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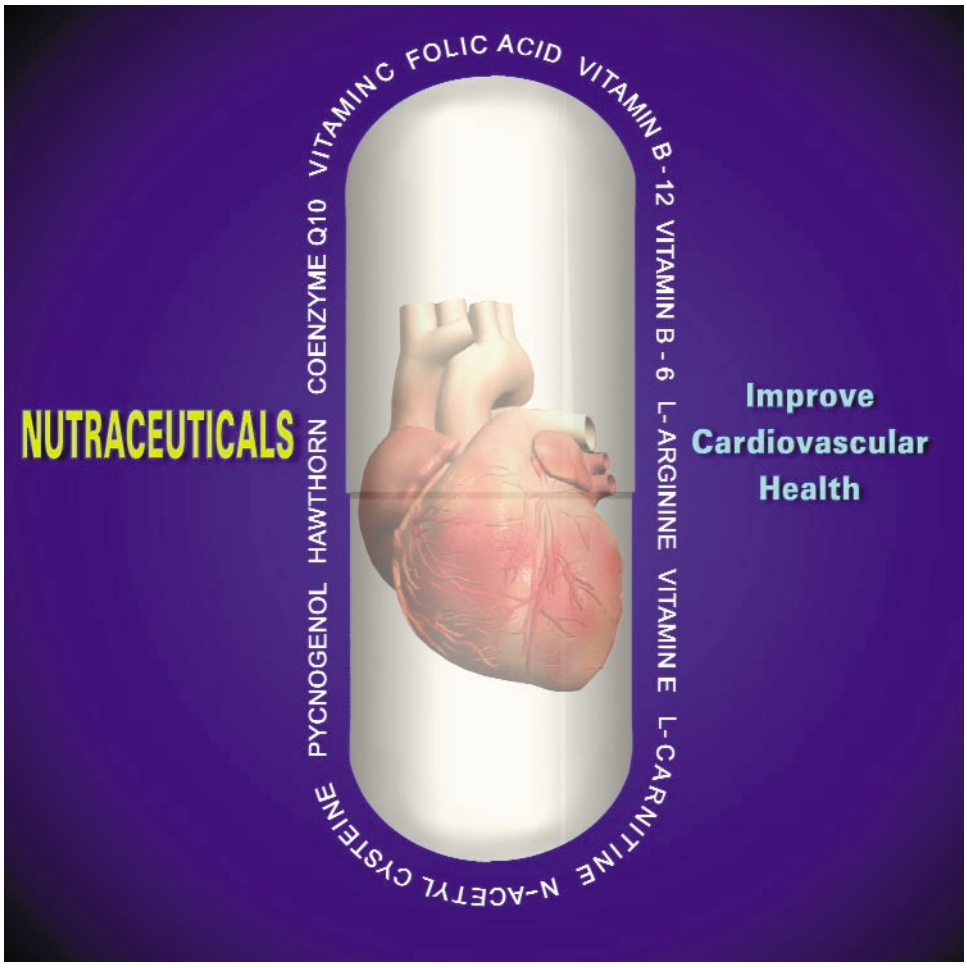

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In This Edition

A Forum for Wellness and Optimal Health

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Journal of the American Nutraceutical Association

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ANA is an alliance of individuals with interest in nutraceutical science, technology, marketing and production. It was established to develop and provide educational materials and continuing education programs for health care professionals on nutraceutical technology and science. ANA publishes a quarterly newsletter, *The Grapevine,* and the *Journal of the American Nutraceutical Association* (*JANA*).

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To order reprints of articles, or additional copies of *JANA*, write to or call:
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We in health care often find it difficult to connect the dots of disease, dysfunction, and gene expression, with the simple tools that can affect outcomes. In this and the fall issues of *JANA*, we will connect some of those dots and make sense out of seemingly unrelated tools.

As we forge ahead into the "genomic century," there is a rising public interest in preventing disease rather than passively allowing it to occur before rescuing ourselves at great cost and loss of autonomy. Nutritional science is teaching us that there are many upstream tools that can assist us to manage functions such as methylation capacity (homocysteine and B vitamins), gut ecology (pre-and probiotics), endocrine messaging (natural hormone replacement), endothelial function (arginine), and novel antioxidants for cellular stress. We have not done nearly enough to help our patients manage their own biology to maintain health rather than attempt to recover it.

Dr. Gitte Jensen works in the new frontier of gut-associated immune functions and the profound ways in which the bowel's ecology influences immune functions far removed from the gut lumen. Dr. Khem Shahani has worked for years to characterize the optimal management of gut flora and gives us a look into the intricacies of species-specific probiotics. While these tools are rarely used in clinical practice, they will be in the future. I encourage you to read this issue's articles by Dr. Jensen and Dr. Shahani.

We spend billions of dollars fixing advanced vascular disease, and manage cholesterol levels only when it comes to prevention. Diet and exercise mantras have been heard for years and are indeed very important. Dr. Kilmer McCully makes a strong case for the need to supplement *nutraceutically* in the significant number of people who have reduced methylating capacity. This variable gene expression places the endothelium at risk for complex oxidative events that may begin the atherogenic process. While cholesterol is important, we are taught here to think symphonically rather than in single notes.

The release of nitric oxide at the endothelial level is one member of the vascular orchestra that appears to go quiet as we age. Many individuals lose the ability to make this all-important mediator of endothelial capacitance as a result of complex deficiencies in gene expression and environmental stresses. Oral intake of a nutraceutical (L-Arginine) may be of tremendous benefit, as Dr. Jack Kleid indicates in his review article.

Oxidative stress appears to be one of the most problematic forces at work in controlling risk for disease and aging. It is becoming increasingly evident that mitochondrial gene expression is most affected when reactive oxidative byproducts are modulated poorly. Simply adding large amounts of vitamin E or C will likely not be adequate for a comprehensive approach as we learn about individual vulnerabilities. New and novel antioxidant compounds abound in foods, and we are just beginning to characterize them. Some may be beneficial as nutraceuticals. An article by Dr. Ram Nimmagudda and colleagues provides useful insights into a class of such compounds.

The results of a clinical study that evaluated the use of a nutraceutical supplement in patients undergoing chemotherapy is presented by Dr. Jerome B. Block and colleagues in this issue of JANA. The results of this pilot study are promising and indicate that some toxic side effects associated with chemotherapy are reduced when patients receive a nutritional supplement prior to and during their chemotherapy regime.

And what happens when we apply these nutraceuticals to patients who are being treated for cancer? A very hot question these days—one that can divide a medical community—involves the propriety of adding helpful compounds that protect cells from the effects of chemo and radiation therapy. What are the risks for treatment failure? How far can we push "well-being" at the expense of killing cancer cells? Dr. Russell Blaylock addresses these difficult issues in an important review article to be published in the fall edition of *JANA*.

Finally, if your practice mirrors my own, not a week goes by without several new patient consultations for natural hormone replacement. Once the complexity of hormone adjustment and replacement is explained, most decide to have an individualized topical mixture compounded for them. In the fall edition of *JANA*, Dr. Deborah Moskowitz provides one of the best reviews I have seen on the subject, one that will guide us in the art and science of helping our patients manage their changing endocrine messengers in a risk-free fashion.

Simple tools that address individuals' needs to optimally and safely manage their own, unique genotypes in order to avert disease endpoints. It's what nutraceuticals are all about.

Chris Foley, MD
Co-editor

Statement by Stephen E. Straus, M.D. Director, National Center for Complementary and Alternative Medicine before the House Committee on Government Reform June 7, 2000

Mr. Chairman and Members of the Committee:

I appreciate the opportunity to appear before you today to address the application of research on complementary and alternative medicine (CAM) to cancer therapy, and the ways that the National Center for Complementary and Alternative Medicine (NCCAM) collaborates with the National Cancer Institute (NCI) to advance our common desire to improve public health.

My presence here today, and NCCAM's very existence, reflects the growing public interest in CAM. By some estimates 42 percent of Americans spent $27 billion on CAM therapies in 1997. In recognition of this growing consumer trend, Congress in 1998 elevated the NIH Office of Alternative Medicine (OAM), expanded its mandate, created the NCCAM, and afforded it administrative authority to design and manage its own research portfolio. The Congress has continued to reflect the growing interest in CAM by further increasing funding for the Center in FY 2000 to $68.4 million. The FY 2001 President's budget requests $72.4 million for NCCAM. We are indeed appreciative of this support.

NCCAM's Broader Mission

As CAM use by the American people has steadily increased, many have asked whether reports of success with these treatments are scientifically valid. A number of practices, once considered unorthodox, have proven safe and effective and been assimilated seamlessly into current medical practice. Practices such as meditation and support groups are now widely accepted as important allies in our fight against disease and disability.

In the absence of definitive evidence of effectiveness, however, some practices may impart untoward consequences. It is critical that untested but widely used CAM treatments be rigorously evaluated for safety and efficacy. Promising new approaches worthy of more intensive study must be identified. In addition, I am energized by this opportunity to help provide the American public the guidance it seeks.

NCCAM's strategy for taking on this challenge is somewhat different from that used by other NIH Institutes and Centers (ICs). While the research of other ICs is usually driven by basic scientific discoveries, NCCAM has chosen to focus most heavily on designing and carrying out definitive clinical trials of widely utilized modalities that, from evidence-based reviews, appear to be the most promising. We are mindful of the responsibility to do so in a manner consistent with established ethical standards and federal guidelines – so as to ensure patient safety and public confidence to the maximum possible extent.

Compelling and rigorous data and not just anecdotes must be provided to the public, and we must educate conventional medical practitioners about the panoply of effective CAM practices, so they can be integrated into medical practice, including cancer care.

NCCAM has developed a draft Strategic Plan – now available for public review and comment on our web site http://nccam.nih.gov/nccam/strategic/ – to ensure that our continued growth, development and research directions are consistent with the challenges set before us. Five strategic areas have been identified as: investing in research; training CAM investigators; expanding outreach; facilitating integration; and practicing responsible stewardship.

Concurrently, along with all other NIH ICs, we are developing a multifaceted effort to eliminate health disparities. Our health disparities plan will focus upon: identifying the extent and nature of CAM use among special populations; study of therapeutic interventions to reduce disparities; increasing participation of minority and under served populations in NCCAM-supported clinical trials; enhancing the ability of minority institutions to support CAM research.

The NCCAM is pleased to have recruited Dr. Morgan Jackson, most recently the director of the Minority Health Program at the Agency for Health Care Research and Quality, to finalize and help implement the plan.

It is to these ends, and in light of the breadth of CAM, that we have established close liaisons with all other NIH components and federal health agencies. Among these, our relationship with the NCI is paramount: my staff and I work closely and on an ongoing basis with the NCI. Early in my tenure as NCCAM Director, and a number of times since, I have met with Dr. Richard Klausner, NCI Director, to discuss prospective collaborations and matters of common interest. I also communicate frequently with Dr. Robert Wittes, who will testify here today. Moreover, our extramural program director and I meet monthly with Dr. Jeffrey White, who directs the NCI Office of Cancer Complementary and Alternative Medicine (OCCAM) and who is accompanying Dr. Wittes today.

St. John's Wort – An Example of NCCAM's Opportunities and Challenges

Already, NCCAM has developed a diverse research portfolio in partnership with the other NIH Institutes and Centers. Among these are some of the largest, and certainly the most definitive Phase III clinical trials ever undertaken for a range of CAM therapies. Allow me to highlight one of these studies.

Extracts of St. John's wort, a flowering plant, have become quite popular as a treatment for depression. In fact, by some accounts, St. John's wort is the number-one-selling nutritional supplement. Because of this intense interest, NCCAM, the National Institute of Mental Health (NIMH), and the NIH Office of Dietary Supplements (ODS) are collaborating on a study of the safety and effectiveness of St. John's wort in treating depression.

A recent report in *The British Medical Journal* showed that St. John's wort is more effective than placebo in treatment of depression, and perhaps as effective as an older generation anti-depressant drug Imipramine. NCCAM's larger and longer-term study compares St. John's wort with placebo and with Zoloft, currently one of the most commonly used anti-depressants.

The potential benefit of St. John's wort, however, comes with previously understudied, and therefore unappreciated risks. An NIH study published February 12th in *Lancet* found that St. John's wort, when taken together with the important HIV protease-inhibiting drug indinavir, increased the rate at which Indinavir was eliminated from the bloodstream, to the extent that blood levels fell below the desired level for effective AIDS treatment. More recent studies have suggested that St. John's wort has a similar effect on some types of birth control medication and on cyclosporin A, a drug used to prevent the rejection of transplanted organs. Other studies have shown that the use of St. John's wort may also increase an individual's sensitivity to exposure to the sun. These findings illustrate vividly both the promise and challenges presented by CAM therapies. Only through rigorous research on these CAM modalities will we be able to determine not only to what extent each is safe or effective, but under what circumstances an effective CAM modality may be contraindicated.

CAM and Cancer

The prospective application of CAM modalities to treat cancer is a major interest of the American public, as reflected in the over 2,000 inquiries which the NCCAM Clearinghouse receives each month. The committee's consideration of the subject today is especially timely, for the NCCAM is pleased to sponsor – along with the NCI, the University of Texas-Houston, and Dr. James Gordon's Center for Mind-Body Medicine – the Comprehensive Cancer Care 2000 conference beginning this week in Arlington, VA. I appreciate and concur with the goals articulated by conference organizers: to bring together "those who are conducting the most innovative research on CAM therapies for cancer...with the most distinguished mainstream oncologists to evaluate promising therapies and how they can be successfully integrated into comprehensive cancer care."

Simply put, CAM-Cancer research, and rigorous, scientific evaluation of CAM therapies for cancer, are among our highest priorities. With this in mind, we recently recruited Dr. Mary Ann Richardson to our extramural program staff. Dr. Richardson comes from the University of Texas – Houston School of Public Health and will direct our research portfolio and stimulate new initiatives in the area of oncology. She brings expertise and experience as director and principal investigator of our first exploratory research center focused solely on cancer and co-sponsored by NCI. In her new role in the NCCAM, Dr. Richardson is meeting today with NCI staff and the National Brain Cancer Foundation. I am confident that she will build upon her developmental and field work and extensive network of conventional and CAM practitioners to move the field forward on a national and international level.

