective antioxidant and anti-inflammatory effects within the cellular cytoplasm and mitochondria have a positive effect in reversing or at least retarding brain aging and consequent decline in cognitive function. Antioxidants such as acetyl-l-carnitine, alpha-lipoic acid, Rhodiola, vinpocetine and huperzine A, and especially the combination of the five, scavenge extra-and intra-cellular reactive oxygen and nitrogen species, including super oxide, hydroxyl and peroxyl radicals, hydrogen peroxide, nitric oxide and peroxynitrite, and may offer ideal synergy for the prevention or amelioration of cognitive decline.

MITOCHONDRIAL DYSFUNCTION

Mitochondria produce 80-90% of the brain and body's primary energy source, ATP (adenosine triphosphate). Mitochondrial function also decays with cellular aging and is a leading theory of aging.[8,11,17] Aging mitochondria membranes lose their membrane potential resulting in depolarization, loss in ATP and increase in ROS generation and cell death. One primary cause is the significant (>50%) reduction in membrane cardiolipin levels, a key lipid that is essential in maintaining adequate (electric) membrane potentials.

Acetyl-l-carnitine has been shown to significantly improve age-associated decline in cardiolipin.

Age-associated accumulation of oxidative damage to mitochondria protein, lipid and nucleic acid leads to eventual neuronal and cognitive dysfunction. Oxidative damage to nucleic acids occurs predominantly in RNA. Dietary administration of nutraceutical ingredients, acetyl-l-carnitine and alpha-lipoic acid has been shown to significantly reduce oxidized mitochondrial RNA, as well as reverse age-associated mitochondrial structural decay.[8,17] Acetyl-l-carnitine and vinpocetine have also been shown to improve lipid, oxygen and glucose delivery for enhanced energy metabolism and generation of ATP.[17,18]

GLUTAMATE-CALCIUM NEUROTOXIC EXCITOTOXICITY

Glutamate is the major excitatory neurotransmitter in the brain. Glutamate receptors, including sub-type NMDA (N-methyl-D-aspartic acid) and AMPA receptors, are prone to excessive excitability, especially within an environment

of reduced blood flow, increased energy deficits and oxidative stress. Glutamate-induced NMDA and possibly AMPA receptor over stimulation drive cellular calcium destabilization, mobilization and subsequent excessive cellular influx and overload. This can especially impair NMDA function within the hippocampus, critical to long- term memory storage. Most seriously, cellular calcium overload can depolarize the mitochondrial membrane, causing impaired ATP synthesis, reduced energy metabolism and increased transmembrane leakage of harmful metabolites of respiration, including over-oxidized enzymes and other reactive oxygen species, ultimately leading to cell death. Perhaps uniquely, acetyl-l-carnitine, huperzine A, vinpocetine, and especially the synergy of the three, downregulate glutamate-induced excitotoxicity by acting as NMDA and AMPA receptor antagonists, thus exerting a major neuroprotective effect and re-normalizing cerebral vascular, cellular and intra-cellular calcium regulation.[4,19,20,21,22] Additionally, vinpocetine supports calcium efflux from cellular cytoplasm and mitochondrial compartments, protecting the mitochondria membrane from calcium uptake and consequent depolarization and death.[23]

COGNITIVE SUPPORT WITH NUTRACEUTICALS

Enhancement of cognitive function in normal and abnormal states has been achieved via pharmaceutical and natural agents addressing: CBF&V enhancement (vinpocetine ginkgo, acetyl-l-carnitine); CMR and CMR02 enhancement (vinpocetine, acetyl-l-carnitine); cholinergic system enhancement via acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine, huperzine A); cholinergic agonists (galantamine, huperzine A); choline donors and ACh precursors (Alpha-GPC, CDP-choline, phosphatidyl choline, choline chloride, citrate and bitartrate); and acetylcholine synthesis enhancers (acetyl-l-carnitine); catecholaminergic system, dopamine and norepinephrine enhancers (methylphenidate, selegilene, Rhodiola, huperzine A, acetyl-l-carnitine and vinpocetine); glutaminergic system, NMDA receptor antagonists (memantine, huperzine A); and antioxidants, anti-inflammatory agents and enzymes (alpha-lipoic acid, acetyl-l-carnitine, ginkgo, Rhodiola, vinpocetine, huperzine A, curcumin, ibuprofen, vitamin E, N-acetyl-cysteine, glutathione, SOD and quercetin).

Following is a review of five natural ingredients that offer promising synergistic potential to address the multiple factors common to most stages of cognitive decline, especially the progressive states of low normally non-medical impairment, e.g., AAMI to possibly MCI and even early AD.

ACETYL-L-CARNITINE

Acetyl-l-carnitine (ALC) is a quaternary amine found in all animal tissue, especially skeletal muscle and liver. ALC functions as a cofactor involved in the transport of long chain fatty acids for oxidation in the mitochondria, thereby generating ATP (adenosine triphosphate).[8,17] ALC also reverse-transports oxidized long, intermediate and short chain lipids out of mitochondria, thus helping to clear away metabolic debris before it accumulates to overload and downregulate mitochondrial function. ALC increases intracellular levels and binding affinity of choline acetyltransferase (ChAT), the enzyme involved in acetylcholine synthesis.[8,17] Levels of ChAT are considered biomarkers of cognitive health, with lower levels typically found in cognitive impairment and correlated to the degree of impairment.

ALC enhances cerebral vascular blood flow,[25] and the functional synthesis and release of acetylcholine via donation of its acetyl moiety. Therefore, ALC helps normalize cholinergic deficits and improve cholinergic function.[26] ALC has also been shown to downregulate the formation of Abeta plaques, a hallmark of cognitive impairment, including AD.[27] ALC is a potent antioxidant[28] and scavenges some of the most neurotoxic oxyradicals, including protection against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity.[29] ALC confers a neuroprotective effect via antagonism of the NMDA receptor.[19] ALC is perhaps most highly acknowledged for its antioxidant and neuroprotective effects within the mitochondria.[8,11] ALC also buffers the brain, especially the hippocampus, against the deleterious effects of the stress hormone, cortisol, apparently by normalizing the HPA (hypothalamus-pituitary axis).[30]

ALC has been repeatedly shown to enhance memory and other cognitive functions in normal and impaired (MCI and AD) individuals.[8,26,31,32] ALC has also been shown to significantly enhance the effect of acetylcholinesterase inhibitors, including in non-responders to AChE inhibitors.[33] ALC has been shown to act as an anti-depressant.[31]

ALPHA-LIPOIC ACID

Alpha-lipoic acid (ALA), also known as alpha-lipoate and thioctic acid, is a disulfide compound and a cofactor in vital energy-producing reactions in the body. ALA is found widely in plant and animal sources. ALA is a broad spectrum antioxidant against reactive oxygen species, such as superoxide radicals, hydroxyl radicals, hypochlorous acid, peroxyl radicals and singlet oxygen.[34]

ALA is a potent, broad spectrum neuroprotective and antioxidant agent that most uniquely expresses its affects within the mitochondria. ALA revitalizes glutathione, one of the mitochondria's most important antioxidant enzymes.[35] ALA also recycles other neuroprotective and antioxidant agents, such as vitamins E and C, and CoQ10.[34]

ALA improves glucose tolerance, lowers insulin resistance, and reduces the glycation of proteins and production of advanced glycation end-product (AGE) plaques.[36] AGEs cause deleterious effects, including the generation of free

radicals and the partial blockage of micro-vessels feeding important neuro-cognitive areas and networks within the brain. ALA has been shown to reverse neuropathy and alcohol liver damage.

ALA's other mechanisms of action include: enhancing ALC's effects at mitochondrial levels;[8] re-enforcement of intra cellular and mitochondrial antioxidant and metabolic enzymes, e.g., carnitine acetyltransferase;[8,11,17] isocitrate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase, NADH-dehydrogenase and cytochrome-c-oxidase;[35] reducing neurotoxic by-products of Abeta plaque[37] inhibition of formation of β-amyloid fibrils from amyloid β-protein[38]; and activation and support of phase II detoxification enzymes.[39]

ALA has been shown to improve memory.[8] ALA alone and especially when combined with acetyl-l-carnitine has demonstrated efficacy in slowing down brain aging at the mitochondrial level, and restoring old brain cells to more youthful levels of health and function.[8,17]

RHODIOLA

Rhodiola (RHO) is an herbal supplement derived from the root of the Rhodiola rosea plant, also known as goldenroot, or roseroot. RHO appears to buffer the brain against the negative effects of stress (cortisol) and other environmental, physical and biochemical stressors such as mental overload and work fatigue.[40-42] RHO is shown to enhance function of the entire catecholamine class of neurotransmitters, bringing about neuro-endocrine balance and downregulation of cortisol. RHO is also a brain monoamine modulator within the limbic as well as the frontal and prefrontal cortex.[43] RHO improves serotonin, dopamine and norepinephrine function. It has also been shown to improve alertness, enhance mood and reduce depression via direct action on neuro-endocrine limbic centers, including amygdala and hypothalmus. RHO reduces hyper excitability of the emotional system by helping to buffer the hypothalamic-pituitary-adrenal (HPA) axis against stress-induced perturbations. Hyperexcitability undermines cognitive function and accelerates oxidative stress and other neurotoxic factors.[44]

Studies have shown RHO to improve mental performance in medical students and pilots under mental, physical and emotional stress.[40-42] RHO was recently shown to have an anti-depressant effect.[45]

VINPOCETINE

Vinpocetine (VIN; ethyl apovincaminate) is a synthetic analog of a major component of vincamine, an extract of the Periwinkle flower, vinca minor. VIN is best known as a potent vasodilator,[46,47] regional cerebral vascular and global cerebromicrovasculature blood flow enhancer.[48,49] VIN is a potent enhancer of cerebral metabolism via upregulation of glucose and oxygen utilization.[49] VIN is a broad-spectrum antioxidant and neuroprotective agent,[23] especially protecting against calcium [Ca2+] overload and neurotoxicity,[4] including the hippocampal CA1 pyramidal and NMDA receptor cells.[22] VIN works at fundamental metabolic levels within the mitochondria's electron transfer chain, specifically upregulating function at ETC complexes II, III and IV, principal sources of electron loss and generation of reactive oxygen species.[50] In addition, vinpocetine has been found to suppress cytokine production by microglia.[51]

Perhaps most unique of vinpocetine's many mechanisms of action is its ability to alter the rheological properties of red blood cells by increasing the erythrocyte's deformability.[48] VIN also decreases platelet aggregation.[52] These two actions combine to enable the blood cells to better penetrate the small, often obstructed vessels of the cerebromicrovasculature, thus delivering adequate supplies of glucose, oxygen and other energy substrates and cell nutrients for improved neuro-cognitive health and function. VIN has also been shown to facilitate the release of oxygen from hemoglobin and increase blood oxygenation.[44,53]

Vinpocetine's neuroprotective action is partially based on its inhibition of voltage-dependent sodium channels, effectively blocking intra-cellular accumulation of sodium, thus decreasing potential damage from ischemia and reperfusion, and the toxic effects of oxidative stress resulting from hypoxia and ischemia.[4,18] VIN has demonstrated an ability to attenuate the oxidative stress and metabolic dysfunction induced by amyloid-beta peptides in PC12 cells.[50]

Like Vitamin E, VIN is an effective scavenger of hydroxyl radicals. It is also able to inhibit lipid peroxidation,[54] a particularly important function since the brain is almost 70% fat, by dry weight, and lipid membranes are highly susceptible to peroxide radicals. Recently it has been shown that (E)-4-hydroxy-2-nonenal (HNE), a metabolite of lipid peroxidation, is extremely neuro-pathologic and may be a primary contributor to accelerated brain aging and neuro-cognitive dysfunction, disease and death.[55] VIN has also been shown to act as a chelating agent for calcium and aluminum in the central nervous system.[56] Vinpocetine is a cerebral vascular, cellular and mitochondrial calcium normalizing agent.[22,53]

VIN has been shown to enhance cortical levels of norepinephrine, a key neurotransmitter for alertness and long-term potentiation for enhanced memory storage. Vinpocetine has been clinically shown to improve memory in both cognitively normal and compromised or impaired groups.[57-9,60]

HUPERZINE A

Huperzine A (HUP) is a potent, reversible, selective inhibitor of AChE with similar or higher potency than donepezil.[20,61] HUP is a weak nicotinic agonist and cerebral

blood flow enhancer.[62] HUP downregulates APP processing and thus reduces generation of Abeta plaques. It also attenuates Abeta induced oxidative damage and neuronal degeneration.[20,63,64] HUP is a potent antioxidant and enhancer of NGF (nerve growth factor),[20,65] a dopamine and norepinephrine modulator,[66] and a NMDA receptor antagonist.[20,21,67] HUP also reduces deleterious lipid peroxidation and hydrogen peroxide formation.[20]

HUP has demonstrated memory enhancing effects in cognitive states ranging from normal to impaired, including AD.[16,20,21,65]

SUMMARY

In light of the aging brain suffering from one or more, and likely many of the above causal and contributory factors to accelerated cognitive decline, it eventually reaches a threshold level of compromise, injury and toxicity.[24] Consequently, the seemingly slightest stressor, such as a head injury, sickness or infection, acute anxiety, Transient Ischemic Attack (TIA), chronic or acute alcohol intake, a traumatic stress event, a disease condition or physical injury, can trigger a breakdown cascade with consequent acceleration of cognitive decline and progression to and through states of cognitive impairment culminating in dementias such as AD.

It would therefore seem prudent to address (intervene) and support the underlying multiple mechanisms of the aging brain as early as possible, before cumulative factors combine to overload the system and trigger breakdowns culminating in the acceleration of normal cognitive decline, impairment and eventual dementia. The combination of nutraceuticals covered herein offer a comprehensive, broad spectrum and synergistic approach to retard cognitive decline.

Mechanisms of Action of Proposed Ingredients & Commonly Used Drugs

		ALC	ALA	VIN	HUP	RHO	GIN	DON	MEM
INCREASED	Cerebral Blood Flow	*		**	*		*		
	Cerebral Blood Volume (circulation)	*		**			*		
	Oxygen Utilization			**					
	rCMR	*		**					
	Acetylcholine	**			**	*	*	**	
	Dopamine	*			*	**			
	Norepinephrine			*	*	**			
	Serotonin					*	*		
	Mitochondria Fnx	**	**	*	*	*			
	Antioxidant Defense	**	**	*	*	*	**		
	Ca Balance			**					
DECREASED	Glutamate Excitotoxicity			*	*				**
	Inflammation		*	*	*	*	*		
	Abeta Neurotoxicity	*	*	*			*		

ALC = Acetyl-l-carnitine
ALA = Alpha-lipoic Acid
RHO = Rhodiola
GIN = Ginkgo Biloba
VIN = Vinpocetine
HUP = Huperzine A
DON = Donepezil HCL (Aricept™)
MEM = Memantine HCL (Nemanda™)

REFERENCES

1. Crook TH, Ferris SH. Age associated memory impairment. *BMJ.* 1992;304;714.
2. Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. *Arch Neurol*. 1999;56(6):760.
3. Martin AJ, Friston KJ, Colebatch JG, Frackowiak RS. Decreases in regional cerebral blood flow with normal aging. *J Cereb Blood Flow Metab.* 1991;(4):684-689.
4. Hadjiev D. Asymptomatic ischemic cerebrovascular disorders and neuroprotection with vinpocetine. The asymptomatic ischemic cerebrovascular disorder (AICVD) is an early manifestation of cerebrovascular disease. *Ideggyogy Sz.* 2003;56(5-6):166-172.
5. Hoyer S, Oesterreich K, Wagner O. Glucose metabolism as the site of the primary abnormality in early-onset dementia of Alzheimer type? *J. Neurol.* 1998;235:143-148.
6. Pettegrew JW, Panchalingam K, Klunk WE, Mcclure RJ, Muenz LR. Alterations of cerebral metabolism in probable Alzheimer's disease: a preliminary study. *Neurobiol Aging.* 1994;15,117:132.
7. Leenders KL, Perani, D, Lammertsma AA, Heather JD, Buckingham P, Jones TR, Healy MJ, Gibbs JM, Wise RJS, Hatazawa J, Herold S, Beaney RP, Brooks, DJ, Spinks T, Rhodes C, Frackowiak RSJ. Cerebral Blood Flow, Blood Volume and Oxygen Utilization. *Brain.* 1990;1131:27-47.
8. Liu J, Killilea DW, Ames B. Age-associated mitochondrial oxidative decay: Improvement of carnitine acetyltransferase substrate-binding affinity and activity in brain by feeding old rats acetyl-L- carnitine and/or R-lipoic acid. *PNAS*. 2002;99(4):1876-1881.
9. Khachaturian ZS. Calcium hypothesis of Alzheimer's disease and brain aging. *Ann N Y Acad Sci.* 1994;747:1-11.
10. Claassen J, Janson R. Cholinergically Mediated Augmentation of Cerebral Perfusion in Alzheimer's Disease and Related Cognitive Disorders: The Cholinergic-Vascular Hypothesis. *J Gerontol A Biol Sci Med Sci.* 2006;61:267-271.
11. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci U S A*.1994;91(23):10771–10778.
12. Reddy PH. Amyloid precursor protein-mediated free radicals and oxidative damage: Implications for the development and progression of Alzheimer's disease. *Jnl Neurochemistry.* 2006;96:1-13.
13. Blalock EM, Chen KC, Sharrow K, Herman JP, Porter NM, Foster TC, Landfield PW. Gene microarrays in hippocampal aging: statistical profiling identifies novel processes correlated with cognitive impairment. *J Neurosci.* 2003 May 1;23(9):3807-19.
14. Velliquette RA, O'Connor T, Vassar R. Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. J Neurosci. 2005;23;25(47):10874-83.
15. Yesavage JA, Mumenthaler MS, Taylor JL, Friedman L, O'Hara R, Sheikh J, Tinklenberg J and Whitehouse. Donepezil and flight simulator performance: Effects on retention of complex skills. *Neurology.* 2002;59:123-125.
16. Sun QQ, Xu SS, Pan JL, Guo HM, Cao WQ. Huperzine-A capsules enhance memory and learning performance in 34 pairs of matched adolescent students. *Acta Pharmacol Sin.* 1999;20(7):601-603.
17. Hagen TM, Ingersoll RTM, Wehr C, Lykkesfeldt J, Vinarsky V, Bartholomew JC, Song M-H and Ames BN. Acetyl-L-carnitine fed to old rats partially restores mitochondrial function and ambulatory activity. *Proc Natl Acad Sci* 1998;95:62-66.
18. Gabryel B, Adamek M, Pudelko A, Malecki A, Trzeciak HI. Piracetam and vinpocetine exert cytoprotective activity and prevent apoptosis of astrocytes in vitro in hypoxia and reoxygenation. *Neurotoxicology*. 2002;23(1):19-31.
19. Forloni G, Angeretti N, Smiroldo S. Neuroprotective activity of acetyl-L-carnitine: studies in vitro. *J Neurosci Res* (USA). 1994;37(1):92-96.
20. Wang R, Yan H, Tang XC. Progress in studies of Huperzine A, a natural cholinesterase inhibitor from Chinese herbal medicine. *Acta Pharmacol Sin*. 2006;27(1):1-26
21. Ved HS, Koenig ML, Dave JR, Doctor BP. Huperzine-A, a potential therapeutic agent for dementia, reduces neuronal cell death caused by glutamine. *Neurorepor*. 1997;8(4):963-968.
22. Zelles T, Franklin L, Koncz I, Lendvai B, Zsilla G. The nootropic drug vinpocetine inhibits veratridine-induced [Ca2+]i increase in rat hippocampal CA1 pyramidal cells. *Neurochem-Res*. 2001;26(8-9):1095-1100.
23. Pereira C, Agostinho P, Moreira PI, Duarte AI, Santos MS, Oliveira CR. Neuroprotection strategies: effect of vinpocetine in vitro oxidative stress models. *Acta Med Port.* 2003;16(6):401-406.
24. Brewer GJ. Neuronal plasticity and stressor toxicity during aging. *Experimental Gerontology.* 2000;35:1165-1183.
25. Postiglione A, Soricelli A, Cicerano U. Effect of acute administration of L-actyl-carnitine on cerebral blood

flow in patients with chronic cerebral infarct. *Pharmacol Res.* 1991;23:241-246.

