The Journal of the American Nutraceutical Association

Vol. 12, No. 1, 2009 www.ana-jana.org

In This Edition

JANA™

A Peer-Reviewed Journal on Nutraceuticals and Nutrition

ISSN-1521-4524

JANA™

Journal of the American Nutraceutical Association

American Nutraceutical Association
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Birmingham, AL 35242
Phone: (205) 980-5710 Fax: (205) 991-9302
Website: www.Ana-Jana.org

ANA is an alliance of healthcare professionals with interest in nutraceutical science, technology, marketing and production. It was established to develop and provide educational materials and continuing education programs for healthcare professionals on nutraceutical technology and science.

The Journal of the American Nutraceutical Association (ISSN-1521-4524) is published three times annually by the American Nutraceutical Association (ANA). Send all inquiries, letters, and submissions to the ANA Editorial Department at 5120 Selkirk Drive, Suite 100, Birmingham, AL 35242.

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Letter to Health Care Professionals From the FDA on the Potential Risk of Severe Liver Injury from the use of Hydroxycut Dietary Supplements

Dear Health Care Professional Colleague:

We are alerting you about a dietary supplement product that we believe presents a serious public health risk. Hydroxycut products are distributed by Iovate Health Sciences Inc., Oakville, Ontario Canada and distributed by Iovate Health Sciences U.S.A., Inc. of Blasdell, NY, and have been implicated in several cases of serious liver injury. The Food and Drug Administration (FDA) has received 23 reports of adverse liver effects in users of Hydroxycut products, ranging from asymptomatic hyperbilirubinemia, jaundice, liver damage, liver transplant, and death. The injuries reported to FDA occurred in persons between 21 and 51 years of age. No other cause for liver disease was identified. In the majority of cases, no preexisting medical condition that would predispose the consumer to liver injury was identified. In some cases, discontinuation of Hydroxycut usage resulted in recovery of liver function. Although the liver damage appears to be relatively rare, FDA believes consumers should not be exposed to unnecessary risk. FDA has also identified several other serious adverse events associated with Hydroxycut, including cases of seizures, rhabdomyolysis, and cardiovascular disorders ranging in severity from palpitations to a heart attack.

Hydroxycut products bear the Iovate or Muscletech Brand name and are multi-ingredient dietary supplements marketed for weight loss, as fat burners, energy enhancers, as low carb diet aids, and to promote water loss. The following products have been recalled by the company:

- Hydroxycut Regular Rapid Release Caplets;
- Hydroxycut Caffeine-Free Rapid Release Caplets;
- Hydroxycut Hardcore Liquid Caplets;
- Hydroxycut Max Liquid Caplets;
- Hydroxycut Regular Drink Packets;
- Hydroxycut Caffeine-Free Drink Packets;
- Hydroxycut Max Drink Packets;
- Hydroxycut Liquid Shots;
- Hydroxycut Hardcore RTDs (Ready-to-Drink);
- Hydroxycut Max Aqua Shed;
- Hydroxycut 24;

This picture shows some of the Hydroxycut products that are being recalled.

- Hydroxycut Carb Control; and
- Hydroxycut Natural.

Based on the information available to FDA, we cannot determine exactly which ingredient(s) or proprietary blends in Hydroxycut may be associated with liver injury, or what other factors, such as health condition, length of use, dosage, or use along with other dietary supplements or drugs, may affect the risk of using Hydroxycut.

FDA is warning consumers to immediately stop use of these products. FDA has issued a consumer warning advising of the potential risks associated with the use of these products and advising consumers to consult their health care provider if they are experiencing symptoms possibly associated with this product, particularly nausea, weakness or fatigue, fever, abdominal pain, or any change in skin color.

We urge you to review your cases of hepatitis in order to determine if any may be related to the use of dietary supplements in these patients. Adverse events associated with the use of dietary supplements should be reported as soon as possible to FDA's MedWatch program by telephone (1-800-332-1088) or Internet (http://www.fda.gov/medwatch).

Department of Health and Human Services

COMMENTARY

Nutrigenomics: Can Folate Decrease the Risk for Hypertension?

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In a recent article[1] published in the *Journal of Nutritional Genomics,* the authors claim that in the presence of a certain genetic variant, the supplementation with dietary folate may lower blood pressure in elderly women.

Why are we focusing our research on dietary folate? What special role do dietary folates play in the cardiovascular disease process? Let's summarize the facts:

Elevated homocysteine (Hcy) is an independent risk factor for cardiovascular disease (CVD), including atherosclerosis, hypertension, stroke and myocardial infarction.[2] Dietary folate and to a lesser extent, vitamins B-12 and B-6, are important determinants of Hcy level. Variation in the many gene products that depend upon these vitamins could also alter Hcy status and modify disease risk. The best known gene variant of folate metabolism is the 5,10-methylenetetrahydrofolate reductase (MTHFR) single nucleotide polymorphism (SNP), in which a C-to-T substitution at nucleotide 677 converts an alanine to a valine in the functional protein.[3] This *677C* 1 *T* -MTHFR SNP and Hcy have both been associated with cardiovascular disease.[4] One of the most significant findings linking folate to hypertension was published in 2005, and shows that elevated dietary folate decreased the risk of hypertension in younger women who consumed at least 1,000 microgram/day of total folate (relative risk 0.54, 95% CI 0.45–0.66; p for trend < 0.001) compared with those who consumed less than 200 microgram/day.[5] Furthermore, reduced folates may interact synergistically with tetrahydrobiopterin metabolism, which is crucial in the synthesis of nitric oxide by endothelialnitric oxide synthase.[6] This step is critical in maintaining the elasticity of the vascular wall. Given the significance of folate-related nutritional genetics in health, and the relative paucity of specific information linking folate-related SNPs to hypertension in otherwise healthy elderly subjects, the authors examined the effect of seven SNPs on the risk for hypertension among 118 subjects (80 normotensive; 38 hypertensive) with an age range of 65–90 years, in an Australian retirement village elderly population sample.

All subjects were scored for the common *677C* 1 *T* -MTHFR, *1298A* 1 *C* -MTHFR, *80G* 1 *A* -RFC, *2756A* 1 *G* -MS, *66A* 1 *G* -MSR, 19bpDHFR and *1561C* 1 T-GCP II SNPs using the polymerase chain reaction (PCR) followed by restriction enzyme digestion, and gel electrophoresis of the cleaved amplicon.

The authors concluded that the *677C* 1 *T* -MTHFR genetic variant is a potential risk factor for hypertension in the elderly. Furthermore, hypertensive subjects have a significantly lower intake of dietary folate than normotensive individuals.

Therefore, given the known role of folate in lowering athero- and thrombogenic homocysteine levels, and the putative role for folate in tetrahydrobiopterin-related nitric oxide metabolism, known to be a critical determinant of

blood pressure, these elderly subjects may be hypertensive due to, at least in part, either or both their folate-related genotype (*677T* -MTHFR) and/or their low dietary folate intake. In females, the higher dietary folate intake is being associated with decreased blood pressure. This finding in an elderly population is consistent with previous studies indicating that higher total folate intake was associated with a decreased risk of hypertension in younger women in particular.

In summary, dietary folate and the *677C* 1 *T* -MTHFR variant may be contributory determinants of blood pressure. Given the increased likelihood of morbidity and mortality associated with hypertension, thought should be given to the possible long-term benefits of increasing dietary folate intake in the elderly. However, this needs to be offset against any risk of masking B-12 deficiency and recent negative findings related to high intakes of vitamins B-12, B-6 and folate in large clinical studies.

The reported study is an example of how research in nutrigenomics can be potentially translated into clinical practice. Such knowledge transfer may represent a first step on how to personalize nutritional interventions and dietary supplementation.

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PERSPECTIVE ARTICLE

Nutraceuticals in the Post-Genomic Era: A Rendezvous of Nutrition and Pharmacology

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ABSTRACT

In recent years, a wealth of molecular, cellular and *in vivo* investigations have elucidated the efficacy and mechanisms of isolated chemical constituents of fruits, vegetables and herbs. As new research continues to reveal the pharmacodynamics, pharmacokinetics and biotransformation of these single agents, the disciplines of nutrition and pharmacology have begun to merge. Pharmacologic concepts such as receptor-ligand interactions and intracellular signal transduction pathways are more relevant to nutrition than ever before, and many principles of pharmacology are applicable in nutraceutical product development and in the strategic clinical use of these products. Pharmacogenomics and pharmacogenetics are rapidly expanding areas of pharmacology that involve the effects of chemicals on the genome and the effects of the genome on pharmacologic parameters, respectively. Nutrigenomics and nutrigenetics are nutritional counterparts to these pharmacologic disciplines and observe the same principles and research methods. The advent of routine genotyping in the clinic will ultimately afford greater precision and individualization of nutraceutical interventions. This and other advances in the molecular etiology of disease will likely result in a more mechanism-based formulation to meet the changing demands of physicians, dietitians and other nutraceutical consumers in the healthcare community.

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INTRODUCTION

Twenty-five centuries have passed since Hippocrates advised, "Let food be thy medicine and medicine be thy food." Although plants and natural products have been used throughout history for medicinal purposes, the chemical characterization of food as medicine is new. Traditionally, the essential nutrients, their roles in normal physiology, and prevention of their deficiency have been the business of nutrition and dietetics. In recent years, however, nutrition has evolved into a biomedical science with tremendous potential for disease prevention and treatment. Epidemiological evidence maintains that fruits and vegetables are protective against cancer and cardiovascular disease. Early speculation of the constituents responsible for these effects focused on the well-characterized antioxidants β-carotene and ascorbic acid. Although more abundant than many low-molecular weight constituents, β-carotene is merely one of at least 220 chemicals in a carrot (Duke, 1992). Indeed, whether β-carotene or ascorbate supplementation can faithfully recapitulate the beneficial effects of a plant-based diet remains unknown. In the last two decades, an expansive repertoire of phytochemicals in plant foods has been chemically and pharmacologically characterized, including phenolics, terpenoids and indoles of enormous structural diversity, and in the case of curcumin, indole 3-carbinol (I3C), and epigallocatechin gal-

late (EGCG), medicinal value has been demonstrated in randomized controlled trials. In the rigorous study of the therapeutic properties, pharmacodynamics, molecular mechanisms, pharmacokinetics and metabolism of isolated dietary agents, the fields of nutrition and pharmacology have begun to fruitfully coalesce.

Three realities have historically divided nutrition and pharmacology. One of these realities is the variability and ambiguity of ingested doses of dietary agents owing to factors such as horticultural methods, cultivar, climate, age, processing and storage. Secondly, over the last decade it has become clear that unlike the canonical drug that binds a singular target with selectivity and often with low-nanomolar affinity, many dietary agents bind more than one molecular target with micromolar affinity. The third and most methodologically daunting distinction is the enormous chemical complexity of a food *versus* a drug. In the oral administration of a whole food, the likelihood of synergy, mutual antagonism and other types of interactions among constituents within the food matrix, in the digestive milieu, and in the body is substantial. Moreover, since foods are usually ingested in combination, the potential for further interactions pose seemingly insurmountable hurdles in the reductionistic pharmacological interrogation of food. In spite of these differences, the systematic scientific inquiry of isolated dietary agents, particularly phytochemicals such as resveratrol from grapes, quercetin from citrus, I3C from cruciferous vegetables, and diallyl disulfide from garlic, has become a major trend attracting the interests and visions of both fields. As the use of nutraceuticals containing these substances has become widespread, we find ourselves asking pharmacologic questions pertaining to mechanisms of action, cognate receptors, pharmacokinetics, biotransformation and toxicity.

RECEPTORS:

THE PILLARS OF PHARMACOLOGY

Receptor-ligand interactions comprise the very cornerstone of pharmacology. The classical definition of a receptor is any molecule within or on the surface of a cell to which a chemical selectively binds, thereby modifying normal activities of the cell. Most of the modern pharmacopoeia consists of xenobiotics that selectively bind a receptor, inducing a conformational change which subsequently modifies a downstream target or cascade of effectors that mediate a coordinated biological response. Food contains many agents that bind receptors, functionally modifying them to incite a reproducible biological response. Among the most illustrious examples are the phytoestrogens genistein and resveratrol, which are estrogen receptor agonists (Pike et al., 1999; Gehm et al., 1997; Dai et al., 2007). Eicosapentaenoic acid (EPA) from fish oil is a peroxisome-proliferator activated receptor ligand (Xu et al., 1999). Diindolylmethane (DIM) from cruciferous vegetables is a competitive androgen receptor antagonist (Le et al., 2003). EGCG from tea is a T-cell receptor CD4 ligand (Williamson et al., 2006) and a dihydrofolate reductase inhibitor (Nevarro-Peran et al., 2005). Theaflavins from tea are inhibitors of antiapoptotic Bcl-2 (Leone et al., 2003). Lycopene from tomatoes is a platelet-derived growth factor inhibitor (Lo et al., 2007) and capsaicin from hot peppers is a vanilloid receptor 1 agonist (Nagy et al., 2004). Flavonoids, the largest class of dietary polyphenols, inhibit the adenosine receptor (Jacobson et al., 2002), the PDGF receptor (Rosenkranz et al., 2002), the thromboxane A(2) receptor (Guerrero et al., 2005) and lipoxygenase (Sies et al., 2005).

Unlike most pharmaceutical drugs, the cognate receptors of many dietary agents remain elusive. However, their effects on intracellular signaling events relevant to disease pathogenesis have been defined. Curcumin, resveratrol, flavopiridol, I3C, zerumbone, EGCG and ursolic acid target the intrinsic apoptotic pathway in tumor cells (Aggarwal and Shishodia, 2006). Genistein (Li et al., 2002), diosgenin (Shishodia et al., 2005), curcumin (Aggarwal et al., 2006) and EGCG (Tang et al., 2003) suppress the activation of the cell survival signaling. Resveratrol (Shen et al., 2003), theaflavin (Aneja et al., 2004) and genistein (Rimbach et al., 2004) target chemokines, attenuating activation of inflammatory cascades. Curcumin (Cho et al., 2005), I3C (Li et al., 2003), and α lipoic acid (Cho et al., 2003) modulate mitogen-activated protein kinase (MAPK) networks that are central homeostatic regulators. At least 80 known dietary compounds inhibit the NFκB pathway, which transcriptionally regulates over 200 genes involved in inflammation, immune function and malignant transformation.