In Fiscal Year 2000, the NCCAM plans to spend over $4 million in support of cancer research studies. This represents a three-fold increase in a single year. We expect to augment our support for cancer studies again in 2001.

Against that backdrop, I would like next to acquaint the committee with our activities involving the integration of CAM and cancer in particular. The portfolio, directed at CAM therapies appropriate to the treatment of cancer as well as its complications, encompasses both the study of cancer interventions and palliative care.

Specialty and Botanical Centers

Specialty Research Centers form an historical foundation for conducting CAM research through the NIH, and provide the setting for ongoing collaborative research. In this regard, our Centers assemble critical masses of basic and clinical scientists to conduct clinical studies into CAM approaches for a variety of health conditions. They also encourage CAM practitioners and researchers to conduct relevant joint research projects. Each focuses on one of several areas, including pediatrics, addiction, cardiovascular disease (CVD), minority aging and CVD, aging, neurological disorders, craniofacial health, arthritis, and chiropractic medicine. Average funding for our new Centers exceeds $1 million annually for five years. In addition, NCCAM supports three Botanical Research Centers in collaboration with the ODS, the National Institute on Environmental Health Sciences (NIEHS), the National Institute on General Medical Sciences (NIGMS), and the Office of Research on Women's Health (ORWH).

Currently, NCI and ODS have joined NCCAM in a solicitation for a new Center to focus on cancer related research issues. This center will focus on basic and clinical studies; Phase I and II clinical trials of botanicals; drug-botanical interactions; unconventional nutritional approaches and dietary supplements that either augment conventional cancer therapies or diminish side effects; and studies of the potential effect of mind-body modalities (e.g., relaxation, imagery, meditation, psychosocial support groups, and the like). I am pleased to report the receipt of a substantial number of applications that will be reviewed this summer, and from which we expect to make as many as two awards.

Various substances present in natural products, including botanicals, have been shown to inhibit cancer in animals. However, little information is available on what may account for their apparent anticarcinogenic effects. Even less is known about interactions among these substances and other dietary components. Research is also needed to provide better understanding of the potential impact of natural products on the treatment of precancerous conditions or early-stage cancerous lesions. Research that examines the potential use of such products for the treatment of conditions which may accompany or follow cancer (pain and loss of appetite, for instance) or side effects of conventional therapies (e.g. nausea, vomiting, and neuropathy) are obvious undertakings for new CAM Cancer Centers.

These Centers are only a part of our expanding research portfolio, which includes a rapidly increasing number of investigator-initiated grants, some of which I will briefly describe.

Studies of Cancer among Specific Populations

The NCCAM is already supporting studies of CAM therapies for cancers which predominantly affect women. According to the CDC, 175,000 women will be diagnosed with breast cancer this year; some 40 percent will die of the disease. A University of Texas study, conducted in collaboration with the National Institute on Nursing Research (NINR), introduces strategies of self-transcendence among support group members to improve well-being and immune function and to increase understanding of the relationship between survival rates and support group participation. Also, the NCCAM-funded Center for Alternative Medicine and Women's Health at Columbia University is supporting trials that evaluate the use of Traditional Chinese Medicine to treat uterine fibroids and breast cancer. At the same time, the Columbia University group is conducting evidence-based reviews of the literature regarding CAM approaches to the prevention and treatment of breast cancer.

Our cancer research portfolio also includes:

- *Studies of shark cartilage that are funded jointly by NCCAM and NCI*. These include an ongoing Phase III clinical trial involving as many as 500 lung cancer patients in both the United States and Canada. A second trial will examine safety and efficacy of shark cartilage in patients with a variety of advanced cancers.
- *Investigations of cancer prevention and treatment strategies*. Clinical trials at the University of Texas Center for Alternative Medicine Research are examining herbal, nutritional, mind-body, and biopharmacologic treatments for lymphoma, lung, and esophageal cancer.
- *Basic research studying the effects of magnetic fields on cancer cell growth*.

Controversial CAM Cancer Regimens

Many CAM approaches are controversial, particularly those used as strict alternatives to conventional regimens for treating life-threatening diseases such as cancer. Nonetheless, NCCAM will pursue rigorous investigations of any such therapy for which there is adequate preliminary data and a compelling public health need. Our commitment is illustrated by our support of a study of the therapy advocated by Dr. Nicholas Gonzalez, in which cancer patients are treated with dietary supplements including pancreatic enzymes, magnesium citrate, papaya plus, vitamins, minerals, trace elements, and animal glandular products, as well as with coffee enemas. There are very preliminary data sug-

gesting the therapy might be effective in prolonging life-expectancy for those individuals suffering from cancer of the pancreas. Given that conventional regimens for pancreatic cancer only moderately prolong life, from a public health standpoint there is sufficient argument to evaluate the Gonzalez protocol in a rigorous scientific fashion. For this reason, the NCCAM and NCI are funding a substantive pilot trial in 90 patients with pancreatic cancer according to Dr. Gonzalez's protocol, at the Columbia-Presbyterian Cancer Center in New York City.

Steps to Expedite Our Research

I am also pleased to report that our National Advisory Council on Complementary and Alternative Medicine (NACCAM) recently approved our proposal to provide supplementary funds to existing NCI Cancer Centers to initiate new CAM research studies. NCI staff are currently considering our offer. This program will encourage communication and collaboration between CAM practitioners and outstanding conventional cancer researchers. Emphasis will be placed, where possible, on the study of minority and under-served populations. Preliminary data from this research will serve as the basis for subsequent, more definitive clinical trials. To be sure, some of the CAM interventions now used to treat cancer will not be validated in those trials, and just as likely some will emerge as important, adjunctive and alternative therapies.

The NCCAM and NCI are also embarking jointly upon a creative, new research grant mechanism – Quick-Trials for Novel Cancer Therapies – designed to simplify the grant application process and provide a rapid turnaround from application to funding. Its features include accelerated peer review, with the goal of issuing new awards within five months of application receipt. Initially announced for a pilot program in prostate cancer, the Quick Trial mechanism provides rapid access to support for pilot, phase I, and phase II cancer clinical trials testing new agents, as well as patient monitoring and laboratory studies to ensure timely development of new treatments.

The NCCAM has also announced our intent to establish the Frontier Medicine Program. This initiative will promote collaborations between conventional and CAM institutions, practitioners, and researchers to study promising and widely used CAM practices – including cancer therapies – that appear to produce benefits but for which there is no plausible explanation or existing scientific support.

CAPCAM

The federally-chartered Cancer Advisory Panel for Complementary and Alternative Medicine (CAPCAM) frames NCCAM's cancer-related activities broadly – and our collaborations with the NCI in particular. Its membership includes CAM practitioners and health care professionals from conventional medicine. CAPCAM represents a unique approach to enabling identification of promising CAM cancer treatments for which scant scientific data are currently available. It is intended to help move into the research stream those practices worthy of scientific study.

CAPCAM advises the NCCAM Director on the assessment of present and future cancer clinical trials and medical interventions, potential research opportunities, and means of communicating research results to key constituencies. The panel affords CAM practitioners world-wide the opportunity to submit retrospective analyses of data of patients treated with a specific modality in order to assess possible therapeutic benefit. This is formally known as the Best Case Series (BCS). The Panel will recommend selected BCS cancer treatments to the NCCAM for further study as appropriate.

The NCI developed the BCS Program in 1991 because most alternative treatments had not been formally evaluated in prospective studies. The CAPCAM process and its predecessor, the Cancer Advisory Panel (CAP) were an outgrowth of the Practice Outcomes Monitoring and Evaluation System (POMES), developed jointly by the former NIH Office of Alternative Medicine (OAM) and NCI. I have already met with the CAPCAM twice, and will next meet in September. Already its members have recommended additional study of a specific dietary supplement as a treatment for non-small cell lung cancer, and further exploration of homeopathic cancer treatments, provided by the PB Homeopathic Research Foundation, Calcutta, India. Moreover, the CAPCAM recently advertised widely in journals and targeted materials its desire to receive best case submissions. We anticipate two additional best case reviews for the next meeting of CAPCAM in September, 2000.

NCCAM's Palliative Care Research

Whether palliative care involves conventional or complementary approaches, its purpose is to add scientifically verified evidence to our base of knowledge about appropriate and compassionate health care. Many of our current studies truly represent palliative care research as they focus on increasing patient comfort, diminishing pain, and rendering disease symptoms less intense or severe. Although some studies do not expressly focus upon cancer patients, research results may be beneficial to them, or others who may be near the end of life.

Our palliative care projects include an examination of the benefits of hatha yoga on cognitive and behavioral changes associated with aging and neurological disorders; evaluation of the effects of acupuncture on persistent pain and inflammation; the aforementioned study of St. John's wort and its effects on major depression; and the effect of acupuncture and moxibustion (heat applied at the acupuncture point).

Palliative care for cancer patients will also be an obvious

interest of our evolving NIH Intramural Research Program. The Director of our program will interact closely with the newly appointed Director for palliative and pain care of the NIH Clinical Center, Dr. Ann Berger, who arrives this summer from the Fox Chase Cancer Center in Philadelphia.

I also want to briefly mention NCCAM's interest and support of the study of certain mind-body research modalities. Although CAM and mind-body medicine only partially overlap, NCCAM is pursuing investigations involving still undocumented CAM techniques; modalities for which there is little evidence in the conventional medical research community; and unorthodox uses for otherwise conventionally-accepted mind-body techniques. In this context, the NCCAM looks forward to evaluating the effectiveness of selected mind-body approaches in cancer treatment. We currently support one such project – a study examining whether self-transcendence strategies affects immune function, well being and survival rates among breast cancer patients.

I note parenthetically that one key aspect of mind-body research involves studies of the "placebo effect." In November, NCCAM, in collaboration with NIDDK, NCI, and other ICs, will convene a major trans-NIH conference on this subject. Goals of the conference include providing a scholarly assessment of the state of the field; identifying areas for which there is scant research, but considerable opportunity; and recommending a formal research agenda to move the field forward, in particular projects to be pursued by interested ICs through individual or joint initiatives with NCCAM. Elucidating the nature of the placebo effect will help us better harness the healing power of the mind.

Integrative Medicine Research Training, and Communications

Medicine is an ever-evolving discipline. It integrates or rejects approaches based on scientific evidence. The results of rigorous research in CAM, including studies of its efficacy in treating cancer, and the disease's many complications will enhance the successful integration of safe and effective modalities into mainstream medical practice. We have initiated a series of specific activities to facilitate this. In particular, NCCAM recently solicited applications to incorporate CAM information, including that which relates to cancer care, into model curricula of medical and allied health schools and continuing medical education programs through Education Project grant awards.

Also, the NCCAM must educate eager students about CAM so that they may knowledgeably guide their future patients toward safe and effective CAM applications. In addition, we must work to overcome the reluctance of conventional physicians to consider validated CAM therapies and to assimilate proven ones into their practice. With this in mind, we established a Clinical Research Curriculum Award (CRCA) to attract talented individuals to CAM research and to provide them with the critical skills that are needed. NCCAM also plans to solicit applications for applied research on identifying barriers to the use of CAM modalities by conventional physicians, including oncologists; strategies to incorporate validated CAM interventions into standard medical practice; and evaluating the effects of this incorporation.

Integrative medicine (of which the field known as "integrative oncology" is a subset) is also a key aspect of NCCAM's planned Intramural Research Program and a component of NCCAM's Specialized Research Centers. Research training is conducted by these Centers, in part to advance our goals in integrative medicine, but also to assist us in building a cadre of skilled CAM investigators. Some of NCCAM's Centers spend as much as ten percent of their budget on training.

Public Outreach and Collaboration with NCI

Specific statutory authority enables NCCAM to reach out directly to the public and practitioners to provide them with critical and valid information regarding the safety and effectiveness of CAM therapies, including cancer. This information dissemination involves extensive and ongoing interaction with NCI.

A focal point for information about NCCAM programs and research findings is the NCCAM Information Clearinghouse, which develops and disseminates information that reflects the state of the science of various CAM modalities. To this end, NCCAM and NCI have undertaken a collaboration to develop – within the coming year – as many as 10 fact sheets which discuss CAM use as therapy for specific cancers.

Assembled by NCCAM from the National Library of Medicine's (NLM) MEDLINE database, the CAM Citation Index (CCI) affords the public access to approximately 175,000 bibliographic citations from the NLM Medline. The CCI is searchable by CAM system, disease, or method. For most types of cancer, the CCI contains many references to alternative medicine research published in the medical literature. Users can access the CCI on the NCCAM Web site at: http://nccam.nih.gov/nccam/resources/cam-ci.

In February 1999, NCCAM joined the federally supported Combined Health Information Database (CHID), which includes a variety of health information materials, including nearly 1,000 CAM citations not available elsewhere. The CAM subfile of CHID contains extensive information on therapies for cancer.

The NCCAM Information Clearinghouse receives more than 250 cancer related inquiries from the public per month. The Clearinghouse identifies the NCI as the Federal Government's lead agency for cancer research and training, and routinely directs consumers and practitioners to the following NCI resources:

• Information specific to CAM, including CAM clinical trials and studies, found in CancerNet on the NCI Web site at: http://cancernet.nci.nih.gov/treatment/cam.shtml

• The information sheet, "Complementary and Alternative Medicine: Treatment Options"

• The NCI Web site: http://www.nci.nih.gov

• The Cancer Information Service at (800) 422-6237

• NCI Public Inquiries Office

• NCI Office of Cancer Complementary and Alternative Medicine (OCCAM)

The NCCAM Web site has already been linked to the new Cancer CAM web site just launched by the OCCAM. It provides the NCI and NCCAM with an interface with the general public, health practitioner and research communities regarding CAM cancer issues. Among other things, the new NCI site states that it is "designed specifically for people with cancer and the people who care about them." I applaud this valuable contribution by the NCI to enhancing public knowledge of CAM and cancer care.