26. Ando S, Tadenuma T, Tanaka Y, Fukui F, Kobayashi S. Enhancement of learning capacity and cholinergic synaptic function by carnitine in aging rats. *J Neurosci Res.* 2001;66(2):266-271.
27. Virmani MA, Caso V, Spadoni A, Rossi S, Russo F, Gaetani F. The action of acetyl-L-carnitine on the neurotoxicity evoked by amyloid fragments and peroxide on primary rat cortical neurones. *Ann NY Acad Sci*. 2001;939:162-178.
28. Rani PJA and Panneerselvam C. Carnitine as a free radical scavenger in aging. *Exp Gerontol.* 2001;36(10):1713-1726.
29. Abdul HM, Calabrese V, Calvani M, Butterfield DA. Acetyl-L-carnitine-induced up-regulation of heat shock proteins protects cortical neurons against amyloid-beta peptide 1-42-mediated oxidative stress and neurotoxicity: Implications for Alzheimer's disease. *Journal of Neuroscience Research.* 2006;84;(2):398-408.
30. Bruno G, Scaccianoce S, Bonamini M, Patacchioli FR, Cesarino F, Grassini P, Sorrentino E, Angelucci L, Lenzi GL. Acetyl-L-carnitine in Alzheimer disease: a short-term study on CSF neurotransmitters and neuropeptides. *Alzheimer Dis Assoc Disord* (USA). 1995;9(3):128-131.
31. Pettegrew JW, Levine J and McClure RJ. Acetyl-L-carnitine physical-chemical, metabolic, and therapeutic properties: relevance for its mode of action in Alzheimer's disease and geriatric depression. *Molecular Psychiatry.* 2000;5;(6);616-632.
32. Montgomery SA, Thal LJ, Amrein R. Meta-analysis of double blind randomized controlled clinical trials of acetyl-L-carnitine versus placebo in the treatment of mild cognitive impairment and mild Alzheimer's disease. *Int Clin Psychopharmacol*. 2003;18(2):61-71.
33. Bianchetti A, Rozzini R, Trabucchi M. Effects of acetyl-L-carnitine in Alzheimer"s disease patients unresponsive to acetylcholinesterase inhibitors. *Curr Med Res Opin*. 2003;19(4):350-3.
34. Packer L, Witta E, Tritschler HJ. Alpha-lipoic acid as a biological antioxidant. *Free Radical Biology and Medicine.* 1995;19(2);227-250.
35. Arivazhagan P, Ramanathan K, Panneerselvam C. Effect of DL-alpha-lipoic acid on glutathione metabolic enzymes in aged rats. *Exp-Gerontol*. 2001;37(1):81-7.
36. Thirunavukkarasu V, Anitha Nandhini AT, Anuradha CV. Lipoic acid improves glucose utilisation and prevents protein glycation and AGE formation. *Pharmazie*. 2005;60(10):772-775.
37. Zhang JM, Hu GY. Huperzine A, a nootropic alkaloid, inhibits N-methyl-D-aspartate-induced current in rat dissociated hippocampal neurons. *Neuroscience*. 2001;105 (3):663-9.
38. Ono K, Mie Yamada H, Yamada M. α-Lipoic acid exhibits anti-amyloidogenicity for β-amyloid fibrils in vitro. *Biochemical and Biophysical Research Communications*. 2006; 341(4);1046-1052.
39. Flier J, Van Muiswinkel F L, Jongenelen CA, Drukarch B. The neuroprotective antioxidant alpha-lipoic acid induces detoxication enzymes in cultured astroglial cells. *Free-Radic-Res*. 2002; 36(6):695-9.
40. Shevtsov VA, Zholus BI, Shervarly VI, Vol'skij VB; Korovin YP, Khristich MP, Roslyakova NA, Wikman GK. A randomized trial of two different doses of a SHR-5 Rhodiola rosea extract versus placebo and control of capacity for mental work. *G. Phytomedicine*. 2003;10(2-3):95-105.
41. Spasov AA, Wikman GK, Mandrikov VB, Mironova IA, Neumoin VV. A double-blind, placebo-controlled pilot study of the stimulating and adaptogenic effect of Rhodiola rosea SHR-5 extract on the fatigue of students caused by stress during an examination period with a repeated low-dose regimen. *Phytomedicine*. 2000 Apr; 7(2):85-9.
42. Darbinyan V, Kteyan A, Panossian A, Gabrielian E, Wikman G, Wagner H. Rhodiola rosea in stress induced fatigue-a double blind cross-over study of a standardized extract SHR-5 with a repeated low-dose regimen on the mental performance of healthy physicians during night duty. *Phytomedicine*, 2000;7(5):365-71.
43. Stancheva SL, Mosharrof A. Effect of the extract of Rhodiola rosea L. on the content of the brain biogenic monamines. *Med Physiol* 1987;40:85-87.

44 Saratikov A, Mar'ina TF, Fisanova LL. Effect of golden root extract on processes of serotonin synthesis in CNS. *Jnl Biological Sciences* 1978:6.

45. Darbinyan V, Aslanyan G, Amroyan E, Gabrielyan E, Malmström C, Panossian A. Clinical trial of Rhodiola rosea L. extract SHR-5 in the treatment of mild to moderate depression. *Nordic Journal of Psychiatry*. 2007;61(5)3;43-348.
46. Bonoczk P, Panczel G, Nagy Z. Vinpocetine increases cerebral blood flow and oxygenation in stroke patients: a near infrared spectroscopy and transcranial Doppler study. *Eur-J-Ultrasound.* 2002;15(1-2):85-91.
47. Gulyás B, Halldin C,Sandell J, Karlsson P, Sóvágó J, Kárpáti E, Kiss B, Vas A, Cselényi Z, Farde L PET studies on the brain uptake and regional distribution of [11C]vinpocetine in human subjects. *Acta Neurologica Scandinavica*, 2002;106(6);325-332.
48. Hayakawa M. Effect of vinpocetine on red blood cell

deformability in stroke patients. *Arzneimittelforschung*. 1992;42(4):425-427.

49. Szilágyi G, Nagy Z, Balkay L, Boros I , Emri M. Lehel., Márián T, Molnár T, Szakáll S, Trón L, Bereczki D, Csiba L Fekete I, Kerényi L, Galuska L, Varga J, Bönöczk P, Vas A, and Gulyás B. Effects of vinpocetine on cerebral blood flow and metabolism in chronic ischaemic stroke patients after a two-week long administration: A PET study. *J. Neur. Sci*.2005;229-230:275-284.
50. Pereira C, Agostinho P, Oliveira CR. Vinpocetine attenuates the metabolic dysfunction induced by amyloid beta-peptides in PC12 cells. *Free-Radic-Res*. 2000;33(5):497-506.
51. Yoshikawa M, Suzumura A, Tamaru T, Takayanagi T, Sawada M Effects of phosphodiesterase inhibitors on cytokine production by microglia. *Multiple Sclerosis.* 1999;5(2):126-133.
52. Akopov SE, Gabrielian ES. Effects of aspirin, dipyridamole, nifedipine and cavinton [Vinpocetine] which act on platelet aggregation induced by different aggregating agents alone and in combination. *Eur J Clin Pharmacol.* 1992;42:257-259.
53. Tohgi H, Sasaki K, Chiba K, Nozaki Y. Effect of vinpocetine on oxygen release of hemoglobin and erythrocyte organic polyphosphate concentrations in patients with vascular dementia of the Binswanger type. *Arzneimittelforschung.* 1990;40(6):640-643.
54. Santos MS, Duarte AI, Moreira PI, Oliveira CR. Synaptosomal response to oxidative stress: effect of vinpocetine. *Free-Radic-Res.* 2000;32(1):57-66.
55. Lovell MA, Xie C, Markesbery WR. Acrolein, a product of lipid peroxidation, inhibits glucose and glutamate uptake in primary neuronal cultures. *Free Radic Biol Med*. 2000;29(8):714-720.
56. Yasui M, Yano I, Ota K, Oshima A. Calcium, phosphorus and aluminium concentrations in the central nervous system, liver and kidney of rabbits with experimental atherosclerosis: preventive effects of vinpocetine on the deposition of these elements. *J Int Med Res*. 1990;18(2):142-152.
57. Polich J, Gloria R. Cognitive effects of a ginkgo biloba/vinpocetine compound in normal adults: systematic assessment of perception, attention and memory. *Hum Psychopharmacol*. 2001;16(5):409-416.
58. Nicholson CD. Pharmacology of nootropics and metabolically active compounds in relation to their use in dementia. Psychopharmacology. 1990;101:147-159.
59. Subhan Z, Hindmarch I. Psychopharmacological effects of vinpocetine in normal healthy volunteers. *Eur J Clin Pharmacol*. 1985;28:567-571.
60. Wollschlaeger B. Efficacy of vinpocetine in the management of cognitive impairment and memory loss. *JANA*. 2001;4:25-30.
61. Liang YQ, Tang XC. Comparative effects of huperzine A, donepezil and rivastigmine on cortical acetylcholine level and acetylcholinesterase activity in rats. *Neuroscience Letters*. 2004;361;(1-3):56-59.
62. Wang LM, Han YF, Tang XC. Huperzine A improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. *Eur J Pharmacol*. 2000;9;398(1):65-72.
63. Xiao XQ, Wang R, Tang XC. Huperzine A and tacrine attenuate-amyloid peptide-induced oxidative injury. *J Neurosci Res*. 2000;61:564-569.
64. Xiao XQ, Zhang HY, Tang XC. Huperzine A attenuates amyloid beta-peptide fragment 25-35-induced apoptosis in rat cortical neurons via inhibiting reactive oxygen species formation and caspase-3 activation. *J Neurosci Res.* 2002;67(1):30-6.
65. Zhang HY, Tang XC. Neuroprotective effects of huperzine A: new therapeutic targets for neurodegenerative disease. *Trends Pharmacol Sci*. 2006;27(12):619-25.
66. Zhu X-D, Giacobini E. Second generation cholinesterase inhibitors: Affect of (L)-huperzine-A on cortical biogenic amines. *Journal of Neuroscience Research*. 2004;41(6):828-835.
67. Gordon RK, Nigam SV, Weitz JA, Dave JR, Doctor BP, Ved HS. The NMDA receptor ion channel: a site for binding of Huperzine A. *J Appl Toxicol.* 2001;21:47-51.

META-ANALYSIS STUDY

The Effect of Vitamin C Supplementation on Blood Pressure with Hypertensive Patients: A Meta-analysis of Randomized Contolled Trials

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Key Indexing Terms: Vitamin C, ascorbic acid, blood pressure, hypertension, meta-analysis.

ABSTRACT

Objective: Hypertension is a common condition with high mortality from associated diseases. Epidemiological evidence suggests that a dietary deficiency of vitamin C may be a risk factor for hypertension. However, it remains unclear whether vitamin C supplementation could reduce blood pressure. Therefore, the purpose of this study was to provide a comprehensive meta-analysis using randomized, controlled trials looking at the effect of vitamin C supplementation on blood pressure in patients with hypertension.

Methods: Nine randomized, controlled trials published between 1966 and August 2007 with a total of 297 participants were identified using MEDLINE and a manual search. Using a random effects model, the effect sizes of vitamin C supplementation on systolic and diastolic blood pressure changes were estimated.

Results: Vitamin C supplementation was associated with a significant reduction in systolic blood pressure with an effect size of -2.37 mm Hg (95 % CI, -3.14 to -1.6 mm Hg). However, the mean change in diastolic blood pressure was non-significant with an effect size of only -0.37 mmHg (95 % CI, -1.5 to 0.76 mm Hg).

Conclusion: Vitamin C supplementation in hypertensive patients appears to possess modest effects on reducing systolic blood pressure.

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INTRODUCTION

Hypertension affects at least one-quarter of the adult population in the United States and is an important determinant of the incidence of coronary heart disease and stroke.[1] Epidemiological evidence suggests that a deficiency of vitamin C may lead to hypertension, and a negative association between plasma vitamin C status and blood pressure has been reported.[2-7] However, this association only suggests and does not prove that the intake of extra vitamin C lowers blood pressure. Clinical trials performed to assess the effect of vitamin C supplementation on blood pressure in hypertensive individuals have met with mixed results.[8-21] It has been stated that there are too few clinical trials to provide confirmatory evidence for a causal relationship.[22] Because of this lack of data from well-designed, randomized, controlled trials, it has been further stated that the use of vitamin C in the management of hypertension should not be recommended.[23] A 2006 publication approved by the American Heart Association Science Advisory and Coordinating Committee stated that it remains unclear whether vitamin C supplementation could reduce blood pressure.[24]

Since no meta-analysis has ever been published in regard to the effect of vitamin C supplementation on blood

pressure, it was believed that such an analysis was necessary. This is especially true in light of the fact that uncertainty exists about vitamin C's efficacy, even though a dozen randomized clinical trials have been conducted. The purpose of this study was to provide a comprehensive meta-analysis of randomized controlled trials to investigate the effect of vitamin C supplementation on blood pressure in patients with hypertension.

METHODS

Selection of Studies

A comprehensive MEDLINE literature search was performed to locate relevant randomized controlled trials published between 1966 through August 2007. The following headings were combined using the following Boolean operation ("vitamin C" OR "ascorbic acid" OR "ascorbate") AND ("blood pressure" OR "hypertension" OR "hypertensive"). The search was restricted to key terms located in the title/abstract, and was also restricted to studies published in English-language journals. Also, only full-length original journal articles were considered, and no attempt was made to include abstracts or unpublished studies. A manual search was also conducted by using reference lists from original research papers and review articles.

To be included in the meta-analysis, a study had to meet the following criteria: (1) the study was conducted using hypertensive human subjects (systolic BP > 130 mm Hg); (2) there was at least single-blinded, random allocation of study participants to either vitamin C treatment or placebo-controlled groups; (3) vitamin C was given orally with a minimum dose of 500 mg per day; (4) the intervention was greater than 4 weeks and less than 12 weeks; (5) the mean blood pressure changes for systolic and diastolic blood pressures in both the treatment and control groups (measured in mm Hg) were reported. The dose and intervention duration cut-offs were chosen based on the observations that 500 mg/day is the required intake for 95 percent of the population to achieve a saturated plasma vitamin C concentration,[25] and that it takes 3 to 4 weeks to reach a plasma steady-state following vitamin C supplementation.[26]

Nine studies met the eligibility criteria and were included in the meta-analysis.[8-16] Although 67 potentially relevant studies were identified and screened, 58 trials did not meet the eligibility criteria. Major reasons for exclusion of studies were (1) utilization of non-oral vitamin C therapies (17 trials); (2) co-intervention with other therapies (17 trials); (3) non-hypertensive subject populations (9 trials); (4) using oral doses of vitamin C less than 500 mg per day (3 trials); (5) study populations overlapped with other published studies (3 trials); (6) a treatment duration of greater than 12 weeks (2 trials); (7) study populations were not randomized (2 trials); (8) lack of utilization of a placebo-controlled group (2 trials); and (9) an absence of data to calculate the net mean change in blood pressure from baseline to end of follow-up (3 trials). Figure 1 shows the number of studies that were identified and excluded at different stages of the selection process.

Figure 1. Study selection process for inclusion in a meta-analysis that investigates the effects of vitamin C supplementation on blood pressure reduction in hypertensive patients.

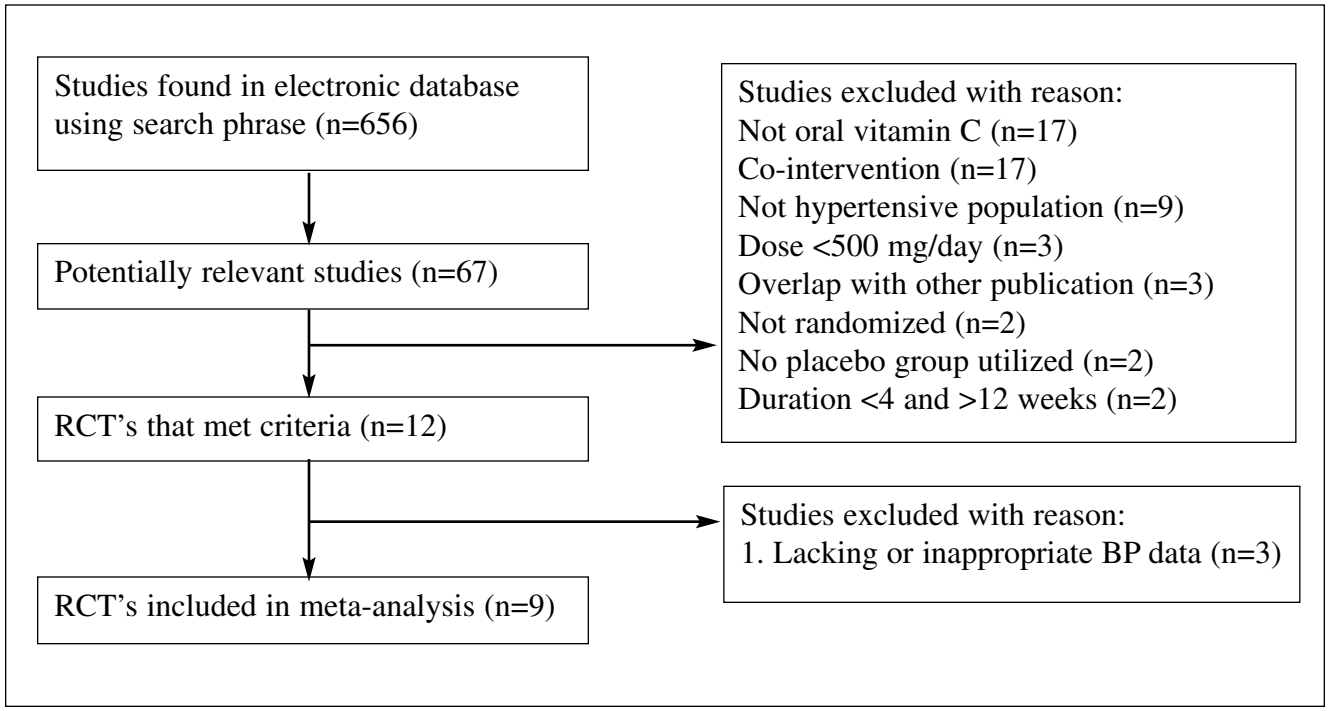


Table 1. Population and baseline characteristics of trials included in the meta-analysis.

Source and Year	Sample Size	Mean Age Y	Male, %	Study Design*	Vitamin C per Day, mg	Duration, wk	Baseline Blood Pressure	
							Systolic mmHg	Diastolic mmHg
Osilesi et al. 1991[8]	12	57.8	25	XD	1000	6	139	81
Ghosh et al. 1994[9]	48	73.7	38	PD	500	6	173.2	90.2
Gokce et al. 1999[10]	46	55.0	91	PD	500	4	145	78
Fotherby et al. 2000[11]	17	72.0	50	XD	500	12	149	84
Duffy et al. 2001[12]	39	48.5	49	PD	500	4	155	87
Darko et al. 2002[13]	35	56.0	66	PD	1500	4	141	80
Mullan et al. 2002[14]	30	59.5	73	PD	500	4	142.1	83.9
Magen et al. 2004[15]	33	52.0	52	PS	500	8	150.6	86.1
Ward et al. 2005[16]	37	61.5	70	PD	500	6	133.6	80.8

* XD, Crossover double blind; PD, Parallel double blind; PS, Parallel single blind

Data Abstraction and Statistical Analysis

Information on sample size, participant characteristics, study design, vitamin C dosage, duration, and treatment results was abstracted from the 9 clinical trials. To calculate the overall effect size, each study was weighted by the reciprocal of the variance for blood pressure changes. Variances for blood pressure net changes between treatment and control groups were provided for two studies, whereas the variances for the remaining seven studies were calculated using the variances at baseline and at the end of follow-up based on the methodology of Follmann et al.[27] In this method, a correlation coefficient of 0.5 between initial and final blood pressures was assumed. Within each trial, equal variance was assumed between the control and intervention groups, as well as between the beginning and end of each trial. For parallel and crossover trials, net changes in blood pressure (BP) were calculated as (BP at end of follow-up in the treatment group – BP at baseline in the treatment group) – (BP at end of follow-up in the control group – BP at baseline in the control group).

Estimates of the mean effect of vitamin C supplementation on blood pressure and the corresponding 95 % CI's were calculated using random-effects models. The assumption of heterogeneity implied by the use of the random-effects model was plausible because of differences between trials in such aspects as duration of the trial, dosages utilized, and sample populations that differed by age and sex. To examine potential publication bias, sample size was plotted against effect size. Data analysis was performed using Comprehensive Meta-Analysis software (version 2.0, Biostat, USA).