GENE REGULATION:

A MECHANISTIC CROSSROADS OF DRUGS AND DIETARY AGENTS

Functional genomics have been instrumental in pharmacology research over the last decade in elucidating global gene expression profiles induced by drugs, generating mechanistic insight and serving as a powerful tool in hypothesis generation. cDNA microarray technology has been employed in pharmacology research and more recently in nutrition research to unveil genes regulated by therapeutic agents that directly or indirectly influence transcription factors or epigenetic programming. The discovery by Haussler and Norman in 1969 of the vitamin D receptor is perhaps the most historically salient example of a transcription factor directly modified by a dietary agent (Haussler and Norman, 1969). More recent examples include genistein (estrogen receptor agonist), EPA and DHA (peroxisome proliferator activating receptor α ago-

nists, Li et al., 2005) and flavonoids (aryl hydrocarbon receptor antagonists, Fukuda et al., 2007). A host of transcription factors are indirectly modulated by dietary agents, including AP-1, a regulator of cell proliferation and malignant transformation and inflammation, which is suppressed by EGCG, quercetin, resveratrol, curcumin, capsaicin, oleandrin and anethole (Manna et al., 2000; Han et al., 2002; Hergenhahn et al., 2002; Han et al., 2001; Manna et al., 1999, Shen et al., 2005). A multiplicity of dietary phytochemicals activate the nuclear factor erythroid-derived 2 transcription factor (Nrf2), which regulates a battery of genes that protect against oxidative stress (Shen et al., 2005). STAT proteins, transcription factors that transmit to the nucleus signals emanating from cytokine receptors, G protein coupled receptors and growth factor receptors, are functionally suppressed by EGCG and curcumin (Bharti et al., 2003; Masuda et al., 2001). The p53 tumor suppressor protein, a transcriptional regulator of apoptosis, cell cycle and genomic stability, is activated by curcumin, resveratrol, I3C and silibinin (Han et al., 1999; Huang et al., 1999; Hong et al., 2002; Gu et al., 2005). In agreement with the emergent multitude of dietary phytochemicals that target transcription factors, a growing number of gene expression profiling studies report gene induction and repression by these agents. I3C, selenomethionine and resveratrol alter expression of gene clusters that regulate cell proliferation and signal transduction (Li et al., 2003; Jones et al., 2005; Goulet et al., 2007). Although they have lower affinity for histone-modifying enzymes than drugs such as valproic acid, several dietary agents act as chromatin-modifying anticancer agents, altering gene expression by affecting histone acetylation status. Examples include short-chain fatty acids, sulforaphane, and diallyl disulfide, inhibitors of histone deacetylases (Myzak et al., 2006; Druesne et al., 2004). Thus, dietary agents can alter gene expression via genomic and epigenetic mechanisms. At the convergence of functional genomics and molecular nutrition, the incipient field of nutrigenomics will continue to illuminate gene regulation by dietary substances and provide unprecedented insight into their pharmacologic mechanisms.

PHARMACOKINETICS AND METABOLISM OF NUTRACEUTICALS

As many nutraceutical products contain high concentrations of one or more compounds, pharmacokinetic concepts such as dosage, bioavailability, distribution, half-life, clearance and elimination are relevant to both product formulation and clinical use. In many instances, information on specific pharmacokinetic interactions among drugs, nutraceuticals, herbal extracts and foods are limited to data from *in vitro* microsomal systems and cultured hepatocytes. Nonetheless, this information can be directly applied to product formulation and prescription in the avoidance of common cytochrome p450 substrates in formulations. Pharmacokinetic principles are valuable in optimizing dosage and mode of administration. In the absence of conclusive clinical studies on a newly characterized natural product, knowledge of molecular weight, lipophilicity and partition coefficient can be used to approximate the propensity of a substance to traverse the blood-brain barrier and other important intercompartmental membranes. In addition, bioavailability, peak plasma concentrations and half-life are highly influenced by genotype. The molecular underpinnings of what has been previously dismissed as "biochemical individuality" are now being delineated by an expanding catalog of genetic polymorphisms affecting enzymes, transporters and other mediators of distribution such as metabolism and clearance. With the development of clinical genotyping methods, clinicians will soon be able to evaluate and adjust dose and schedule of nutraceuticals to maximize absorption and distribution to the target tissues and to accommodate individual pharmacokinetic genotypes. With these new capabilities in individualized medicine, there will be a growing demand among healthcare practitioners for nutraceuticals that can be applied in genotype-specific therapies.

PHARMACODYNAMICS:

WHY MECHANISMS MATTER IN PRODUCT FORMULATION AND CLINICAL USE

By definition, pharmacokinetics describe the effects of the body on a drug, while pharmacodynamics describe the effects of the drug on the body. The latter encompasses events such as receptor-ligand interactions and signaling pathways. A working pharmacological acumen on the part of the clinician allows sound interpretation of the rapidly expanding body of molecular nutrition literature. These interpretations facilitate the prediction of mechanism-based interactions, substitution of nutraceutical agents for reasons of availability or cost, rational selection of polymechanistic combination therapies, and strategic individualization of diet and supplement regimens. A grasp of pharmacology on the part of the nutraceutical product development team enables a more rational approach to product design to meet the changing demands of the practitioners who prescribe them.

Many receptors and enzymes involved in the pharmacodynamics and pharmacokinetics of dietary agents are encoded by polymorphic genes. In the coming years, large sets of data pertaining to the influence of genotypes on dietary requirements, disease risk, and the efficacy and safety of specific interventional strategies in subpopulations will become accessible to clinicians. Because a single allele usually encodes a single protein, consideration of individual genotype in designing personalized nutrition strategies requires knowledge of receptor and post-receptor pharmacology of dietary agents. For example, PPARγ and glutathione sulfotransferase (GST) are molecular targets of

dietary fatty acids and isothiocyanates, respectively (Xu et al., 1999; Brooks et al., 2001). The Pro12Ala polymorphism of the PPARγ2 gene influences the efficacy of EPA and DHA in diabetic subjects (Ylonen et al., 2007). GST polymorphisms explain, at least in part, the inter-individual variation in response to isothiocyanates (Lampe and Peterson, 2002). Gingerols, shogaols and arylalkanes from ginger rhizome, a widely used spice and herbal antiemetic, are 5HT3 serotonin receptor antagonists (Abdel-Aziz et al., 2005; 2006), and polymorphisms in the subunits of this receptor contribute to the highly variable efficacy of antiemetic drugs that act through 5HT3 receptor blockade (Ho and Gan, 2006). Many phytochemicals in food down-regulate or inhibit cyclooxygenase-2 (COX-2), including resveratrol, curcumin, flavopiridol, ellagic acid, ursolic acid, oleandrin, EGCG, silibinin, limonene and dibenzoylmethane (reviewed by Aggarwal and Shishodia, 2006), and several allelic variants of COX-2 are known to influence the efficacy of COX-2 inhibitory drugs (Lee et al., 2006), suggesting the efficacy of these dietary agents may be compromised by variant COX-2 genotypes.

CONCLUSION

With the pharmacologic characterization of singular dietary agents and their widespread use in the form of nutraceuticals, the tenets of pharmacology have assumed an unprecedented value in product formulation and clinical use. Despite the growing body of information on diet and disease prevention, there remains a paucity of human clinical data on pharmacokinetics, pharmacodynamics and metabolism of isolated dietary agents. However, consumer demand for alternative therapies and preventive nutritional medicine remains strong. With the limited information available, basic and clinical pharmacology principles can be applied in nutraceutical product development to assess the viability of prospective ingredients and products, and in the clinic to assess risk and benefit as accurately as possible, based on preclinical data. Sound interpretation of the expanding body of pharmacology and molecular biology literature requires knowledge of basic receptor pharmacology, pharmacodynamics, pharmacokinetics and drug metabolism. It also demands a grasp of intracellular signaling pathways relevant to disease and dietary intervention such as mitogenic, inflammatory, cell cycle and apoptotic pathways. As the fields of nutrigenetics and nutrigenomics continue to elucidate diet/gene interactions and effects of dietary agents on gene expression, integration of these biomedical arenas into nutraceutical product design and clinical use will afford a more rational and individualized approach to nutraceutical design and application.

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Key Gaps Remain in Understanding Health Effects of Vitamin D

Special Journal Issue Summarizes What We Know and What We Need to Learn

Despite considerable progress in research to understand the health effects of vitamin D, experts convened by the NIH to review the available data found major gaps in the evidence. The data are strongest in the area of bone health among elderly men and post-menopausal women, suggesting that increased vitamin D intake can improve bone health and prevent falls. For other age groups and health issues, though, it is too early to say conclusively whether more vitamin D might be beneficial.

An in-depth review of current research on the health effects of vitamin D was published as the proceedings of the NIH conference, "Vitamin D and Health in the 21st Century: An Update", which appeared in an August 2008 supplement to the *American Journal of Clinical Nutrition*.

Intriguing findings from research conducted in recent years have led to increased interest in vitamin D among health care providers, researchers, and the general public, including concern about possibly widespread deficiency, calls for supplementation, and even use of large doses of vitamin D as treatments for a variety of conditions.

"Given recent findings, it's easy to see why people are so enthusiastic about the potential power of vitamin D, but we must recognize the limitations of any study and exercise caution when making broad public health recommendations," said Mary Frances Picciano, Ph.D., a senior nutrition research scientist in the NIH Office of Dietary Supplements, who co-authored an overview of the conference included in the journal supplement. "This is a very complex set of issues and there is still a lot we don't know about how vitamin D levels affect health, especially across different age groups and ethnic populations."

"It's tempting to think that an essential nutrient is safe at any level — that if some is good, more is better," said Paul M. Coates, Ph.D., director of the Office of Dietary Supplements. "We've learned that this isn't always true, and there are potential harms associated with high levels of many nutrients."

Participants in the NIH conference identified a number of limitations of the existing evidence on vitamin D, including:

- Many studies have failed to control for factors that could confuse study findings, such as diet, baseline vitamin D status, age, disease, season (as relevant to sun exposure), and physical activity.
- Few studies have examined the effects of vitamin D independent of calcium or other nutrients.
- Reliable data on the vitamin D content of foods is not available.
- Existing laboratory tests used to measure vitamin D levels in blood vary widely.
- Preliminary research findings suggest a role for vitamin D in preventing chronic diseases such as diabetes, immune function, and cancer, but further study is needed.
- Research has not identified the vitamin D levels needed to achieve desired health outcomes in people at various life and reproductive stages and in dark-skinned individuals.

Vitamin D is an essential component in bone health that helps ensure that the body absorbs calcium, which is critical for building strong, healthy bones. People get this nutrient from three sources: sunlight, dietary supplements, and foods. Most people meet their vitamin D needs through exposure to sunlight, but questions remain about what amount of sun exposure would yield beneficial levels of vitamin D without unacceptably elevating skin cancer risk. Very few foods naturally contain vitamin D, so much of the vitamin D in Americans' diets comes from fortified foods such as milk and cereal. The flesh of certain fish such as salmon, tuna, and mackerel and fish liver oils are among the best naturally-occurring sources. Small amounts of vitamin D are found in beef liver, cheese, and egg yolks.

It is possible to get the currently recommended amounts of vitamin D from diet. Two glasses of vitamin D-fortified milk per day, for example, provides enough vitamin D for a healthy person under age 50. But individuals who are not consuming vitamin D-rich or fortified foods, or getting regular sun exposure may want to consult a health care provider about taking supplements to ensure adequate intake. To learn about vitamin D intake recommendations for different age groups, read the Office of Dietary Supplements' vitamin D fact sheet at http://dietary-supplements.info.nih.gov/factsheets/vitamind.asp.

Without sufficient vitamin D, bones can become thin, brittle, or misshapen. Vitamin D deficiency can lead to rickets in children and osteomalacia (softening of the bones) in adults. Together with calcium, vitamin D also helps protect older adults from developing osteoporosis. However, excess vitamin D intake can also cause harmful side effects, including nausea, vomiting, diarrhea, constipation and development of kidney stones. Healthcare providers may check vitamin D blood levels in individuals at increased risk for deficiency such as breastfed infants, older adults, people with limited sun exposure, people with dark skin, people with fat malabsorption, and people who are obese.

Investigations of vitamin D's health effects are expanding and areas of promising research include its role in type 1 diabetes, some cancers, autoimmune diseases such as multiple sclerosis, and infectious diseases such as tuberculosis.

In light of recent research, some advocates and researchers have called for a review of the U.S. Dietary Reference Intakes for vitamin D. Current recommendations for daily vitamin D intake were developed in 1997 by the Food and Nutrition Board of the Institute of Medicine. The U.S. Department of Health and Human Services, in collaboration with the U.S. Departments of Agriculture and Defense, and Health Canada are currently in discussions with the Institute of Medicine to revisit the recommendations.

The *American Journal of Clinical Nutrition* supplement is available to subscribers at http://www.ajcn.org/, and contains an overview of the conference, invited papers from many of the conference speakers, and a summary of the roundtable discussion held following the conference. The supplement may be accessed via the ODS website, at http://ods.od.nih.gov/news/AJCN2008.aspx. The American Journal of Clinical Nutrition is published by the American Society for Nutrition (http://www.nutrition.org/).

To learn more about vitamin D or other dietary supplements through fact sheets, databases, and other research resources, please visit the ODS website (http://dietary-supplements.info.nih.gov/index.aspx).