CONCLUSION

In closing, I would like to share with the Committee my vision of where I expect complementary and alternative medicine to be in the years to come. NCCAM's leadership will stimulate both the conventional and CAM communities to conduct compelling and open-minded scientific research. Several therapeutic and preventative modalities currently deemed elements of CAM will prove effective. Based on rigorous evidence, these interventions will be integrated into conventional medical education and practice, and the term "complementary and alternative medicine" will be superseded by the concept of "integrative medicine." The field of integrative medicine will be seen as providing novel insights and tools for human health, and not as a source of tension that insinuates itself between and among practitioners of the healing arts and their patients. Modalities found to be unsafe or ineffective will be rejected readily by a well-informed public.

My vision is an optimistic one. However, I am confident that the NCCAM, building on a foundation of superb science and consumer service, and collaborating with such outstanding partners as the National Cancer Institute, will be a world leader – not only in complementary and alternative medicine as a whole, but in addressing the painful and tragic disease of cancer that touches the lives of every American family.

Vitamins When Combined with Anti-oxidants Reduce Risk of Heart Disease, Stroke According to New Study

The benefits of taking a daily multivitamin in combination with one of the antioxidant vitamins A, C or E, were confirmed in a study published in the July edition of *the American Journal of Epidemiology* (2000;152:149-162). The report confirmed that the greatest benefit is in the reduction of risk of dying from heart disease and stroke. However, this vitamin regimen may also increase the risk of dying from cancer in male smokers, the authors report.

The findings "warrant corroboration," according to Margaret L. Watkins and colleagues from the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia. They call for further studies to examine the role that vitamins and vitamin combinations may play in dying from heart disease, cancer and stroke—the three leading causes of death in the US.

In the study, which included more than 1 million adults aged 30 and older, researchers compared death rates of those who used multivitamins alone; vitamin A, C or E alone; or a multivitamin with vitamin A, C or E; with death rates of people who did not take vitamins, over a 7-year period.

The investigators found that adults who took a multivitamin with an antioxidant vitamin had a 15% lower risk of dying from heart disease and stroke than people who did not take vitamins. Risk appeared to fall with time as individuals took the vitamin combination.

However, there was no survival advantage among people who took a multivitamin alone. "One explanation is that there may be a minimum dose of a single vitamin or combination of supplements necessary for risk reduction," the researchers suggest.

In other findings, the risk of death from cancer was the same among vitamin users and non-users. Use of multivitamins alone or with other vitamins seemed to increase the risk of dying from all cancers, compared with using vitamin A, C or E alone, the report indicates.

Male smokers who used multivitamins alone or in combination with other vitamins had a higher risk of dying from cancer than nonsmoking males who took vitamins. What's more, men who smoked and took vitamins had a higher risk of dying from prostate cancer than male smokers who did not take vitamins.

These findings support previous research demonstrating that high doses of beta-carotene can increase the risk of dying from lung cancer in male smokers. The authors suggest that the use of vitamin A, C or E may counterbalance the observed elevated cancer mortality risk among men who used multivitamins.

They also note that adults who took vitamins tended to be more educated and less overweight than those who did not. Vitamin-takers were also more likely to eat vegetables and drink wine or liquor.

SOURCE: *American Journal of Epidemiology* 2000;152:149-162.

White House Commission on Complementary and Alternative Medicine Policy: A Historic Opportunity

Candace Campbell*
Executive Director, American Preventive Medical Association

These are exciting times in health care! Complementary and alternative medicine (CAM) has moved out of the shadows and onto center stage, eliciting wounded screams along the way from many of the conventional folks who are being forced to accommodate that change. In the process, the complaints about inadequate science, lack of regulation, and questionable education standards are prodding the emerging CAM industry to do some serious and much-needed self-evaluation. As a result, standards are rising and debates have begun on the topic of professional credentialing and good manufacturing standards.

Now, because of the dramatic increase in CAM usage by the American public and the increasing furor over integration, the Clinton administration has agreed that the time has come to investigate what the federal government is doing, or should be doing, to ensure safe, appropriate, and accessible CAM delivery. Through the same legislation that created the National Center for Complementary and Alternative Medicine (NCCAM) at the National Institutes of Health two years ago, a White House Commission on CAM Policy was also established. Recently empanelled with 13 health care professionals, its ranks will soon swell to 20. The Commission is in charge of reporting to the President on legislative and administrative recommendations for assuring that public policy maximizes the benefits of CAM for Americans. Under the microscope are the education and training of CAM health care practitioners; the coordination of research on CAM practices and products; the provision of reliable and useful information to practitioners and patients; and appropriate access to and delivery of CAM.

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www.apma.net

"Within five to ten years, complementary therapies will be a part of the care in every major hospital and clinic across the country, and our definition of medicine will be far larger than it is today," said Dr. James Gordon, chairman of the commission and director of the Center for Mind-Body Medicine. "The questions are not 'if', or even 'when', this will happen. The questions that must be answered, and the ones the commission will address, are how can we find out which of these therapies are truly effective; how can clinicians be trained in using them and the public educated about them; and how will those therapies that do prove effective be safely integrated into a truly comprehensive and humane care for all Americans."

The commission could be the proverbial double-edged sword. On one hand, a thorough, unbiased report that reflects the range of policy changes necessary to achieve a more integrated health care system would benefit all Americans. It would illuminate for Congress and the agencies the areas that need attention, the regulations that need amendment, and the roadblocks to access and research created by stagnant or biased policy. This will take courage on the part of both commission members and those identified as agents of change. On the other hand, a negative report that discourages change and supports the status quo could significantly damage any hopes for real health care integration in the foreseeable future.

As with all research, the answers will depend on how the questions are asked. Through a series of town hall meetings around the country, the commission will be gathering the information needed to prepare its report to the President, which is due by March 2002. All of these meetings, as well as commission meetings, will include time for public comment. In addition, written comments may be submitted for consideration by the members. In the near future, the commission will also have a web site where visitors can read the minutes of all meetings.

The original 13 members seem excited about the task at hand and willing to press for answers. The breadth of their experience, from natural products manufacturing to insurance coverage, from oriental medicine to oncology, bodes well for a balanced perspective to the investigation. While several major CAM professions are not yet represented, that shortcoming may be addressed through the final seven appointments.

If the final report is to reflect the needs and concerns of the CAM community, however, it will be incumbent upon consumers and professionals alike to provide as much information as possible to the commission, and the caliber of that information will be paramount. As Peter Reinecke, legislative director for US Senator Harkin noted at the first commission meeting, how the final report is perceived depends significantly on the degree to which the commission succeeds in gathering public comment. How it is used to promote future change will also be dependent on the response of the CAM community. Will it be yet another blue ribbon panel report that gathers dust soon after being released? Will it adequately reflect the policy changes necessary to complete a paradigm shift? Will it be embraced by the CAM community and used to press Congress and the next Administration to address the changes recommended by the commission? We will know soon enough. Only one thing is certain: this commission's investigation into federal CAM policy presents an historic opportunity to affect dramatic changes in the country's health care environment. If the CAM community does not aggressively participate in the education of the commission, it will surely be disappointed in the results.

Readers interested in more information about the Commission can contact executive director Stephen C. Groft, PharmD, or executive secretary Michele M. Chang, MPH, at 301-435-7592.

Candace Campbell is executive director of the American Preventive Medical Association, a nonprofit advocacy organization dedicated to increasing Americans' health care freedom through legislative and regulatory changes at the federal level. APMA led the effort to establish NCCAM and the White House Commission.

BOOK REVIEW

The Ghen and Rains Physicians' Guide to Pharmaceutical Compounding
Authors: Mitchell J. Ghen, DO, PhD, and John R. Rains, RPh, FACA

IMPAKT COMMUNICATIONS, 2000, 430 PAGES, $54.95

This one-of-a-kind book is a must for pharmacists and physicians concerned with the personalized compounding of medications. It provides practical information about pharmaceutical compounding and converges the paths of alternative and conventional medicine into one single meeting place. Dr. Ghen's approach to medicine transports the reader back to a time when disease and patient were treated simultaneously. Of particular interest are the sections identified as Personal Experience where patients share personal reflections of their illness and its treatments. This focus highlights the importance of psychotherapy as well as pharmacotherapy and points to the necessity of patient-specific treatment plans.

The book includes an extensive listing of specific formulations and monographs, and discusses the effective use of flavors, sweeteners, textures, and colors. Safety and effectiveness are addressed, with chapters dedicated to preservatives, sterilization, and stability. Dosage form discussions vary from the preparation of vaginal suppositories to that of pediatric lollipops.

The physician is also provided with a list of criteria used to evaluate compounding services. Many pharmacists perceive simple "mixing" as "compounding"; this book helps to distinguish between the two. The authors also review equipment needed to successfully compound medicines.

Chapters on hormone replacement, pain management, rehabilitation, herbal products, nutraceuticals, homeopathic treatments, and hospital compounding are clinically relevant and uniquely realistic as they relate to the clinical practice of the year 2000 (intranasal insulin is discussed). Each section includes a compounder's advantage tip that describes the most appropriate method to complete a particular task.

This well-written book is a welcome addition to a physician or pharmacist's collection as it reinforces the importance of patient-specific medicine amidst a revolution of standardization and managed care.

Reviewers; Vince Dolce, PharmD Candidate, Lisa Colodny, PharmD BCNSP, Broward General Medical Center Drug Information Center, Fort Lauderdale, Florida

ORIGINAL RESEARCH

Results of a Study to Evaluate the Use of Propax™ to Reduce Adverse Effects of Chemotherapy

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ABSTRACT

Objective: To assess the effectiveness of the dietary supplement Propax™ with NT Factor™ in reducing chemotherapy-induced fatigue, nausea and vomiting, and other selected clinical side effects associated with chemotherapy.

Methods: Using a crossover placebo-controlled, randomized, double-blinded design, 36 patients with cancer were enrolled in the 12-week pilot study. Simultaneously, an open-label trial of Propax™ in 22 other patients with cancer was similarly implemented. Recommended daily dose of the study product was 12 tablets and 3 softgel capsules daily.

Results: The consumption of the recommended daily dosage of the dietary supplement Propax™ with NT Factor™ resulted in an improvement or no change or worsening in chemotherapy-related side effects of fatigue, nausea, impaired taste, diarrhea, general tiredness, constipation, and insomnia. Other chemotherapy-induced toxicities (mouth sores, skin changes, and decreased appetite) were not similarly benefited and progressively became more severe throughout the progression of the study. These results were assessed by standard Quality of Life (QOL) questionnaires completed by patients and nurses in the offices of oncologists participating in the study.

No reported adverse drug events considered to be severe were reported in the study, and when present most were related to mild non-specific gastrointestinal discomfort.

CONCLUSION

Fatigue is one of the most common complaints in cancer patients. The results of this pilot study, both open-label and double-blinded placebo-crossover in design, indicate that patient perception of benefit with Propax™ supplementation to chemotherapy is significant in reducing fatigue and other chemotherapy-induced toxicities. As chemotherapy toxicity is expected to worsen or progress with continued therapy, such improvement or lack of worsening of side effects is an important outcome. The results from the blinded study were very similar to the results from the unblinded study both with regard to patient documentation of their symptoms, and nurses' assessments.

SUMMARY

Propax™ with NT Factor™ supplementation to standard chemotherapy regimens had beneficial impact on several quality of life parameters with a high degree of patient acceptance of the supplementation regimen that was also confirmed by nurse observations.

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INTRODUCTION

Cancer patients frequently encounter numerous adverse reactions associated with the administration of chemotherapy. Fatigue is one of the most common complaints.[1-5] Though common, it is not well understood, and there are numerous theories regarding its severity and prevalence during a patient's daily routine.[6] While over 75% of cancer patients reported that fatigue adversely affected their lives, only 32% of oncologists recognized this symptom in their patients.[6] Of more importance, both patients and physicians reported fatigue to be a more prominent adverse event than pain. This is especially interesting given that 74% of patients believed fatigue was untreatable and must simply be endured.[6] Depending on the chemotherapeutic regimen chosen, the degree of fatigue reported by patients varies widely, from mild[2] to severe.[4] It is not surprising then, that Buckingham et al. reported fatigue as the most common and troublesome side effect in ovarian cancer patients who received carboplatin for treatment.[7] Similarly, fatigue was reported as a significant toxicity in 33% of patients treated with docetaxel for pancreatic cancer.[1] Cancer cachexia, a paraneoplastic syndrome due often to cytokine liberation, is also associated with increasing fatigue.[8]

The difference among cancer types that affect the level of fatigue in patients with cancer has not been formally recognized. However, based on the results of a quality of life survey in 1997, Pater et al. concluded that those patients with metastatic disease and those with poor performance status were more likely to experience fatigue, while older patients and female patients with breast cancer reported less fatigue, as did those whose gastrointestinal responses were controlled by antiemetics; patients with ovarian and lung cancer experienced greater degrees of fatigue.[9] Modulating fatigue is the level of anemia, commonly present as a result of chronic disease, and secondary to bleeding due to cancer and/or chemotherapy and secondary to radiation injury to the bone marrow.