RESULTS

Participant Characteristics and Study Design

Participant and study design characteristics for the 9 randomized, controlled trials included in the meta-analysis are presented in Table 1. Collectively, the 9 trials conducted between 1991 and 2005 included a total of 297 subjects (141 in the vitamin C supplementation group and 156 in the control group). All trials were conducted with adults, with an age range of 48.5 to 73.7 years. Men were the majority in 5 of the 9 trials, with the pooled population made up of 60 % males. Six trials had a parallel double blind design, two used a crossover double blind design and one used a parallel single blind design. The study duration varied from 4 to 12 weeks, with a median length of 6 weeks. Vitamin C supplementation for 7 of the 9 trials was 500 mg/day, while one trial used 1000 mg/day, and the remaining trial used 1500 mg/day. Average pretreatment blood pressure ranged from 139 to 173.2 mm Hg for systolic and 78 to 90.2 mm Hg for diastolic blood pressure.

Net Change in Blood Pressure

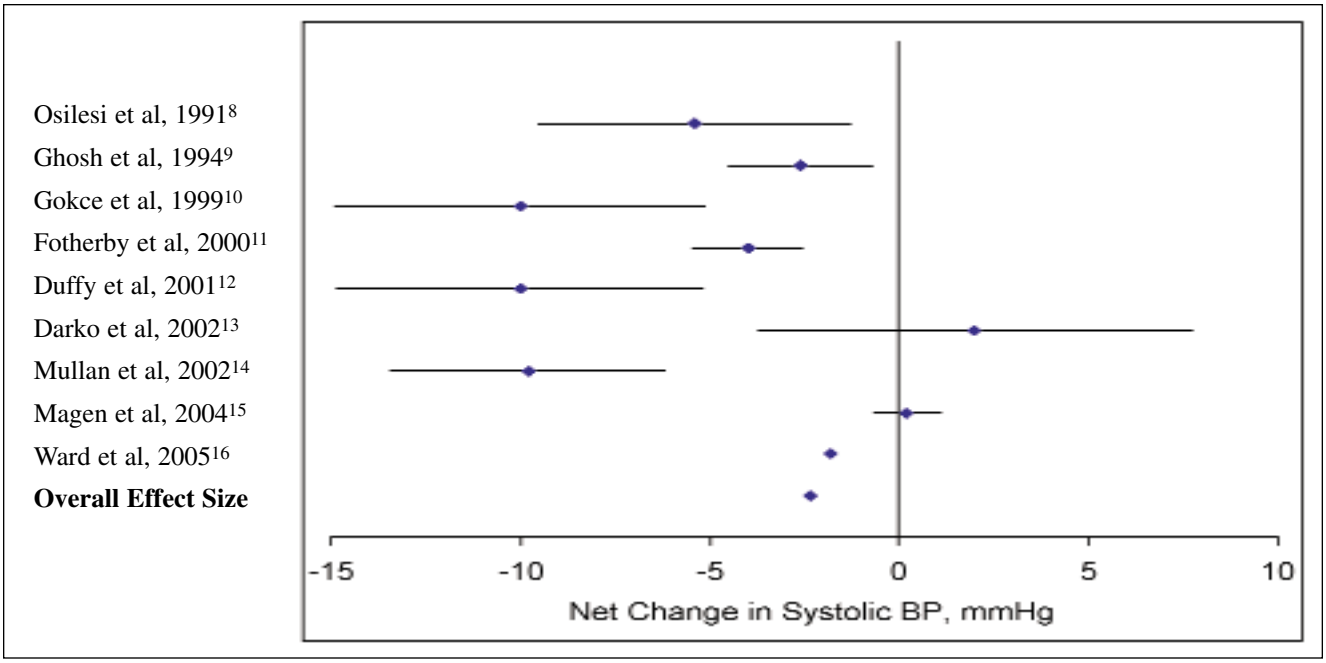
The mean net changes in systolic and diastolic blood pressure after vitamin C supplementation are presented in Table 2. Seven of the 9 trials had an intervention-related trend toward a reduction in systolic blood pressure, with all seven trials showing a statistically significant reduction ($p<0.05$) in blood pressure when compared to the control group (Figure 2). For diastolic blood pressure, a trend toward intervention-related reduction was observed in only 4 of the 9 trials, with 3 of the 9 trials showing a statistical-

Table 2. Mean change in systolic and diastolic blood pressure after vitamin C supplementation.

Source and Year	Sample Size	Net Change in Blood Pressure*			
		Systolic mmHg	P Value §	Diastolic, mmHg	P Value §
Osilesi et al. 1991[8]	12	-5.4	0.017	0.6	NS
Ghosh et al. 1994[9]	48	-2.6	0.010	-1.2	NS
Gokce et al. 1999[10]	46	-10.0	0.000	1.0	NS
Fotherby et al. 2000[11]	17	-4.0	0.000	-2.0	0.011
Duffy et al. 2001[12]	39	-10.0	0.000	-4.0	0.000
Darko et al. 2002[13]	35	2.0	NS	0.0	NS
Mullan et al. 2002[14]	30	9.8	0.000	-4.4	0.000
Magen et al. 2004[15]	33	0.2	NS	2.8	0.000
Ward et al. 2005[16]	37	-1.8	0.031	1.5	0.017

* For parallel trials, the net change is (intervention final BP – baseline BP) – (control final BP – baseline BP); For crossover trials the net change is intervention final BP-control final BP. § The P value was calculated by the author.

Figure 2. Net change (and 95% CI) in systolic blood pressure associated with vitamin C supplementation. The overall effect size is weighted by the inverse of the total variance of each trial.

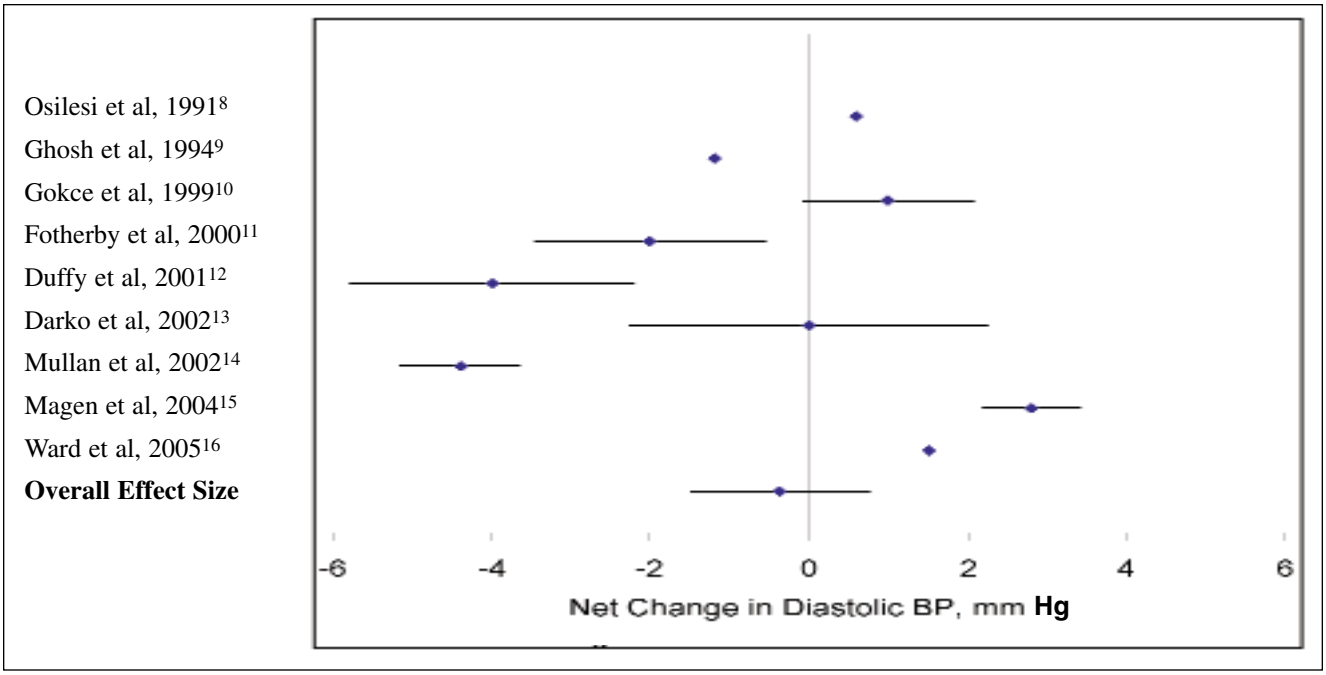


ly significant reduction in blood pressure when compared to the control group (Figure 3). However, two trials showed a statistically significant increase in diastolic blood pressure after the vitamin C intervention.

The effect size along with the 95 % CI's for each clinical trial, as well as the overall effect size on systolic and diastolic blood pressure in hypertensive patients, are presented in Figures 2 and 3. The overall pooled estimate of the effect of vitamin C supplementation on systolic blood pressure was -2.37 mm Hg (95 % CI, -3.14 to -1.60; p = 0.000). The overall pooled estimate of the effect of vitamin C supplementation on diastolic blood pressure was -0.37 mm Hg (95 percent CI, -1.50 to 0.75; p = NS).

The plot of sample size versus effect size showed a typical "funnel" shape with little variation in effect size for large sample studies, and an increasing spread of effect size

Figure 3. Net change (and 95% CI) in diastolic blood pressure associated with vitamin C supplementation. The overall effect size is weighted by the inverse of the total variance of each trial.



with smaller sample sizes. The distribution of effects sizes seen in the individual studies was symmetrically distributed around the pooled mean effect size.

DISCUSSION

This is the first meta-analysis to provide a comprehensive examination of the effect of vitamin C supplementation on blood pressure and is based on nine randomized, controlled clinical trials involving 297 participants. The overall effect size estimates observed from the meta-analysis were a 2.37 mm Hg reduction in systolic blood pressure and a 0.37 mm Hg reduction in diastolic blood pressure. Although the change in diastolic blood pressure was insignificant, the significant reduction in systolic blood pressure could potentially translate to a 7 percent reduction in stroke and a 4 percent reduction in coronary heart disease.[28-30] The effects of vitamin C supplementation on systolic blood pressure is consistent with epidemiologic observations that there exists an inverse relationship between blood pressure and plasma vitamin C status. This meta-analysis supports that vitamin C supplementation has the potential to positively impact both cardiovascular and cerebrovascular disease rates.

A possible explanation for the non-significant observable differences with diastolic blood pressure may be because baseline levels were starting below 90 mm Hg (except for one study), so there was no significant room for improvement when subjects were supplemented with vitamin C. The one study that started with a baseline diastolic blood pressure of 90.2 mm Hg, resulted in a mean net change of -1.2 mm Hg when compared to the control group, which was not statistically significant.

When compared to other proposed nutritional supplements, Vitamin C may be as beneficial for reducing systolic blood pressure. A meta-analysis of potassium supplement studies suggests a reduction in both systolic blood pressure of approximately 3.1 mm Hg, and a meta-analysis of calcium supplement studies suggests and even smaller effect on systolic blood pressure of 1.7 mm Hg.[31-32] Vitamin C supplementation is also comparable to the effects of alcohol reduction and aerobic exercise on systolic blood pressure, as these two meta-analyses showed reductions of 3.3 and 3.8 mm Hg respectively.[33-34]

In regard to mechanism of action, hypertension is associated with higher than normal lipoperoxidation and an imbalance in antioxidant status, suggesting that oxidative stress is an important driving factor in the pathogenesis of hypertension.[35] Hypertension has been shown to be associated with impaired nitric oxide (NO) production.[36] Vascular endothelium derived nitric oxide synthase (eNOS) plays a critical role in the regulation of vascular tone, and it appears that vitamin C can improve endothelium vasodilation by augmenting NO bioavailability.[37] Because superoxide radicals can degrade NO, it has been proposed that vitamin C improves blood pressure by scavenging superoxide radicals and thereby preventing the inactivation of

NO.[38-39] However, it has been reported that supraphysiological concentrations of vitamin C are required to prevent the superoxide radical destruction of NO.[40]

It is also known that oxidized LDL inhibits NO release from endothelial cells, and therefore vitamin C may preserve NO by preventing the oxidation of LDL.[41-42] Vitamin C may also increase NO production by enhancing eNOS activity.[43] This effect appears to be mediated through increasing the intracellular content of tetrahydrobiopterin.[44] Tetrahydrobiopterin is a cofactor for eNOS that requires vitamin C for stabilization as well as protection from oxidation. These effects were evident as an increase in the half-life of tetrahydrobiopterin inside the endothelial cell has been observed.[45]

A major limitation of this study is the pooling together of clinical trials that include a considerable amount of heterogeneity in design and population characteristics. Average subject age varied between 48.5 and 73.7 years, and it is known that vitamin C concentration in serum decreases with aging, while a concomitant increase in blood pressure occurs due to an increase in arterial stiffness.[46] In a comparative baseline study, it was found that vitamin C supplementation with an elderly population in their late 70's reduced systolic blood pressure by approximately 18.5 mm Hg more when compared to the change observed in an adult population in their mid 50's.[47] Also, differences in dietary characteristics may result in unevenly matched baseline plasma vitamin C concentrations. In the 6 studies that observed baseline plasma vitamin C concentrations, the range varied between 44.6 to 74 mol. This may confound both the starting baseline blood pressures as well as the absorbability of vitamin C supplementation, which is dependent upon pre-absorption plasma concentrations. Also, not having evenly matched baseline blood pressures could confound the results, as populations with higher baseline blood pressures could possibly exhibit more of a hypotensive effect with vitamin C supplementation. Confounders also included differences between studies with vitamin C supplementation dose (ranged between 500 to 1500 mg/day) and study duration (ranged between 4 to 12 weeks).

CONCLUSION

In summary, this meta-analysis has shown that supplementation with at least 500 mg/day of oral vitamin C, for a minimum of 6 weeks, can lower systolic blood pressure. Although the reduction in systolic blood pressure was modest, any small reduction can have beneficial effects on the incidence of coronary heart disease and stroke, especially in light of the low cost and absence of toxicity when vitamin C supplementation is within the ranges of 500 to 1000 mg/day.[48]

REFERENCES

1. Burt VL, Whelton P, Roccella EJ, et al. Prevalence of hypertension in the US adult population. Results from the Third National Health and Nutrition Examination Survey, 1988-1991. *Hypertens*. 1995;25(3):305-313.
2. Yoshioka M, Matsushita T, Chuman Y. Inverse association of serum ascorbic acid level and blood pressure or rate of hypertension in male adults aged 30-39 years. *Int J Vitam Nutr Res.*1984;54(4):343-347.
3. Bulpitt CJ. Vitamin C and blood pressure. *J Hypertens.* 1990;8(12):1071-1075.
4. Jacques PF. Relationship of vitamin C status to cholesterol and blood pressure. *Ann N Y Acad Sci.* 1992;669:205-213
5. Jacques PF. A cross-sectional study of vitamin C intake and blood pressure in the elderly. *Int J Vitam Nutr Res.* 1992;62(3):252-255.
6. Moran JP, Cohen L, Greene JM, et al. Plasma ascorbic acid concentrations relate inversely to blood pressure in human subjects. *Am J Clin Nutr.* 1993;57(2):213-217.
7. Ness AR, Khaw KT, Bingham S, Day NE. Vitamin C status and blood pressure. *J Hypertens.* 1996;14(4):503-508.
8. Osilesi O, Trout DL, Ogunwole JO, Glover EE. Blood pressure and plasma lipids during ascorbic acid supplementation in borderline hypertensive and normotensive adults. *Nut Res.* 1991;11:405-412.
9. Ghosh SK, Ekpo EB, Shah IU, Girling AJ, Jenkins C, Sinclair AJ. A double-blind, placebo-controlled parallel trial of vitamin C treatment in elderly patients with hypertension. *Gerontol.* 1994;40(5):268-272.
10. Gokce N, Keaney JF Jr, Frei B, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circ.* 1999;99(25):3234-3240.
11. Fotherby MD, Williams JC, Forster LA, Craner P, Ferns GA. Effect of vitamin C on ambulatory blood pressure and plasma lipids in older persons. *J Hypertens.* 2000;18(4):411-415.
12. Duffy SJ, Gokce N, Holbrook M, et al. Effect of ascorbic acid treatment on conduit vessel endothelial dysfunction in patients with hypertension. *Am J Physiol Heart Circ Physiol.* 2001;280(2):528-534.
13. Darko D, Dornhorst A, Kelly FJ, Ritter JM, Chowienczyk PJ. Lack of effect of oral vitamin C on blood pressure, oxidative stress and endothelial function in Type II diabetes. *Clin Sci (Lond).* 2002;103(4):339-344.
14. Mullan BA, Young IS, Fee H, McCance DR. Ascorbic acid reduces blood pressure and arterial stiffness in type 2 diabetes. *Hypertens.* 2002;40(6):804-809.

15. Magen E, Viskoper R, Mishal J, et al. Resistant arterial hypertension and hyperlipidemia: atorvastatin, not vitamin C, for blood pressure control. *Isr Med Assoc J.* 2004;6(12):742-746.

16. Ward NC, Hodgson JM, Croft KD, Burke V, Beilin LJ, Puddey IB. The combination of vitamin C and grapeseed polyphenols increases blood pressure: a randomized, double-blind, placebo-controlled trial. *J Hypertens.* 2005 Feb;23(2):427-434.

17. Koh ET. Effect of vitamin C on blood parameters of hypertensive subjects. *J Okla State Med Assoc.* 1984;77(6):177-182.

18. Lovat LB, Lu Y, Palmer AJ, Edwards R, Fletcher AE, Bulpitt CJ. Double-blind trial of vitamin C in elderly hypertensives. *J Hum Hypertens.* 1993;7(4):403-405.

19. Eriksson J, Kohvakka A. Magnesium and ascorbic acid supplementation in diabetes mellitus. *Ann Nutr Metab.* 1995;39(4):217-223.

20. Rolla G, Brussino L, Carra R, Garbella E, Bucca C. Hypertension and ascorbic acid. *Lancet.* 2000;355(9211):1271-1272

21. Hajjar IM, George V, Sasse EA, Kochar MS. A randomized, double-blind, controlled trial of vitamin C in the management of hypertension and lipids. *Am J Ther.* 2002;9(4):289-293.

22. Ness AR, Chee D, Elliott P, Vitamin C and blood pressure--an overview. *J Hum Hypertens.* 1997;11(6):343-350.

23. Wexler R, Aukerman G. Nonpharmacologic strategies for managing hypertension. *Am Fam Physician.* 2006;73(11):1953-1956.

24. Appel LJ, Brands MW, Daniels SR, Karanja N, Elmer PJ, Sacks FM. American Heart Association. Dietary approaches to prevent and treat hypertension: a scientific statement from the American Heart Association. *Hypertens.* 2006;47(2):296-308.

25. Brubacher D, Moser U, Jordan P. Vitamin C concentrations in plasma as a function of intake: a meta-analysis. *Int J Vitam Nutr Res.* 2000;70(5):226-237.

26. Levine M, Conry-Cantilena C, Wang Y, et al. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci.* 1996;93(8):3704-3709.

27. Follmann D, Elliott P, Suh I, Cutler J. Variance imputation for overviews of clinical trials with continuous response. *J Clin Epidemiol.* 1992;45(7):769-773.

28. Franklin SS, Larson MG, Khan SA, et al. Does the relation of blood pressure to coronary heart disease risk change with aging? The Framingham Heart Study. *Circ.* 2001;103(9):1245-1249.

29. Bowman TS, Gaziano JM, Kase CS, Sesso HD, Kurth T. Blood pressure measures and risk of total, ischemic, and hemorrhagic stroke in men. *Neurol.* 2006;67(5):820-823.

30. Menotti A, Lanti M, Nedeljkovic S, et al. The relationship of age, blood pressure, serum cholesterol and smoking habits with the risk of typical and atypical coronary heart disease death in the European cohorts of the Seven Countries Study. *Int J Cardiol.* 2006;106(2):157-163.

31. Whelton PK, He J, Cutler JA, et al. Effects of oral potassium on blood pressure. Meta-analysis of randomized controlled clinical trials. *JAMA.* 1997;277(20):1624-1632.

32. Allender PS, Cutler JA, Follmann D, Cappuccio FP, Pryer J, Elliott P. Dietary calcium and blood pressure: a meta-analysis of randomized clinical trials. *Ann Intern Med.* 1996;124(9):825-831.

33. Xin X, He J, Frontini MG, et al. Effects of alcohol reduction on blood pressure: a meta-analysis of randomized controlled trials. *Hyptertens.* 2001;38(5):1112-1117.

34. Whelton SP, Chin A, Xin X, He J. Effect of aerobic exercise on blood pressure: a meta-analysis of randomized, controlled trials. *Ann Intern Med.* 2002;136(7):493-503.

35. Russo C, Olivieri O, Girelli D, et al. Anti-oxidant status and lipid peroxidation in patients with essential hypertension. *J Hypertens.* 1998;16(9):1267-1271.