ORIGINAL RESEARCH

A Randomized, Double-blind, Placebo Controlled Study Examining the Effects of a Combination Nutraceutical Formula on Cognitive Functioning and Mood

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ABSTRACT

The combination nutraceutical formula consisting of Huperzine A, Vinpocetine and Acetyl-L-Carnitine was examined in a 30-day double blind, placebo controlled clinical trial assessing a range of cognitive and mood variables. Seventy-four healthy participants, 43 in the combination nutraceutical formula group and 31 in the placebo group, with a mean age of 48 years, completed the study. Statistically significant improvements in several variables relative to placebo could be attributed to the 30 days administration of the combination nutraceutical formula, including working memory accuracy ($p < .03$), long term memory consolidation ($p < .02$) and mood ($p < .02$), suggesting an improvement in complex cognitive processes. The cognitive processes assess both the initial stages of holding information in conscious awareness as well as the encoding and retrieval of recently learned material. The mood measures assessed levels of anger and depression. Interestingly, improvements in speed of memory retrieval suggest functioning equivalent to age cohorts of approximately 10 or more years younger. A range of more simple measures of information processing speed, such as reaction time and other indices of mood, were not improved. The results suggest the promise of special nutraceutical formulations for improving cognition. There were also no side effects or adverse reactions reported by the participants.

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INTRODUCTION

With increasing life expectancies and the maturation of the "baby boom" generation, adapting to the challenges posed by the ageing population has been identified as one of the major issues facing contemporary western societies. Human ageing has significant societal, economic, health and personal costs. Increasing age is associated with a cluster of illnesses involving oxidative stress, including cardiovascular and respiratory disease, as well as neurological conditions such as Parkinson's disease (PD) and Alzheimer's disease (AD).

A time-honored and much empirically supported method of promoting optimal health throughout the lifespan has been through the adoption and maintenance of an appropriate, healthy diet. Recent research suggests that this

principle not only applies to protection from "physical ailments" such as cardiovascular problems, but may also extend to ameliorating the effects of cognitive decline associated with increased age. The maintenance of brain health underpinning intact cognition is a key factor to maintaining a positive, engaged and productive lifestyle. In light of this fact, the role of diet, including supplementation with nutritional and even pharmacological interventions capable of ameliorating the declining neurocognitive changes that occur with age, constitute vital areas of research. Accordingly, there is considerable interest in whether natural supplements or nutraceuticals can improve cognition.

The combination nutraceutical formula has been developed to improve cognitive functioning, particularly in an age group showing normal age related cognitive decline. The combination nutraceutical formula is a compound of Huperzine A, Vinpocetine and Acetyl-L-Carnitine ingredients, which individually have been extensively studied in experimental animal and human clinical trials. We provide a brief summary of these studies below. Additionally, a recent review in *JANA*[1] has outlined the evidence for their mode of action on the human brain with a particular focus on improving cognition and memory.

Huperzine A (HupA)

Huperzine A (HupA), isolated from the Chinese herb Huperzia serrata, has been suggested to be a promising compound to treat Alzheimer's disease.[2] Consistent with this view are the results of studies revealing that HupA functions as a reversible inhibitor of acetylcholinesterase,[3] which increases the amount of synaptic acetylcholine available for neurotransmission. Additionally, based on observations in the rat cerebral cortex,[4] studies have proposed that HupA functions as a non-competitive antagonist of NMDA receptors. HupA has been found to reverse or attenuate cognitive deficits in a broad range of animal models.[5] Numerous clinical trials have demonstrated that Hup A is effective in relieving memory deficits associated with college students,[6] the elderly and Alzheimer's disease without any serious adverse side effects,[5] and is considered to be a safe supplement.[7]

Vinpocetine (VIN)

Vinpocetine (VIN), derived from the Periwinkle plant (*Vinca minor*)[8] is widely used as a neuroprotective agent.[9, 10, 11, 12] The primary action of VIN is to enhance cerebral vascular blood flow, brain energy metabolism[12, 13, 14, 15] and increase the neuronal uptake of glucose and oxygen.[12, 16, 17] Due to these beneficial actions, VIN has been used in the prevention and treatment of conditions and diseases associated with compromised cognitive function.[12, 20] VIN has been shown to improve the speed of memory learning and recall in cognitively healthy subjects[18, 19] and in cognitively compromised subjects.[8]

Acetyl-L-Carnitine (ALC)

Acetyl-l-Carnitine (ALC) is naturally synthesized in the human brain, liver and kidney[21] and may have beneficial properties in treating age related disorders such as Alzheimer's dementia.[22, 23, 24] Further, studies have shown that ALC may be beneficial in the treatment of depression,[25, 26] attention deficit disorders[27, 28] and cognitive impairment induced by alcohol.[29] ALC plays an essential role in energy production by facilitating the uptake of Acetyl CoA into the mitochondria during fatty acid oxidation[30, 31] and increases ATP energy production.[32] ALC also enhances acetylcholine synthesis[33, 34] and cerebral vascular blood flow,[35, 36] which are implicated in age related normal and abnormal states of cognitive decline, such as Alzheimer's disease.

We hypothesized that the combination nutraceutical formula compound would have a strong effect on improving cognitive functioning, attention, energy levels, stress adaptation and mood states. We tested this hypothesis by conducting a randomized double blind placebo controlled human trial with 90 participants.

METHOD AND MATERIALS

Participants

Ninety participants (45 in each of the two groups) were initially enrolled into the 30 day chronic study. Seventy-four (74) participants completed the 30 days and were tested at both baseline and at 30 days (43 in the combination nutraceutical formula group and 31 in the control group). The mean age of the combination nutraceutical formula group was 49.5 years (SD = 1.6 22-66 years) and the mean age of the placebo group was 47.1 years (SD = 1.9 24-62) years. There was no significant difference in the number of males or females who participated in the study.

Inclusion/Exclusion criteria

Each participant underwent an individual screening appointment with a registered nurse. Screening incorporated a medical history and cognitive assessment. Participants were eligible if they were aged between 22 and 66 years of age. Exclusion criteria included the following: not currently taking prescription drugs affecting the brain or nervous system (e.g., Modafinil, acetylcholinesterase inhibitors, anticholinergics, stimulants, L-dopa, MAO inhibitors, NMDA receptor antagonists, methylphenidate, amphetamine, pseudo-ephedrine, SSRIs and other anti-depressant medication); not currently taking OTC medications affecting the brain (e.g., ephedra based diet pills); those who have not used any supplements within the past 30 days that have an effect on cognitive function, memory, anxiety, depression (e.g. Ginseng, Gingko, Vinpocetine, 5HTP, Tryptophan, St. John's Wort, ephedrine (ephedra), alpha GPC, Citicoline, phosphatidylserine, acetyl-l-carnitine, Focus Factor™); not active smokers; not taking the following: anti-coagulant drugs (Warfarin, Heparin, Plavix); anti-cholinergics or acetylcholinesterase inhibitors (bethanechol, Ureholine),

donepezil (Aricept), rivastigmine (Exelon), galantamine (Reminyl), edrophonium (Enoln, Reversol, Tensilon), neostigmine (Prostigmin); do not have any of the following health conditions: AIDS, HIV, chronic fatigue syndrome, Epstein-Barr, fibromyalgia, lupus, multiple sclerosis, thyroiditis, ulcerative colitis, Crohn's disease, irritable bowel syndrome, dementia including Alzheimer's and Parkinson's disease, Type 1 or 2 diabetes, insomnia or sleep apnea, narcolepsy; no history of head trauma; no neurological deficits; not pregnant or lactating; not anticipating any planned changes in lifestyle (e.g. exercise regimen) for the duration of the study; and no known allergies to nuts.

Study Design

The study was a randomized double blind placebo-controlled design in which participants were allocated either a daily dose of the combination nutraceutical formula or placebo for 30 days. The dose was 1,515 mg per day and each participant was instructed to take 3 pills per day. The combination nutraceutical formula, known as ProceraAVH, was provided by 20/20 Brain Power Partners, LLC (Founders of Brain Research Labs), Laguna Beach, California.

Measures

Several cognitive and psychological measures were assessed at baseline and at 30 days.

Cognitive Testing

The CDR® program is an automated computerized cognitive assessment system, which has been used in more than 250 published clinical drug studies. The CDR system comprises a battery of cognitive tests that are sensitive to the effects of psychopharmacological substances.[37] The CDR system profiles and assesses a range of cognitive functions, including attention, information processing, sub-loops of working memory, reasoning, secondary memory and skilled coordination. All tasks in the battery are computer controlled, with information being presented on high-resolution monitors, and the responses recorded via a response module containing two buttons, one marked 'YES,' the other marked 'NO.' The versions of the tests specified for elderly participants were employed. The selected battery took the participants around 30 minutes to complete and parallel forms of the tasks were presented at subsequent testing sessions. The cognitive tests used from the battery are presented in Table 1.

The Profile of Mood States (POMS) is a self-report designed to measure six dimensions of mood: tension/anxiety; depression/dejection; anger/hostility; vigor/activity; fatigue/inertia; and confusion/bewilderment.[38]

Table 1. The CDR (Cognitive Drug Research) Computerized Cognitive Assessment System (Areas of Cognition Measured)

Level I: Attention

The ability to select, evaluate and respond to appropriate environmental information

Task	Cognitive States and Processes Assessed
Simple Reaction Time	Alertness, power of concentration Primary stage of information processing
Choice Reaction Time	As above, plus stimulus discrimination; response organization
Digit Vigilance	Intensive vigilance; sustained attention; ability to ignore distraction

Level II: Short Term or Working Memory

The ability to temporarily store the information relevant to ongoing tasks

Task	Cognitive States and Processes Assessed
Numeric Working Memory	Sub-vocal rehearsal of digit sequences Articulatory loop sub-system of working memory
Spatial Working Memory	Ability to temporarily retain spatial information Visuo-Spatial sub-loop of working memory

Level III: Long Term or Episodic Secondary Memory

The ability to register, store and retrieve information over any period required

Task	Cognitive States and Processes Assessed
Word Recall*	Ability to store and recall verbal information; capacity for un-cued retrieval of words; episodic secondary verbal recall
Word Recognition	Ability (speed and sensitivity) to discriminate novel from previously presented words; episodic secondary recognition
Picture Recognition	Ability to discriminate novel from previously presented pictorial information; episodic secondary non-verbal visual recognition
Face Recognition*	Ability to discriminate novel from previously presented faces; episodic secondary face recognition

* Face recognition task and word recall task were not administered in this study.

Procedure

At baseline each participant completed a general health assessment, which included blood pressure, height and weight, and were then randomly allocated into one of the two treatment groups. They then completed a CDR practice session, which is required in order to become familiar with the tests. After completing the practice session, they were administered the cognitive and psychological tests. Thirty days after their first visit, they completed cognitive and psychological testing again.

RESULTS

A series of repeated measures (ANOVAs) were calculated to examine the changes between baseline and 30 days administration of the combination nutraceutical formula and placebo on the cognitive and psychological measures. As this was the first clinical trial combining the three components in the combination nutraceutical formula, we report the statistically significant analyses ($p < .05$) as well as the analyses approaching statistical significance ($p > .05 \leq .10$), which will help the design of future studies assessing this compound.

(1) COGNITION

Non-significant changes in simple reaction time, digit vigilance and choice reaction time, spatial working memory and picture recognition, (long term memory consolidation of objects), were observed over the 30 days administration of the trial. However, 30 days administration of the combination nutraceutical formula (compared to placebo) improved a range of cognitive processes. Means and SDs for these variables are reported in Table 2.

Numeric Working Memory

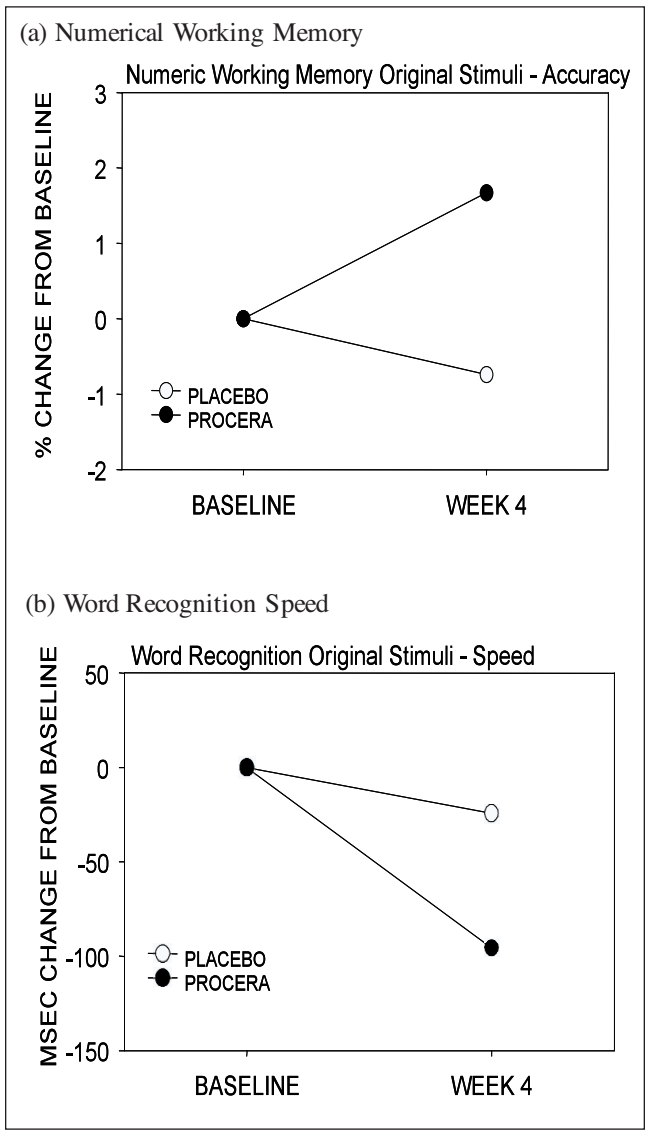
Participants on the combination nutraceutical formula treatment showed statistically significant improvement ($p < .03$) in numeric working memory accuracy compared to placebo participants. A statistically significant improvement in holding numbers in working memory (immediate

Table 2: Means and SDs for significant outcome variables at baseline and again at 30 days for the combination nutraceutical formula (ProceraAVH) and placebo groups.