Nausea and vomiting are also common complaints of oncology patients who receive chemotherapeutic agents.[10-15] Depending on the regimen, the degree of nausea and emesis reported may vary, ranging from mild with minimal emetogenic regimens to severe with substantial emetogenic regimens. Although significant progress has been made in developing more effective means of preventing nausea and vomiting induced by chemotherapy, incomplete or uncontrolled emesis remains a problem for a significant percentage of cancer patients.[10,16]

Chemotherapy-induced vomiting may actually be classified into acute and delayed phases. The acute phase includes emesis up to 24 hours after chemotherapy is completed; the delayed phase is emesis anytime thereafter.[11] While newer agents such as the 5H3 receptor antagonists (ondanesetron, granisetron, and dolasetron) are very effective in controlling emesis during the acute phase, they are less effective during the delayed phase.[11]

The mechanism of chemotherapy-induced nausea and vomiting is not completely understood. However, interaction between chemotherapeutic agents and the nausea trigger zone has been implicated as a probable cause.[17] Other proposed mechanisms may be related to tumor metabolism itself, the metabolic response of the body to cytokine release, as well as to surgery, chemotherapy, or radiation.[18]

In addition, several risk factors may predispose a patient to chemotherapy-induced nausea and vomiting. Risk factors include being female, being between the ages of 6 and 50 years, and being someone who drinks little or no alcohol.[20] Regardless of etiology, chemotherapy-induced nausea and vomiting remain the most feared side effects of many chemotherapy regimens.[12] All chemotherapy-related toxicities directly and indirectly affect the nutritional health of the oncology patient. As a consequence, malnutrition is a common complication that significantly affects both quality of life and survival.[21]

Nutritional improvement for the oncology patient may inhibit deterioration, improve nutritional and immunologial parameters, and may help avoid complications.[21] The protective effects of fat-soluble and other natural antioxidants are well known.[8] These antioxidant defenses are important in determining immune cell integrity and the functionality of membrane lipids, cellular proteins, and nucleic acids. Additionally, antioxidants are believed to control signal transduction and gene expression in immune cells.[9] There are several stages where antioxidants may control the progression and malignancy of disease. Antioxidants may also provide protection even when cancer-infected viral activity is present.[18] Dietary introduction of these nutrients may stimulate host immunological defenses and damage malignant cells directly by cycling with consequent oxygen radical production.

The unique dietary supplement, Propax™ with NT Factor™ (Chart A) was developed to address the nutritional concerns of oncology patients. The formulation is a nutrient tablet base supported by a broad range of vitamins, minerals and micronutrients. NT Factor™ is a proprietary nutrient complex designed to maintain normal cell function. In an animal study conducted on NT Factor™ at the Henry Ford Health System, it was shown that rats fed a diet containing NT Factor™ showed a 20% improvement in mitochondria function over those animals fed the identical diet without NT Factor™, as measured by the Rhodmine flow cytometry.[19] NT Factor™ is comprised of growth factors and specific foods of bifido and lactal species bacterium to promote and maintain a healthy gut and support nutrient absorption. It also contains a specific fraction of phosphoglycolipids extracted from soy that provides an exogenous supply of polyunsaturated phosphatidyl-

choline(PPC). PPC may function importantly in the repair and maintenance of the cell membrane, a fundamental requirement for normal cell function.

The properties of Propax™ and NT Factor™ may be of benefit in treating the fatigue and malaise commonly seen in patients with immunosuppressive diseases. This is supported by the recent work of Lilleby et al. who focused on the importance of overall well-being of patients. In fact, quality of life issues such as physical and emotional function and fatigue were of greater significance to the patient than other issues like sexuality or probability of infection.[27] Lovely and colleagues concluded similar results by reporting an inverse relationship between quality of life and fatigue.[28]

Regardless of underlying causes of the nutritional imbalance commonly observed in oncology patients, impact on a patient's quality of life and survival has been extensively analyzed.[18,29] Celaya et al. reported that the cancer itself might negatively affect nutrition through tumor metabolism and metabolic responses of the body to cytokine release.[21] The nutritional status of the patient may already be impaired long before the onset of radiation or chemotherapy.[30] Therefore, effective nutritional support may be beneficial in this group of patients reflected in enhanced wound healing, augmented visceral function, and improved cellular immunity.[31] This is supported by Chuntrasakul et al. who reported significant improvement in nutritional and immunologic parameters in immunocompromised patients who received supplementation with arginine, glutamine, and omega-fatty acids.[32] Similarly, Henquin concluded that the health of patients with poor nutritional status during chemotherapy deteriorated, while patients with good nutritional profiles maintained good clinical status.[33] Therefore, prevention or reduction of nutritional deficiencies by adequate therapies may contribute to a reduction in morbidity and mortality in this population.[34]

Given the negative repercussions of nausea and vomiting, fatigue, compromised nutritional status, and other chemotherapy-related toxicities, agents that decrease these presentations by maintaining normal cell lines on an adequate and fully functioning level will improve the quality of life for the cancer patient. Towards that end this pilot study of the nutraceutical supplement Propax™ with NT Factor™ was launched to investigate its potential efficacious effects in chemotherapy-induced fatigue, nausea and vomiting, and other clinical toxic side effects.

OBJECTIVE

The primary objective of this pilot study, both open-label and double-blinded, was to assess the effectiveness of Propax™ with NT Factor™, administered before and during a 12-week regimen of standard chemotherapy for advanced cancer, on the symptoms and side effects of drug toxicity. Efficacy was evaluated via standard instruments that measure quality of life. The secondary objective was to compare the results between a placebo-controlled blinded study and the open-label study to evaluate if the study results are impacted by the trial design.

DATA COLLECTION

Data was collected for both the blinded and open-label studies by a designated nurse in each oncologist's office.

As patients entered the study, nurses established baseline information about their symptoms and perceptions of quality of life (QOL) issues via a Nurse Review Questionnaire. The same questions were asked and answers recorded at the time of each chemotherapy administration.

Patients completed a Patient Wellness Questionnaire when they first entered the study, and were asked to complete this QOL questionnaire each week during the study. The patients were given stamped, self-addressed envelopes to return the questionnaires to the physician's office, or they could return them in person on a weekly basis.

STUDY DESIGN

The unblinded study was an open-label application of Propax™, Nutritional Therapeutics, Inc., Hauppague, New York. Patients were placed on nutritional supplementation 5 to 7 days prior to chemotherapy treatments and continued throughout the first 3 months of chemotherapy treatment. The unblinded study obtained Human Use Committee approval and each patient completed an informed consent form prior to entry into the study.

The blinded study was a double-blind, placebo-crossover trial also requiring Human Use Committee approval and informed consent for patient entry. Patients were randomized to either a placebo or supplement 5 to 7 days prior to the initiation of chemotherapy. After 6 weeks of chemotherapy, patients were then crossed over to the other product (ie, placebo to Propax™, or Propax™ to placebo). Questionnaires were then evaluated based on improvement or worsening of symptoms from chemotherapy-induced toxicities including fatigue and other QOL issues.

STUDY POPULATION

Patients for both studies were selected from outpatient chemotherapy centers in California, Florida, Maine, Massachusetts, New Jersey, and New York, for patients with colon, rectal, or pancreatic cancers with identical 5-FU/Leukovorin regimens. Patients with advanced unresectable non-small cell or small cell lung cancer not receiving radiation were also eligible as long as carboplatin and etoposide were used for treatment. The initial study protocol was amended to include patients with sarcoma, breast, ovarian, or other cancers as long as a 3-month survival period was anticipated.

INCLUSION / EXCLUSION CRITERIA

Patients were excluded from both the blinded and open-label study when they were less than 21 years of age, women of childbearing age, mentally incompetent, or renally impaired (SCr > 2.0 mg/dL). Patients were also excluded when serum bilirubin was 2.0 mg/dl or greater, when weight loss greater than 15% of normal body weight had occurred within the last 4 months, or when the patient spent more than 50% of waking time in bed. All patients concurrently consuming vitamin or nutritional supplements were also excluded.

Patients were eligible for study entry with pathologically-confirmed diagnosis of cancer, appropriate informed consent, and absence of requirement for radiation treatment. No patients in the blinded or unblinded study received radiation therapy during the course of the study. Standard measures for management of chemotherapy clinical toxicities (antiemetics, growth factors, fluids, etc.) were permitted with a physician's order.

METHODOLOGY

The dose of Propax™ was 1 packet administered 3 times a day. Each packet contains 4 tablets and 1 softgel capsule. The vitamin and mineral supplements (tablets) were administered simultaneously and with food to limit potential gastrointestinal upset. The softgel capsule containing essential fatty acids (EFA) was taken 30 minutes to 1 hour after the tablets to avoid interference with absorption of the vitamin and mineral-containing supplements.

Questionnaires were sorted by study into patient group and nurse group and again by chronological order of administration. Baseline, midpoint, and final values of both patient and nurse questionnaires were documented for all patients for whom at least 3 questionnaires were completed. The data was then evaluated by patient response and criteria response for the efficacy of Propax.™ Patients graded wellness from 0 to 4 based on the length of time specific quality of life indicators were adversely affected by chemotherapy (0 = unaffected, 1 = affected part of the time, 2 = affected half of the time, 3 = affected most of the time, and 4 = affected all the time). Nurses graded quality of life indicators on a scale from 0 (unaffected) to 4 (severely effected).

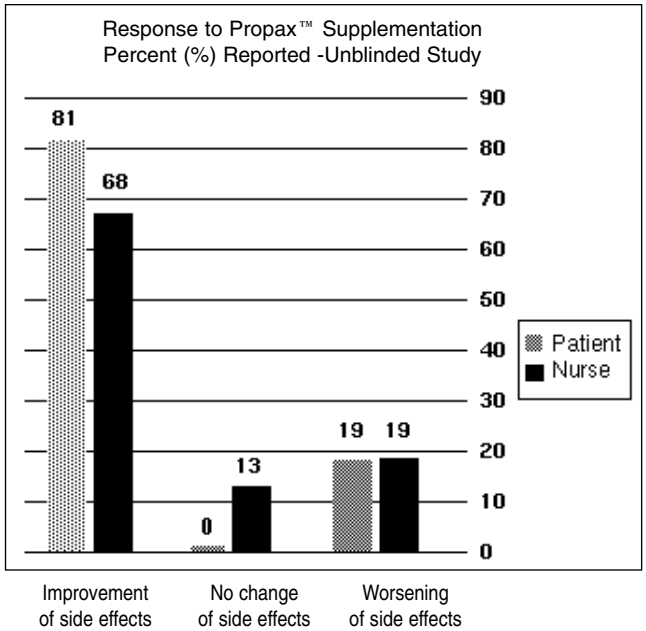
RESULTS OF UNBLINDED STUDY

Thirty-six patients completed informed consent forms. Of these 36, 22 were enrolled into the unblinded trial with 16 completing the study with at least 3 completed nurse questionnaires to compare baseline and final QOL indicators. The reasons given by patients who initially consented and then chose to not participate in the study were (1) a worsening of their disease, (2) failure to begin chemotherapy, and (3) a general feeling that they could not take the suggested daily dosage of the study product. Patient identification numbers were assigned by the study evaluator to preserve patient confidentiality, and have no impact on study design or methodology. For the 16 patients who completed the study, baseline severity scores were compared to final severity scores assigned by both patient and nurse. Since a grade of 4 was considered severe and 0 was considered less severe, a shift towards the lower (or a negative) number implies improvement in a QOL indicator; a shift towards the higher (or positive) number would indicate a worsening of the criteria. Patients receiving Propax™ indicated an improvement in episodes of nausea, diarrhea, constipation, mouth sores, skin changes, and raw mouth/throat. Patients reported significant improvements (> 0.5 change in score) in fatigue, sense of taste, tiredness, insomnia, and overall side effects of chemotherapy. Conversely, worsening of scores was documented by the patients for feeling sad and sick. Patients reported no changes (either improvement or worsening) in the occurrence of throat sores. Nurses reported no change in episodes of diarrhea, worsening of muscle weakness, and vomiting, and noted improvements in appetite (nausea), confusion, constipation, dermatoxicity (rashes), insomnia, stomatitis, and thrush.

Of the patient questionnaires in the open-label arm of the study, 81% of the patients reported overall improvement in quality of life indicators, and 19% reported a worsening of side effects related to chemotherapy toxicity. Nurses reported that 68% of the patients experienced decreased chemotherapy-induced toxicity, 13% experienced no worsening of these side effects when Propax™ was administered, and 19% experienced increased toxicity. (Table 1)

While there was a 13% difference in the patient reporting and nurses reporting on overall improvement, the nurs-

Table 1



es noted that there was improvement or no worsening of side effects in 81% of the patients completing the open-label study. The nurses and patients both reported the same percentage of worsening of side effects related to chemotherapy treatment (19%).

RESULTS OF BLINDED STUDY

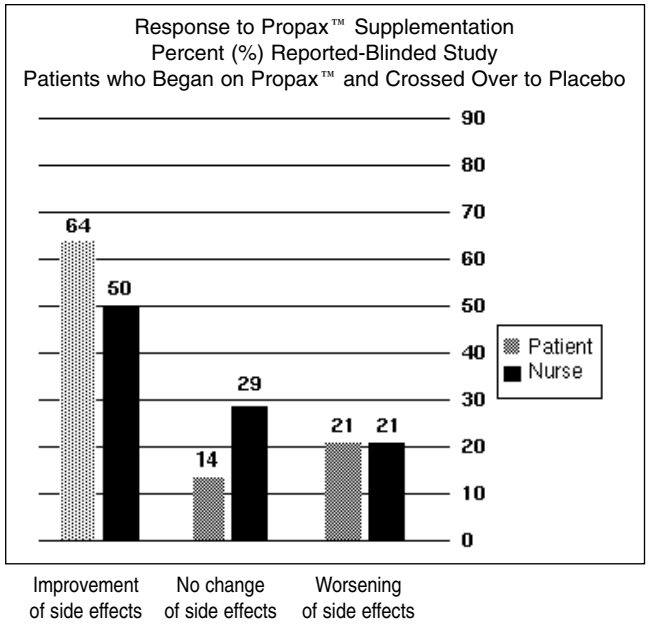
Of the 39 patients who initially completed informed consent forms, 3 choose to not enter the study for various reasons: (1) decision to withdraw from chemotherapy treatment, (2) required daily dosage of supplements was too great, and (3) the patient condition worsened prior to entering the study. Of the 36 patients who entered the study, 22 completed treatment and 14 dropped out prior to completion.