36. Kedziora-Kornatowska K, Czuczejko J, Pawluk H, et al. The markers of oxidative stress and activity of the antioxidant system in the blood of elderly patients with essential arterial hypertension. *Cell Mol Biol Lett.* 2004;9(4A):635-641.

37. Levine GN, Frei B, Koulouris SN, Gerhard MD, Keaney JF Jr, Vita JA. Ascorbic acid reverses endothelial vasomotor dysfunction in patients with coronary artery disease. *Circ.* 1996;93(6):1107-1113.

38. Taddei S, Virdis A, Ghiadoni L, Magagna A, Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circ.* 1998;97(22):2222-2229.

39. Jackson TS, Xu A, Vita JA, Keaney JF Jr. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ Res.* 1998;83(9):916-922.

40. Sherman DL, Keaney JF Jr, Biegelsen ES, Duffy SJ, Coffman JD, Vita JA. Pharmacological concentrations of ascorbic acid are required for the beneficial effect on endothelial vasomotor function in hypertension. *Hypertens.* 2000;35(4):936-941.

41. Frei B, England L, Ames BN. Ascorbate is an outstanding antioxidant in human blood plasma. *Proc Natl Acad Sci.* 1989;86(16):6377-6381.

42. Retsky KL, Freeman MW, Frei B. Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. *J Biol Chem.* 1993;268(2):1304-1309.

43. Huang A, Vita JA, Venema RC, Keaney JF Jr. Ascorbic acid enhances endothelial nitric-oxide synthase activity by increasing intracellular tetrahydrobiopterin. *J Biol Chem.* 2000;275(23):17399-406.

44. Baker TA, Milstien S, Katusic ZS. Effect of vitamin C on the availability of tetrahydrobiopterin in human endothelial cells. *J Cardiovasc Pharmacol.* 2001;37(3):333-338.

45. Heller R, Unbehaun A, Schellenberg B, Mayer B, Werner-Felmayer G, Werner ER. L-ascorbic acid potentiates endothelial nitric oxide synthesis via a chemical stabilization of tetrahydrobiopterin. *J Biol Chem.* 2001;276(1):40-47.

46. Griffiths LL, Brocklehurst JC, Scott DL, Marks J, Blackley J. Thiamine and ascorbic acid levels in the elderly. *Gerontol Clin.* 1967;9:1-10.

47. Sato K, Dohi Y, Kojima M, Miyagawa K, Takase H, Katada E, Suzuki S. Effects of ascorbic acid on ambulatory blood pressure in elderly patients with refractory hypertension. *Arzneimittelforschung.* 2006;56(7):535-540.

48. Rivers JM. Safety of high-level vitamin C ingestion. *Int J Vitam Nutr Res Suppl.* 1989;30:95-102.

PILOT STUDY

Oral Tolerability of Cysteine-Rich Whey Protein Isolate in Autism–A Pilot Study

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ABSTRACT

Purpose: To examine the tolerability of non-denatured whey protein isolate (NWPI) in children with autism. Many children with autism are low in glutathione and have higher levels of oxidative stress. NWPI can raise glutathione levels and reduce oxidative stress. However, anecdotal reports suggest that NWPI may be problematic in children with autism because it contains cysteine and other sulfurated amino acids.

Methods: A 6-week open-label trial was conducted, supplementing 10 children with autism or autism spectrum disorder (ASD), 3-15 years of age, with NWPI (Immunocal®). To measure possible side effects, procedures that examined the frequency, intensity, and types of side effects, as well as behavioral measures, were completed at baseline, and at days 3, 14, 30, and 45.

Results: Seven of the ten children took the supplement over the six-week trial and tolerated it well. Two children discontinued after two weeks due to possible side effects: one due to gastrointestinal disturbance and one due to being less responsive to parents. Another child discontinued due to difficulty of administering the product.

Conclusion: This study suggests that NWPI can be used as a supplement for this small population of children with autism without high rates of side effects, which means that further studies to determine its safety and efficacy in larger populations might yield the same promising result. Larger studies are planned to determine its efficacy in raising glutathione levels.

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INTRODUCTION

Five recent studies showed that oxidative stress and/or lipid peroxidation are increased in autism,[1-6] including research by James et al[4,5] that suggests that glutathione (GSH) is lower in children with autism than in control children, and that a higher fraction of their glutathione is oxidized. For a more complete review of the possible role of oxidative stress and toxicity in the pathology of autism, see Kern and Jones.[7]

A safe supplement that can normalize glutathione levels and reduce oxidative stress could potentially be benefi-

cial in autism. To raise glutathione levels, humans require the building blocks or precursors of glutathione because oral glutathione is considered ineffective.[8,9] Glutathione (GSH), or 2-amino-5-{[2-[(carboxymethyl)amino]-1-(mercaptomethyl)-2-oxoethyl]amino}-5-oxopentanoic acid, is a small protein made up of three amino acids: glycine, cysteine, and glutamic acid. GSH is a thiol and thus contains sulfur.[10,11] The side-chain sulfhydryl residue (-SH) that is in the cysteine part of the molecule is what provides most of its physiological properties.[10,11] Cysteine is the rate-limiting substrate for GSH production.[12]

Non-denatured whey protein isolate is cysteine-rich in the form of cystine (two cysteine molecules linked by a disulfide bond). The term "non-denatured" is preferable to the less precise "un-denatured" to describe a protein that has been preserved in its native state and has retained its original physical characteristics and conformation. Changing a milk protein's native conformation can alter its biological activity.[13] Cystine (reduced cysteine) is much more stable than cysteine and accounts for 90% of the amino acid in the plasma; once it crosses the cell membrane, it is oxidized to cysteine and then used to make glutathione.[10,14] Non-denatured whey protein concentrates and isolates have been shown to increase glutathione levels in many diseases and disorders such as acquired immune deficiency syndrome (AIDS), cystic fibrosis, lung disease, chronic fatigue syndrome, hepatitis B, and cancer (e.g., colon, liver, breast, pancreas), without any toxicity or adverse events.[10,14-26] There have been only occasional cases of gastrointestinal upset reported.[14]

Non-denatured whey protein isolate use has not been examined in autism; and although reports are conflicting, some anecdotal reports suggest that some children with autism may have problems with ingestion of cysteine-rich or any sulfur-rich compound, such as whey protein. Whey protein concentrates (>70% protein) which contain the milk protein caseine can be further problematic. The problems anecdotally reported are worsening of behavior and gastrointestinal disturbance with dysbiosis (specifically, an increase in yeast). In this population, food intolerances and GI disturbance are commonly reported.[27] Because of the anecdotal reports that suggest that children with autism may not tolerate sulfur-rich compounds, combined with the evidence that non-denatured whey protein isolate is safe and effective in raising glutathione levels and reducing oxidative stress in other disorders and diseases, it was determined that a trial using non-denatured whey protein in autism to examine its tolerability in this population was necessary. This study used Immunocal®, a non-denatured whey protein isolate (>90% protein), which is a medically recognized option for raising glutathione levels.

The study was conducted to determine if: (1) the children would take this supplement, which is a bland-tasting powder, and (2) the supplement would be tolerated even though it is a sulfur-rich compound.

METHODS

Design: The study, which used Immunocal®, was a six-week, open-label clinical trial with ten children previously diagnosed with autism or ASD. Informed consent and HIPAA forms were obtained from every child's parent. Children were always in the presence of one or both parents. At baseline, information regarding demographics, formal diagnosis, age at diagnosis, age of apparent onset, information regarding delay or regression, any current medical issues, medications, and allergies was obtained on each child. A Childhood Autism Rating Scale (CARS)[28] was also completed at baseline. After a stool specimen was collected (by the parent at home) for analysis, each child was started on Immunocal®. The stool specimen was completed on seven of the ten children before the treatment to acquire a baseline for dysbiosis (three parents were not able to obtain usable specimens). Other measures examining behavior and side effects were used as repeated measures and were completed periodically by the parents to examine any clinical change in the child. These measures are listed in the measures section.

Location: This study took place at the Mood Disorders Research Program and Clinic at the University of Texas Southwestern Medical Center (UTSW) in Dallas, Texas. The study protocol received Institutional Review Board (IRB) approval from the University of Texas Southwestern Medical Center. All parents signed a consent and Health Insurance Portability and Accountability Act (HIPAA) form and all received a copy.

Subjects: Subjects for this study were recruited from autism societies and physicians from the Dallas/Fort Worth area, and via the Internet (by posting on the UTSW study site). The children were three to fifteen years of age. Six had a formal diagnosis of autism and four had a formal diagnosis of ASD. The CARS ranged from 30 to 43 with a mean of 35.4 (SD= 4.4), thus ranging from mild to severe autism. There were nine males and one female. Eight children were Caucasian and two were African-American. The exclusion criteria were: (1) already taking whey protein or starting any new drug or therapy; and (2) a comorbid diagnosis of Fragile X disorder, tuberous sclerosis, phenylketonuria (PKU), Lesch-Nyhan syndrome, fetal alcohol syndrome, or a history of maternal illicit drug use.

MEASURES

Comprehensive Parasitology (CP3): The CP3 panel includes a standard microbiology (bacterial culture), yeast culture and speciation, microscopic evaluation for yeast (KOH stain), and parasites. This test was completed before treatment to acquire a baseline for possible dysbiosis.

Childhood Autism Rating Scale: The CARS is a 15-item behavioral rating scale developed to identify autism as well as to quantitatively describe the severity of the disor-

der.[28] Independent reports on CARS indicate that it has high validity. Eaves and Milner[29] found that it correctly identified 98 percent of autistic subjects and correlated (r = 0.67) with the Autism Behavior Checklist. In another similar study, 92 percent of subjects were correctly classified, and the CARS correlated with the Real Life Rating Scale.[30] The CARS was completed by the Principal Investigator by observing the subjects and interviewing parents at baseline. The CARS was also completed by a parent on study days 1, 3, 14, 30, and 45.

Frequency and Intensity of Side Effect Rating (FISER)/Global Rating of Side Effect Burden (GRSEB)/: The FISER/GRSEB surveys include global measures, each using a 7-point Likert-type scale rated 0 to 6. One rate is anchored for frequency, another rates the intensity of side effects encountered in the prior week that the caregivers believe were due to the treatment, and the third asks caregivers to estimate the overall burden or degree of interference in day-to-day activities and function due to side effects attributable specifically to the treatment.[31] The survey was completed by a parent on study days 3, 14, 30, and 45.

Patient Rated Inventory of Side Effects (PRISE)—modified: The PRISE lists a variety of possible side effects to choose from and a scale to rate the specific side effect. The list also includes gastrointestinal side effects. In addition, the measure has a place to list any side effects not previously listed. The survey was completed by a parent on days 3, 14, 30, and 45.

Aberrant Behavior Checklist, Subscales I to V (ABC): The ABC scale rates inappropriate and maladaptive behavior.[32] The ABC was designed to monitor the behavioral effects of psychotropic drugs. The scale was completed by a parent at baseline, and at days 3, 14, 30, and 45.

Clinical Global Impression Scale (CGI): The CGI three-item scale asks the caregiver to mark the patient as better (1), the same (2), or worse overall (3). The scale was completed by the parent at baseline and at days 3, 14, 30, and 45.

Treatment Adherence Measure (TAM): The TAM is a ten-item, Morisky-type self-report on treatment adherence that asks specific questions regarding the dose and frequency of use. The TAM was used to determine the level of adherence to the treatment. Morisky-type adherence measures have been used widely and have shown good reliability for a self-report measure.[33] The measure was completed by a parent at days 3, 14, 30, and 45.

Product Used: A medically recognized option for raising glutathione levels, Immunocal® is a bovine milk serum protein or non-heated non-denatured whey protein isolate manufactured in a way that preserves the native protein configuration of the whey. It is a white powdery substance that comes in an airtight and humidity resistant envelope. Immunocal® contains <1 percent lactose, <1 percent fat, and minimal amounts of caseine (*Physician's Desk Reference; PDR*).

Dosing and Administration: Recommended dosing for children is 10g per day for those over 40 pounds and 0.5 grams per kilo (2.2 pounds) of body weight for children under 40 pounds. Due to reports of intolerance of sulfur-rich foods in this population, the dosing of the non-denatured whey protein isolate was cautiously titrated. Dosing began at one-fourth dose for 5 days, then one-half dose for 5 days, then three-fourths dose for 5 days, and finally a full dose by approximately the third week. Immunocal® was taken with a light meal or on an empty stomach once or twice per day. The powder was mixed with fruit juice or food. It was not heated or blended in a power blender. Parents gave the children Immunocal® mixed in a variety of ways (e.g., orange juice, Hershey's chocolate syrup, ice cream, chocolate milk, Carnation Instant Breakfast, applesauce, peanut butter and jelly, yogurt, and Boston Chicken's Caesar salad dressing).

RESULTS

Seven out of the ten children took the supplement over the full six-week trial and tolerated the supplement. Three children discontinued after two weeks of the trial. One child discontinued because of gas and bloating. This occurred at a higher dose level, but not at a lower dose level. This child's baseline bowel flora was within normal limits, but the child did have a history of chronic diarrhea. No other children had any gastrointestinal changes, even the two children who had dysbiosis at baseline. One child discontinued the study due to being less responsive to parents. Another discontinued because one parent had difficulty administering the whey product. Of the completers, two parents verbally reported that their children became more hyperactive initially; however, the hyperactivity resolved in a few weeks.

Data were collected on a number of behavioral measures, the Childhood Autism Rating Scale, the Aberrant Behavior Checklist, and a Clinical Global Impression measure. Means for the three non-completers and seven completers are presented below (Table 1). These measures were used to examine any clinical change in the child that we would need to be concerned about based on anecdotal reports. The data were insufficient for statistical analysis. It is worth noting that the changes in the behavioral measures appear to be in a positive direction (Figure 1). Caution should be used in interpreting these findings since any meaningful test would require a larger study and a control group.

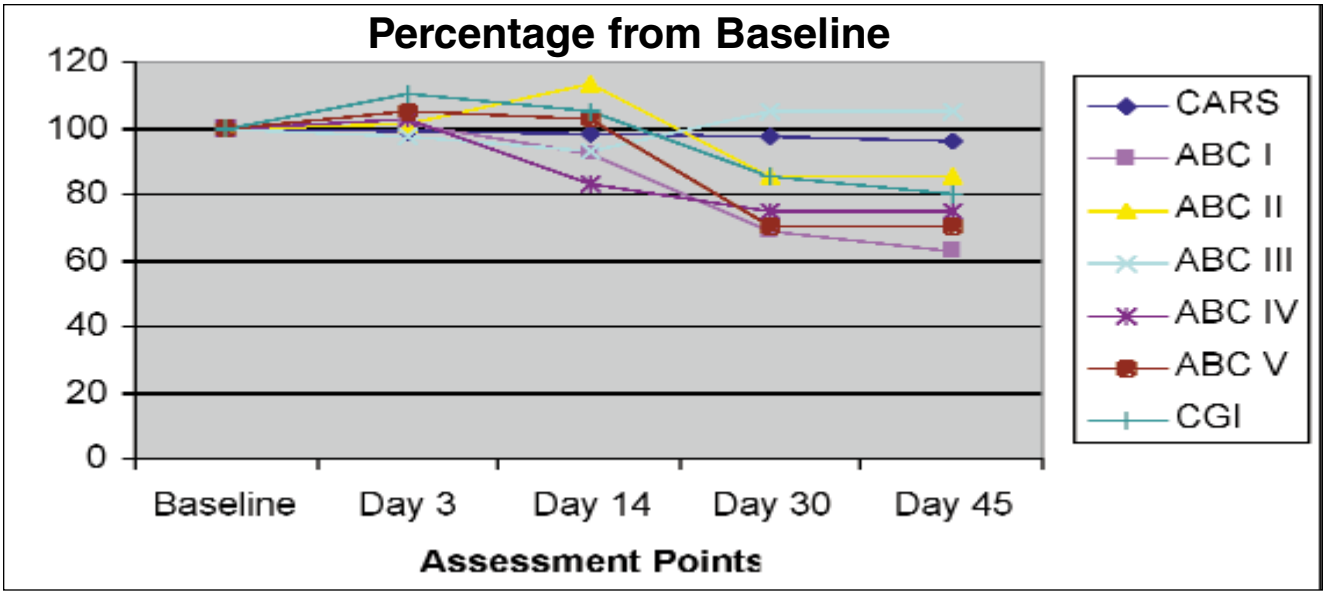
Treatment adherence in the completer group was good, with most participants taking their doses 75 to 100 percent of the time. Parents were allowed to mix the whey with anything as long as it was not blended with a power blender or heated (to avoid denaturization). Some parents had more

Table 1.

Measures	**Baseline** Mean (SD) n=10	**Day 3** Mean (SD) n=10	**Day 14** Mean (SD) n=9	**Day 30** Mean (SD) n=7	**Day 45** Mean (SD) n=7
CARS	33.1 (6.6)	33.4 (6.36)	32.6 (4.8)	32.1 (5.3)	31.7 (5.5)
ABC I	10.6 (7.6)	10.7 (7.5)	9.8 (6.6)	7.3 (5.4)	6.7 (5.0)
ABC II	8.2 (8.5)	8.3 (8.4)	9.3 (8.3)	7.0 (7.3)	7.0 (7.3)
ABC III	4.1 (3.2)	4.0 (3.2)	3.8 (3.3)	4.3 (3.9)	4.3 (3.9)
ABC IV	16.5 (13.3)	17.0 (13.5)	13.7 (9.6)	12.4 (11.2)	12.4 (11.2)
ABC V	3.7 (3.1)	3.9 (2.8)	3.8 (3.1)	2.6 (1.6)	2.6 (1.6)
CGI*	2	2.2 (0.4)	2.1 (0.3)	1.7(0.5)	1.6 (.5)

This table shows the mean and standard deviation (SD) of the parent-rated measures on all participants (completers and non-completers), pre-treatment (baseline), and post-treatment. A decrease in score represents improvement. *Note that all participants started as a 2 on the CGI.

Figure 1.



This figure shows the percentage change (lower is better) over time on CARS, ABC I-V, and CGI.

difficulty getting their child to take the whey than others, with some parents having to try several different ways to mix it and some having no difficulty at all.

A stool specimen (CP3) was completed on seven of the ten children (three parents were not able to obtain usable specimens) before the treatment to acquire a baseline for dysbiosis. Two children had dysbiosis at baseline: one child had moderate yeast overgrowth and one child had *Staphylococcus Aureus* (a bacterium). Neither of these two children had any GI changes with treatment.

DISCUSSION

The study suggests that, for the most part, children with autism do not have problems tolerating cysteine in this form (cystine/whey protein isolate). The results are limited due to fact that the study was conducted on very small sample and for a relatively short period of time. Therefore, a larger and longer study would be necessary to get specific estimates of tolerability and to measure the possible benefits.

As mentioned, one child discontinued the study because of gas and bloating. A variety of medical conditions is often reported in children with autism, including different GI symptomatology.[34] In this study, for example, four children were reported to have GI function within normal limits, three were reported to have chronic constipation, and three were reported to have chronic diarrhea (prior to the start of the study and did not change). This variation suggests that children with autism may tolerate some supplements or treatments better than others. It can be hypothesized that reactions to whey proteins in these individuals may be due to the presence of casein, another milk protein that can be present in whey concentrates or other whey products. Casein-elimination diets have been reported as beneficial in ASD.[35]

Also, as mentioned, one child discontinued the study due to being less responsive to his parents. Overall, the parental reports and measures suggest that, generally, there was no worsening of behavior. Interestingly, four parents reported improvements in their children's ability to communicate and willingness to interact with others, and the CARS, ABC, and CGI all showed a trend toward the positive. Figure 1 shows that trend. However, since the trial was not a blinded trial, it is difficult to determine what was placebo effect and what was treatment effect.

The main purpose of this study was to establish some level of understanding of the safety and tolerability in the use of a cystine-rich (sulfur-rich) compound in the treatment of autism. Ultimately, it is important to know whether non-denatured whey protein can be used to raise GSH levels in these children.

As mentioned in the Introduction, non-denatured whey protein isolate has been shown to increase glutathione levels and improve the healing process in many diseases and disorders, such as cystic fibrosis, lung disease, AIDS and other types of immune deficiencies, chronic fatigue, hepatitis B, and cancer, with no toxicity or adverse events.[14-26] Immunocal®, a non-denatured whey protein isolate, has been shown to augment GSH levels and to improve clinical parameters.

For example, a study by Grey et al.[26] showed that, compared to casein, Immunocal®, 10 g twice a day, increased glutathione levels in patients with cystic fibrosis. An overabundance of oxidants relative to antioxidants is associated with cystic fibrosis.[26] Glutathione functions in the lung as a major frontline defense against oxidative stress.