	Condition	Mean	Std. Deviation	P
Numeric Working Memory Original Stimuli - Accuracy - baseline	Procera AVH	93.76	5.14	
	Placebo	95.77	5.26	
Numeric Working Memory Original Stimuli - Accuracy - week 4	Procera AVH	95.43	3.39	P < .03
	Placebo	95.03	3.94	
Word Recognition Original Stimuli - Speed: Mean - baseline	Procera AVH	853.12	184.99	
	Placebo	774.99	122.44	
Word Recognition Original Stimuli - Speed: Mean – week 4	Procera AVH	757.52	138.40	P < .02
	Placebo	750.54	7.03	
Depression/Dejection baseline (POMS)	Procera AVH	8.00	9.42	
	Placebo	5.19	8.54	
Depression/Dejection - week 4 (POMS)	Procera AVH	4.32	5.12	P < .06
	Placebo	4.35	6.57	
Anger/Hostility baseline (POMS)	Procera AVH	7.83	7.56	
	Placebo	4.48	5.80	
Anger/Hostility - week 4 (POMS)	Procera AVH	4.16	4.30	P < .03
	Placebo	3.80	6.12	
Total Mood Disturbance score BL (POMS)	Procera AVH	65.93	30.50	
	Placebo	52.90	23.86	
Total Mood Disturbance score - week 4 (POMS)	Procera AVH	47.55	17.16	P < .02
	Placebo	46.51	21.42	

memory) was shown from baseline to day 30 due to the combination nutraceutical formula treatment (see Figure 1).

Figure 1: Changes in Numeric Working Memory Accuracy Changes and Word Recognition Speed (long term memory) for placebo and the combination nutraceutical formula (ProceraAVH).



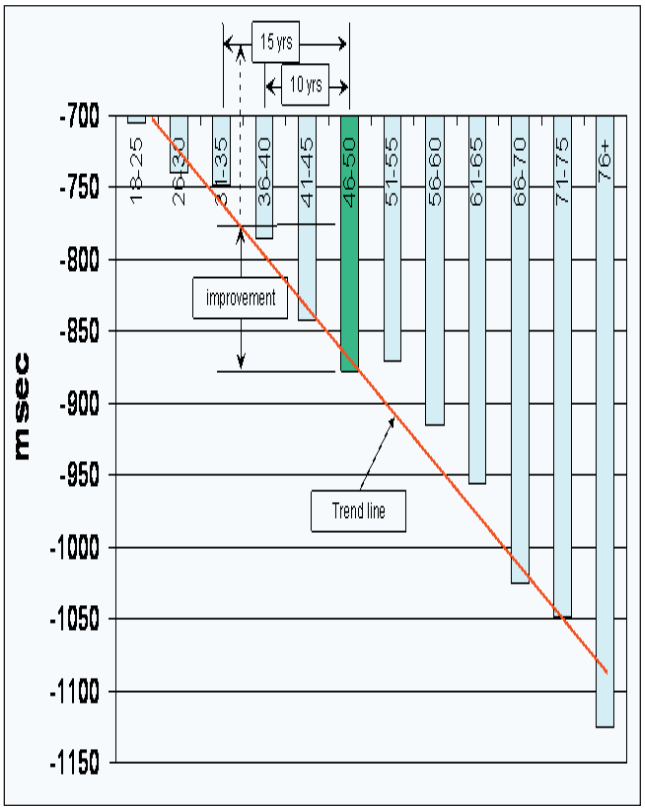
Spatial Working Memory

There was also a trend towards statistical significance ($p < .09$) for the number of outliers during the spatial working memory task. Outliers indicate lapses in concentration over the duration of the task. Participants in the combination nutraceutical formula treatment group showed less mean number of such lapses during the task and were therefore better able to focus and concentrate/process during the spatial working memory task, which is a complex cognitive task.

Word Recognition

The speed of performance during the word recognition task was significantly improved ($p < .02$) for participants on the combination nutraceutical formula treatment compared to the placebo treatment over the 30 days of administration (Table 1). This indicated that the combination nutraceutical formula significantly improved memory consolidation processes and in particular, the speed at which a participant was able to consolidate and access new memories into long term storage (Figure 1). As extensive age related normative data are available for the speed of recognition task from the CDR battery, it was possible to calculate the approximate improvement in relative age related functioning due to the combination nutraceutical formula treatment. An improvement in RT of approximately 100 msec was seen in the Procera group compared to approximately 20 msec in the placebo group. Given that the mean age of the group was 48 years, a net improvement of approximately 80 msec on this task (measuring speed of long term memory retrieval) equates to approximately the functioning of age bands some 10-15 years younger (Figure 2).

Figure 2: Age related improvements in speed of memory retrieval with the combination nutraceutical formula (ProceraAVH). Improvements in RT due to 30 days administration of the combination nutraceutical formula, (compared to placebo), equate to between 10 and 15 years of normal age-related decline in speed of memory retrieval (80 msec), given the mean age of the sample (48 years). Graph adapted with the kind permission of Cognitive Drug Research (CDR UK).



(2) MOOD

Depression

The combination nutraceutical formula group showed a decrease in depression scores relative to the placebo group ($p < .06$). This suggests that the combination nutraceutical formula may improve depressive mood conditions.

Anger/Hostility

The combination nutraceutical formula group showed a statistically significant ($p < .03$) decrease in anger/hostility over the 30 days trial relative to the placebo group. This indicates that 30 days of treatment with the combination nutraceutical formula significantly improves feelings of anger and hostility. This result is supportive of the decrease in depression scores.

Confusion

Participants in the combination nutraceutical formula group also showed a decrease in confusion over the 30 days trial ($p < .06$), which was greater than in the placebo participants. Again, this result is consistent with the decrease in depression and anger/hostility shown by the combination nutraceutical formula participants over the 30 days trial. A decrease in confusion may be best understood in terms of improving mental clarity.

Vigor

A non-significant change in vigor scores were observed across the 30 days of the trial ($P < .10$). An improvement in vigor may be best understood in terms of increased mental energy.

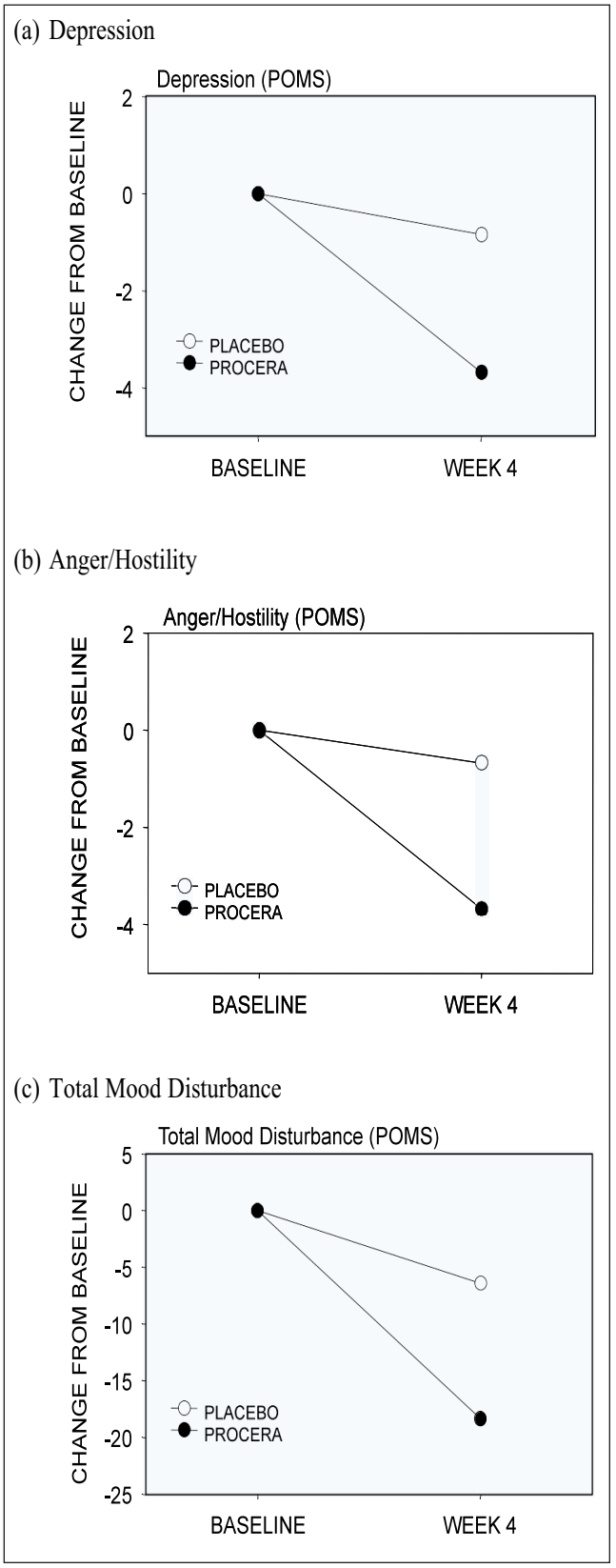
Total Mood Disturbance

The main factor score relating to negative moods on the POMS is total mood disturbance. This factor may be regarded as a highly reliable indicator of changes in negative emotions or moods over the 30 days of supplementation. There was a highly significant change in the total mood disturbance over the 30 days in favor of the combination nutraceutical formula group compared to the placebo group ($p < .02$). This improvement in mood due to the combination nutraceutical formula is consistent with the observed changes in depression, anger/hostility, confusion and vigor. The result also suggests that changes in mood due to the combination nutraceutical formula are highly noticeable in the participants. Figure 2 displays the changes from baseline over the 30 days treatment for the combination nutraceutical formula and placebo groups.

(3) Safety

There were no statistically significant differences in side-effects between the two conditions after 30 days of administration.

Figure 3: Changes in mood variables for placebo and the combination nutraceutical (ProceraAVH) formula conditions relative to baseline scores.[7]



DISCUSSION

This was the first double blind placebo controlled study to examine the effect of 30 days administration of the combination nutraceutical formula, called ProceraAVH, on cognitive and mood variables. The data from this trial provide evidence that this compound improves a range of cognitive and mood variables in healthy adults. The cognitive changes were observable using a highly standardized and reliable battery of cognitive tasks, and the mood changes were readily observed and reported by the participants.

In terms of the cognitive variables, there is evidence that the unique ProceraAVH nutraceutical formula mainly improves functioning during complex cognitive tasks that assess memory (working and long term, or consolidation) rather than in simple discrimination tasks such as choice reaction time. Interestingly, the cognitive processes that appear to be improved relate to the middle (working memory; consolidation) and late stages of memory functioning (memory retrieval of newly learned material). This may suggest a specific focus of effect on the human brain, incorporating frontal and temporal circuits that underpin working memory and long term memory consolidation.

In terms of mood, the combination nutraceutical formula appears to improve mental clarity and mental energy, and to help repair mood disturbances. Mood disturbances are commonly experienced from time to time by all adults, together with confusion and low level depressive symptoms. The combination nutraceutical formula appears to improve these moods, which may be important in improving cognitive processes. Memory in particular is very sensitive to disturbances in mood such as anxiety and other negative emotions that fall under the umbrella of terms such as depression.

Overall, the results suggest that the combination nutraceutical formula, ProceraAVH, exerts beneficial effects on both cognition and mood. The results of the present study confirm the results and conclusions of the extensive literature examining Vinpocetine, Huperzine A and Acetyl-L-Carnitine singly on cognition and mood in animals and humans. Future larger scale trials on this formulation should be undertaken as a matter of priority in order to explore additional possible areas and conditions of impact. Of particular merit is the question of whether the combination nutraceutical formula may have the greatest effect on the elderly, in whom cognition is most challenged.

ACKNOWLDGEMENT

Approximately 50% of the cost of this trial was provided by 20/20 Brain Power Partners, LLC (Founders of Brain Research Labs) as an external grant to Swinburne University in order for us to conduct the study. There are no other potential conflicts of interest.

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ORIGINAL RESEARCH

A Single-Center, Double-Blind Placebo Controlled Study to Evaluate the Efficacy of Kre-Celazine®, an Oral Buffered Creatine-Cetylated Fatty Acid Compound, in its Ability to Reduce Site-specific Inflammation and Pain

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KEY WORDS

Kre-Celazine,® buffered-creatine compound, fatty acids, joint and muscle inflammation.

ABSTRACT

In order to determine whether an oral, alkali buffered-creatine – cetylated fatty acid compound was capable of reducing site-specific chronic joint and muscle related inflammation/pain with equal effectiveness, 35 subjects, each fulfilling the entrance criteria, were divided into 2 groups – Group A ("Test Compound" group) and Group B ("Placebo group"). Each participant took the same number of capsules irrespective of their group assignment, for 30 consecutive days. Efficacy was based on the final evaluation of pre and post blood tests, physical examinations (entrance and exit) and participants' "Pain Journal" comments. Results indicated that approximately 100% of ankle/foot pain, 80 - 85% of neck/ shoulder/elbow/wrist and hand pain, 71% of knee pain, respondents in Group A rated their "compound" better than/as good as a prescription product in its ability to reduce/eliminate pain. Hip and back pain scores for Group A were no better than placebo scores. Group A experienced a modest increase in mobility (35%), but no measurable increase in range of motion over and above that experienced in Group B. The alkali buffered-creatine – cetylated fatty acid compound exerted its greatest impact on areas of inflammation/pain in the extremities, as well as in the neck and shoulder region.