Patients who began on Propax™ and crossed over to placebo (Table 2)

For patients who began on Propax™ and ended on placebo, 9 patients (64%) reported improvement in chemotherapy-related side effects, 2 (14%) reported no change or worsening of side effects, and 3 (21%) reported a worsening of side effects at study conclusion. As reported by patients, the side effects that showed improvement or did not worsen after beginning chemotherapy included fatigue, nausea, impaired taste, diarrhea, general tiredness, constipation, and insomnia. Other chemotherapy-induced toxicities (mouth sores, skin changes, and decreased appetite) became more severe throughout the progression of the study, in contrast to the previously-noted toxicity parameters.

Nurses reported similar results: 7 patients (50%) experienced overall improvement, 4 patients (29%) encountered no overall worsening of side effects with supplementation, and 3 (21%) suffered a worsening of side effects.

Table 2



Patients who began on placebo and crossed over to Propax™

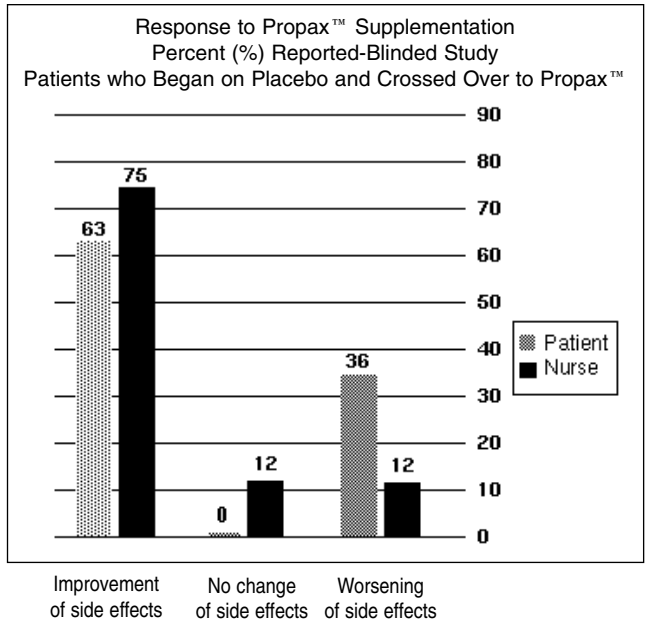
Patients in the blinded group who began on placebo were crossed over to Propax™ after week 6 of the study. At the end of the 12-week study, following crossover to Propax™, patients reported improvements in nausea, impaired taste, tiredness, appetite, sick feeling, and sad feeling.

The group of patients who were randomized to initial placebo use began the study with a collective baseline score of 44. This increased to 51 (indicating a worsening of toxicity symptoms) up to the midpoint of the study, resulting in a net mean score increase of .875 points per patient while on placebo. When crossed over to Propax™, the patients reported a collective 10 point decrease in their toxicity scores, or a net mean change of -1.25 points per patient, which indicates positive improvement of toxicity symptoms following the crossover to Propax™ from placebo.

At the conclusion of the study of those patients who began on placebo, 5 patients (63%) reported an improvement in chemotherapy-related side effects after switching to Propax™ and 3 patients (36%) reported a worsening of side effects.

The nurses reported several findings for patients who began on placebo. Patients had a group score at the beginning of the study of 29. At midpoint of the study, the group score in this arm of the study decreased 21% to 23, or a net mean change of -.75 points per patient. This would indicate an improvement in toxicities related to treatment for those patients who began on placebo prior to crossover to Propax™. However, following crossover from placebo to Propax™, the patients group score decreased 35% to 15 indicating a greater decrease in chemotherapy- related side effects following crossover to Propax™. Following

Table 3



crossover to Propax™, the net mean change was -1.0 per-patient as compared to -.75 per patient while on placebo.

The nurses reported that at the end of the study 6 patients (75%) in this arm of the study showed a decrease in side effects, 1 patient (12%) had no change or worsening of side effects, and 1 patient (12%) experienced a worsening of side effects related to treatment at the end of the study.

In summary, results reported by patients and nurses indicated benefit from the Propax™ and placebo arms in the blinded study. As reported above, the patients initially randomized to placebo showed a greater improvement in quality of life indicators following the crossover at week 6 of the study to Propax™.

DROPOUTS

Unblinded arm of study:

Six patients (27%) of the 22 randomized into the unblinded study dropped out before study completion citing as reasons the nausea, abdominal cramps, difficulty swallowing pills, and constipation, though none were reported to be severe. In most cases, the overall medical condition of these patients worsened, and it was not always possible to determine if the symptoms for withdrawal were the result of the study product or expected side effects of the chemotherapy and the disease itself.

Blinded arm of study:

Of the 39 patients who initially signed informed consent forms, 14 patients (36 %) dropped out of the blinded study. An additional 3 patients (8%) who initially consented chose not to enter the study due to a worsening of their condition. Reasons given for withdrawing from the study were: GI discomfort (1), nausea (3), stopped chemotherapy treatments (2), depression or worsening of disease condition (2), admitted into hospice (1), no reason provided (2), difficulty in swallowing pills (2), and constipation (1).

Adverse drug reactions

During the unblinded study, 4 patients experienced adverse drug reactions (ADR). Most were gastrointestinal in nature and included GI discomfort, soft stool, constipation, and flatulence. Other reported reactions included fatigue, difficulty in swallowing, dry skin, runny eyes, insomnia, and peripheral edema. It was difficult to determine if these reactions were related to the study product or events related to the chemotherapy treatments. None were considered severe or warranted withdrawal from study.

During the blinded study, 4 patients in the group that began on placebo reported ADR events. Of those patients who began on Propax™, 11 reported an adverse drug reaction. The ADRs reported by patients included GI discomfort, rash, nausea, indigestion, increased bowel movement, sore throat, unpleasant taste in mouth, headache, diarrhea, and dry skin. The majority of the adverse events were related to GI upset, and none were considered severe or warranted withdrawal from study. For those patients who experienced GI upset, they were advised to cut back on the dosage of the study product to one packet of the tablets daily for 3 days, and then titrate the dosage over the next four days, increasing the dosage daily until they were back at the suggested daily dosage of three (3) packets. This procedure worked for those who complained initially of GI upset, and following this procedure there were no further complaints of GI upset by those patients who remained in the study. For those who complained of unpleasant taste in their mouth, this was determined to be related to the clear gel capsule (EFA) which has a fishy taste as described by some patients. Nurses reported that they could not attribute each adverse event to the study product as some complaints may have been related to the chemotherapy treatment.

CONCOMITANT MEDICATIONS

In addition to standard chemotherapeutic protocols that included 5-FU/Leukovorin regimens, carboplatin, and etoposide, patients also received Medrol dosepack, compazine, acetaminophen, and Ibuprofen as prescribed by their physician for clinically-vindicated conditions. These medications can cause GI upset and may induce weakness as well. One patient in each study documented the use of filagrastim, a granulocyte-stimulating factor.

DISCUSSION

Many factors can influence the nutritional status of cancer patients, including cachexia, nausea, vomiting, decreased caloric intake, or the specific choice of oncology therapies.[23] Although the influence of these factors on nutrition is not well defined, the relationship has been extensively studied. Tonosaki et al. reported skinfold thickness as a nutritional indicator was significantly influenced by nausea and vomiting and also by infectious processes associated with elevated temperatures.[24] Similarly, Sarna et al. reported a parallel relationship between decreased calorie consumption and functional status in lung cancer patients over a 6-month period.[25] In both the blinded and open-label arms of this study, both patients and nurses reported improved quality of life scores for appetite changes and nausea. Improved appetite and decreased nausea may positively affect quality of life by nutritionally optimizing the gastrointestinal conditions and decreasing the body's response to chemotherapy-induced toxicities. This is consistent with the work of Grunberg et al. who proposed that decreased episodes of nausea/vomiting will result in a significant improvement in quality of life indicators. Additionally, the correlation between decreased nausea and decreased cost for total care has been documented.[26]

The results of this pilot study, both open-label and double-blinded placebo-crossover in design, indicate that

patient perception of benefit with Propax™ supplementation to chemotherapy is significant. Benefit was seen predominantly in nausea, fatigue, and diarrhea. The improvements warrant further study as most oncology patients report persistent fatigue,[35] nausea,[36] dry mouth,[22] and taste changes[22,37] throughout treatment modalities. As chemotherapy toxicity is expected to worsen or progress with continued therapy,[22] such improvement or lack of side effects worsening is an important outcome.

It is interesting to note that the results from the blinded study were very similar to the results from the unblinded study. Patients in the unblinded arm reported an 81% improvement in quality of life indicators and 19% reported a worsening of side effects related to chemotherapy toxicity. At the conclusion of the blinded arm, 64% of the patients who began on Propax™ reported an improvement in quality of life indicators and decrease in chemotherapy-related side effects, 14% reported no change or worsening of side effects, and 21% reported a worsening of quality of life indicators at study conclusion. Of those patients who began on Propax™ and crossed over to placebo, 63% reported an improvement in quality of life indicators and a decrease in chemotherapy-related side effects, and 36% reported a worsening of side effects. Three (3) patients who were crossed over to placebo after beginning on Propax™ requested to return to the original product after reporting a worsening of their side effects following crossover.

These patient-reported results were supported by nurse reports. At the end of the unblinded study, nurses reported that 75% of the patients showed an increase in quality of life indicators and a decrease in side effects related to chemotherapy treatment, 12% of the patients had no change or worsening in quality of life indicators and side effects, and 12% of the patients experienced a worsening of side effects related to their treatment.

In the blinded study, nurses reported that for those patients who began on placebo and crossed over to Propax™ 75% experienced an improvement in quality of life indicators and a decrease in chemotherapy-related side effects, 12% had no change in quality of life indicators nor worsening of side effects, and 12% reported a worsening of side effects. For those patients who began on Propax™ and crossed over to placebo, nurses reported that 64% reported an improvement in quality of life indicators and decrease in side effects, 14% reported no change nor worsening of side effects, and 21% reported a decrease in quality of life indicators and an increase in side effects related to chemotherapy.

While it was initially anticipated that a larger percentage of patients might experience a decrease in quality of life indicators and increase in side effects following crossover from Propax™ to placebo, one possible explanation may be that the duration of the study and the rapid crossover design may not have permitted adequate separation from the Propax™ effects from any subsequent placebo effects. The

Chart A. Contents of Propax™ with NT Factor™
Each serving pack (4 tablets and 1 softgel capsule) provide the following nutrients

Vitamin A (as acetate)	4375 IU
Vitamin A (as natural beta-carotene)	3750 IU
Vitamin C (as calcium ascorbate)	150 mg
Vitamin D-3 (as cholecalciferol)	32 IU
Vitamin E (as d-alpha tocopherol)	145 IU
Vitamin K (as phytonadione)	2.5 mcg
Vitamin B-1 (thiamin HCl)	6.25 mg
Vitamin B-2 (as riboflavin/ribose-5-phosphate)	30 mg
Vitamin B-3 (as niacinamide)	60 mg
Vitamin B-6 (as pyridoxine/P-5-P)	40 mg
Folic Acid (as folate)	200 mcg
Vitamin B-12 (cyanocobalamin)	25 mcg
Biotin	25 mcg
Pantothenic Acid (as d-calcium pantothenate)	25 mg
Calcium (as phosphate, ascorbate, citrate, sulfate, borogluconate)	360 mg
Iodine (as kelp)	18.75 mcg
Magnesium (as carbonate, oxide, glycinate, sulfate)	160 mg
Zinc (as methionate)	12.5 mg
Selenium (as selenomethionate)	75 mcg
Copper (as tyrosinate)	300 mcg
Manganese (as glycinate)	2.5 mg
Chromium (as nicotinate)	50 mcg
Molybdenum (as glycinate)	20 mcg
Potassium (as citrate)	12.8 mg
Bioflavonoids (as citrus, rutin, rosehips, quercetin)	165 mg
Boron (as calcium borogluconate)	500 mcg
Co Enzyme Q10 (ubiquinone)	4 mg
Creatine (monohydrate, phosphate)	122.5 mg
Grape Seed Extract (proanthocyanidins)	5 mg
Inositol (inositol/inositol nicotinate)	25 mg
Lactoferrin	4 mg
Pantethine (as coenzyme A precursor)	70 mg
Vanadium (as vanadyl sulfate)	12.5 mcg
Alpha-Keto Glutarate	125 mg
Glutathione (as reduced)	5 mg
L-Tyrosine	60 mg
N-Acetyl-L-Cysteine	25 mg
Taurine	110 mg
Green Tea Extract	50 mg
Horsetail (as silica)	12.5 mg
Phosphoglycolipids	160 mg
EPA (as eicosapentaenoic acid)	180 mg
DHA (as docosahexanoic acid)	120 mg
NT Factor (as tablet base)	1400 mg

NT Factor is a proprietary food tablet base comprised of defatted rice bran, arginine, beet root fiber, black strap molasses, glycine, magnesium sulfate, enriched polyunsaturated phosphatidyl choline (phospholipids), saponin (glycolipids), para-amino benzoate, leek, pantethine (bifidus growth factor) taurine, garlic, calcium borogluconate, omega-6 fatty acids, omega-3 essential fatty acids, artichoke, barley malt, potassium citrate, calcium sulfate, spirulina, bromelain, natural vitamin E, calcium ascorbate, alpha-lipoic acid, oligosaccharides, B-6, niacinamide, riboflavin, inositol, niacin, calcium pantothenate, thiamin, B-12, bifidus, acidophilus, folic acid, chromium picolinate.

duration of any Propax™ effects after discontinuation is not known nor addressed in this study.

Although this initial pilot study involved small numbers of patients, the results of the study were confirmed in both the open-label and double-blinded, placebo-controlled arms of the trial. Benson et al. noted in their study that there was little evidence that estimates of treatment effects in observational studies published after 1984 were either consistently larger than or qualitatively different from those obtained in randomized, controlled trials.[38]

Concato et al. also reached the same conclusion in a recent study.[39] After looking at randomized controlled trials, observational studies, and the hierarchy of research designs published in five major medical journals from 1991 to 1995, Concato et al. concluded that the results of well-designed observational studies (with either a cohort or a case-control design) do not systematically overestimate the magnitude of the effects of treatment as compared with those in randomized, controlled trials on the same topic.