A Canadian clinical trial using Immunocal® was conducted in children (8 months to 15 years old) with AIDS and wasting syndrome. Immunocal® was administered for six months. All of the children experienced weight gain. Six of the ten patients had improved anthropometric parameters (skin-folds/triceps/mid arm circumference). The GSH-promoting activity of the whey supplement showed in six out of ten subjects.[14]

Watanabe et al.[20] showed that Immunocal® improves GSH levels, as well as liver and immune function, in hepatitis B patients. Serum alanine aminotransferase activity decreased, lipid peroxide levels significantly decreased, and interleukin 2 levels and natural killer activity increased.

A study of performance enhancement with Immunocal was conducted with 10 healthy males and 10 healthy females by Lands et al.[36] Lymphocyte GSH was significantly higher in the treatment group. Likewise, the supplemented subjects had a significantly improved peak power and 30-second work activity as compared to placebo.

Because this product is safe and effective in raising glutathione levels in other disorders and diseases, it is possible that it can be used in autism. This current trial suggests that non-denatured whey protein isolate can be used as a dietary supplement for children with autism without high rates of GI disturbance or other side effects. However, because autism involves a heterogeneous population, parents and clinicians should observe each child individually and understand that it may be better tolerated in some children than others. Since it shows acceptable tolerability, further research is needed and planned to determine its efficacy in raising GSH levels in those with autism and its effect on behavior in autistic children.

ACKNOWLEDGEMENT:

POTENTIAL CONFLICTS OF INTEREST

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REFERENCES

1. Yorbik O, Sayal A, Akay C, Akbiyik DI, Sohmen T. Investigation of antioxidant enzymes in children with autistic disorder. *Prostaglandins Leukotrienes Essential Fatty Acids.* 2002;67*:*341-343.
2. Chauhan A, Chauhan V, Brown WT, Cohen I. Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferring—the antioxidant proteins. *Life Sci*. 2004:75*:*2539-2549.
3. Zoroglu SS, Armutcu F, Ozen S, Gurel A, Sivasli E, Yetkin O, Meram I. Increased oxidative stress and altered activities of erythrocyte free radical scavenging enzymes in autism*. Eur Arch Psychiatry Clin Neurosci.* 2004;254:143-147.
4. James J, Cutler P, Melnyk S, Jernigan S, Janak L, Gaylor DW, Neubrander JA. Metabolic biomarkers of oxidative stress and impaired methylation capacity in children with autism. *American J Clin Nutri.* 2004;80*:*1611-1617.
5. James SJ, Melnyk S, Jernigan S, Cleves MA, Halsted CH, Wong DH, Cutler P, Bock K, Boris M, Bradstreet JJ, Baker

SM, Gaylor DW. Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. *Am J Med Gen, Part B, Neuropsychiatric Genetics: the Official Publication of the International Society of Psychiatric Genetics.* 2006;141:947-956.

6. Sogut S, Zoroglu SS, Ozyurt H, Yilmaz HR, Ozugurlu F, Sivasli E, Yetkin O, Yanik M, Tutkun H, Savas HA, Tarakcioglu M, Akyol O. Changes in the nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiology mechanisms in autism. *Clin Chim Acta.* 2003;33:111-117.

7. Kern JK, Jones AM. Evidence of toxicity, oxidative stress, and neuronal insult in autism. *J Toxicol Environ Health, Part B*. 2006;9:485-499.

8. Dalhoff K, Ranek L, Mantoni M, Poulsen HE. Glutathione treatment of hepatocellular carcinoma. *Liver.* 1992;12:341-343.

9. Witschi A, Reddy S, Stofer B, Lauterburg BH. The systemic availability of oral glutathione. *Eur J Clin Pharmacol.* 1992;43:667-669.

10. Gutman J. *Glutathione—Your Body's Most Powerful Protector*, 3rd ed. Montreal: Communications kudo.ca Inc.; 2002.

11. Sen CK. Nutritional biochemistry of cellular glutathione. *J Nutr Biochem*. 1997;8:660-672.

12. Bounous G. Whey protein concentrate (WPC) and glutathione modulation in cancer treatment. *Anticancer Res*. 2000;20:4785-4792.

13. Kinsella JE. Milk protein: physiochemical and functional properties. Crit Rev Food Sci Nutr. 1984;21:197-262.

14. Baruchel S, Viau G, Oliver R, Wainberg, MA. Nutraceutical modulation with a humanized native milk serum protein isolate, Immunocal®: application in AIDS and cancer. In L. Montagnier, R. Olivier, C. Pasquier, eds. *Oxidative Stress in Cancer, AIDS and Neurodegenerative Diseases.* New York, NY: Marcel Dekker Inc. 1998;447-461.

15. Bounous G, Molson J. The antioxidant system. *Anticancer Res*. 2003;23:1411-1415.

16. Bounous, G, Batist G, Gold P. Immunoenhancing property of dietary whey protein in mice: role of glutathione. *Clin Invest Med*. 1989;12:154-161.

17. Bounous G, Gold P. The biological activity of undenatured dietary whey proteins: role of glutathione. *Clin Invest Med.* 1991;14:296-309.

18. Bounous G, Gervais F, Amer V, Batist G, Gold P. The influence of dietary whey protein on tissue glutathione and the diseases of aging. *Clin Invest Med.* 1989;12:343-349.

19. Lothian B, Grey V, Kimoff RJ, Lands LC. Treatment of obstructive airway disease with a cysteine donor protein supplement: a case report. *Chest.* 2000;117:914-916.

20. Watanabe A, Okada K, Shimizu Y, Wakabayashi H, Higuchi K, Niiya K, Kuwabara Y, Yasuyama T, Ito H, Tsukishiro T, Kondoh Y, Emi N, Kohri H. Nutritional therapy of chronic hepatitis by whey protein (non-heated). *J Med*. 2000;31:283-302.

21. Kennedy R, Konok G, Bounous G, Baruchel S, Lee TD. The use of a whey protein isolate in the treatment of patients with metastatic carcinoma: A phase 1-II clinical study. *Anticancer Res*. 1995;15:2643-2650.

22. Micke P, Beeh KM, Schlaak JF, Buhl, R. Oral supplementation with whey proteins increases plasma glutathione levels of HIV-infected patients. *Eur Clin Invest.* 2001;31:171-178.

23. Micke P, Beeh KM, Buhl, R. Effects of long-term supplementation with whey proteins on plasma glutathione levels of HIV-infected patients. *Eur J Nutr.* 2002;41:12-18.

24. Agin D, Kotler DP, Papandreou D, Liss M, Wang J, Thornton J, Gallagher D, Pierson RN Jr. Effects of whey protein and resistance exercise on body composition and muscle strength in women with HIV infection. *Ann N Y Acad Sci*. 2000;904:607-609.

25. Marshall K. Therapeutic applications of whey protein. *Alt Med Rev.* 2004;9:136-156.

26. Grey V, Mohammed SR, Smountas AA, Bahlool R, Lands LC. Improved glutathione status in young adult patients with cystic fibrosis supplemented with whey protein. *J Cystic Fibrosis.* 2003;2:195-198.

27. Jyonouchi H, Sun S, Itokazu N. Innate immunity associated with inflammatory responses and cytokine production against common dietary proteins in patients with autism spectrum disorder. *Neuropsychobiol.* 2002;46:76-84.

28. Schopler E, Reichler RJ, Renner BR. *The Childhood Autism Rating Scale, 2004.* Western Psychological Services, 12031 Wilshire Boulevard, Los Angeles, CA 90025-1251.

29. Eaves R, Milner B. The criterion-related validity of the Childhood Autism Rating Scale and the Autism Behavior Checklist. *J Abnorm Child Psych*. 1993;21:481-491.

30. Sevin J, Matson J, Coe D, Fee V, Sevin BM. A comparison and evaluation of three commonly used autism scales. *J Autism Dev Disord.* 1993;21:417-432.

31. Wisniewski SR, Rush AJ, Balasubramani GK, Trivedi MH. Self-rated global measure of the frequency, intensity and burden of medication side effects. *J Psychiatric Pract.* 2006;12:71-79.

32. Aman M, Singh N. *Aberrant Behavior Checklist*. New York: Slosson Educational Publications, Inc., 1986.

33. Morisky DE, Green LW, Levine DM. Concurrent and predictive validity of a self-reported measure of medication adherence. *Med Care.* 1986;24:67-74.

34. Kern JK, Miller VS, Evans PA, Trivedi MH. Efficacy of porcine secretin in children with autism and pervasive developmental disorders. J Autism Dev Disord. 2002;32:153-160.

35. Christison GW, Ivany K. Elimination diets in autism spectrum disorders: any wheat amidst the chaff? *J Dev Behav Pediatr*. 2006;27:S162-171.

36. Lands LC, Grey VL, Smountas AA. Effect of supplementation with a cysteine donor on muscular performance. *J Appl Pych Physiol*.1999;87:1381-1385.

ORIGINAL RESEARCH

A Comparison of Injected and Orally Administered β-glucans

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ABSTRACT

β-glucans have been extensively studied for their pharmacological effects. Despite in-depth research, little is known about the optimal dose and/or optimal route of application. In this paper, we are reporting the result of comparing the immunostimulating activities of four commercially available glucans differing both in their solubility and source. In addition, we compared intraperitoneal and oral application, and the differences between a single versus repeated doses.

Our data showed strong differences in activities of individual glucans, with glucan yeast-derived #300 being the best, and grain-derived ImmuneFiber being the worst. Furthermore, we demonstrated that oral delivery of glucan resulted in significant immunological activity, which albeit slightly lower, corresponded with injectable application. Depending on the applied dose, the effects of individual glucans were long-lasting and in some cases, lasted up to two weeks.

In conclusion, our report represents further evidence about differences among commercial glucans and shows that these biological response modifiers can be similarly active when used in both injectable and oral form.

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INTRODUCTION

Natural products, useful in treating or preventing various diseases, have been sought throughout the history of mankind. Most of these natural products are plagued with a common problem, i.e., the fact that they often represent a complex mixture of individual ingredients, each of which can contribute to their biological activities. Natural (1,3)-β-D-glucans from yeast, grain and mushrooms are well-established biological response modifiers,[1,2] representing highly conserved structural components of cell walls in yeast, fungi, seaweed, or grain seeds.

Numerous types of glucans have been isolated from almost every species of yeast, grain and fungi. (1,3)- β-D-glucans have been extensively studied for their immunological and pharmacological effects. More than 2,000 papers describing the biological activities of glucans exist in the literature.[3] Another advantage of glucans is the fact that all sufficiently purified polysaccharidic immunomodulators distinguish themselves by very low toxicity (*e.g.*, for mouse lentinan has LD_{50} > 1600 mg/kg[4]).

Despite detailed knowledge of the activities of many glucans, limited information is available regarding the mechanisms of action by orally delivered glucans. For some time, there were even suggestions that orally administered glucans have no activity at all. Only recently has more information about the mechanisms of action of orally delivered glucans become available.[5,6]

The limited number of papers dealing with the problems of glucan transfer through the gastrointestinal tract mainly focus on the fact that fluorescent-labeled glucan can be detected in cells isolated from various tissues.[7] The studies of Ross's group indicated that orally-administered (1,3)-β-D-glucan is taken up by gastrointestinal macrophages

and subsequently shuttled to the reticuloendothelial system and bone marrow. Recent observation found that both insoluble glucans and soluble seaweed-derived Phycarine have similarly pronounced effects when applied via intraperitoneal or oral administration.[7,8,9]

The aims of the present study were to follow up our previously published comparison of commercial β-glucans[10] and to test the effect of different commercially available glucans on both the cellular and humoral branches of immune reactions using different routes of administration.

MATERIAL AND METHODS

Animals

Female, 6 to 10 week old BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal work was done according to the University of Louisville IACUC protocol. Animals were sacrificed by CO_2 asphyxiation.

Materials

RPMI 1640 medium, sodium citrate, dextran, ovalbumin, Ficoll-Hypaque, antibiotics, sodium azide, bovine serum albumin, Wright stain, Limulus lysate test E-TOXATE, Freund's adjuvant and Concanavalin A were obtained from Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was from Hyclone Laboratories (Logan, UT).

β -1,3 glucans

The glucans used in this study were purchased from the following companies: NOW BETA glucan from NOW FOODS (Bloomingdale, IL), Krestin from Biotec (Kureha Chemical Industries, Tokyo, Japan), Glucan #300 from Transfer Point (Columbia, SC), and ImmunoFiber from (Whole Control, Arvada, CO).

Glucan treatment

Individual glucans were applied either intraperitoneally or orally. The samples were collected at different intervals after either single or three ip. injections (100 mg of glucan/mouse) or after one day or a fourteen-day feeding with glucan-containing diet. All diets (Laboratory Rodent Diet 5001 enhanced with various doses of glucan) were formulated and prepared by Purina (Richmond, IN). Diet ingredients for all groups were identical except for the proportion of glucan.

Antibodies

For fluorescence staining, the following antibodies have been employed: anti-mouse CD4, CD8 and CD19, conjugated with FITC, which were purchased from Biosource (Camarillo, CA).

Flow cytometry

Cells were stained with monoclonal antibodies on ice in 12 x 75-mm glass tubes using standard techniques. Pellets of $5x10^5$ cells were incubated with 10 µl of FITC-labeled antibodies (1 to 20 µg/ml in PBS) for 30 minutes on ice. After washing with cold PBS, the cells were re-suspended in PBS containing 1% BSA and 10 mM sodium azide. Flow cytometry was performed with a FACScan (Becton Dickinson, San Jose, CA) flow cytometer and the data from over 10,000 cells/samples were analyzed.

Phagocytosis

The technique that employs phagocytosis of synthetic polymeric microspheres was described earlier.[11,12] Briefly: peritoneal cells were incubated with 0.05 ml of 2-hydroxyethyl methacrylate particles (HEMA; $5x10^8$/ml). The test tubes were incubated at 37° C for 60 min., with intermittent shaking. Smears were stained with Wright stain. The cells with three or more HEMA particles were considered positive. The same smears were also used for evaluation of cell types.

Evaluation of IL-2 production

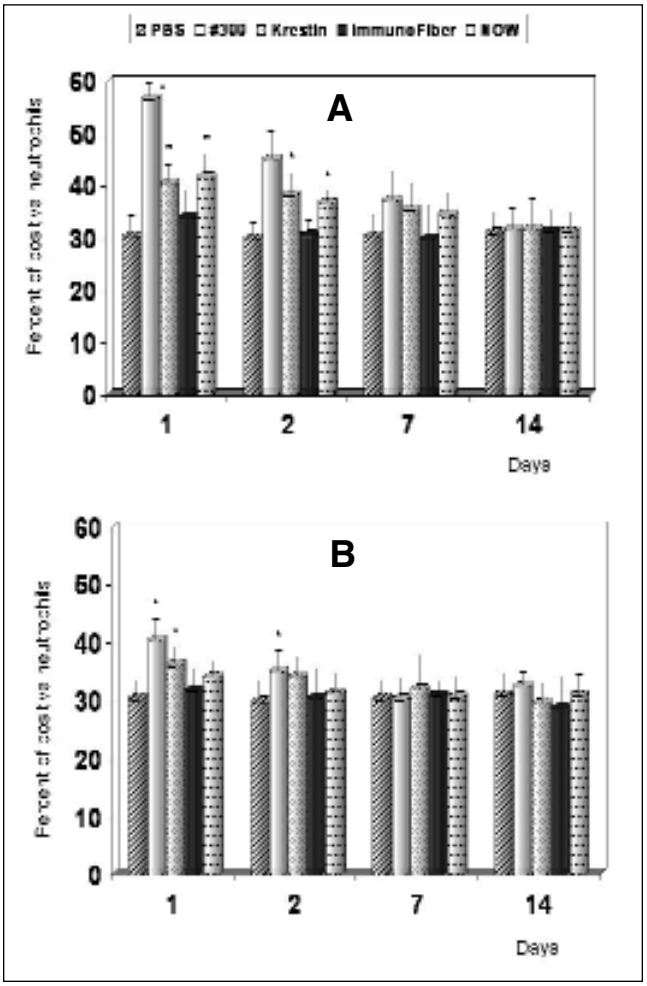
Purified spleen cells ($2x10^6$/ml in RPMI 1640 medium with 5% FCS) were added into wells of a 24-well tissue culture plate. After the addition of 1 mg of Concanavalin A into positive-control wells, cells were incubated for 72 hrs. in a humidified incubator (37°C, 5% CO_2). At the endpoint of incubation, supernatants were collected, filtered through 0.45 mm filters and tested for the presence of IL-2. Levels of the IL-2 were measured using a Quantikine mouse IL-2 kit (R&D Systems, Minneapolis, MN).

RESULTS

Most published studies describe effects of injected β-glucans (either ip., iv. or sc.). However, it is necessary, in the event of clinical practice, to evaluate the possibility of oral delivery.

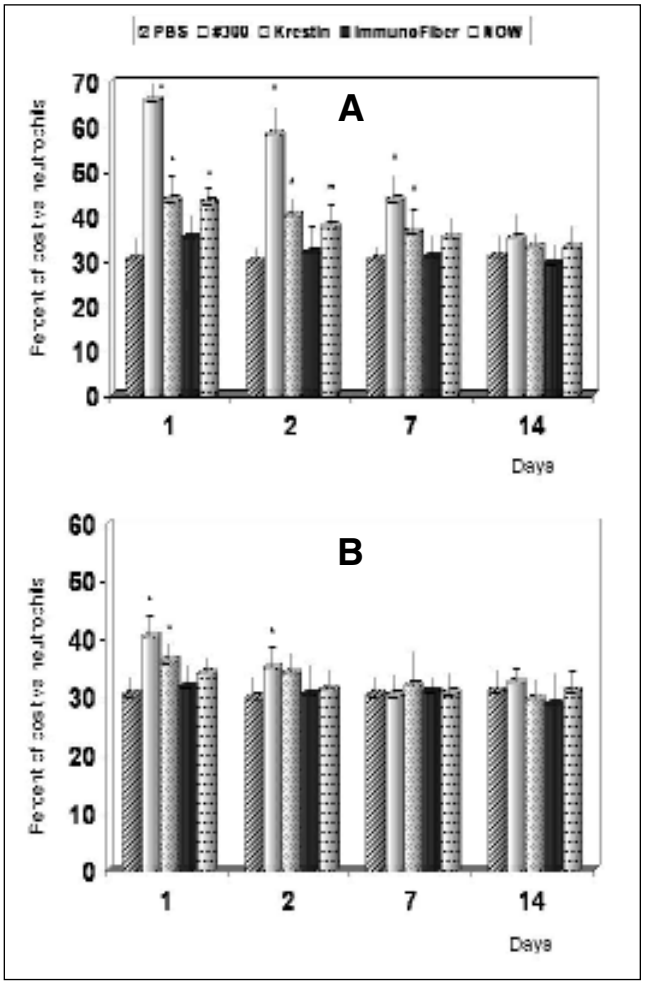
Phagocytosis is one of the biological activities traditionally connected with effects of immunomodulators, including glucans. Therefore, we started our study by comparing the effects of orally and intraperitoneally applied glucans. When used as a single dose, ip. application showed more profound effects than oral application (Figure 1 A,B). In addition, some glucans (such as NOW and Krestin) exhibited either longer effects or were effective only after injection. When we repeated the glucan administration for three consecutive days, we found not only higher phagocytic activity, but that it also lasted significantly longer (in the case of #300 and Krestin, up to 7 days). Oral delivery also showed higher effects, but similar to a single dose, signifi-

Figure 1.



*Effect of an administration of 100 μg of different glucan samples on phagocytosis by peripheral blood granulocytes (A intraperitoneally, B orally). Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.*

Figure 2.



*Effect of an administration of 100 μg of different glucan samples on phagocytosis by peripheral blood granulocytes (A intraperitoneally, B orally). Glucans were applied three times. Each value represents the mean ± SD. *Represents significant differences between control (PBS) and glucan samples at P ≤0.05 level.*

cant effects were observed only in the case of #300 (Figure 2 A,B).