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INTRODUCTION

Chronic inflammation and muscle pain affects the body's ability to execute fluid motion. Ensuing joint stiffness restricts range of motion (ROM), which in turn negatively impacts quality of life (QOL). Chronic inflammation is a primary reason for doctor visits and increased costs in our healthcare system.[1] Thousands of "Baby Boomers" born between 1946 and 1950, are now transitioning through age 60 and beyond. Along with the prospect of living to the century mark, comes the reality that osteoarthritis, sports and non-sports related injuries also increase.[2] This year, the Arthritis Foundation has estimated that immune-related joint degenerative conditions are expected to strike more then 27 million Americans during the next decade,[3] with additional untold numbers afflicted with ligament weakness, fibromyalgia, idiopathic pains and muscle trauma.[4-5] Pain reducing medications are utilized daily,

essentially to combat the symptoms of immune-related aging issues. Unfortunately, prescription drugs are not without side effects and many consumers are turning or have already turned to over-the-counter (OTC) substances in the hopes of obtaining pain relief without those side effects.[6]

Cetylated fatty acids (as a group) have been reported to exhibit an anti-inflammatory activity in joint/muscle/ligament regions. These promising results were based primarily on animal study data,[7] while the number of human studies showing any conclusive anti-inflammatory effect is limited.[8-9] Cetylated fatty acids are believed to act by inhibiting the cyclooxygenase pathway in test animals, however, it is unknown whether the same mechanism is functioning in humans.[10]

The National Institutes of Health (NIH) has funded several meta-analyses in the attempt to help identify and validate anti-inflammatory OTC materials.[11] Of the more unexpected findings, unsaponifiables (oils),[12-14] and creatine monohydrate were found capable of modulating certain aspects of cell surface/pro-inflammatory reactant interactions.[15] Creatine monohydrate is a supplement primarily used by athletes who are engaged in high-energy demand activities.[16] It has been extensively researched for its safety and ergo-dynamic supportive function. Until recently, however, very little attention has been paid to its ability to influence cell surface interactions.

Based on research data, we hypothesize that a compound composed of a buffered creatine in combination with cetylated fatty acids might result in a safe, effective form of OTC pain relief.

MATERIALS AND METHODS

Test Subjects

Prospective participants were recruited from Billings Montana and the surrounding suburbs through local advertisements. Respondents who indicated they had chronically (>6 months) experienced at least one area of localized pain/stiffness, underwent an extensive pre-screening process that included a preliminary interview, comprehensive questionnaire and blood tests, before being admitted to the study. A total of 35 subjects (21 males, 14 females) ranging from ages 23 to 88, were admitted. Participants experiencing at least one and occasionally multiple isolated areas of chronic joint/muscle inflammation/pain, were divided into 2 groups designated "Group A" (n = 24) and "Group B" (n = 11). Individuals in Group A were assigned four capsules of an oral, alkali buffered-creatine – cetylated fatty acid compound called the "Test Compound" (product name: Kre-Celazine,® manufactured by All American Pharmaceutical and Natural Foods Corporation, Billings, Montana) daily, Group B, an equal number of placebo capsules daily. The capsules were prepared by the manufacturer in a manner so as to preclude either the physicians or the participants from identifying the contents. The investigator, examining physicians, testing laboratory staff and the participants did not know to which group an individual had been assigned. Each individual's participation lasted 30 consecutive days. Only complete data from those who were considered "100% compliant" was considered "valid." To be considered compliant during this period, each participant was required to take the assigned capsules, comply with the blood draws and examination schedule, fill out a "Pain Journal" according to the protocol provided, and return the original bottle with any remaining capsules, after the last day of their participation.

Study Material Preparation

The "Test Compound" was a proprietary oral preparation consisting of an alkali-buffered creatine – cetylated fatty acid compound, called Kre-Celazine®. Capsules and bottles for the study were prepared in such a manner as to preclude any participant, study physician or blood lab technician from knowing which individual was receiving either material. The identity codes for the participant assignments were kept confidential until after the study.

Blood Sampling

Blood sampling was done at a Laboratory Corporation of America blood collection lab according to standard protocols.

Study Protocol

The entire study was conducted over a period of 90 days. Applications were accepted from both men and women, ages 21 years or older. Prospective subjects were pre-screened according to the following protocol:

- A recruitment Inclusion/Exclusion Criteria form.
- A fasting blood draw completed for creatinine and AST (SGOT) and C-reactive protein.

To be eligible for inclusion in the study, prospective participants were required to fulfill the following inclusion criteria prior to admission:

- Have normal fasting serum levels of creatinine (<1.3mg/dl F; <1.6 mg/dl M), and AST(SGOT) (<41 IU/L - M/F).
- Be at least 21 years of age or older.
- Sign an Informed Consent form.

Prospective participants were excluded from participation if they fulfilled any one or more of the following exclusion criteria:

- Serum creatinine level of >1.5 mg/dl M; >1.2 mg/dl F.
- Serum AST (SGOT) level >41 IU/L.
- Pregnant or breastfeeding.

- Digestive-related or fat-malabsorption disorder.
- Taking a lipid-absorbing drug (excluding a statin).
- Chronic disease state (i.e. hepatitis, cirrhosis, diabetes, cancer, organ failure).
- Taking multiple medications for numerous medical conditions.
- Steroid anti-inflammatory usage.
- Current methotrexate usage.
- Smoker.
- Alcoholic.
- Multi-cups (coffee or tea) drinker daily.
- Taking numerous nutritional supplements.
- Having a medical condition that would preclude participation.

Each study participant was provided with an instruction sheet. After completion of the entrance examination by a study physician, participants were instructed to self-administer the capsules, two in the morning and two in the late afternoon-evening, on an empty stomach, swallowing each capsule with water. No other liquid was permitted for use in swallowing the capsule. Participants were reminded to avoid caffeinated drinks for at least five hours after taking any capsules, and score pain in a Pain Journal. At the end of the study (day 30), participants were required to have a repeat blood draw and complete an exit examination prior to returning the bottle to the company with the remaining capsules. In order to insure compliance with the capsule administration portion of the protocol, each participant had a specific excess number of capsules in his or her bottle. Only data from those participants who were considered 100% compliant was used.

Physicians' Entrance/Exit Exam Protocol

All participating physicians were provided with an examination protocol to be completed, which included the following information:

- Participant Number.
- Gender.
- Blood Test Results [Creatinine; AST/(SGOT); C-Reactive Protein].
- Comment section for blood tests results.
- General Health Assessment section.
- Location and extent of joint or muscle related pain.
- Arthritis or fibromyalgia related pain (specify if possible).
- Define limits of mobility (a joint/or limb).
- Range-of-motion test (ROM).
- [Exit Exam Question] Has this participant's pain changed (better/worse) since the entrance exam?
- [Exit Exam Question] General mobility assessment – what has changed since the initiation of the study?

RESULTS

Participant compliance was high. Complete data from a total of 31 of the original 35 participants was available. Dropouts occurred only in "Group A" and were the result of loss of interest (two persons), adverse/untoward reactions rated significant/serious (edema/pain in one person), final blood data lost at the lab (one person). All 31 participants returned bottles with the correct number of capsules. The gender distribution changed for "Group A" due to the withdrawal of 3 female participants and the loss of data for one male participant. Adverse reactions, rated mild/minor (gas/upset stomach) were reported by both groups (Group A = 20%; Group B = 9%). The participants' lab and questionnaire data are shown in **Table 1**. Final evaluation of any changes in mobility, ROM and inflammation/pain were scored during the exit exams and is shown in **Table 2.**

DISCUSSION

With the prospect of longevity well beyond the 90 year range, the search for anti-inflammatory pain relief that is without the burden of serious side effects, grows more urgent. Cetylated fatty acids (as a group) are under consideration as a substance having potential anti-inflammatory properties with the ability to suppress pro-inflammatory cytokines. Prior to this study, much of the supporting anti-inflammatory data was based on a limited number of human studies and animal data. Equally interesting is creatine monohydrate – a fairly new contender to the anti-inflammatory field. Research suggested that in its phosphorylated form, creatine supplementation may be capable of positively affecting endothelial permeability, thereby inhibiting potentially inflammatory stimulating molecules from adhering and expressing their action on endothelial cells.

Our findings indicate that an oral buffered-creatine monohydrate and cetylated fatty acid compound is effective in improving pain/stiffness and mobility scores in the extremities, as well as in the shoulder and neck region. This finding is of particular interest. While cetylated fatty acid formulations, both topical and oral, have tentatively been cited for their potential to improve knee related stiffness/pain, there has been no significant mention of substantial improvement to those additional areas positively impacted by the formulation used in this study. If the purported anti-inflammatory activity for the cetylated fatty acid family (cetyl myristoleate, cetyl myristate, cetyl palmitoleate, cetyl laureate, cetyl plamitate and cetyl oleate) is primarily directed toward (or confined to) the

Table 1. Participants' Lab and Questionnaire Data.

		Group A (Kre-Celazine®)		**Group B** (Placebo)	
Participants:	Initial	24		11	
	Completing	20		11	
Gender		**17 males/3 females**		**4 males/7 females**	
Age		**23 – 88 years**			
Dropouts (M/F)		**3 F**		**0**	
Entrance blood tests:		**Overall average**			
Creatinine		0.9 mg/dl		0.8 mg/dl	
AST(SGOT)		23 IU/L		22 IU/L	
C-reactive Protein		4.1 mg/L		6.2 mg/L	
Exit blood tests:		**Overall average**			
Creatinine		0.9 mg/dl		0.8 mg/dl	
AST(SGOT)		25 IU/L		21 IU/L	
C-reactive Protein		4.2 mg/L		5.1 mg/L	
Pain Relief: (Percentage of participants rating their treatment**,** "as good as" or "better than" their usual OTC or prescription pain reliever)		**Per Area:**			
	Ankle/Foot –	100% (2/2)		0%	
	Knee (and Leg) –	71+% (5/7)		0%	
	Hip –	33% (1/3)		33% (1/3)	
	Back –	50% (5/10)		0%	
	Neck/Shoulders –	85+% (6/7)		33% (1/3)	
	Elbow/Wrist/Hand	80% (4/5)		33% (1/3)	
No Pain Relief: (Percentage of participants rating their treatment, "not as good as" or "didn't work" compared to their usual OTC or prescription pain reliever)		**Per Area:**			
	Ankle/Foot –	0%		100% (3/3)	
	Knee (and Leg) –	29% (2/7)		100% (5/11)	
	Hip –	67% (2/3)		66% (2/3)	
	Back –	50% (5/10)		100% (1/1)	
	Neck/Shoulders –	15% (1/7)		66% (2/3)	
	Elbow/Wrist/Hand	20% (1/5)		66% (2/3)	
Personally said they experienced reduced pain/increased mobility		**Overall Average**			
		Yes	**No**	**Yes**	**No**
		60% (12/20)	40% (8/20)	27% (3/11)	73% (8/11)
Experienced an adverse event:					
Minor or mild (gas/upset stomach)		20% (4/20)		9% (1/11)	
Significant/serious (edema/pain)		4%* (1/21)*		0% x	
Blood Pressure:		**Overall Average**			
		Systolic/Diastolic		**Systolic/Diastolic**	
Entrance		**127/83**		**129/82**	
Exit		**125/75**		**119/77**	

* Due to an adverse event, this participant withdrew before the end of the study.

Table 12. Physicians' Reports.

	Group A (Kre-Celazine®)	**Group B** (Placebo)
ROM:		
Increase	20% (4/20)	18% (2/11)
Decrease	10% (2/20)	36% (4/11)
No Change	75% (15/20)	45% (5/11)
Mobility:		
Increase	35% (7/20)	9% (1/11)
Decrease	5% (1/20)	36% (4/11)
No Change	15% (3/20)	—
Data not Reported	45% (9/20)	54% (6/11)
Pain:		
Decrease	90% (18/20)	36% (4/11)
No Change	10% (2/20)	55% (6/11)

Information for Physicians' Reports was obtained during examinations/interviews.

knee region, this would suggest that the additional neck/shoulder/extremity areas, positively impacted by Kre-Celazine® may have resulted from the additional buffered creatine compound in the mix.

Anecdotally noted and of interest, is the small decrease in diastolic blood pressure over that observed for the placebo. This effect on blood pressure has not heretofore been mentioned in creatine or cetylated fatty acid studies, and may warrant further investigation.

CONCLUSION

The findings presented in this study suggest that Kre-Celazine® is a moderately effective non-prescription material for the reduction of pain and stiffness of the extremities, neck and shoulder regions in humans. It should be noted that the study group was small in number and additional studies are needed to confirm the results from this initial study.

ACKNOWLEDGEMENT

We wish to thank the following Administrative Assistants: Sandy Bates from All American Pharmaceutical Company, and Bonnie Rogness and Sally Wenz from The Yellowstone Naturopathic Clinic in Billings, Montana, for their tireless efforts and organizational support throughout the project. We would also like to thank the participants for making this project possible.

This study was made possible by a grant from the Montana Department of Commerce Research and Commercialization Grant Program, and co-funding was provided from the All American Pharmaceutical and Natural food Corporation.

The first author is the founder, principal investigator and research director of the All American Pharmaceutical and Natural food Corporation and the patent holder of Kre-Celazine.®

None of the secondary authors from the Yellowstone Naturopathic Clinic in Billings, Montana, have any financial interest/stake in the All American Pharmaceutical Company, its products or its grant monies.

The last author, Wendy Jones, works for Royal Knight Incorporated, a research consulting company, which was retained by All American Pharmaceutical Company to assist in the planning, execution and technical writing of their studies and data.