CONCLUSIONS

Based on the results of this initial pilot study, additional well-designed clinical studies with larger patient populations are justified and encouraged in order to draw further conclusions on the effectiveness of nutritional supplements like Propax™ in cancer treatment. This initial pilot study provides evidence that the use of a nutritional supplement such as Propax™ may correlate with positive results for decreased fatigue, vomiting, nausea, and diarrhea, as well as with an improvement in overall well-being; effects such as mucositis, skin toxicity, and appetite were not similarly noted. Larger studied numbers of patients are required to obtain firm statistical support for these noted early encouraging findings. Future studies with a larger patient population will also help determine if a dose reduction of chemotherapy can be avoided with the use of a nutritional supplement such as Propax™, which could permit toleration of higher levels of drug therapy. The cost-effectiveness of such strategies should also be evaluated relative to decreasing treatment costs by avoiding episodes of neutropenia, sepsis, emesis, and other chemotherapy-induced side effects.

The dropout rates in the blinded study appeared more related to the number of pills required for adequate dosage and a worsening of the actual cancer itself rather than to any direct Propax™ side effects.

In summary, Propax™ supplementation to standard chemotherapy regimens had beneficial impact on several quality of life parameters with a high degree of patient acceptance of the supplementation regimen.

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ORIGINAL RESEARCH

Identification of Novel Anti-Oxidant Potential in Peptide Hydrolysates Using Cell-Based Models of Human Metabolism†

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ABSTRACT

While traditional antioxidant potential has been ascribed to hydrogen donor properties of compound classes such as tocopherols and flavonoids, it has become apparent that the mitigation of redox stress in biological systems can be effected by broader classes of molecular entities acting through a variety of mechanisms including delocalization of compartmental intracellular capacitance changes. To explore whether such mechanisms can be effected through non-traditional $H^{\bullet}$ donor antioxidants, an oxidative cell-based screening system was employed to assess the efficacy of putative protein and peptide-based antioxidants. Herein we report upon the use of oxidative cell-based screening for the identification of peptide-based antioxidants and the antioxidant potential of two wheat-derived high glutamine content peptides in cellular and non-cellular antioxidant assays. Results obtained through this screening methodology indicate: 1. Of several hundred peptides evaluated for generalized cytoprotective properties during oxidative stress, two protein hydrolysates showed potential to protect cells from injury secondary to oxidative/metabolic stress. 2. Subsequent evaluation of the peptide GPX in a functional biological model employing muscle contractility suggested a 28.5% functional recovery, relative to untreated controls, secondary to administration of 2,4-dinitrophenol. 3. Further qualification of the molecular basis of cytoprotection by maintenance of intracellular ATP levels indicated that of the two peptides that effected generalized cytoprotection, only one, GPX, effected maintenance of 95% intracellular ATP, relative to untreated controls. 4. Free glutamine, and GPX precursors failed to yield cytoprotective effects in either of the oxidative cell-based assays employed. The data suggest that oxidative cell-based models of human metabolism described herein may find utility in the qualification of peptide-derived non-traditional $H^{\bullet}$ donor antioxidants for circumstances where oxidative and/or metabolic stress compromise normal physiologic function.

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INTRODUCTION

As the link between diet and disease becomes ever so apparent, the pace of technical advances in our understanding of the molecular basis of nutrition science is accelerating. Of interest to consumers, regulatory agencies, and scientists, is the specific class of nutrients referred to broadly as antioxidants. While the consumption of fruits and vegetables has been associated with lower incidence and

decreased mortality rates of cancer[1-6] and cardiovascular disease,[7-12] significant controversy exists about recommendations for diet supplementation with antioxidants. The debate over supplementation can be in part attributed to the absence of biologically contextual-relevant assays that elucidate the molecular basis of antioxidant action as a function of an individual's specific needs. Additional confounding variables exist about the responder basis predicated upon host genotype and phenotype. Recently, there have been several investigations from Cao et al reporting the measurements of the antioxidant capacity of some common fruits and vegetables using the Oxygen Radical Absorbance Capacity assay.[13-16] Data presented therein suggested that 80% of the total antioxidant capacity of fruits and vegetables may be exceeded by that derived from non-vitamin C, E, and beta-carotene sources. Using similar methodology, a screening of commercially available natural dietary antioxidant supplements and a comparison to the amounts offered by fruits and vegetables have also been reported.[17] To further understand the molecular basis for antioxidant potential and to broaden our understanding of the non-vitamin antioxidant potential associated with common foods, we undertook an examination of the antioxidant potential of protein-derived chemical entities using oxidative cell-based screening technology. Herein we report, consistent with the Oxygen Radical Absorbance Capacity observations, that peptides can effect antioxidant activity. It is further suggested that while such peptides may effect their cytoprotective action through $H^{\bullet}$ donor properties, the maintenance of intracellular ATP levels during severe oxidative stress may suggest multiple convergent mechanisms of action associated with conventional antioxidant potential.

MATERIALS AND METHODS

Chemicals: Cell culture reagents were obtained from Life Technologies (Life Technologies, Inc., Rockville, Md) and all other assay reagents from Sigma (Sigma-Aldrich, St. Louis, Mo). Native proteins, peptides, and hydrolysates were obtained from DMV International and other commercial peptide vendors.

Cell Culture: Cell assays were conducted as described below under standard cell culture conditions. Contractility studies were performed on primary isolated rat cardiomyocytes.[18] Cell viability measurements were performed on a cardiac-derived cell line subpopulation selected for oxidative characteristics obtained from the American Type Culture Collection.

ATP measurements: Measurement of changes in cellular adenosine triphosphate (ATP) content was accomplished employing the ENLITEN ATP Assay System (Promega, Madison, Wis). Cardiac-derived cells were plated into 96 well plates in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) media and grown for 24 hours prior to use. Quantitation of intracellular ATP was conducted as follows. Cells were cultured as described and energetically challenged to deplete ATP using antimycin, an inhibitor of mitochondrial function, suspended in a HEPES-based buffer. The cells were exposed to the antimycin containing buffer with or without putative peptide protectants. These condidtions were maintained at 37°C for 2 hr. Intracellular ATP was assessed in a 96 well plate format employing ENLITEN ATP Assay System and a Labsytems Ascent FL Luminescent microplate reader (Labsytems Oy, Helsinki, Finland). Protection against oxidative stress is expressed as percentage ATP remaining relative to untreated controls.

Contractility measurements: Measurement of cardiomyocyte contractility was made using FLUO-4, a fluorescent calcium probe (Molecular Probes Inc., Eugene, Ore). Freshly isolated cardiomyocytes were plated into culture plates in DMEM media and grown for 2 days prior to testing. Cardiomyocytes were subjected to energetic stress using an uncoupler of oxidative phosphorylation, 2,4- dinitrophenol (DNP). Cells were suspended in 2,4-DNP containing solution and treated with and without test article suspended in a HEPES-based buffer. Viability was assessed through measurement of recovery of calcium transients. Specifically, following the addition of 2,4-DNP, cardiomyocytes were pre-loaded with the calcium dye, stimulated to contract by increasing extracellular calcium, and the fluorescence of collective calcium transients measured.

TABLE 1: PROTECTIVE EFFECTS OF PROTEIN HYDROLYSATES ON CELL CONTRACTILITY

Test Article†	% Recovery of Cell Contractility
Protein hydrolysate 1 (GPX)	28.5 ± 4.7 ‡‡
WGE 80 GPN (glutamine peptide)	28.7 ± 4.6 ‡‡
WGE 80 GPA (glutamine peptide)	7.2 ± 1.2
WE 80 B (commercial WPH)	1.64 ± 0.5
Protein hydrolysate 3 (WPH -2)	2.14 ± 0.92
Protein hydrolysate 5.2	1 ± 0.3
Protein hydrolysate 5.3	6 ± 1.2
Protein hydrolysate 5.5	1.5 ± 0.4
Protein hydrolysate 5.8	- 10 ± 5
Protein hydrolysate 5.9	-15 ± 5.6
Protein hydrolysate 5.11	- 10 ± 2.4
Protein hydrolysate 5.12	0.56 ± 0.2
Protein hydrolysate 5.13	-23 ± 7.8
Protein hydrolysate 4.6	2 ± 0.6
Protein hydrolysate 4.10	-1.5 ± 0.3
Protein hydrolysate 4P12	14.5 ± 1.67

†Protein hydrolysates incubated at 10 mg/ml in the test medium. Values are expressed as a percent of uninjured control and are mean ± sd (n=3).

‡‡ Recovery of cell contractility equal to or greater than 20% is assumed to be significant activity.

Recovery of contractility of the cardiomyocytes from 2,4-DNP challenge was recorded and results expressed as a percentage recovery, relative to untreated control.

RESULTS

The ability of a particular protein hydrolysate to effect cytoprotection in response to oxidative stress was assessed using transformed and oxidative cell-based models of human cardiac function. Of the 16 protein-derived samples evaluated (Table 1), only GPX and WGE 80 GPN effected protection as evidenced by percent contractile recovery values exceeding 25%.

TABLE 2: PROTECTIVE EFFECTS OF PROTEIN HYDROLYSATES ON CELL ENERGY

Test Article†	% Protection of Cell Energy	% Recovery of Cell Contractility
Protein hydrolysate 1 (GPX)	96 ± 4‡‡	28.5 ± 4.7
Protein hydrolysate 3 (WPH –1)	1.3 ± 1	2.14 ± 0.92
WGE 80 GPN (glutamine peptide)	41.7 ± 3.8	28.7 ± 4.6
Glutamine	0.28 ± 0.1	-6.2 ± 2.2

†Protein hydrolysates incubated at 5 mg/ml for the ATP assay and 10mg/ml for the contractility assay. ATP and contractility measurements were run as described in the Methods and Materials section. Values are expressed as a percent of non-ischemic control and are mean ± sd (n=3).

‡‡ Protection of cellular energy equal to or greater than 50% is assumed to be significant activity.

To determine whether the observed cytoprotective attributes were secondary to the high glutamine content, or referable to the native peptide, comparisons were made with the peptide precursor and free glutamine. The peptide hydrolysate precursor and glutamine failed to effect cytoprotection as evidenced by contractility recovery values below 5% (Table 2).

To begin to elucidate a mechanism of action of GPX, initial studies were performed to assess whether maintenance of contractile function correlated with other standard viability assays commonly used to assess vital function following challenge. Given the central role of ATP in maintaining contractile function and the clear evidence for ATP depletion following treatment with inhibitors of mitochondrial function, it was of interest to examine the recovery of intracellular ATP levels as a proxy for more physiologically-relevant cytoprotection. Data obtained suggested that in addition to maintaining contractile function, GPX also afforded significant maintenance of intracellular ATP in a dose dependent manner from 1.25 through 5 mg/mL (Figure 1). A similar effect of dose-protective recovery on contractile function was also observed for GPX (Figure 2).

DISCUSSION

The evolutionary co-opting of oxygen detoxification and energy coupling has afforded numerous biological advantages. These relate to several processes about efficiency of energy extraction from biological fuels. However, these evolutionary pathways are not without liabilities. One liability, addressed within, is the toxic exposure not only to molecular oxygen from the atmosphere, but also to the partial reduction products of molecular oxygen and other electron-accepting species. Collectively, both plants and animals have evolved vital mechanisms that preferentially mitigate adventitious oxidative and metabolic stress. Several agents have received considerable attention, eg, tocopherols, owing to their well-characterized activity and central role in mitigating redox stress. It is becoming increasingly clear in recent times that numerous other phytochemicals, eg, flavonoids, also participate in mitigating biological oxidative stress. Given the ubiquity of such phytochemicals and other putative antioxidant nutrients, a more complete understanding of antioxidants is desired to devise alimentation strategies that effect beneficial end-user results.

Underlying one mechanism of oxidative stress, is the partial reduction of molecular oxygen to yield a host of reduced molecular and atomic oxygen species. These collectively are referred to as reactive oxygen species. Reactive Oxygen Species have been implicated in the pathogenesis of numerous conditions, a partial list of which includes aging, stroke, cardiovascular disease, apoptosis, and immune impairment.[19] Human defense mechanism to ROS include enzyme-based systems, eg, superoxide dismutase, catalase, glutathione peroxidase, and non-enzymatic detoxification agents, eg, albumin, ceruloplasmin, ferritin, ascorbic acid, a-tocopherol, b-carotene, reduced glutathione, uric acid, and bilirubin.[20]

As there are potentially hundreds of biologically-relevant free radicals that can in principle effect concordant damage, it is not surprising that an equally diverse set of