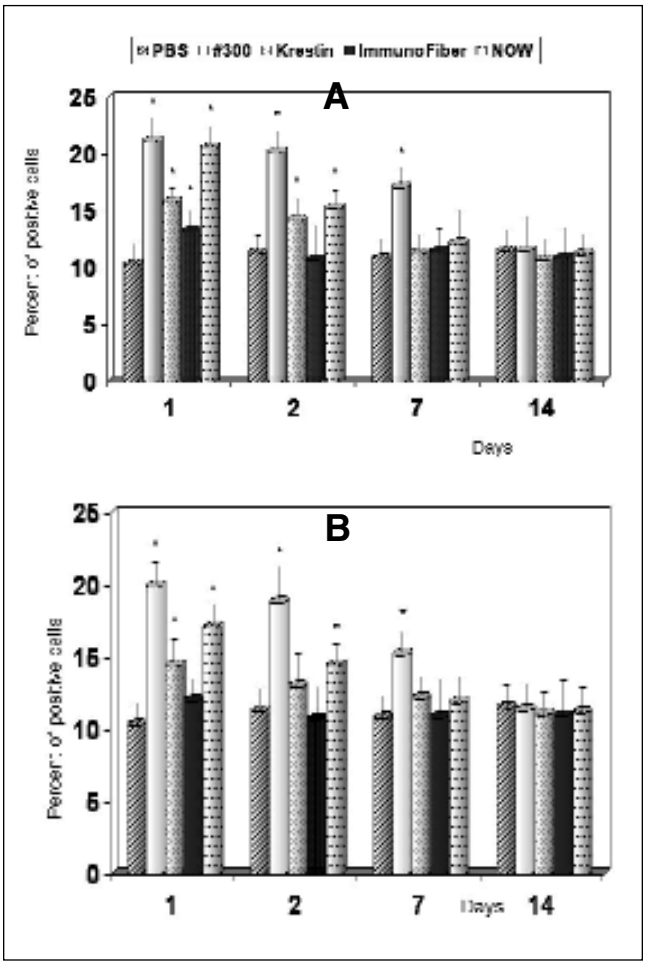
Next, we evaluated the effects of our glucans on the expression of some immunologically important surface markers on spleen lymphocytes isolated from mice stimulated with individual glucans. Using ip. injection, we found that 24 hrs. later, all glucans increased expression of CD4, but this effect was long-lasting only in the case of #300 (Figure 3A). When testing CD8 expression, the effects of three active glucans, #300, NOW and Krestin, were observed for 48 hrs. (Figure 4 A). None of the tested glucans affected the number of CD19-positive cells (B lymphocytes (Figure 5A).

A similar situation has been found in orally-stimulated mice; the only exception was no activity of ImmunoFiber (Figures 3B,4B). Again, no effects on expression of CD19 (Figure 5B).

Production of IL-2 belongs to the valuable indicators of the immune activities. Therefore, we compared the effects of tested glucans on the secretion of IL-2 by spleen cells isolated from glucan-treated mice. The IL-2 production was measured after a 72 hr. *in vitro* incubation of cells. The results, summarized in Figure 6, showed that even when all tested glucan stimulated IL-2 production, there were huge differences between individual glucans (i.e., #300 stimulated IL-2 secretion 3.5 times more than ImmunoFiber). The activity of all tested glucans slowly decreased with time, but was still measurable 14 days after injection (Figure 6A). Virtually identical, albeit lower, results were found in the case of orally-treated mice (Figure 6B).

When we evaluated the IL-2 production after repeated stimulation with glucans, we found a higher overall secretion of IL-2. Using ip. injection, #300 was more active than

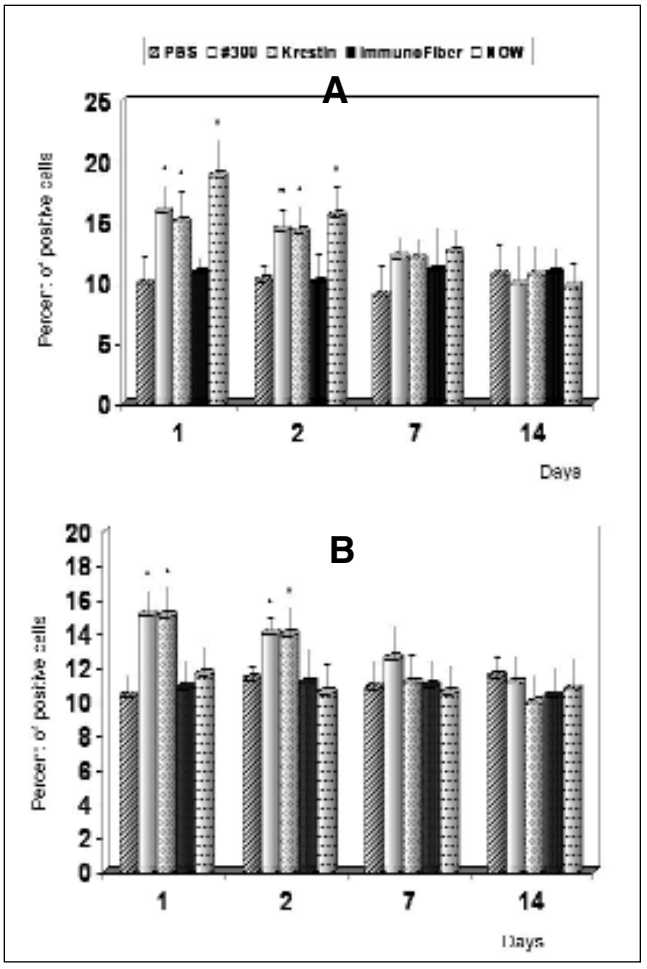
Figure 3.



Effect of application of 100 μg of tested glucans on the expression of CD4 marker by spleen cells (A intraperitoneally, B orally). The cells from three donors at each time interval were examined and the results given represent the means ± SD.

**Represents significant differences between control (PBS) and samples at P ≤ 0.05 level.*

Figure 4.



*Effect of application of 100 μg of tested glucans on the expression of CD8 marker by spleen cells (A intraperitoneally, B orally). The cells from three donors at each time interval were examined and the results given represent the means ± SD. *Represents significant differences between control (PBS) and samples at P ≤ 0.05 level.*

Concanavalin A up to seven days after last application, with both Krestin and NOW showing strong stimulation (Figure 7A). The same situation was found after oral application, where we discovered significant stimulation in each glucan even two weeks after the last application (Figure 7B). It is important to note that the secretion of IL-2 by control (i.e., non-stimulated cells) was almost zero; therefore, all glucans yielded statistically significant stimulations. When compared to stimulation with Con A, #300 showed stronger effects, Krestin and NOW were comparable, and ImmunoFiber showed lower activity.

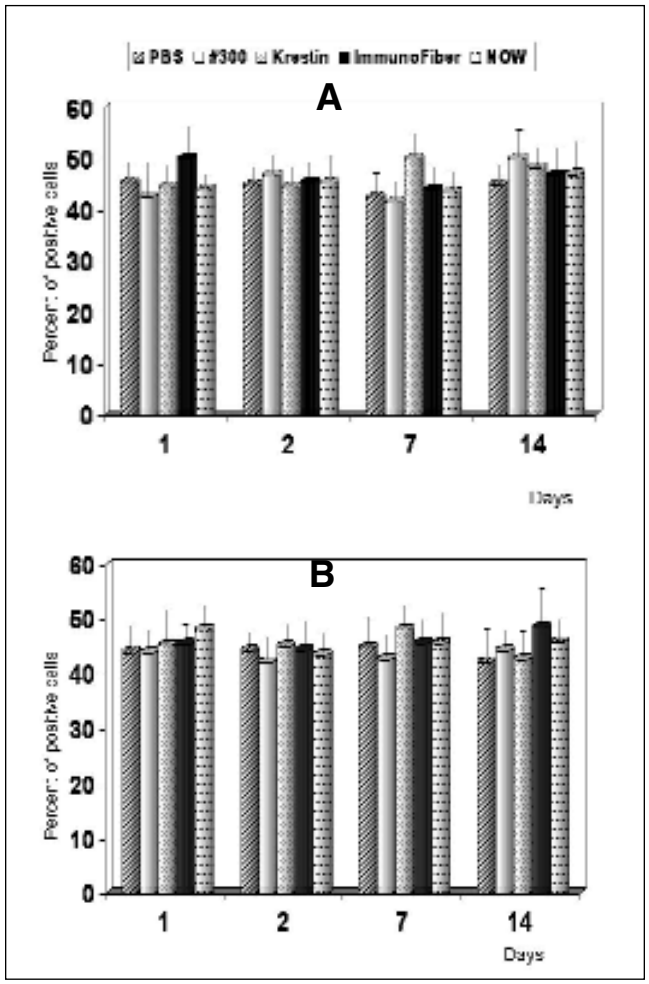
Glucans are usually considered more as stimulators of the cellular branch of immune reactions; however, some glucans can act as nonspecific adjuvant. Using an experimental model of ovalbumin immunization, we applied glucan either intraperitoneally together with two doses of antigen (Figure 8A), or orally for two weeks (Figure 8B). In both cases, only glucans #300, Krestin and ImmunoFiber showed stimulation of antibody response.

Finally, we evaluated whether the glucan feeding was reflected in changes of weight of individual organs. As seen in Table 1, there were no differences in the weight of any tested organs. In addition, the ip. injection had no effects (results not shown).

DISCUSSION

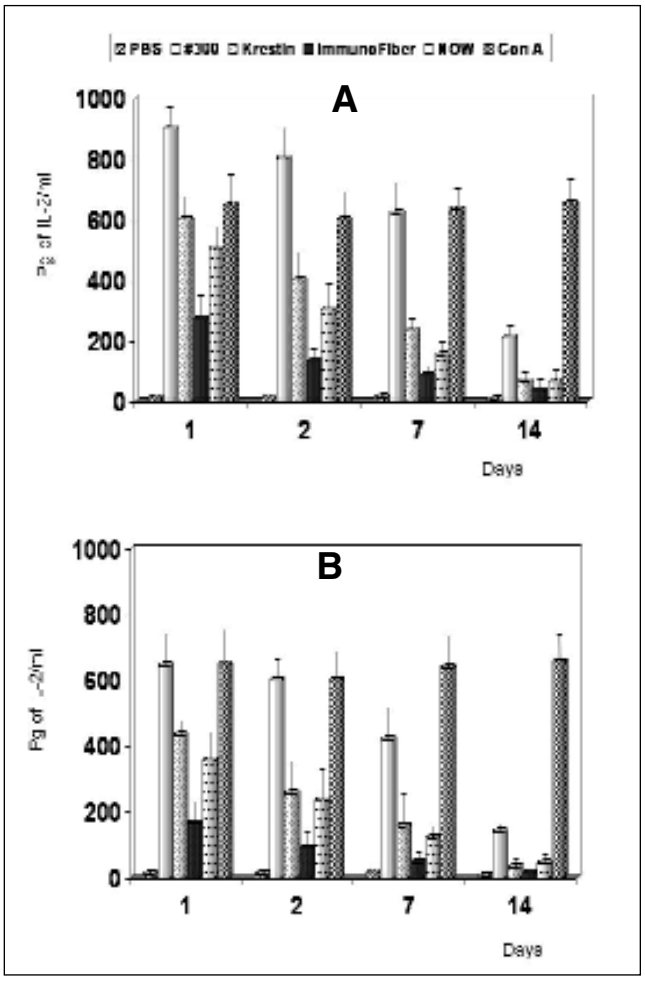
β-Glucans show notable physiological effects, which is the main reason why so much attention has been devoted to them. They belong to a group of physiologically active

Figure 5.



Effect of application of 100 ?g of tested glucans on the expression of CD19 marker by spleen cells (A intraperitoneally, B orally). The cells from three donors at each time interval were examined and the results given represent the means ± SD.

Figure 6.

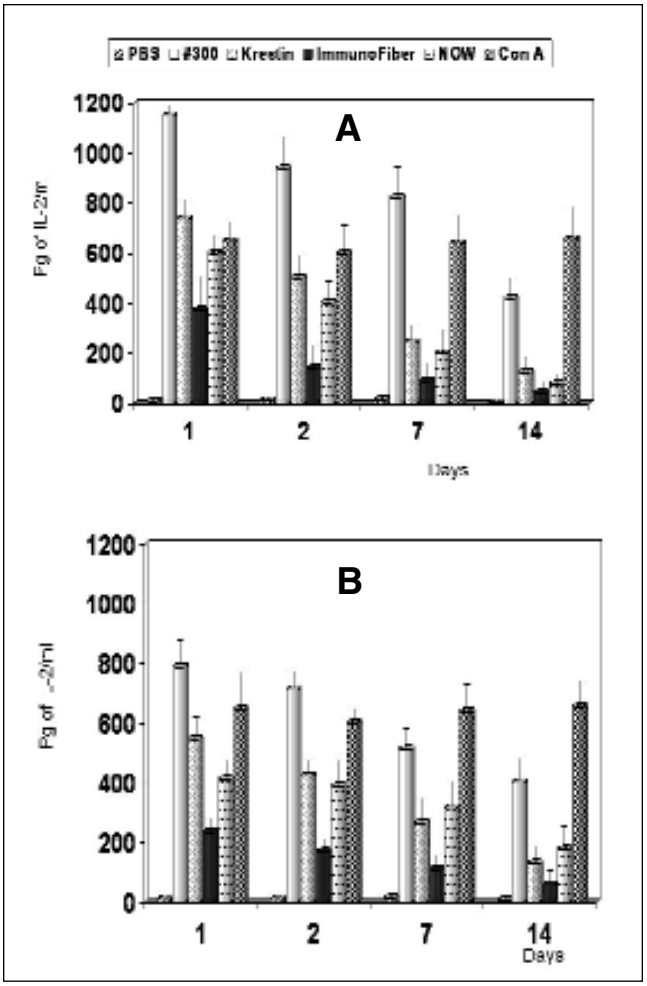


Effects of glucans on Con A-stimulated secretion of IL-2 by spleen cells (A intraperitoneally, B orally).

Table 1.

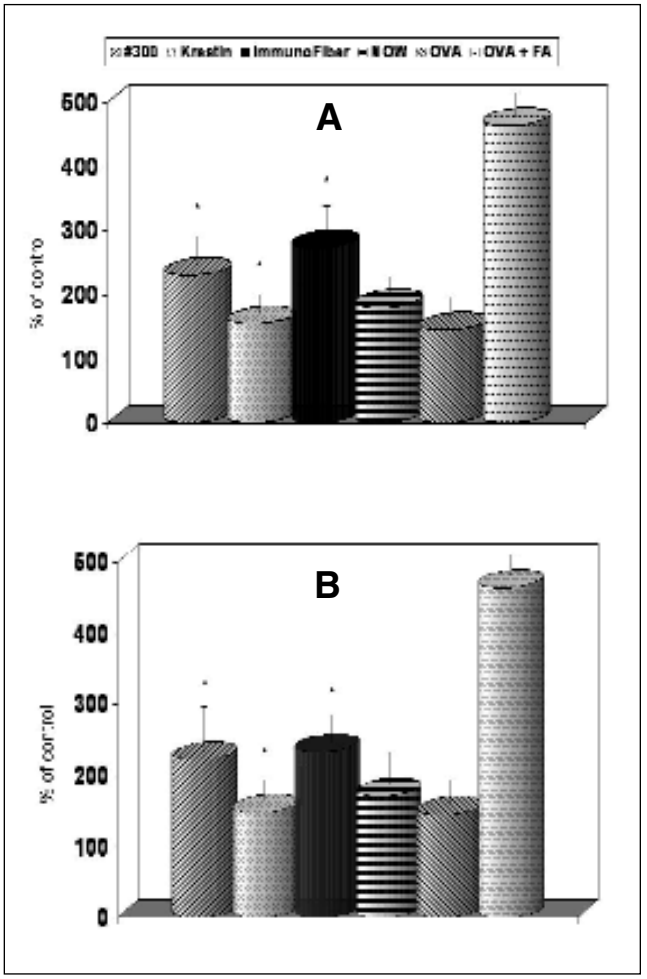
	Control	#300	Krestin	ImmunoFibre	Now
Total weight	23.33 ± 1.06	24.34 ± 1.27	24.11 ± 1.48	24.99 ± 4.11	26.99 ± 2.77
Liver	1.77 ± 0.23	1.49 ± 0.31	1.55 ± 0.19	1.54 ± 0.38	1.47 ± 0.35
Spleen	0.16 ± 0.09	0.17 ± 0.06	0.14 ± 0.05	0.17 ± 0.07	0.16 ± 0.03
Thymus	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.08 ± 0.02
Heart	0.16 ± 0.06	0.17 ± 0.10	0.18 ± 0.08	0.16 ± 0.03	0.17 ± 0.03
Kidneys	0.14 ± 0.10	0.38 ± 0.07	0.40 ± 0.12	0.40 ± 0.15	0.42 ± 0.05
Lung	0.16 ± 0.02	0.17 ± 0.02	0.15 ± 0.02	0.18 ± 0.03	0.18 ± 0.05

Figure 7.



Effects of glucans on Con A–stimulated secretion of IL-2 by spleen cells (A intraperitoneally, B orally). Glucans were applied three times.

Figure 8.



*Effects of two ip. injections (A) or two week oral delivery (B) of tested glucans on formation of antibodies against ovalbumin. Mice were injected twice (two weeks apart) with antigen and the serum was collected 7 days after last injection. Level of specific antibodies against ovalbumin was detected by ELISA. As a positive control, Freund's adjuvant was used. *Represents significant differences between control (ovalbumin alone) and samples at $P \leq 0.05$ level.*

compounds, collectively termed biological response modifiers. Thus far, among many known and tested immunomodulators of the first order, polysaccharides isolated from different microorganisms and plants hold a formidable place. A large number of such polysaccharides, that act only as immunopotentiators are well known.[13]

Binding of β-glucan to specific receptors (either CR3 or Dectin-1) activates macrophages. The activation consists of several interconnected processes including increased chemokinesis, chemotaxis, migration of macrophages, degranulation leading to increased expression of adhesive molecules, and adhesion to the endothelium. In addition, β-glucan binding triggers intracellular processes, characterized by the respiratory burst after phagocytosis of invading cells (formation of reactive oxygen species and free radicals), the increase of content and activity of hydrolytic enzymes, and signaling processes leading to activation of other cells and secretion of cytokines. For an excellent review regarding interaction of glucans with macrophages, see Schepetkin and Quinn.[14]

Regarding the question as to whether glucans are similarly active when administered orally, we compared the oral and intraperitoneal applications. To allow our experiments more relevancy in the use of natural immunostimulants, we compared the effects of a single application with repeated doses.

The rationale for the choice of glucans parallels what was stated in our previous paper.[10] We chose four glucans widely sold and available in the US, Europe, and the Far East, representing grain-, mushroom- and yeast-derived glucans in soluble and insoluble form. Briefly, #300 is insoluble yeast-derived glucan; Krestin is soluble mushroom-

derived glucan; ImmunoFiber represents soluble grain-derived glucan; and NOW is a mixture of both insoluble glucans from yeast and soluble glucans from mushrooms.

There are very few comprehensive reviews focused on biological properties of glucans from various existing sources. The comparative reviews focus mainly on the reflection of chemical characteristics of glucans on their biological and immunological properties.[15,16]

In this paper, we continued the comparison of several commercially important glucans.[10] Glucans are well known for their ability to stimulate the innate immunity and the cellular branch of immune reaction.[13] Therefore, our initial focus was phagocytic activity with the use of peripheral blood neutrophils and synthetic microspheres as a model. Our results confirmed our previous studies showing that glucan #300 was one of the most active glucans, regardless of the route of application.[8,10,17-19] Additional data showed that the duration of these effects depends on the strength and timing of the glucan treatment since repeated doses clearly resulted in stronger and longer action stimulation.

We then turned our attention to the effect of glucans on surface markers. In the case of CD4-positive lymphocytes, one injection of any of the glucans was enough to increase the influx of these cells. In the case of oral application, the data were similar with the exception of ImmunoFiber, which showed no activity. Similar data were observed in the case of CD8-positive splenocytes. In both cases, only #300, Krestin and NOW showed longer effects — two days for Krestin and NOW, and up to one week for glucan #300. The number of CD19-positive cells (B lymphocytes) did not change. These findings were in agreement with previous data established using Phycarine[20] or lentinan.[21] When we measured repeated doses of glucan, the results were identical to those shown in Figures 3 to 5, and due to the restricted space, were not included in this report.

It is assumed that glucan application results in signaling processes leading to activation of macrophages and other cells, and subsequent secretion of cytokines and other substances initiating inflammation reactions (*e.g.*, interleukins IL-1, IL-2, IL-6, and TNF-α).[22-24] We found that all tested glucans stimulated splenocytes to produce IL-2, with #300 and Krestin showing the strongest and longest effects. Our findings were similar to previously published data.[8,10,20]

As some recent studies established that glucans can also support the humoral branch of the immune reaction by serving as adjuvant,[25] we compared the adjuvant activities of tested glucans with Freund's adjuvant. Our results showed that even when the activities were always lower than those of Freund's adjuvant, they were nevertheless significant, with the higher activity found in the previously almost inactive ImmunoFiber. These data correlate well with the previous finding of significant adjuvant activity with grain-derived glucans.[10] It is important to note that in these experiments, we applied the glucans either two times ip. (together with the antigen) or for a full two weeks (in case of oral application).