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ORIGINAL RESEARCH

Inhibitory Effects of a Novel Nutrient Mixture on MMP Secretion and Invasion on Human Thyroid Cancer Cell Line SW 579

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ABSTRACT

Thyroid cancer is the most common endocrine malignancy. Mortality from thyroid cancer results from tumor invasion with local and distant metastases. Degradation of extracellular matrix is the hallmark of metastasis and is mediated by matrix metalloproteinase (MMP) enzymes. A novel Nutrient Mixture (NM) containing ascorbic acid, lysine, proline, and green tea extract has exhibited significant anticancer activity in other cancer cell lines. In this study, we investigated the effects of NM on a thyroid cancer cell line on proliferation (MTT Assay), MMP secretion (Gelatinase-Zymography), Matrigel™ invasion, and morphology (H&E). Zymography demonstrated that NM inhibited MMP-2 secretion, with virtually total inhibition at 1000 mg/mL. Matrigel™ invasion was inhibited at 50, 100, and 500 mg/mL by 42%, 63%, and 100%, respectively. NM was nontoxic to thyroid cancer cells below 500 mg/mL, and H&E staining did not show morphological changes.

CONCLUSION

NM significantly inhibited critical steps in cancer progression by blocking MMP-2 enzymes and Matrigel™ invasion.

KEY WORDS

Thyroid cancer, MMPs, Matrigel™ invasion, nutrients, green tea extract, ascorbic acid, lysine.

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INTRODUCTION

Thyroid cancer is the most common of all endocrine cancers. American Cancer Society estimates approximately 33,550 new cases of thyroid cancer will be diagnosed in the United States in 2007, and approximately 1530 deaths are estimated due to thyroid cancer. Some of the risk factors for developing thyroid cancer are: white race, female gender, low intake of iodine, and previous radiation exposure. Thyroid cancer will constitute 4% of all female cancers in 2007. Women between ages of 40 and 70 are at higher risk; however, women as young as 19[1] have been reported to be diagnosed with this disease. The hormone estrogen is thought to increase the rate of thyroid cancer in women.[2,3,4] Radiation, especially therapeutic radiation for Hodgkin's disease in childhood, increases chances of developing thyroid cancer later in life. Lowering the doses of radiation has not decreased this risk.[5]

Thyroid cancers are divided into three categories: differentiated, medullary, and anaplastic cancers. Well-differentiated thyroid cancers (papillary and follicular) grow slowly, are rarely fatal, but may recur. Medullary cancers developing from C cells of the gland metastasize faster and are resistant to radioactive iodine. Anaplastic thyroid cancers are relatively uncommon, yet the most dangerous, of malignant tumors, and have the worst prognosis.

The accepted methods of treatment for all types of thyroid cancers include surgery, radioactive iodine, thyroid hormone supplementation, external beam radiation therapy, and chemotherapy.[6] The best suggested approach by the American Cancer Society is a combination of two or more of these methods. In most cases, surgery is followed by radioactive iodine treatment, with or without supplemental thyroxin. Chemotherapy has very limited use and is only used as palliative care.[7] Despite this combination approach, anaplastic

thyroid carcinoma responds very poorly and has uniformly dismal prognosis with median survival of 3 to 7 months.[8, 9]

This indicates that at this point there is no radical cure available for thyroid cancer, and multiple treatment combinations are tried in the hope of achieving maximum survival.[10] The standards of treatment, including surgery, radiation, chemotherapy, or even a combination of them, have not addressed the aspect of metastases, marking these approaches less effective at improving survival in the majority of cases.[11,12] Clearly there is a need for a new, safer, and more effective approach for thyroid cancer.

Previous studies have shown that proteolytic degradation of extracellular matrix (ECM) is the key factor in stromal invasion of the tumors and eventual metastasis. Matrix metalloproteinases (MMPs) are a group of calcium-dependent, zinc-containing endopeptidase enzymes that are responsible for the tissue remodeling and degradation of the extracellular matrix, including collagen and elastin. MMPs are excreted by a variety of connective tissue cells.[13,14] These MMPs participate in normal tissue remodeling, such as embryonic development, angiogenesis, and wound healing. Studies have shown that the aggressiveness of the cancer indicated by invasiveness, grade, and stage, is highly correlated with the expression of MMPs.[15] Though several proteolytic enzymes are postulated to play a role in this process, MMPs, especially MMP-2 and MMP-9, are identified as the most important.[16]

Rath and Pauling postulated that nutrients such as lysine and ascorbic acid (Vitamin C) could act as natural inhibitors of ECM proteolysis and, therefore, by stabilizing the connective tissue, have the potential to modulate tumor growth and metastasis.[17] These nutrients can utilize their antitumor potential through several mechanisms, including the inhibition of MMPs, as well as strengthening of connective tissue surrounding cancer cells, which is also known as "Tumor encapsulating effect". Our previous studies have also confirmed this approach.[18, 19]

In our current study, we have investigated the antitumor potential of an in vitro Nutrient Mixture (NM) on thyroid cancer in the SW 579 cell line by measuring cell proliferation, modulation of MMP secretion, cancer cell invasive potential, and morphology. The Nutrient Mixture (NM) is a combination nutrients formulated to target the key physiological pathways in cancer progression and metastasis.

MATERIALS AND METHODS

2.1 Cell Culture

Human Anaplastic Thyroid Carcinoma Cell line SW 579, obtained from ATCC (American Type Culture Collection, Rockville, MD), was grown in Leibowitz medium with 10% feta bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL) in 24-well tissue culture plates (Costar, Cambridge, MA). Cells were incubated with 1 mL of medium at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO_2. At near confluence, the cells were treated with Nutrient Mixture (NM) dissolved in media at 0, 10, 50, 100, 500, and 1000 mg/mL. The plates were then returned to the incubator.

2.2 MTT Assay

Cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After incubating for 24 hours, the cells were washed with phosphate-buffered saline (PBS) and 500 ml of MTT (Sigma Catalog No. M-2128), 0.5 mg/mL media was added to each well. The plates were covered and returned to the 37°C incubator for 2 hours, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the well, the formazan product was dissolved in 1 mL DMSO (Dimethyl sulfoxide), and absorbance was measured at 570 nm in Bio Spec 1601 Shimadzu spectrometer. The OD_{570} of the DMSO solution in each well was considered to be proportional to the number of cells. The OD_{570} of the control (treatment without supplement) was considered to be 100%.

2.3 Gelatinase Zymography

MMP secretion in conditioned media was determined by gelatinase zymography. Gelatinase zymography was performed in 10% polyacrylamide precast Novex gel, sodium dodecyl sulphate (Invitrogen Corp.), in the presence of 0.1% gelatin under non-reducing conditions. Culture medium (20 ml) was loaded, and sodium dodecyl sulphate (SDS)-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with Tris-Glycerine SDS buffer as described by the manufacturer (Novex). Samples were not boiled before electrophoresis. After electrophoresis, the gels were washed with 5%_Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in the presence of 50 mM Tris-HCl, 5 mm $CaCl_2$, 5 mM $ZnCl_2$ at pH 7.5, stained with Coomasie Blue R 0.5% for 30 minutes, and destained. Protein standards were run concurrently, and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

2.4 Matrigel™ Invasion Studies

Invasion studies were conducted using Matrigel™ (Becton-Dickinson) inserts in 24-well plates. Suspended in medium, human thyroid cancer cells SW 579 were supplemented with nutrient, as specified in the design of the experiment and seeded on the insert in the well. Thus, both the medium on the insert and in the well contained the same

supplements. The plates with the inserts were then incubated in a culture incubator equilibrated with 95% air and 5% CO_2 for 24 hours. After incubation, the media from the wells were withdrawn. The cells on the upper surface of the inserts were gently scrubbed away with cotton swabs. The cells that had penetrated the Matrigel™ membrane and had migrated onto the lower surface of the Matrigel™ were stained with hematoxylin and eosin and visually counted under the microscope.

2.5 Morphology

Morphology of cells cultured for 24 hours in the test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy.

2.6 Composition of Nutrient Mixture (NM)

Stock solution of the Nutrient Mixture (NM) prepared for testing was composed of the following: Vitamin C (as ascorbic acid, and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetylcysteine 200 mg; standardized green tea extract 1000 mg (green tea extract was derived from green tea leaves obtained from US Pharma Lab.)

The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate [EGCG] 35%, and caffeine 1.0%, selenium 30 mg, copper 2 mg, and manganese 1 mg.

The Nutrient Mixture (NM) was formulated based on targeting different physiological processes involved in cancer progression and metastasis. For example, the ECM integrity is dependant upon adequate collagen formation and its stability. In this aspect, ascorbic acid and the amino acids lysine and proline are necessary for the formation and optimum structure of collagen fibers. Manganese and copper are also essential cofactors in the collagen formation process. Collagen stability can be controlled by lysine[17] and also by N-acetylcysteine through its inhibitory effect on MMP-9 activity[20] and invasive activities of tumor cells.[21, 22] Selenium has also been shown to interfere with MMP expression and tumor invasion,[23] as has migration of endothelial cells through ECM.[21] Ascorbic acid has been shown to inhibit cell division and growth through production of hydrogen peroxide.[24] Green tea extract has shown to be a promising agent in controlling angiogenesis, metastasis, and other aspects of cancer progression.[25,26] Because arginine is a precursor of nitric oxide (NO), any deficiency of arginine can limit the production of NO, which has been shown to act predominantly as an inducer of apoptosis, as in breast cancer cells.[26]

Based on the evidence available in literature and our own research, we have postulated that metabolic effects of a combination of ascorbic acid, lysine, proline, green tea extract, arginine, N-acetylcysteine, selenium, copper, and manganese would result from their synergy. For example, we found that a combination of ascorbic acid, lysine, and proline used with EGCG enhanced the anti-invasive activity of 20 mg/mL EGCG to that of 50 mg/mL.[27] Thus, by including N-acetylcysteine, arginine, selenium, manganese, and copper with ascorbic acid, proline, lysine, and EGCG, we could obtain significant reduction in cell invasion at a much lower concentration of EGCG or other components. Also, the combined effects of these individual nutrients on decreasing proliferation of neoplastic cells were superior to the effects of their individual components or when they were randomly combined.[28]

2.7 Statistical Analysis

The results were expressed as means ± SD (Standard Deviation) for the groups. Data was analyzed by independent sample "t" test.

RESULTS

3.1 Cell Proliferation Study

The Nutrient Mixture (NM) had no significant toxic effect on human thyroid carcinoma SW 579 cell proliferation as shown in Fig.1.

3.2 Gelatinase Zymography Study

Gelatinase Zymography study shows only one band corresponding to MMP-2. MMP-9 is not secreted by SW 579 cell line. The expression of MMP-2 was decreased with increasing concentration of NM as shown in Fig. 2A. Densitometry analysis as seen in Fig. 2B shows that with the increasing concentrations of NM from 10, 50, 100, 500, and 1000 mg/ml, the expression of MMP-2 decreased from 107, 97, 85, and 20% respectively to almost complete inhibition at 1000 mg/ml, with R^2 value being 0.8007.

3.3 Invasion studies

The Nutrient Mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel™ in a dose dependant fashion, as seen in Fig. 4, with 42% inhibition at 50 mg/mL, 63% inhibition at 100 mg/mL, and 100% at 500 mg/mL (P=0.0002) NM, as shown in Fig. 3.

3.4 Morphology study (H&E staining)

NM showed no effect on morphology of thyroid cancer cell line SW 579 up to 100mg/ml. However, significant changes were seen at higher doses, from 500 to 1,000 mg/mL, as seen is in Fig. 5-A through 5-F.

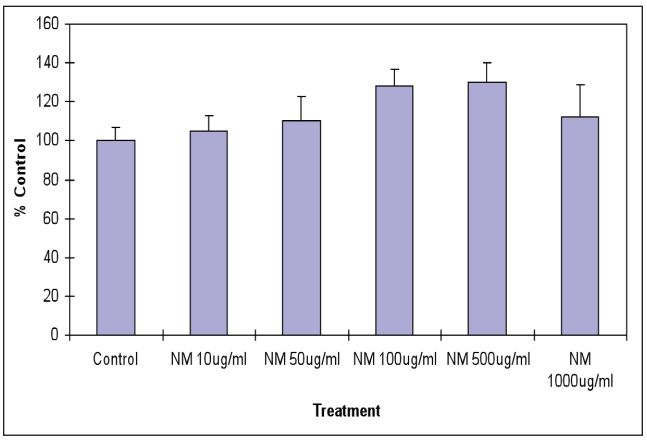
DISCUSSION

In the current study, we analyzed the effects of NM on anaplastic thyroid cancer cell line SW 579. The results indicate that NM is effective in inhibition of Matrigel™ invasion, in a dose dependant manner, by thyroid cancer cells SW 579. In addition, we also noticed that NM decreases MMP-2 secretion by thyroid cancer cells in a dose depen-

dant fashion. NM did not seem to affect the morphology of cells SW 579 below 500 mg/mL. The results obtained with this thyroid cancer cell line corroborate with our previous data evaluating NM effects on other cancer cell lines[29,30,31] proving that the inhibitory actions of the Nutrient Mixture (NM) on MMP secretion and Matrigel™ invasion are similar to most other cancer cell lines.