FIGURE 1: PROTECTIVE EFFECT OF GPX AGAINST CELLULAR ATP DEPLETION

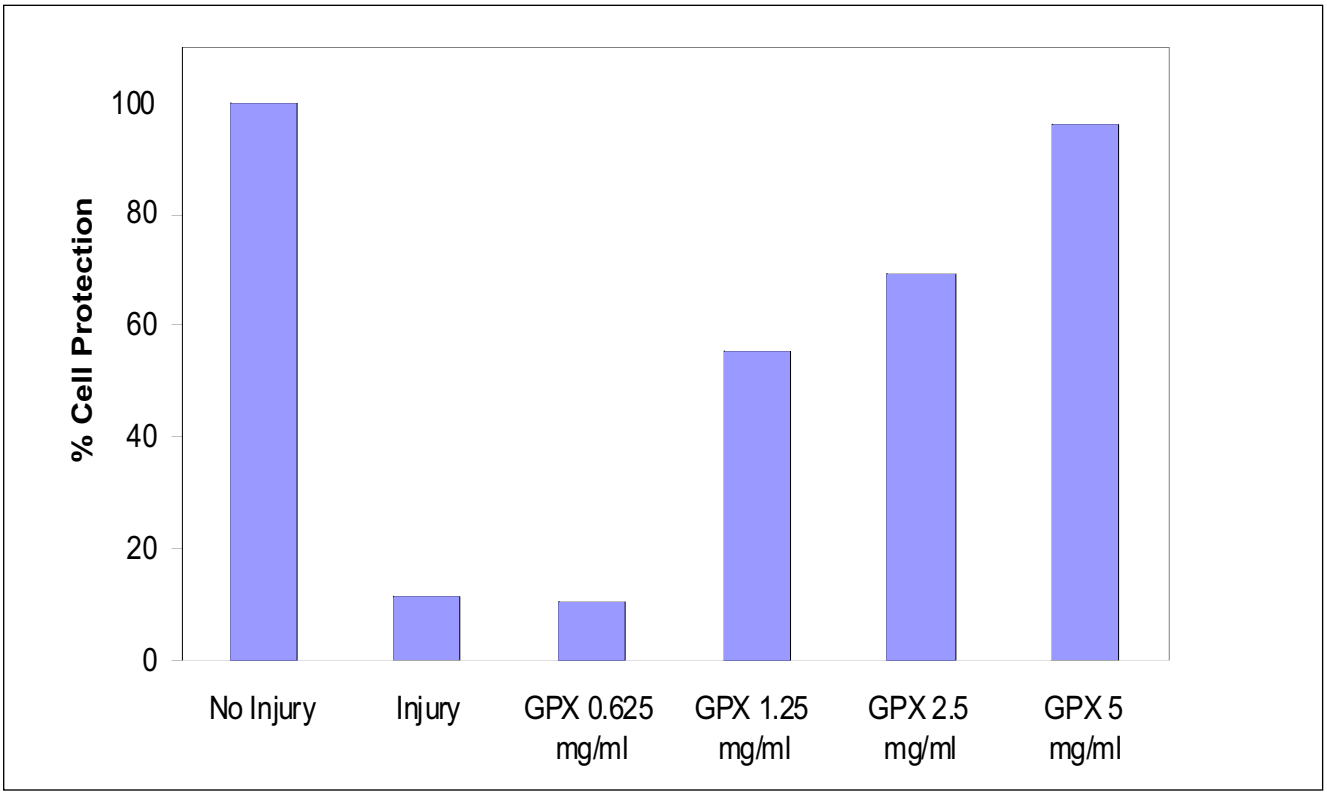
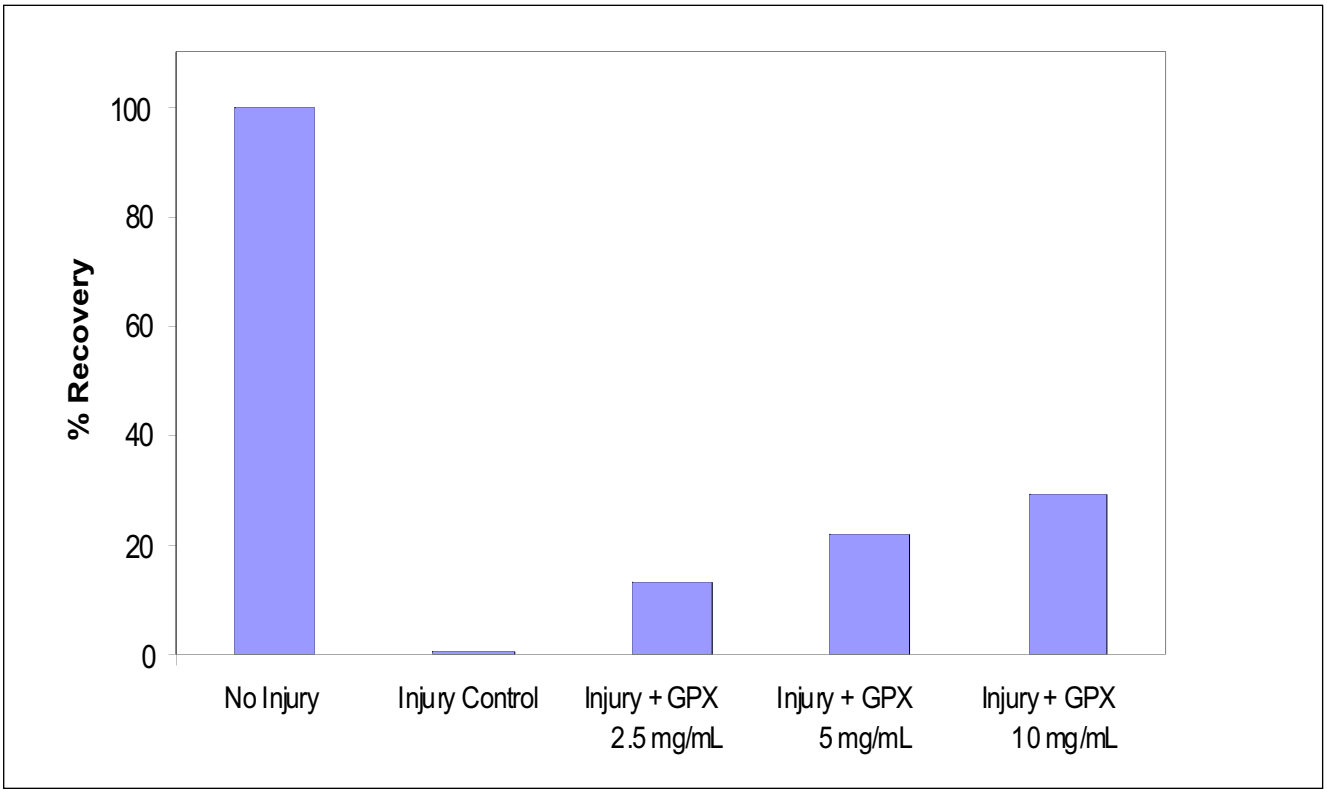


FIGURE 2: PROTECTIVE EFFECT OF GPX ON RECOVERY OF CONTRACTILITY FUNCTION



neutralizing agents has evolved in part to mitigate the attendant damage. To expedite the identification of such a diverse set of compounds and enzymes involved with this global protective system, several methods have been advanced to score antioxidant potential as a means to understand mechanism of action and biological relevance.[21-25]

Of recent we have identified several peptide hydrolysates that effect cytoprotective properties as characterized by *in vitro* cell-based biological assays. Given this observation, it was of further interest to investigate the molecular basis of the activity of these peptides and to compare their antioxidant potential with other assays commonly employed. Reported within is the data obtained from the screening of 16 commercially available protein hydrolysates for their antioxidant capacities based on their cytoprotective effect secondary to antimycin-mediated redox stress. Additional qualification of peptide-mediated oxidative stress cytoprotection was effected by measuring intracellular ATP levels as a proxy for long-term viability. Of the 16 peptide hydrolysates examined, three exhibited functional recovery in a myocyte contractility assay. Interestingly, only one (GPX) effected both generalized cytoprotection and maintenance of ATP levels. Similarly available peptides or protein precursors containing an elevated percentage of glutamine failed to exhibit both the cytoprotective and ATP-preserving effects, suggesting a unique composition or structure of peptide hydrolysate GPX. That the cytoprotective effect is referable to unique peptide structure, and not to free glutamine, was suggested by the absence of a cytoprotective effect of ATP preservation by free glutamine.

Several questions remain unanswered. These include: 1. What intracellular or extracellular receptor-mediated mechanism is responsible for the exhibited cytoprotective effect? 2. What specific peptide structure is responsible for the observed action? 3. What is the role of radical scavenging versus dissipation of intracellular compartmentalized redox charge imbalance? 4. What is the bioavailability of peptides for non-gut-based targets? and 5. The convergence of the GPX cytoprotective effect with multiple modes of action including beneficial effects of glutamine (not related to cytoprotection).

While efforts are actively ongoing to address these important questions, several early-stage conclusions are submitted: 1. Peptides and peptide hydrolysates, thought to possess varying topographic morphologies, may effect benefit through an as of yet unknown mechanism beyond their utility as fuel sources. 2. Data presented herein suggests that a classic view of antioxidants as $H^{\bullet}$ donor species may need to be extended to include receptor or target-based mechanisms that ameliorate compartmentalized redox stress. 3. As peptides are central to maintenance of normal physiologic function and the treatment of illness, more work needs to be performed to understand the physiologic relevance of peptides such as GPX that may confer specific advantages. 4. Tools such as cell-based screening technology that allow a window into the mechanism may offer advantages as a bridge between low therapeutic index peptides and human or animal testing. 5. The subtle effects of structure-activity relationships in multi-component peptide mixtures warrant investigation. 6. Given the role of glutamine in immunocompetence and the importance of host antioxidant defense mechanisms, further work is warranted to investigate the clinical utility of GPX and GPX-like peptide hydrolysates.

Abbreviations: ATCC, American Type Culture Collection; ATP, adenosine triphosphate; DMEM, Dulbecco's modified Eagle's medium

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ORIGINAL RESEARCH

Some Physical Properties of Ginkgo Biloba Extracts Important for Tableting and Encapsulation[2]

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ABSTRACT

Objective: To evaluate the flow and deformation properties of *Ginkgo biloba* extracts (GBEs) donated from multiple sources as received and dried.

Methods: Heckel analysis was performed to determine mean yield pressures at two punch speeds. Strain rate sensitivity and long term elastic recovery were assessed. Flow properties were assessed via minimum orifice diameter and compressibility index. The influence of moisture on flow and mean yield pressure was determined.

Results: Significant differences (α=0.05) were found between 'fast' and 'slow' yield pressure values for three sources. No conclusive elastic recovery data could be obtained. Significant differences (α=0.05) were found between the mean yield pressure values of dried GBEs versus GBEs as received for four sources. All sources exhibited poor compressibility and flowability indices. Dried GBE had a lower compressibility index than material as received.

Conclusions: Relative to reference materials, all brands tested appear to be plasto-elastic in nature. Under test conditions, significant differences in 'fast' and 'slow' punch speeds revealed possible strain rate sensitivity for three of the five *Ginkgo biloba* sources. GBEs with reduced moisture maintained plasticity. GBEs analyzed have variable flow properties. Reducing free moisture resulted in a lower compressibility index. This information may be useful in formulation and process development of *Ginkgo biloba*.

Key words: *Ginkgo biloba*, Compressibility, Flow, Deformation, Tableting, Encapsulation

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INTRODUCTION

Nutraceuticals represent a multibillion dollar industry with an estimated 12 billion dollars spent in the U.S. on vitamins, minerals, herbs, and supplements in 1997, and the trend does not appear to be reversing.[1] In fact, the popular herb *Ginkgo biloba*, is used to improve mental functioning as well as alleviate many symptoms ranging from intermittent claudication to vertigo and tinnitus, is among the most widely prescribed drugs in France and Germany.[2]

With the large impact of nutraceuticals on the national economy and implications for health care, standardization and quality control of active ingredients are a large concern. In addition, guidelines for the delivery system are important, as many workers have reported inconsistent or poor quality supplements.[1,3,4] Formulation clearly plays a large role in dosage form performance of nutraceuticals as with any drug product; however, formulation of nutraceuticals and in particular, botanical extracts, may be problematic.[5] The raw material may be in any form ranging from crude leaves or roots, to a powdered extract. In addition, the doses may be relatively high. Botanical extracts may be hygroscopic, sticky, exhibit poor flow, have variable particle size distribution, and low bulk densities.[5-8]

Foremost among formulation challenges are powdered extract rheologic characteristics. Flow properties are extremely important for processing powders, especially on high-speed tableting or encapsulation equipment. Many factors depend upon adequate flow, not the least of which are fill weight variation and content uniformity of tablets or capsules, problems that have plagued the nutraceutical industry.[1,4] Particle-particle interactions such as frictional forces, van der Waals interactions, surface irregularities, electrostatic effects, and moisture all affect powder flow.[9] Frictional forces are a result of the normal force perpendicular to the plane of shear and the coefficient of friction of the powders. van der Waals interactions are true cohesional forces that develop between like particles. Structural cohesion occurs due to surface irregularities whereby the particle shape hinders flow. Electrostatic effects occur when a powder is agitated so that there is a disturbance in the surface charge. The altered charge distribution may result in cohesion-like effects that are independent of van der Waals effects. To a certain degree, adsorbed moisture facilitates flow by reducing electrostatic effects by increasing surface conductivity. However, when there is too much moisture, liquid bonds may form with negative curvature, creating a suction. The result is a surface tension effect. Also, the viscosity of the liquid may hinder powder movement. Methods to assess the flowability of powders and indirectly, the factors outlined above that influence flow, range from simple angle-of-repose tests, to elaborate annular shear cell studies. Several methods should be used to assess flow since no single method adequately addresses all of the influential factors.[9]

An equally important extract property for the formulation of solid dosage forms is the ability to prepare coherent tablets and/or plugs. Formation of a tablet necessitates both compression and consolidation of powder within the die cavity. Conversely, preparation of a powder plug by encapsulation often requires compression only, and may or may not involve some degree of consolidation of the powder bed. During the initial compressive stages, a mechanical force is applied to the powder bed by a tablet punch or a capsule machine tamping pin. The particles rearrange to allow closer packing, reducing the powder bed bulk volume. In tableting, the volume is generally reduced sufficiently so that further rearrangement is impossible.[10, 11]

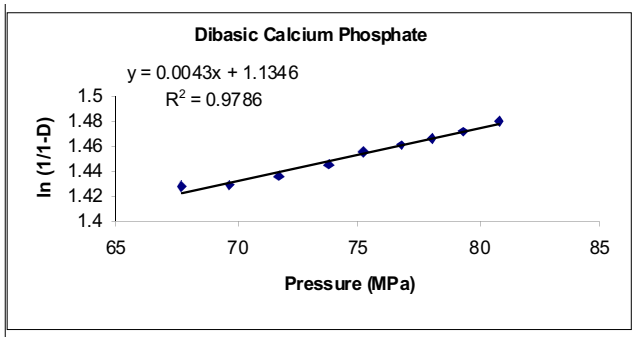
The consolidation or bond formation stage begins when stress (stress=force/area) at particle point contacts increases greatly and these point contacts begin to deform. If the elastic limit of the material is not exceeded, the material will spontaneously reverse to its original shape when the compressive load is removed. However, if the yield pressure or elastic limit is reached, an irreversible deformation begins. If the compressive load is removed after the yield pressure is exceeded, the material will not spontaneously regain its original shape. Irreversible changes usually occur by a combination of plastic deformation and brittle fracture, with one mechanism often predominating. In plastically-deforming materials, the material may behave as a fluid when the yield pressure is exceeded and the bulk volume of the powder bed and internal stresses are decreased by plastic flow. Plastic deformation is characteristic of materials with a shear strength less than the tensile strength. Conversely, if the material fails in tension more easily than in shear (i.e. shear strength> tensile strength), the predominant mechanism of deformation is brittle fracture. With these materials, bulk volume of the powder bed may be further reduced by particle fracture. The smaller particles begin to fill void spaces between larger particles, which may then continue to fracture into smaller particles.