The present paper represents yet another proof of vast differences among commercially available glucans. To conclude — glucan #300 was again a highly active glucan with a sufficiently broad range of action. We demonstrated that oral application is comparable to the intraperitoneal route, and that the somehow lower effects after oral stimulation can be easily overcome by repeated oral doses.

ACKNOWLEDGEMENT

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DISCLAIMER

The authors of this study have no significant financial interest in any of the products or manufacturers mentioned in the article. No external funding was provided for this study.

REFERENCES

1. Borchers AT, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity. *Proc Soc Exp Biol Med*. 1999; 221:281-293.
2. Brown GD, Gordon S. Fungal b-glucans and mammalian immunity. I*mmunity*. 2003; 19:311-315.
3. Novak M, Vetvicka V. Beta-glucans, history and present. *Alt Med Rev* 2007, in press.
4. Chihara G, Maeda YY, Hamuro J. Current status and perspectives of immunomodulators of microbial origin. *Int J Tis React.* 1982; IV:207-225.
5. Hong F, Yan J, Baran JT, Allendorf DJ, Hansen RD, Ostroff GR, Xing PX, Cheung NK, Ross GD. Mechanism by which orally administered b-1,3-glucans enhance the tumoricidal activity of antitumor monoclonal antibodies in murine tumor models. *J Immunol*. 2004; 173:797-806.
6. Vetvicka V, Dvorak B, Vetvickova J, Richter J, Krizan J, Sima P, Yvin JC. Orally administered marine (1->3)- b-D-glucan Phycarine stimulates both humoral and cellular immunity. *Int J Biol Macromol*. 2006; 40:291-298.
7. Li B, Allendorf DJ, Hansen R, Marroquin J, Ding C, Cramer DE, Yan J. Yeast b-glucan amplifies phagocyte killing of iC3b-opsonized tumor cells via complement receptor 3-Syk-Phosphatidylinositol 3-kinase pathway. *J Immunol*. 2006; 177:1661-1669.
8. Allendorf DJ, Baran JT, Hansen RD, Subbarao K, Walsh D, Hong F, Marroquin J, Yan J. Orally administered b-glucan functions via anti-tumor mAbs and the complement system to recruit $CR3^+$ neutrophils and

macrophages that produce tumor regression and tumor-free survival. *Mol Immunol*. 2003; 40:195-196.

9. Yan J, Allendorf DJ, Brandley B. Yeast whole glucan particle (WGP) beta-glucan in conjunction with antitumor monoclonal antibodies to treat cancer. *Expert Opin Biol Ther*. 2005; 5:691-702.
10. Vetvicka V, Vetvickova J. An evaluation of the immunological activities of commercially available b1,3-glucans. *JANA*. 2007; 10:25-31.
11. Vetvicka V, Fornusek L, Kopecek J, Kaminkova J, Kasparek L, Vranova M. Phagocytosis of human blood leukocytes: a simple micromethod. *Immunol Lett*. 1982; 5:97-100.
12. Vetvicka V, Holub M, Kováru H, Siman P, Kováru F. Alpha-fetoprotein and phagocytosis in athymic nude mice. *Immunol Lett*. 1988; 19:95-98.
13. Whistler RL, Bushway AA, Singh PP, Nakahara W, Tokuzen R. Noncytotoxic, antitumor polysaccharides. *Adv Carbohydr Chem Biochem*. 1976; 32:235-275.
14. Schepetkin IA, Quinn MT. Botanical polysaccharides: macrophage immunomodulation and therapeutical potential. *Int Immunopharmacol*. 2006; 6:317-333.
15. Yadomae T. Structure and biological activities of fungal b-1,3-glucans. *Yakugaku Zasshi*.2000; 120:413-431.
16. Kogan G. (1-3,1-6)-b-D-glucans of yeast and fungi and their biological activity. In: Atta-ur-Rahman, (ed): Studies in Natural Products Chemistry. Amsterdam, *Elsevier*. 2000: 107-152.
17. Kurashige S, Akuzawa Y, Endo F. Effects of Lentinus edodes, Grifola frondosa and Pleurotus ostreatus administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N-butanolnitrosoamine. *Immunopharmacol Immunotoxicol*. 1997;19:175-183.
18. Vetvicka V, Vetvicka J. Physiological effects of different types of β-glucan. Biomed. Pap. 2007;151:1-7.
19. Vetvicka V, Vetvickova J. Immunostimulating properties of two different b-glucans isolated from Maitake mushrooms (*Grifola frondosa*). *JANA*. 2005; 8:33-39.
20. Vetvicka V, Yvin JC. Effects of marine beta-1,3 glucan on immune reactions. *Int Immunopharmacol*. 2004; 4:721-730.
21. Arinaga S, Karimine N, Takamuku K, Nanbara S, Nagamatsu M, Ueo H, Akiyoshi T. Enhanced production of interleukin 1 and tumor necrosis factor by peripheral monocytes after lentinan administration in patients with gastric carcinoma. *Int J Immunopharmacol*. 1992; 14:43-47.
22. Adachi Y, Okazaki M, Ohno N, Yadomae T. Enhancement of cytokine production by macrophages stimulated with (1->3)- b-D-glucan, Grifolan (GRN), isolated from *Grifola frondosa*. *Biol Pharm Bull*. 1994; 17:1554-1560.
23. Abel G, Czop JK. Stimulation of human monocyte b-glucan receptors by glucan particles induces production of TNF-a and IL-1b. *Int J Immunopharmacol*. 1992; 14:1363-1373.
24. Vetvicka V, Terayama K, Mandeville R, Brousseau P, Kournikakis B, Ostroff G. Pilot study: orally administered yeast b1,3-glucan prophylactically protects against anthrax infection and cancer in mice. *JANA*. 2002; 5:1-6.
25. Cook JA, Holbrook TW. Immunogenicity of soluble and particulate antigens from *Leishmania donovani:* effect of glucan as an adjuvant. *Infect Immun*. 1984; 40:1038-1043.

ORIGINAL RESEARCH

A Standardized *Withania Somnifera* Extract Significantly Reduces Stress-Related Parameters in Chronically Stressed Humans: A Double-Blind, Randomized, Placebo-Controlled Study

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ABSTRACT

Withania somnifera (WS) has historically been used in Asia for treating stress-related health conditions. In this study, we investigated the effects of standardized WS root and leaf extract (WSE) in chronically stressed humans in a modern clinical trial. Participants were randomly assigned to WSE (125 mg QD, 125 mg BID, or 250 mg BID) or placebo groups. Stress levels were assessed at Days 0, 30, and 60 using a modified Hamilton anxiety (mHAM-A) scale. Biochemical and clinical variables were measured at Days 0 and 60. Of 130 subjects enrolled, 98 completed the study. Between Days 0 and 60, the WSE 125 mg QD group decreased significantly more than placebo for mean mHAM-A score, serum cortisol, serum C-reactive protein, pulse rate and blood pressure, and increased significantly for mean serum DHEAS and hemoglobin. Other WSE treatment groups had greater dose-dependent responses in these parameters and had significantly greater responses compared to placebo in mean fasting blood glucose, serum lipid profiles and cardiac risk ratios. Participants and dropouts reported no adverse effects. Therefore, this study provides evidence that the consumption of WSE significantly reduces experiential and biochemical indicators of stress without adverse effects.

Key words: *Withania somnifera*, antistress, withanolides, sitoindosides, cortisol, C-reactive protein.

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INTRODUCTION

Stress is a major component of modern life, causing adverse physiological conditions such as cognitive deficiencies, impaired glucose and lipid homeostasis, immunosuppression, sexual dysfunction, gastric ulceration, and alteration in serum cortisol and dehydroepiandrosterone sulfate (DHEAS) levels.[1] Development of active management and treatment protocols that control stress-related symptoms with minimum adverse effects would be of great benefit.

Withania somnifera Dunal (Solanacea) (WS) has traditionally been used in Asia for safely managing and treating stress. Also known as Ashwagandha, Indian ginseng and winter cherry, it belongs to a *rasayana* (vitalizer) group of medicinal plants that stabilize and revitalize systemic functions. The Ayurvedic system of medicine claims that it promotes stress relief, health and longevity by potentiating the immune system, arresting premature aging, restoring homeostasis and increasing resistance to adverse environ-

mental factors, collectively known as the antistress-adaptogenic effect.[2-4] However, its healing effects on chronically stressed individuals have never been evaluated in a randomized, controlled clinical trial.

Several bio-actives, including withanolide glycosides (also known as sitoindosides) and withanolide aglycones, are considered to be responsible for the medicinal properties of WS.[5,6] Traditional medicinal use of WS has employed root powder derived from wild plants,[7] which are relatively low in concentration of bioactives.[5] Cultivated WS varieties differ morphologically from wild WS,[8] and have higher levels of bioactive components.[5,9] Methods to further concentrate levels of bioactives in standardized WS extracts (WSE) have also been developed.[9]

The objective of this study was to investigate, in chronically stressed adults, the impact of WSE on experiential and biochemical indicators of stress and anxiety as well as cardiovascular risk, and to evaluate WSE tolerance.

MATERIALS AND METHODS

This double-blind, randomized, placebo-controlled study was conducted from November 2004 to October 2006 at the Central Research Institute (Ayurveda), Ministry of Health and Family Welfare, Bidhan Nagar, Kolkata, India (CRI), in accordance with the World Health Organization's Guideline for Good Clinical Practice and the World Medical Association Declaration of Helsinki.[10, 11] The CRI Ethics Committee approved the protocol. Patients identified as stressed in the CRI outpatient department were assessed clinically (blood pressure, resting heart rate, reflexes, and neurological and psychological status) and completed a questionnaire assessing the severity of stress symptoms (cognitive, mood and behavioral) based on a Bengali version of a modified Hamilton anxiety (mHAM-A) scale for stress.[12, 13]

In the questionnaire, patients rated symptoms of anxiety (fatigue, flushing, perspiration, loss of appetite, headache and muscle pain, feelings of impending doom, palpitations, dry mouth, sleeplessness, forgetfulness, irritability and inability to concentrate) on a 5-point scale (0 = no symptoms; 1 = occasional; 2 = mild/poor; 3 = moderate; 4 = severe). The total score was calculated by adding the score from individual questions.

Men and women aged 18 to 60 years were eligible for the study if they had a mHAM-A score of 24 to 42. Exclusion criteria were any concomitant serious physical disorder(s) or antistress treatment (antidepressants, anxiolytic) that was ongoing or had occurred during the previous month. Exercise as well as drugs that lower serum lipids, blood pressure or blood sugar were not considered exclusion criteria, but this was not considered a significant bias in the study because only two participants (1.5% of the total) were taking drugs of this nature (hypertensive) and they were allocated randomly into different treatment groups. Participants provided written informed consent in English and Bengali, the local language, and were randomly divided into four groups using a computer-generated random number list: WSE 125 mg QD, WSE 125 mg BID, WSE 250 mg BID, and placebo.

The WSE used in this study {trade names Sensoril® (Natreon Inc., New Brunswick, New Jersey) and Essentra® (NutraGenesis, LLC, Brattleboro, Vermont)} was derived from a withaferin A and corresponding withanolide glycoside-predominant, genetically uniform chemotype, which was cultivated in the central and northern provinces of India. WS root and leaf material was processed using a water-based extraction protocol and assessed using high performance thin layer chromatography analysis of fractions against standard references (CAMAG Linomat V applicator, CAMAG TLC Scanner, and WinCats software version 1.3.4; CAMAG, Sonnenmattstr. Muttenz, Switzerland) in accordance with US Patent 6,713,092. The single lot of WSE used in the study had a composition that was standardized to a minimum of 8% withanolide glycosides and 32% oligosaccharides, and a maximum of 2% withaferin A.

WSE (125 mg or 250 mg) plus excipients or excipients only (placebo) were placed in coded hard-gelatin capsules identical in size, shape, color (opaque white) and texture. Participants received two bottles of capsules and were told to take one capsule from Bottle 1 before lunch and one capsule from Bottle 2 before dinner for 60 days. Participants in the WSE 125 mg QD group took a 125 mg WSE capsule before lunch and a placebo capsule before dinner. Participants in the other groups took two of the same capsule (corresponding to the group name, e.g., the placebo group took two placebo capsules, etc.) each day. At each of four visits, participants received a 15-day supply of capsules. Compliance was monitored by counting the remaining pills at each follow-up visit and at the end of the study. Information about tolerance (i.e., treatment-emergent adverse effects) was obtained by questioning the participants and clinically examining them at each visit.

Experiential feelings of stress and anxiety were assessed by calculating the sum of scores from the mHAM-A questionnaire taken at baseline (Day 0), Day 30 and Day 60. To measure biochemical markers of stress and anxiety, participants fasted overnight prior to visits at baseline and Day 60 to avoid diurnal variations (particularly in serum cortisol concentration[14]). Blood samples (6–10 mL) were collected in vacutainer tubes (BD Vacutainer Systems Medical Supplies, Plymouth Devon, UK) between 9 a.m. and 11 a.m., stored at 4°C, and assayed for serum concentrations of cortisol, dehydroepiandrosterone sulfate (DHEAS), C-reactive protein (CRP), fasting blood glucose (FBG), total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), very low-density

lipoprotein cholesterol (VLDL-C), high-density lipoprotein cholesterol (HDL-C), and hemoglobin at a laboratory (Doyen Diagnostic & Research Foundation, 59 Bhupen Bose Avenue, Kolkata-700004) accredited by the National Accreditation Board for Testing and Calibration Laboratories, Dept. of Science and Technology, Government of India. Pulse rate and blood pressure were also determined.

Difference scores were calculated by subtracting sum values at 30 days from baseline, 60 days from baseline, and 60 days from 30 days for each variable individually and for the total anxiety score. Difference scores were then compared among groups using 1-way analyses of variance with post hoc pairwise comparisons of the three treatment groups with the placebo group using the least significant difference method. Percent changes were expressed as the difference between the means of the baseline and treatment phases divided by the mean of the baseline phase multiplied by 100. Cardiovascular risk ratios (TC:HDL-C and LDL-C:HDL-C) were calculated as TC divided by HDL-C and LDL-C divided by HDL-C, respectively. Values between 3.5 and 5 for TC:HDL-C and between 1.1 and 3.6 for LDL-C:HDL-C indicate average risk of developing coronary artery disease and heart disease.[15] Sample size calculations were not done for this study. $P < 0.05$ was considered statistically significant.

RESULTS

Out of 160 eligible participants, 130 (95 men, 35 women; mean age= 39.8 years) initially enrolled in the study (Table 1) and 32 (26 men, 6 women) dropped out (WSE 125 mg QD group, 11; WSE 125 mg BID group, 5; WSE 250 mg BID group, 1; and placebo group, 15). Reasons for study withdrawal were protocol violations, 6; being lost to follow-up, 10; physician's decision, 4; and lack of efficacy, 12. This dropout rate (and the fact that lack of efficacy was the largest category of dropouts) is fairly common in clinical trials of psychiatric drugs and is well within professionally recognized limits.[16] Because dropouts were not included in the final analysis, per-protocol analysis was followed and intent-to-treat analysis was not performed.[17]

Participants in all WSE treatment groups experienced improved well being at Day 30 and Day 60 (Figure 1). The 125 mg QD group decreased significantly (P<0.001) in mean sum mHAM-A score from baseline (29.9) to Day 30 (18.1; -39.5%) and to Day 60 (11.3; -62.2%) compared to the placebo group, which showed no significant mean change in sum mHAM-A score throughout the study. Mean sum mHAM-A scores for the other WSE groups decreased even further than for the 125 mg QD group in a dose-dependent manner. The mean sum mHAM-A score for the placebo group at baseline, 27.6, was lower than those of the WSE-treated groups (which ranged between 29.2-29.9), but not significantly. Mean scores for individual questions on the mHAM-A questionnaire also decreased significantly (P<0.001) at Day 30 and Day 60 for all WSE treatment groups versus the placebo group (Table 2).

Mean values (SD) of biochemical and clinical parameters investigated for each treatment group are summarized in Tables 3 and 4. Between baseline and Day 60, the WSE 125 mg QD group decreased significantly (P<0.05) more than the placebo group for mean serum cortisol (-14.5%), serum VLDL-C (-8.9%), systolic BP (-1.6%), diastolic BP (-5.6%), and (P<0.001) serum CRP (-31.6%) and pulse rate (-6.0%), and increased significantly (P<0.05) more than the placebo group for mean serum DHEAS (13.2%) and hemoglobin (6.3%). For the same period, the other WSE treat-

Table 1. Baseline demographic and clinical characteristics of study participants (N = 130).[z]

Characteristic	Group			
	WSE 125 mg QD (n = 30)	WSE 125 mg BID (n = 35)	WSE 250 mg BID (n = 35)	Placebo (n = 30)
Age, mean (SD), y	37.8 (12.4)	39.4 (12.6)	40.0 (9.9)	42.1 (9.6)
Sex, no. (%)				
Men	22 (73.3)	27 (77.1)	23 (65.7)	23 (76.7)
Women	8 (26.7)	8 (22.9)	12 (34.3)	7 (23.3)
Weight, mean (SD), kg	58.9 (9.9)	60.5 (9.0)	59.1 (9.2)	59.1 (6.6)
Height, mean (SD), cm	161.8 (9.3)	161.5 (7.7)	162.7 (7.3)	159.4 (6.7)

WSE = *Withania somnifera* extract.

[z] No significant between-group differences were found.

Table 2. Mean (SD) scores by treatment group for individual symptoms of stress and anxiety based on a modified Hamilton anxiety scale[z] at Day 0 (baseline) and after 30 and 60 days of treatment with *Withania somnifera* extract (WSE) or placebo (n = 98).

	Symptom																	
	Fatigue			Flushing			Perspiration			Loss of appetite			Headache and muscle pain			Feelings of impending doom		
Group	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
WSE 125 mg QD (n = 19)	3.2 (0.7)	1.9 (0.7)†	1.5 (0.7)†‡	2.1 (1.0)	1.1 (0.8)†	0.4 (0.6)†	2.4 (1.2)	1.6 (0.8)†	1.2 (0.7)†	1.3 (0.6)	1.1 (0.8)†	0.9 (0.7)†‡	2.4 (1.0)	1.1 (0.8)†	0.6 (0.6)†	3.2 (0.8)	1.7 (0.7)†	0.8 (0.6)†‡
WSE 125 mg BID (n = 30)	2.9 (0.9)	1.3 (1.0)†	0.6 (0.6)†‡	1.6 (1.1)	0.8 (0.9)†	0.4 (0.6)†	1.9 (1.1)	1.3 (0.6)†	0.9 (0.4)†	1.7 (1.1)	0.8 (0.7)†	0.5 (0.6)†	1.7 (1.1)	0.8 (0.7)†	0.5 (0.6)†‡	3.6 (0.6)	2.0 (0.9)†	0.9 (0.8)†‡
WSE 250 mg BID (n = 34)	3.0 (1.0)	1.4 (0.8)†	0.4 (0.5)†‡	1.8 (1.0)	0.7 (0.7)†	0.4 (0.5)†	1.8 (1.3)	1.2 (0.4)†	0.8 (1.3)†	1.4 (0.7)	1.0 (0.4)†	0.8 (0.4)†‡	1.7 (1.0)	0.5 (0.6)†	0.3 (0.5)†‡	3.1 (0.7)	1.4 (0.6)†	0.5 (0.7)†‡
Placebo (n = 15)	2.9 (1.0)	2.9 (0.9)	2.7 (0.8)	1.9 (1.0)	1.7 (1.2)	1.5 (0.9)	1.5 (0.9)	1.5 (1.1)	1.5 (0.7)	1.2 (0.6)	1.5 (0.5)	1.5 (0.5)	2.1 (0.8)	1.9 (0.8)	1.8 (0.8)	3.1 (1.0)	3.0 (1.0)	3.2 (0.9)

	Symptom																	
	Palpitations			Dry mouth			Sleeplessness			Forgetfulness			Irritability			Inability to concentrate		
Group	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60	Day 0	Day 30	Day 60
WSE 125 mg QD (n = 19)	1.9 (0.8)	1.1 (0.8)†	0.6 (0.5)†	1.4 (1.0)	0.8 (0.8)†	0.5 (0.6)†	3.1 (0.9)	1.9 (0.8)†	0.9 (0.8)†‡	2.7 (0.7)	1.8 (0.5)†	1.4 (0.6)†‡	3.1 (0.7)	1.9 (0.9)†	0.9 (0.5)†‡	3.2 (0.5)	2.1 (0.7)†	1.6 (0.8)†‡
WSE 125 mg BID (n = 30)	2.2 (1.1)	0.9 (0.9)†	0.6 (0.6)†	1.5 (0.8)	0.7 (0.7)†	0.4 (0.5)†	3.1 (1.0)	1.9 (1.1)†	1.0 (0.8)†‡	2.8 (0.8)	1.8 (0.8)†	1.2 (0.6)†‡	3.4 (0.6)	1.9 (0.7)†	0.8 (0.6)†‡	3.3 (0.7)	1.6 (0.8)†	0.8 (0.6)†‡
WSE 250 mg BID (n = 34)	2.2 (1.1)	0.8 (0.8)†	0.6 (0.7)†	1.5 (1.0)	0.4 (0.7)†	0.0 (0.0)†	3.2 (1.1)	1.6 (0.7)†	0.5 (0.7)†‡	2.8 (0.7)	1.1 (0.5)†	0.9 (0.5)†‡	3.2 (0.7)	1.7 (0.6)†	0.4 (0.6)†‡	3.1 (0.9)	1.5 (0.7)†	0.5 (0.6)†‡
Placebo (n = 15)	2.2 (0.7)	2.1 (0.7)	1.9 (0.7)	1.5 (0.9)	1.5 (0.9)	1.4 (0.7)	2.7 (1.2)	2.7 (1.2)	2.7 (1.1)	2.8 (0.4)	2.5 (0.5)	2.5 (0.5)	2.7 (1.1)	2.8 (1.1)	2.9 (1.1)	3.0 (0.9)	3.0 (1.0)	3.0 (0.9)

[z] Scale: 0 = never; 1 = occasional; 2 = mild/poor; 3 = moderate; 4 = severe.