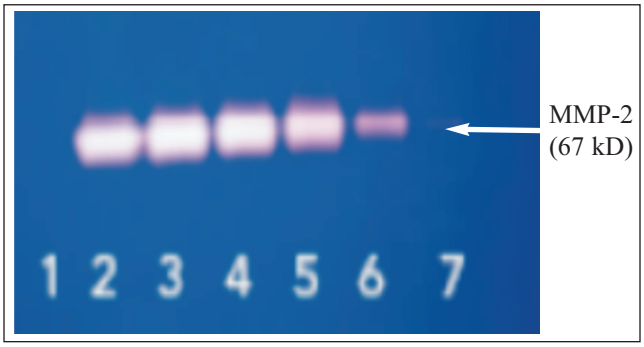
Anaplastic thyroid carcinomas constitute 1 to 5% of all thyroid cancers and are an extremely malignant tumor of the elderly population. Long-standing and well-differentiated thyroid cancers, such as papillary and follicular types, frequently coexist with the diagnosis of anaplastic thyroid cancer in 23 to 90% of the cases, and are also thought to be a predisposing factor for development of anaplastic thyroid carcinoma.[32] Prolonged goiter with Thyroid Stimulating Hormone (TSH) treatment may be responsible for such changes.[33] Anaplastic thyroid carcinoma is assumed to be one of the most destructive of all human malignancies. Fatalities attributed to this cancer are due to local and distant metastasis. Local invasion in the trachea and esophagus is as dangerous as distant metastasis in lungs, bones, and brain. Therefore, controlling this process of metastatic is the topmost priority. Many studies have indicated the importance of MMP enzymes in tissue remodeling and tumor progression through extracellular matrix (ECM) degradation. Of all MMP types, MMP-2 and MMP-9 are indicated to cause the highest level of collagen IV destruction. Collagen is the main component of cellular basement membrane and therefore plays a critical role in tumor progression and invasion. Researchers have shown, especially in papillary thyroid cancer, that plasma Vascular Endothelial Growth Factor (VEGF) and MMP-9 are significantly increased and are very good indicators of its metastatic potential.[16] It has also been proven that MMP-2 and its tissue inhibitors play a vital role in the pathogenesis

Figure 1. Effect of NM on thyroid cancer cells SW 579 cell proliferation: MTT Assay 24 hours.



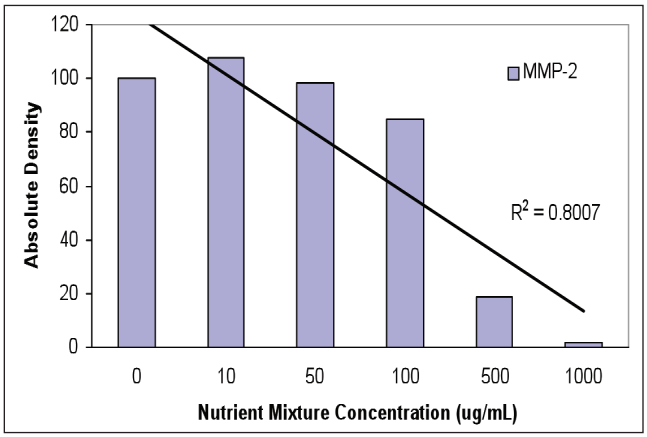
The nutrient mixture (NM) had no significant toxic effect on human thyroid carcinoma SW 579 cell proliferation.

Figure 2. Effect of NM on MMP secretion of thyroid cancer cell line SW 579: 2A- (Legend: 1- Markers, 2- Control, 3-7 NM 10, 50, 100, 500, 1000 µg/ml).



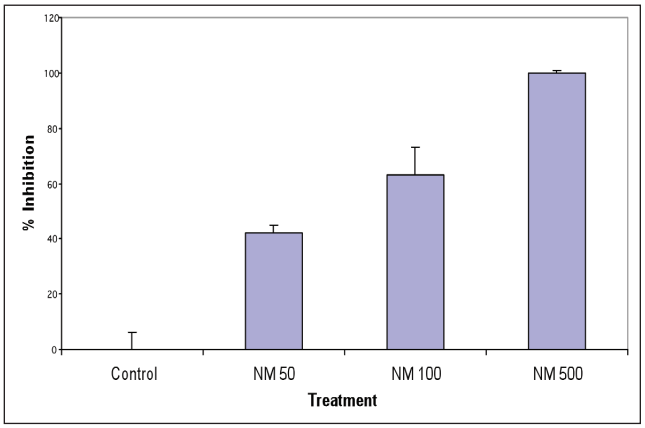
Cells were cultured, challenged with different concentrations of NM and condition media was applied for Zymography.

Figure 2B. Densitometry Analysis: Effect of NM on relative activity of MMP-2 in human thyroid cancer cells SW 579.



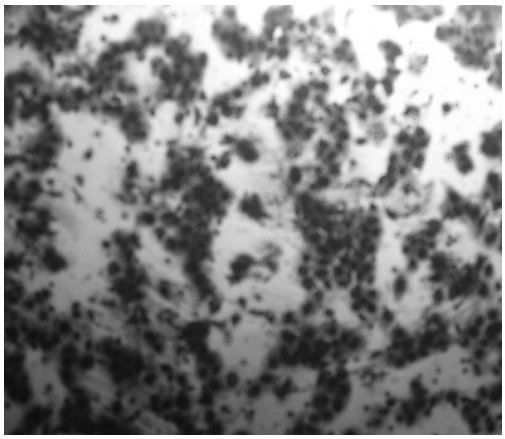
The expression of MMP-2 is decreasing with increasing concentration of NM.

Figure 3. Effect of NM on Matrigel Invasion of thyroid cancer cells SW 579.

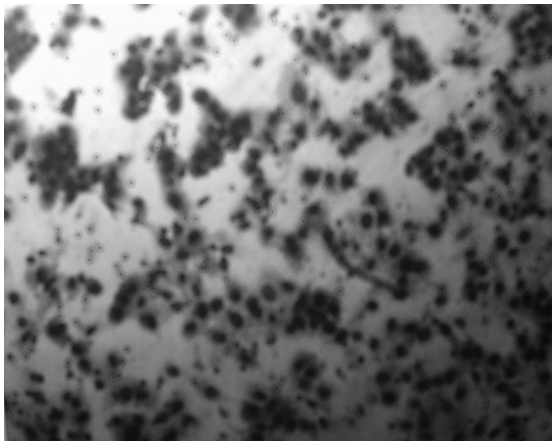


The nutrient mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel in a dose dependant fashion.

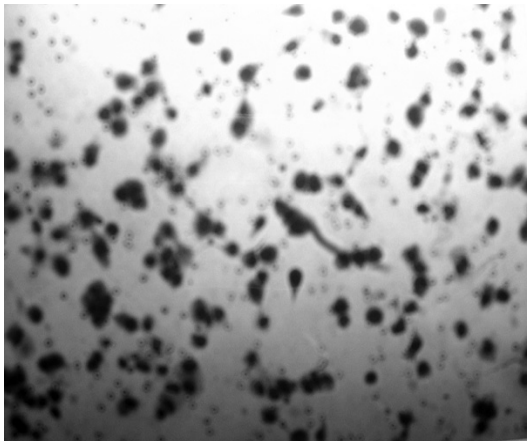
Figure 4. Thyroid Cancer cells SW 579 Invasion Photomicrographs.



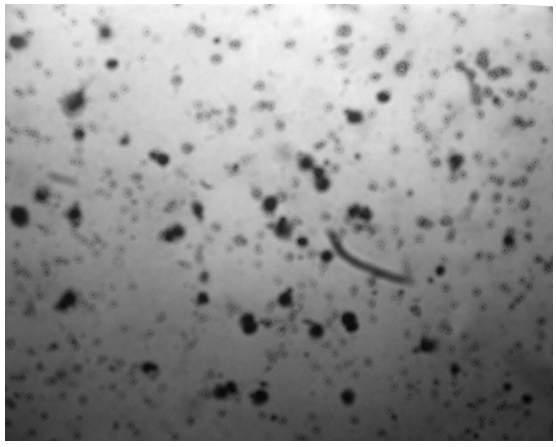
4A - Control



4B - NM 50µg/ml



4C - NM 100µg/ml

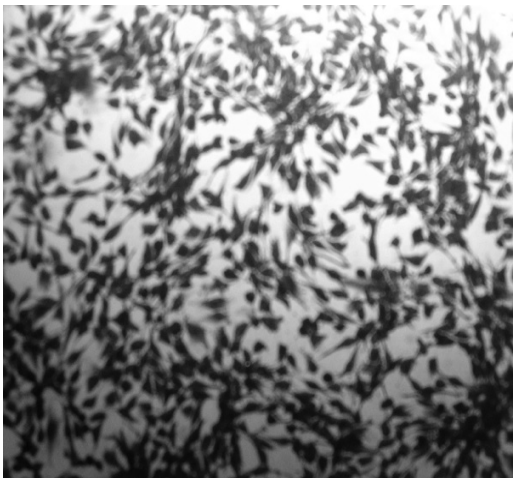


4D - NM 500µg/ml

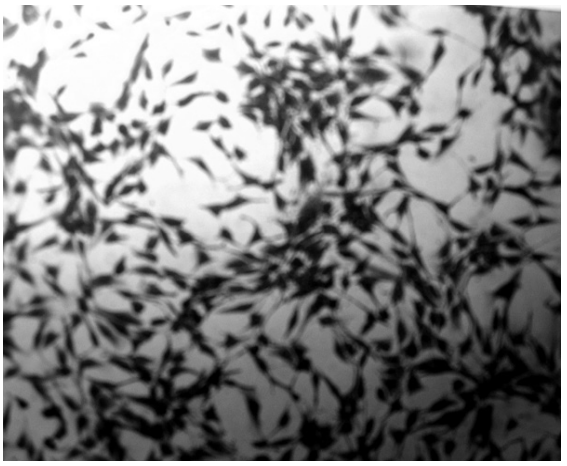
4E - NM 1000µg/ml

The nutrient mixture (NM) significantly reduced the invasion of thyroid carcinoma cells through Matrigel in a dose dependant fashion.

Figure 5. Thyroid Cancer cell SW 579 cell Morphology Photomicrographs (H&E Staining).



5A - Control

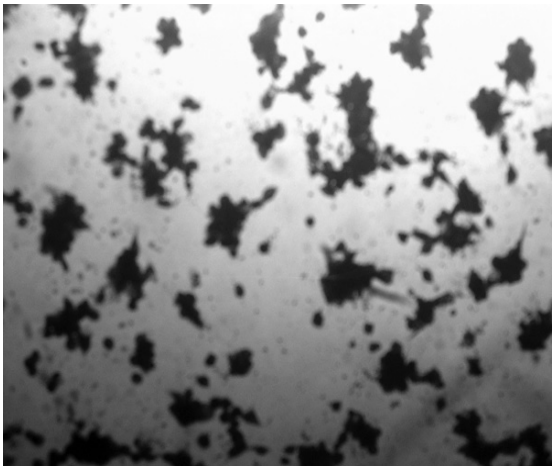


4B - NM 10µg/ml

5C - NM 50µg/ml

5D - NM 100µg/ml

5E - NM 500µg/ml



5F - NM 1000µg/ml

NM was not toxic to thyroid cancer cells below 500 µg/ml

of thyroid cancer.[34] Higher expression of both MMP-2 and MMP-9 is significantly correlated with larger tumor size, lymph node metastasis, higher clinical stage, and increased potential for intra-thyroidal and vascular invasion.[53] Increased MMP-2 secretion can also be used as a diagnostic marker to differentiate papillary versus other thyroid neoplasms. Therefore, it can be inferred that MMP enzymes are critical in the mechanism of invasion, angiogenesis, and metastasis of thyroid cancers.[36]

Control of proteolytic activity in ECM provides an opportunity to address common mechanisms of metastasis, angiogenesis, and tumor growth. It has been postulated that MMP inhibitors could be effective antitumor agents for the treatment of aggressive thyroid carcinomas.[37,38] Considerable evidence has accumulated regarding development of synthetic and specific MMP blockers as medications.[39] However, it seems that one of the most promising approaches to cancer would be targeting universal pathomechanisms involved in cancer growth and metastasis, such as encapsulating the tumor, strengthening of connective tissue, preventing angiogenesis, and blocking MMP activity. Rath and Pauling have suggested that lysine and lysine analogues are effective blockers of MMP-2 and MMP-9, and that they also strengthen the surrounding connective tissue to prevent matrix invasion and thus contribute to the encapsulation of the tumor.[17] Extracellular matrix integrity is dependant upon adequate collagen formation and stability, which is supported by lysine along with vitamin C and proline. Optimization of synthesis and structure of collagen fibrils depend on hydroxylation of proline and lysine residues in collagen fibrils. Ascorbic acid is essential for this hydroxylation and for regulating the collagen synthesis at a transcription level. Furthermore, lysine prevents cell migration by preventing collagen-digesting enzymes from binding to plasminogen-active sites, thereby blocking the activation of plasmin by plasminogen.[17] However, suboptimal levels of ascorbic acid and lysine are possible in various pathological stages and in deficient diets as these nutrients are not produced in the human body.

In this study, the dose dependant reduction of MMP-2, and also the significant reduction of Matrigel™ invasion by thyroid cancer cells SW 579, indicate that NM effectively blocks MMP-2 enzymes and supports collagen formation, thus preventing metastasis. This is especially significant in light of the fact that, at present, there is little or no cure available for thyroid cancer, and prolonging survival of the patient is the only goal of treatment combinations. While the death toll from thyroid cancer continues to mount every year, physicians are advised to combine two or more best-suited approaches for their patients.[16,40,41] Surgery is the most common choice of treatment for thyroid cancers; it does not, however, address metastasized cancers.[42] In addition to the inherent risks involved in any major operation, thyroid surgeries carry the additional risk of injury to the laryngeal nerve, leaving the person with permanent hoarseness of voice. Accidental removal of the parathyroid gland during surgery could lead to imbalances in calcium metabolism, with serious implications. In most cases, chemotherapy is used as a palliative measure. Although well-differentiated types, such as papillary and follicular, are the most common thyroid cancers, they have a high recurrence rate and are predisposing factors for anaplastic cancer. Even the most targeted radiotherapy indiscriminately attacks surrounding healthy cells as well as cancer, causing considerable cellular and ECM damage. In contrast to that, NM does not affect normal cells, even at high concentrations, indicating the safety of the combination. Cancer mortality results mainly from the tumor invading local tissues and metastasizing to vital organs, such as liver, lungs, and brain. As mentioned previously, degradation of ECM is hallmark of these events and is mediated by MMPs. Therefore, safe and effective inhibition of MMPs is the target of treatment.

CONCLUSIONS

While additional animal studies and clinical trials are required, the results of our study suggest that this formulation of Nutrient Mixture is a very good candidate for therapeutic evaluation in thyroid cancer as it inhibits MMP secretion and invasion of cancer cells, assuring safety for normal cells.

ACKNOWLEDGEMENTS:

Julio Monterrey performed thyroid zymograph scans and densitometry analysis and calculations. Dr. Rath's Research Institute provided funding for this research.

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ORIGINAL RESEARCH

Fears of Genetic Discrimination with Nutrigenetic Testing Can Be Diminished Through Professional Education in Genetic Testing

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ABSTRACT

Purpose: Genetic screening for disease susceptibility is becoming increasingly more common, but studies show that primary care physicians fear that this form of testing poses certain risks for genetic discrimination. A survey of first and fourth trimester chiropractic students was performed to determine if education in genetic testing can reduce the fear of genetic discrimination.

Methods: First trimester chiropractic students (N=66) enrolled in a clinical biochemistry laboratory, attended a new two-hour lecture on the theoretical constructs of genetic testing and performed a genetic fingerprinting lab exercise. A survey was later given to the first trimester class asking them to give consent to having the results of their genetic test entered into their personnel file in the registrar's office. A separate survey asking the same question was also given to a fourth trimester class (N=33). The comparison of attitudes between the first and fourth trimester classes is unique because the fourth trimester class had neither the formal lecture on genetic testing nor the opportunity to perform the genetic fingerprinting lab.

Results: Overall, 6.1% of the first trimester students (4 of 66) denied consent to having their results entered into their personnel file, while 84.8% of the fourth trimester students (28 of 33) denied consent ($p<.001$). With respect to subjective comments, 53% of the students who denied consent commented on issues regarding privacy of their genetic information, while 25% commented on their fears of genetic discrimination and concerns over the future use and abuse of their stored genetic material.

Conclusion: As expected, a significant majority of the students in the fourth trimester class, with no formal education in genetic testing, expressed concerns over confidentiality and genetic discrimination. To reduce fears of genetic discrimination, which could otherwise have a negative impact on the utilization of genetic testing for public health benefits, this investigation suggests that education in genetic testing is necessary.

Key Indexing Terms: genetic testing; genetic discrimination; primary care; education

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INTRODUCTION

The completion of the Human Genome Project has ushered in a new era of personalized health care and prevention by evaluating individual genetic susceptibility to disease.[1,2] As a result of the expansion of genomics into human health applications, an increasing number of gene tests are becoming commercially available. Genetic tests for approximately 1,500 disease related genes are currently available or in research development.[3] Although most available tests are for rare diseases, tests to identify inherited risk for common diseases such as breast and colorectal cancer, diabetes, coronary heart disease, rheumatoid arthri-

tis, asthma and Alzheimer's disease have also been developed. These tests will play an important role in the diagnosis, monitoring and treatment of diseases. More importantly, genetic testing could encourage high-risk individuals to reduce their harmful environmental exposures, to increase their surveillance for early disease or to consider preventative therapy via nutritional supplementation.[4]

Unfortunately, according to the conclusions based on studies by Freedman et al. and Hayflick et al., a majority of primary care physicians do not feel qualified to provide genetic counseling or to recommend genetic testing for their patients.[5,6] Recent studies by Suther and Goodson and Burke et al. also suggest that the underutilization of genetic services by primary care physicians is due to a lack of knowledge, low levels of confidence and lack of adequate up-to-date genetics information.[7,8] Although primary care physicians perceived genetics as an important and increasingly relevant topic in primary care, Watson et al. found that primary care physicians expressed a lack of genetic knowledge and referral skills as a barrier to providing genetic services.[9] Based upon the conclusions from these various studies, it has been suggested that clinical genetics training must be given more attention in both undergraduate and postgraduate education.[7]

Primary care physicians have also expressed concerns regarding the potential for genetic discrimination as an adverse consequence of genetic testing.[10] They envision that many ethical dilemmas will arise as a consequence of genetic screening, especially those relating to informed consent, confidentiality and possible "genetic discrimination" against patients.[11] More than half of the physicians surveyed by Freedman et al. reported that it is difficult to ensure that a patient's genetic test results will remain confidential, and the majority believed that patients with a positive test result would be at risk for insurance discrimination.[6]

It has been stated that clinical genetics training for both physicians and other healthcare professionals must be given more attention in undergraduate education.[12] Although such training will inform physicians about the utilization of genomic medicine, the question still remains as to whether or not such formal education can deal with their concerns over the issues of genetic discrimination. To address this very important issue in genomic medicine, a survey of first and fourth trimester chiropractic students was performed in order to determine if educating students about genetic testing can reduce their fears of genetic discrimination.

METHODS

Two separate classes of chiropractic students were presented with a one-question survey. The two groups included 66 first trimester students enrolled in a clinical biochemistry course and 33 fourth trimester students enrolled in a medical genetics course. The two groups of students were unique because the students in the first trimester clinical biochemistry class were being presented with clinical genetics material that they had never been taught before. The new material included a 2-hour lecture on the theory and practicality of Polymerase Chain Reactions (PCR) and its utilization in genetic screening for disease. This lecture was then followed by a 2- hour laboratory exercise, at which time the students obtained a genetic fingerprint of their own D1S80 anomalous gene loci using PCR. The genetic material was obtained through the use of a cheek swab. It is probably important to stress that what makes this particular investigation unique, is that the new material had never been presented or discussed with the current fourth trimester students in any previous trimester.

On the week following, a survey was presented to both classes consisting of a single question, which could be answered by circling either yes or no. If a student circled "no" he or she was then asked to write a short statement as to why. The survey question presented to the two classes differed based on the didactic difference between the two classes.

The question presented to the first trimester class read:

The DNA sample provided as part of your lab will be analyzed and your genetic fingerprint will be entered into your personnel file in the Register's Office. In order to comply with privacy issues, we will require your consent. Please indicate your consent by circling the appropriate response and signing this document in the space provided.

The question presented to the fourth trimester class read:

We wish to obtain a sample of your DNA (via cheek swab) to obtain a genetic fingerprint, which will be entered into your personnel file in the Register's Office. Prior to participating in this data collection, we require your consent. Please indicate your consent by circling the appropriate response and signing this document in the space provided.

The data from the surveys were entered into an Excel spread sheet, which was designed to exclude the personal identities of the students. The data from the "yes or no" question was tabulated and compared statistically using a chi square. The students' statements explaining the reasoning for declining to have their genetic fingerprinting entered into their personnel file was categorized by the author based on the emergent themes of the responses. The frequency of the various responses within each of the themes was tabulated, and upon tabulating the students' responses, the descriptions from both the first and fourth trimester classes were combined.

RESULTS

The first trimester class was comprised of 66 chiropractic students (50% female) with an average age of 25.0 years. The fourth trimester class was comprised of 33 chiro-

practic students (54% female) with an average age of 25.2 years. Overall, 6.1% of the first trimester students (4 of 66) denied consent to having their results entered into their personnel file, while 84.8% of the fourth trimester students (28 of 33) denied consent. The percentage of first trimester students who denied consent was significantly smaller (χ^2=62.43, p<.001) when compared to the fourth trimester class.

The 32 combined first and fourth trimester students' statements explaining the reasoning for declining to have their genetic fingerprinting entered into their personnel file was categorized and the frequency of the various responses within each of the themes is provided in Table 1. Note that an individual statement could fit into more than one category.

Of the 32 chiropractic students who denied consent to provide a genetic fingerprint, 17 stated reasons pertaining to privacy issues. Some students stated outright that this was a violation of their right to privacy, while other students who stated concerns over privacy expressed less extreme views. One student simply stated: *"I personally do not think the school needs to know about my genes,"* while a second student wrote: *"I would not want this information known by anyone."* This last statement expressed a common theme shared by a number of students regarding the possible release of this information beyond the intended registrars' office. The privacy concerns over the release of their genetic information can be expressed by three separate students who wrote: *"I do not want the world to know what my genes hold," "What else will be done with this information?"* and *"What will it be used for now and in the future?"*

The chiropractic students' concern regarding the privacy and accessibility of their genetic information by outside organizations lends itself to another commonly expressed theme, that of confidentiality. Seven of the 32 students expressed direct concerns over confidentiality and the protection of their genetic information stored in their personnel files. One student expressed fears over confidentiality by asking: *"How do you know that this information will not slip into someone else's hands?*. Another student wrote: *"Would my DNA profile be used or sold to other companies?"* The opinions about confidentiality were well summarized by one student who said: *"I do not believe that this information can maintain its secrecy."* Concerns over accessibility of a student's genetic profile are well warranted, as a lack of confidentiality could result in the abuse of this information, ultimately leading to some form of discrimination. This sentiment is adequately expressed by a student who states: *"I do not want my DNA available on record because I do not know what it contains, and if there is something in there that could lead to discrimination I do not want anyone to have access to it."*

Eight of the 32 chiropractic students voiced concerns regarding the potential abuse of their genetic information, and eight students also stated that they feared their genetic information could be used against them resulting in some form of genetic discrimination. One student expressed this concern by stating: *"This information seems harmless initially, but this world has many individuals, whom, if this type of information gets in their possession, they may cause problems and troubles that I and others would not want to deal with."* Two other students were more explicit in expressing their fears, with the first student asking: *"Would my DNA possibly exclude me from being a student?"* while the second wrote: *"I have enough problems getting health insurance, I do not need the insurance company to have any other reasons to deny me."* Some students possessed a more proactive attitude on discrimination as observed by one student who stated: *"I wish to retain privacy and control over the results of any genetic information that may be used for insurance or other purposes."*

Table 1. Reasons given for the 32 students who declined having their genetic fingerprint entered into their personnel file.

Students' Statement Categories	Number	% of 32
Stated that genetic make up was private matter.	17	53
Questioned why such an exercise was performed.	16	50
Concerns over the future use and abuse of their stored genetic material.	8	25
Fear of genetic discrimination.	8	25
Concerns over confidentiality regarding potential uses of the obtained genetic material.	7	22
There was a lack of informed consent.	7	22
Genetic test would not provide any beneficial personal outcome.	6	19
I would rather live in a state of "blissful ignorance" regarding my genetic make up.	5	16
Would rather use other forms of personal identification.	3	9
Denied testing on the grounds of religious beliefs.	2	6
Campus security is not an issue and therefore would deny being tested.	2	6

DISCUSSION

In this study, a question asking if they would agree to have a small segment of their genetic code placed into their personnel file, was posed to both a first and fourth trimester chiropractic class. Such a question is not so farfetched as the State of Illinois has legislated that all students entering allopathic, osteopathic and chiropractic schools in 2006 provide a conventional fingerprint and have a criminal background check.[13] As expected, a significant majority (84.8%) of the students in the fourth trimester class, with no formal education in genetic testing, expressed concerns over confidentiality and genetic discrimination. These findings are not too different from Geetter who reported that 80% of surveyed respondents expressed concern that insurance companies would be able to access genetic information.[14] Also, these results are similar to those of Hall & Rich and Matloff et al., who both cited that genetic discrimination had been a major deterrent for physicians performing genetic testing.[15, 16] However, contrary to these findings, it is believed that the fear of genetic discrimination is far out of proportion with the amount of discrimination actually experienced.[17,18] Finally, although the fear of genetic discrimination and lack of confidentiality has been shown to be high in primary care physicians, it is even more so with physicians who lack formal education in genetics testing.[9]

To reduce fears of genetic discrimination, which could otherwise have a negative impact on the utilization of genetic testing for public health benefits, this investigation suggests that education in genetic testing is necessary for health practitioners. This finding supports previous studies, which observed that primary care physicians who felt well qualified to recommend testing were twice as likely to have ordered genetic testing, compared with those who felt unqualified.[19] Accurate knowledge about genetic testing may help physicians make informed decisions about the risks and benefits, and may reduce barriers to such testing. Francis Collins, director of the National Human Genome Research Institute, expressed an urgent need for formal education in genetic testing by stating: "Every attempt needs to be made to reform current medical school curricula so that the next generation of physicians will be ready to appropriately use genetic technologies, and to address associated ethical, legal, psychological and social implications."[20] To facilitate this need, genetic specialists, primary care educators, government and professional societies have recently joined to plan and disseminate several professional and public genetic education initiatives. For example, the Genetics in Primary Care (GPC) project was developed with the goal of enhancing the training of medical students and primary care residents.[21] Furthermore, the National Coalition for Health Professional Education in Genetics (NCHPEG) has developed guidelines for genetic education and defined core competencies in genetics for healthcare providers.[22]

A small population of students who are educated in genetics may still go on to exhibit concerns regarding genetic discrimination and lack of confidentiality, as was observed in 4 of the 66 students within the first trimester class. Primary care physicians educated in genetics could still feel very suspicious of such information being collected, fearing that abuse and discrimination is always a possibility. These concerns were illustrated by one of the 4 first trimester students who noted: "*The speed at which everyone in this world is being catalogued, tagged and stored scares me and I don't want to be part of it.*"

In regard to the outcome of this study, caution should be exercised since the design of this one- question survey lends itself to a number of biases. For example, the fourth trimester class may have been put into a position to deny giving up their genetic information since they were in effect "taken by storm" with this question; whereas students in the first trimester class had one week to adjust to the issues prior to being asked to give up their genetic information.

Many primary care physicians believe genetic applications in primary care will be most fruitful in the field of disease prevention, since individuals with higher genetic risk will have greater lead time for making behavioral changes that could prevent and decrease the severity of specific diseases.[11] As genetic testing for multi-factorial diseases becomes ever more clinically applicable, the increasing number of patients seeking this testing will require that primary care physicians become more involved in this form of healthcare delivery. It is therefore reasonable to assume that the demands on primary care physicians will increase substantially once they are required to provide information on genetic tests to their patients. Francis Collins sums this up by stating, "This 'next revolution in medicine' will fall on the shoulders of physicians who provide primary care."[23] Hence, the findings from this qualitative investigation highlights the need to implement clinical genetics training into the undergraduate education of physicians in order to minimize concerns and promote the utilization of genetic testing as a public health tool.

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