†P < 0.001 versus Placebo Group, ‡P < 0.05 versus 30 days of treatment.

ment groups had even greater responses in these parameters than the WSE 125 mg QD group in a dose-dependent manner. In addition, the WSE 125 mg BID group had significantly (P<0.05) greater reductions, compared to the placebo group, in mean FBG (-4.7%), serum TC (-7.0%), serum TG (-9.5%), and serum LDL-C (-9.0%). The WSE 250 mg BID group had similar to greater responses in mean FBG, and serum TC, TG and LDL-C than the WSE 125 mg BID group, and also had a significantly (P<0.001) greater increase in mean serum HDL-C compared to the placebo group (17.3%). Cardiac risk ratios, at the higher end of the average risk range at Day 0 for all treatment groups, improved for the two higher dosage WSE groups at Day 60 by decreasing significantly (P<0.05) compared to the placebo group (data not shown).

No significant difference was observed in the placebo group between baseline and Day 60 for any biochemical or clinical parameter measured. In addition, no study participant or dropout experienced any adverse effects or withdrawal effects, regardless of the dosage or frequency.

DISCUSSION

Several studies have been carried out during the last few decades on the chemical constituents of WS[18] and its biological activities, mostly using powder (or non-standardized extracts) derived from the roots of wild plants. Experimental studies of WS have assessed its antistress,[19] antioxidant,[20] immunomodulatory,[21, 22] anticancer,[23] antitumor,[24] cardioprotective,[25] antiosteoarthritis[26] and antiaging[27] activities. Our study is the first to evaluate the therapeutic benefits of standardized WSE in human subjects using modern clinical trials.

Our findings that WSE reduces experiential feelings of stress and anxiety at all dosage levels tested supports the traditional claims of WS's antistress-adaptogenic effect. All WSE-treated groups showed improvement in mHAM-A stress and anxiety scores at both Day 30 and Day 60 as a result of participants feeling less fatigue, flushing, perspiration, loss of appetite, headache and muscle pain, feelings of impending doom, palpitations, dry mouth, sleeplessness, for-

Table 3. Mean (SD) values and percentage changes by treatment group of biochemical variables measured at Day 0 (baseline) and after 60 days of treatment with *Withania somnifera* extract (WSE) or placebo (n = 98).

	Biochemical Variables															
	Serum Cortisol µg/dL			Serum DHEAS µg/dL			CRP mg/L			FBG mg/dL			Serum TC mg/dL			
Group	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	
WSE 125 mg QD (n = 19)	13.1 (3.0)	11.2 (2.1)	–14.5*	167.4 (37.8)	189.5 (40.8)	13.2*	3.8 (1.2)	2.6 (1.1)	–31.6†	88.1 (12.5)	87.6 (11.7)	–0.6	176.4 (30.0)	173.5 (27.6)	–1.6	
WSE 125 mg BID (n = 30)	12.8 (3.9)	9.7 (2.4)	–24.2†	152 (48.9)	200.7 (54.6)	32.2†‡	4.1 (1.0)	2.6 (1.0)	–36.6†	94.4 (16.5)	90.0 (13.6)	–4.7*	176.0 (33.4)	163.7 (30.9)	–7.0*	
WSE 250 mg BID (n = 34)	14.1 (3.3)	9.8 (2.4)	–30.5†‡	159.6 (61.7)	212.1 (74.7)	32.5†‡	5.4 (1.4)	3.5 (2.6)	–35.2†	91.9 (11.2)	86.3 (8.7)	–6.1*	193.5 (27.2)	168.1 (27.5)	–13.1†‡#	
Placebo (n = 15)	13.5 (3.2)	14.1 (3.3)	4.4	166.7 (32.9)	149.3 (35.6)	–10.8	6.4 (3.7)	6.0 (3.7)	–6.3	91.2 (12.2)	93.1 (11.1)	2.1	171.1 (18.6)	172.7 (22.7)	0.9	
	Serum TG mg/dL			Serum LDL-C mg/dL			Serum VLDL-C mg/dL			Serum HDL-C mg/dL						
Group	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ				
WSE 125 mg QD (n = 19)	119.4 (45.5)	114.2 (41.6)	–4.4	122.7 (25.0)	114.9 (18.3)	–6.4	25.9 (8.9)	23.6 (6.0)	–8.9*	37.9 (4.9)	39.0 (5.0)	2.9				
WSE 125 mg BID (n = 30)	135.3 (40.5)	122.4 (34.9)	–9.5*	118.1 (26.2)	107.5 (22.7)	–9.0*	32.5 (12.2)	27.1 (10.4)	–16.6†	37.3 (6.4)	39.0 (5.2)	4.6				
WSE 250 mg BID (n = 34)	132.8 (46.0)	117.3 (35.7)	–11.7†‡	134.7 (20.4)	111.2 (14.0)	-17.4†‡#	32.7 (7.8)	24.9 (7.0)	–23.9†‡	34.6 (7.0)	40.6 (6.4)	17.3*‡#				
Placebo (n = 15)	127.9 (31.4)	133.9 (33.3)	4.7	119.5 (28.6)	118.8 (26.9)	–0.6	27.1 (7.8)	29.4 (8.8)	8.5	39.3 (4.9)	39.1 (5.5)	–0.5				

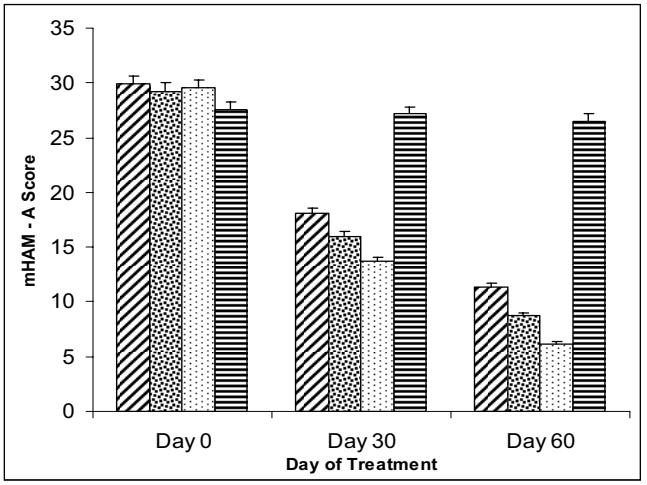
DHEAS = dihydroepiandrosterone sulfate; CRP = C-reactive protein; FBG = fasting blood glucose; TC = total cholesterol; TG = triglycerides; LDL-C = low-density lipoprotein cholesterol; VLDL-C = very low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol. * P<0.05 versus Placebo Group, † P<0.001 versus Placebo Group, ‡ P<0.05 versus WSE 125 mg QD Group, # P<0.05 versus WSE 125 mg BID Group.

getfulness, irritability and inability to concentrate. The placebo group did not display changes in mHAM-A score throughout the study. Feelings of frustration that treatment was not helping their stress may have contributed to the higher dropout rate in this group. The placebo group had nine participants drop out due to lack of efficacy compared to three for all WSE-treated groups combined (data not shown).

WSE's therapeutic activity may be attributed, at least in part, to its effect on the hypothalamic-pituitary-adrenal axis, which regulates serum cortisol concentration.[28] The link between adaptogenic effects and cortisol has previously been described.[29] In fact, the mean serum cortisol concentration in all three WSE-treated groups, while being in the normal range throughout the study, declined between baseline and Day 60. In individuals with normal circadian rhythm, serum cortisol concentration is high in the morning and tends to decrease throughout the afternoon, reaching its lowest point around 11 p.m.[14] One of the effects of chronic stress is that serum cortisol concentration peaks in the afternoon, rather than becoming lower.[28] Supplementation with WSE may offset this afternoon cortisol peak in stressed individuals, although future studies are needed to confirm this.

Many types of physical and emotional stress, particularly those that are chronic in nature, reduce serum DHEAS

Figure 1. Mean sum (SD) total stress and anxiety scores based on a modified Hamilton anxiety (mHAM-A) scale[z] by group for Day 0 (baseline), Day 30 and Day 60 of treatment with *Withania somnifera* extract (WSE) or placebo (n = 98).



[z]Scale: 0 = never; 1 = occasional; 2 = mild/poor; 3 = moderate; 4 = severe.

Table 4. Mean (SD) values and percentage changes by treatment group of clinical variables measured at Day 0 (baseline) and after 60 days of treatment with *Withania somnifera* extract (WSE) or placebo (n = 98).

	Clinical Variables											
	Hemoglobin g/dL			Pulse rate Beats/min			Systolic Blood Pressure (BP) mm Hg			Diastolic Blood Pressure (BP) mm Hg		
	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ	Day 0	Day 60	%Δ
WSE 125 mg QD (n = 19)	12.7 (1.2)	13.5 (1.5)	6.3*	76.4 (4.6)	71.8 (1.4)	–6.0†	121.6 (11.7)	119.6 (7.0)	–1.6*	83.5 (10.1)	78.8 (5.8)	–5.6*
WSE 125 mg BID (n = 30)	12.6 (1.3)	13.1 (1.4)	4.0*	80.7 (6.4)	74.1 (3.7)	–8.2†	126.3 (13.6)	122.0 (8.6)	–3.4†	82.8 (7.1)	78.7 (4.4)	–5.0*
WSE 250 mg BID (n = 34)	12.1 (1.2)	13.2 (1.1)	9.1†	78.8 (7.7)	73.6 (4.3)	–6.6†	120.0 (15.2)	116.1 (9.8)	–3.3†	81.6 (9.5)	76.4 (7.1)	–6.4†
Placebo (n = 15)	12.7 (1.2)	12.5 (1.2)	–1.6	78.4 (2.3)	80.0 (3.4)	2.0	118.7 (11.4)	125.6 (8.0)	5.8	83.5 (6.7)	86.3 (4.5)	3.4

* P<0.05 versus Placebo Group, † P<0.001 versus Placebo Group.

concentration, which can be used as a marker of stress.[30] In the present study, the WSE-treated groups displayed increased serum DHEAS concentrations by the end of the study, compared to the placebo group. The normalizing action of WSE may also be due to neuroprotective properties of withanolide glycosides and withaferin A that help reduce the stress-induced generation of reactive oxygen species in various parts of the brain.[22, 31-33]

Chronic stress has been found to be associated with higher than normal levels of serum CRP, a systemic marker of inflammation that is associated with increased risk for a host of chronic diseases.[34] Use of WSE at all doses and frequencies in this study resulted in a decrease in mean serum CRP concentration, indicating that systemic inflammation may have declined in members of each WSE-treated group. Ingestion of WS root powder has decreased inflammation in animal models.[35] Together these findings suggest that WSE use might contribute to decreased risk of chronic disease, an idea meriting further investigation.

Reduction in fasting blood glucose concentration in the two highest dosage WSE-treated groups was also found. This finding may be related to the observed concomitant reduction in serum cortisol concentration. Cortisol is a glucocorticoid hormone that performs several functions, including regulation of blood sugar levels.[36]

CONCLUSIONS

This study determined that daily consumption of standardized WSE at three dosages (125 mg QD, 125 mg BID, and 250 mg BID) reduced experiential feelings of stress and anxiety, serum concentrations of cortisol and CRP, pulse rate and blood pressure; and increased serum concentration of DHEAS in the chronically stressed adults who completed the study. The WSE 125 mg BID and WSE 250 mg BID dosages also improved fasting blood glucose levels and lipid profiles for study participants in those groups. Cardiac risk ratios improved for the two higher dosage WSE groups. Although the 25% dropout rate may have partially skewed results, the observed dose-dependent, significant trends in most variables evaluated support the view that daily use of WSE would benefit people suffering from the effects of stress and anxiety without any adverse effects.

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POTENTIAL CONFLICTS OF INTEREST

Dr. Auddy is an employee of Natreon Inc., which is the patent holder of *Withania somnifera* extract sold under the trade names Essentra® and Sensoril®.

Dr. Abedon is an employee of NutraGenesis LLC, which sells *Withania somnifera* extract exclusively under the trade names Essentra® and Sensoril®.

Dr. Ghosal was an unpaid adviser to Natreon Inc. at the time the study was conducted.

REFERENCES

1. Elliot GR, Eisdorfer C. *Stress and Human Health*. Springer Publishing, New York. 1982.
2. Mukhopadhya B, Chakraborti A, Ghosal S. Immunomodulatory properties of some Indian medicinal plants. In: Mori A, Satoh T, eds. *Emerging Drugs*. Vol I. PJD Publications, Westbury, USA. 2001:445–460.
3. Weiner MA, Weiner J. Ashwagandha (Indian ginseng). In: *Herbs that Heal*. Quantum Books, Mill Valley, CA. 1994:70–72.
4. Archana R, Namasivayam A. Antistressor effect of *Withania somnifera*. *J Ethnopharmacol*.1999;64:91–93.
5. Ghosal S. In pursuit of standardization of Ayurvedic drugs. *Ann Natl Acad Ind Med*. 1986;1:1-14.
6. Sangwan RS, Cahurasiya ND, Misra LN, et al. Phytochemical variability in commercial herbal products and preparations of *Withania somnifera* (Ashwagandha). *Current Sci*. 2004; 86(3):461-465.
7. Bector NP, Puri AS, Sharma D. Role of *Withania somnifera* (Ashwagandha) in various types of arthropathies. *Ind Jour Med Res*.1968;56:1581-1583.
8. Ray AB, Gupta M. Withasteroids, a growing group of naturally occurring steroidal lactones. In: Herz W, Kerby GW, Moore RE. eds. *Progress in the chemistry of organic natural products*. Wein-Springer-Verlag, New York. 1994; 63:1-106.
9. Ghosal S. *Withania somnifera* composition. Method for obtaining same and pharmaceutical, nutritional and personal care formulations thereof. 2004. US Patent 6,713,092.
10. International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use – Guideline for Good Clinical Practice, ICH Topic E6. Geneva, Switzerland. 1996; Available at http://www.ich.org/LOB/media/MEDIA482.pdf. Accessed Dec. 7, 2007.
11. World Medical Association Declaration of Helsinki: Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Subjects. WMA, Ferney-Voltaire, France. 1989; Available at http://www.wma.net/e/policy/b3.htm. Accessed Dec. 7, 2007.
12. Hamilton M. The assessment of anxiety states by rating. *Br J Med Psycho*l. 1959;32:50–55.
13. Hamilton Anxiety Scale. [Family Practice Notebook Website]. Available at http://www.fpnotebook.com/PSY86.htm. Accessed Dec. 7, 2007.
14. Van Cauter E, Leproult R, Kupper DJ. Effects of gender and age on the levels and circadian rhythmicity of plasma cortisol. *J Clin Endocrinol Metab*. 1996;81:2468–2473.
15. Wallach J. *Interpretation of diagnostic tests, 6th ed.* Little Brown & Co., New York.1996:482.
16. Kemmler G, Hummer M, Widschwendter C, et al. Dropout rates in placebo-controlled and active-control clinical trials of antipsychotic drugs: A meta-analysis. *Arch Gen Psychiatry.* 2005;62:1305-1312.
17. Armitage P. Exclusions, losses to follow-up, and withdrawals in clinical trials. In: Shapiro SH, Louis TA, eds. *Clinical Trials: Issues and Approaches*. Marcel Decker, New York. 1983:99–113.
18. Upton, R, Graff A, Evans F, et al. Ashwagandha root (*Withania somnifera*) analytical, quality control, and therapeutic monograph. In: Upton, R., ed. *American Herbal Pharmacopoeia and Therapeutic Compendium*. 2000:1-25.
19. Bhattacharya SK, Muruganandam AV. Adaptogenic activity of *Withania somnifera*: an experimental study using a rat model of chronic stress. *Pharmacol Biochem Behav*. 2003;75:547–555.
20. Bhattacharya SK, Satyan KS, Ghosal S. Antioxidant activity of glycowithanolides from *Withania somnifera*. *Indian J Exp Biol*. 1997;35:236–239.
21. Davis L, Kuttan G. Immunomodulatory activity of *Withania somnifera*. *J Ethnopharmacol*. 2000;71:193–200.
22. Ghosal S, Lal J, Srivastava RS, et al. Bioactive phytosterol conjugates. 7:Immunomodulatory and CNS effects of sitoindosides IX and X, two new glycowithanolides from *Withania somnifera*. *Phytother Res*. 1989;3:201–206.
23. Prakash J, Gupta SK, Dinda AK. *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. *Nutr Cancer*. 2002;42:91–97.
24. Jayaprakasam B, Zhang Y, Seeram NP, et al. Growth inhibition of human tumor cell lines by withanolides from *Withania somnifera* leaves. *Life Sci*. 2003;74:125–132.
25. Dhuley JN. Adaptogenic and cardioprotective action of ashwagandha in rats and frogs. *J Ethnopharmacol*. 2000;70:57–63.
26. Kulkarni RR, Patki PS, Jog VP, et al. Treatment of osteoarthritis with a herbomineral formulation: a double-blind, placebo-control, cross-over study. *J Ethnopharmacol*. 1991;33:91–95.
27. Bhattacharya SK, Kumar A, Ghosal S. Effects of glycowithanolides on an animal model of Alzheimer's disease. *Phytother Res*. 1995;9:110–113.
28. Rosmond, R. Stress induced disturbances of the HPA axis: a pathway to Type 2 diabetes? *Med Sci Monit*. 2003;9:RA35-39.
29. Singh A, Saxena E, Bhutani KK. Adrenocorticosterone alteration in male, albino mice treated with *Trichopus zeylanicus*, *Withania somnifera* and *Panax ginseng* preparations. *Phytother Res*. 2000;14:122–125.
30. Yen SS, Morales AJ, Khorram O. Replacement of DHEA in aging men and women: potential remedial effects. *Ann NY Acad Sci*. 1995;774:128–142.
31. Bhattacharya SK, Goel RK, Kaur R, et al. Antistress activity of sitoindoside VII and VIII, new acylsterylglycosides from *Withania somnifera*. *Phytother Res*. 1987;1:32–37.
32. Ahmad M, Saleem S, Ahmad AS, et al. Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. *Hum Exp Toxicol*. 2005;24:137–147.
33. Jesberger JA, Richardson JS. Oxygen free radicals and brain dysfunction. *Int J Neurosci*. 1991;57:1–17.
34. Liepa GU, Basu H. C-reactive proteins and chronic disease: what role does nutrition play? *Nutrition in Clinical Practice*. 2003;18:227-233.
35. Anbalagan K, Sadique, J. Influence of an Indian medicine (Ashwagandha) on acute-phase reactants in inflammation. *Indian J Exp Biol*. 1981;19:245-249.
36. Fruehwald-Shultes, B, Kern W, Bong W, et al. Supraphysiological hyperinsulinemia acutely increases hypothalamic-pituitary-adrenal secretory activity in humans. *J Clin End Met*. 1999, 84:3041-3046.