The type of permanent deformation that occurs depends on material properties such as the lattice structure and resultant shear strength versus tensile strength, as well as the rate of strain.[10, 11] Typically, the components of real formulations are neither ideally plastic nor ideally elastic; rather, they exhibit both characteristics and often deform viscoelastically or plastoelastically. Such materials are often said to exhibit plastic flow. Unlike ideal plastic deformation, plastic flow is time dependent. If the strain is applied at a sufficiently slow rate to such materials, the material can deform to relieve internal stresses by plastic flow. However, if the rate of strain is very fast, there may not be sufficient time to relieve stresses by plastic flow and the particles will be less deformable (i.e., have greater yield stress) and may exhibit fracture. Such materials are therefore said to exhibit strain rate sensitivity.[12]

Consolidation or the formation of bonds results from intermolecular interactions, fusion at point contacts, and, to a negligible extent, structural effects. The mechanical strength of the compact is a function of these bonding forces and the area over which they act. Therefore, deformation of the material to form large clean surfaces is extremely important for lasting bond formation.[10, 11] Hiestand has reviewed various mechanisms of deformation and concluded that plastic deformation is the predominant mechanism by which large clean surfaces are created for formation of bonds.[13] A combination of plastically deforming matrix to relieve internal stresses and brittle material for clean, strongly bonding surfaces is desirable to form mechanically-strong compacts.[10]

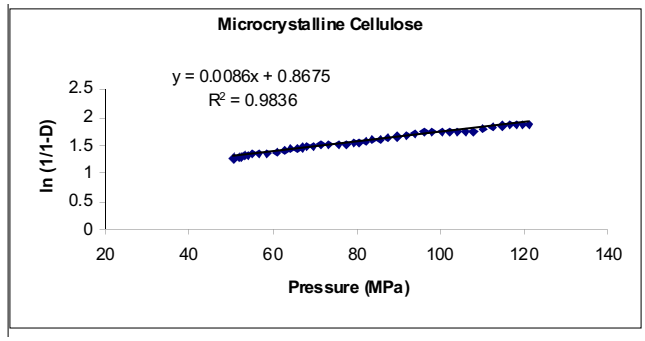
Pressure-volume studies may be performed to determine the relative plastic or brittle nature of an extract and thereby enable the formulator to anticipate potential compaction properties. The Heckel relationship assumes that the decreasing porosity of a compact with applied pressure is a first-order process.[14] The Heckel equation may be used to relate the relative density (D) of a powder bed during compaction to the applied pressure: $\ln (1/1-D)=KP + A$. P is the applied pressure, A is a constant, and D is the relative density of the powder bed and is calculated from the bulk

Figure 1. Heckel plot of material that deforms primarily by brittle fracture.



Dibasic calcium phosphate deforms primarily by brittle fracture. The slope is low and therefore the calculated yield pressure is relatively high (Py=232.6 MPa).

Figure 2. Heckel plot of material that deforms primarily by plastic deformation.



Microcrystalline cellulose deforms primarily by plastic deformation. The slope is high and therefore the calculated yield pressure is relatively low (Py=116.3 MPa).

density divided by the true particle density. The constants K (slope) and A (intercept) may be determined from the extrapolated linear portion of a plot of ln (1/1-D) versus P. The inverse of the slope, K, is taken as the mean yield pressure, Py.[15] This slope represents the rate of densification with applied pressure. Materials that are relatively brittle, such as dibasic calcium phosphate have high yield pressures (Figure 1). Materials that are relatively plastic, such as microcrystalline cellulose, have low yield pressures (Figure 2) [15]. Heckel analysis may be used to determine the strain rate sensitivity of a powder by comparing Py determined at slow and fast punch speeds.[12]

$$StrainRateSensitivity = \frac{Py(fast) - Py(slow)}{Py(fast)}$$

As previously mentioned, plastically deforming materials may be prone to strain rate sensitivity. Strain Rate Sensitivity measurements help formulators anticipate problems of scaling up to high speed tableting. Materials that exhibit a high degree of strain rate sensitivity may produce highly acceptable tablets in the laboratory on slow speed development presses, but make very poor tablets when run on high speed production equipment. Stored elastic energy during compaction and subsequent recovery upon the removal of axial pressure has been related to the tendency of tablets to cap. Shear stresses developed during compaction that cannot be relieved by plastic flow have also been implicated in elastic recovery.[15]

After extensive literature review, there appears to be a dearth of information regarding basic flow and compression properties of botanical extracts. Flow and compaction properties of various direct compression formulations of liquid extracts loaded on fumed silica have been reported.[6] Flow properties of neat powdered plant extracts as well as formulated extract has been reported for *Hamamelis virginiana.*[7] Flow properties and final tablet physical properties for various formulations of a powdered plant extract were reported.[8] However, there have been no studies addressing flow properties of neat powdered plant extract from multiple sources. In addition, no deformation properties of any plant extract have been reported.

The objectives of this study were to evaluate flow properties of various *Ginkgo biloba* extracts donated from multiple sources and to determine their relative plastic or brittle nature. In addition, an attempt was made to assess strain rate sensitivity and elastic recovery of neat GBEs. Further, the effect of moisture content on flow properties and mean yield pressure was determined.

MATERIALS AND METHODS

Five representative *Ginkgo biloba* dry powder extracts (P, I, B, D, T) were donated from multiple sources. No crude materials were evaluated. All extracts are hydroalcoholic extracts except source D, which is an aqueous extract. Although information regarding additional excipients used during extract processing was unavailable, a common industry practice is to add maltodextrin, silica, or other excipients to facilitate handling. Emcompress® (Lot # SO2G) brand dibasic calcium phosphate (Penwest, Patterson, NY), and Avicel PH-101® (Lot # 1603) brand microcrystalline cellulose (FMC Corp, Philadelphia, Pa) were utilized as reference materials in the compression studies.

Flow Studies

Minimum orifice diameter studies were conducted on neat GBEs by the method of Gioia.[16] A Flodex Powder Flowability Tester (Hanson Research Corporation, Northridge, Calif)) was utilized to determine the minimum orifice through which the powder could freely flow. The flowability index is the diameter of the smallest hole through which the powder flows freely three times out of

three. A small flowability index is indicative of a freely flowing powder.[16] Each experiment was performed in triplicate. The compressibility index, C, is an indirect measure of flow and is determined from:

$$C = \left(\frac{\rho t - \rho o}{\rho t} \right) \times 100$$

ρt = tapped density
ρ = bulk density

Compressibility indices <15% are indicative of freely flowing powders, whereas indices > 40% are indicative of very, very poor flow.[17] This method is highly dependent on how the loose bulk density is measured, as any disturbance of the powder bed will result in a false reading for loose bulk density. Therefore, the loose bulk densities were obtained by the poured density method and the volumeter method described in the Pharmacopeial Forum.[18] A 100 ml graduated cylinder and a Mettler PE 300 electronic balance were used to determine the poured density. A Scott Volumeter (Fisher Scientific, Springfield, NJ) and Mettler PE 300 electronic balance were used to determine bulk density by the volumeter method. The tapped density was determined with a Stampf Volumeter (J. Engelsman, Ludwigshafen, a. Rh., distributed by Shandon Southern Instruments, Inc, Sewickley, Pa) by the method outlined in the Pharmacopeial Forum[18] except the cylinder was dropped 1000 times. Each density was determined in triplicate (bulk and tapped) as well as each compressibility index.

The compressibility index study was repeated using the poured bulk density to determine the effect of moisture content on flow properties of source D, the GBE that exhibited the worst flow by both compressibility index and minimum orifice diameter studies. A Computrac Max 2000 (Arizona Instruments, Phoenix, Ariz) was utilized to determine source D moisture content under ambient storage conditions, to dry the material, and to determine moisture content at the completion of the study. The experiment was performed in triplicate. Source D was dried at 105^{O}C and the average compressibility index.[18] and moisture content for the dried samples were determined.

Deformation Studies

Tablet-in-die Heckel analysis was performed to determine mean yield pressures at relatively fast and relatively slow punch speeds for the neat GBEs. In addition, Avicel PH-101 brand microcrystalline cellulose (plastic material) and Emcompress® brand dibasic calcium phosphate (brittle material) were analyzed to determine reference yield pressures. True densities of GBEs were determined with Multivolume 1305 Pycnometer (Micromeritics, Norcross, Ga). A fully instrumented Colton 321 single station press was utilized at punch speeds nominally set for 'fast' (58 tablets/min) and 'slow' (10 tablets/min). Prior to analysis, the raw data were treated by Fast Fourier Transform (Igor Pro™ software). The linear portion was determined from the second derivative and linear regression. Strain rate sensitivity indices (SRS) were calculated by the method of Roberts and Rowe.[12] Long-term elastic recovery was assessed by measuring tablet height with a micrometer screw immediately after ejection and 24 hours post compression.

The Heckel analysis was repeated with materials with reduced moisture content to determine the effect of moisture content on mean yield pressure. A Computrac Max 2000 (Arizona Instruments, Phoenix, Ariz) was utilized to determine GBE moisture content under ambient storage conditions, to dry the materials, and to determine moisture content after storage with desiccant. The GBEs were dried at 105^{O}C (T, D, P, B). Source I degraded at 105^{O}C and was therefore dried at 90^{O}C. After drying each GBE, four quantities of the GBE necessary to prepare a zero porosity compact were quickly weighed and placed in a dessicator apparatus. The compression study was rapidly performed in triplicate at a punch speed of 10 tablets/minute. Afterwards, the fourth quantity was analyzed by loss on drying to determine the maximum amount of moisture contained in the samples during the compression process.

RESULTS

According to the minimum orifice diameter studies, source I exhibits the best flow properties. Two of the GBEs (P and D) would not flow at all through the largest orifice (34 mm). The remaining two (T and B) exhibited mass flow with a cylindrical 'rat hole' type appearance left in the cylinder with even the largest (34 mm) orifice. The ranking of flow from best to worst is: I, T=B,P=D. It is obvious that these botanical extracts are too cohesive for minimum orifice diameter tests to give valid results. This device may only be used to show relative improvement in flow.

The compressibility indices calculated from both Scott Volumeter or poured bulk densities indicate that the flowability of the various sources of GBE may be ranked from best to worst in the following order: I, P, B, T, D. According to Carr's ranking of relative flow, all sources have very poor (C = 33-38) to very, very poor (C $\geq$ 40) flow.[17] These results concur with those obtained by minimum orifice diameter with source I exhibiting the most promising flow properties. In addition, the compressibility index of dried source D exhibited statistically significant (α=0.05) improvement.

Deformation Properties (Table 2)

Student's t-test revealed significant differences (α=0.05) between 'fast' and 'slow' yield pressure values for GBEs B, T, and P. Comparative strain rate sensitivity indices (%) were as follows: Dibasic calcium phosphate

Table 1. Flowability of various *Ginkgo biloba* extracts

Ginkgo Biloba Extract	Compressibility Index ± % Coefficient of Variation			Flowability Index (mm)
	Poured Density As Received	Poured Density Dried	Scott Density As Received	
T	43.7±2.7		56.1±2.4	>34
B	41.5±4.2		51.9±0.9	>34
P	39.7±1.3		49.7±0.7	>>34***
D	46.4±2.9	36.6±5.9*	**	>>34***
I	32.9±3.6		39.9±1.8	26

* Average final moisture content = 0.25%.

** This material was very cohesive and it bridged the glass baffles of the Scott Volumeter.

*** This material was very cohesive and it bridged the largest orifice of the Flodex.

(Emcompress®) (-4.6 or ≅ 0); microcrystalline cellulose (Avicel PH-101®) (9.6); B (12.3); P (9.4); T (12.7); I (9.4); and D (3.8). However, maximum punch speed attainable on the press was not sufficient to reveal the well-documented time dependent deformation of microcrystalline cellulose. Attempts to determine long-term elastic recovery were complicated by swelling due to moisture sorption. Therefore, no conclusive elastic recovery data could be obtained. Student's t-test also revealed significant differences (α=0.05) between the yield pressures of dried extracts with reduced moisture contents, versus extracts with moisture contents as received for GBEs B, I, P, and T.

CONCLUSIONS

Results of the flow studies agree with the premise that many botanical extracts exhibit poor flow. It may be concluded that Ginkgo biloba extracts from the various sources selected exhibit poor flow. However, dynamic measurements (minimum orifice diameter) alone may not give valid results and should be used in conjunction with other tests such as compressibility index or shear cell analysis. When a dynamic measurement is performed, such as mass flow rate, mechanical energy (e.g. vibration) may need to be provided to facilitate flow of these highly cohesive extracts. Reducing the free moisture in poorly-flowing extracts may enhance flow properties significantly.

The results of the initial deformation studies suggest that the neat GBEs are plastic in nature and will probably form coherent capsule plugs or tablets. Although there were limitations to the maximum attainable punch speed, strain rate sensitivity indices support the premise that the GBEs are relatively plastic with time dependent characteristics. Further work will require the use of a compaction simulator. In addition, although the yield pressures were higher for reduced moisture extracts, the GBEs remained relatively plastic with yield pressures substantially lower than the brittle material, dibasic calcium phosphate.

Reducing free moisture in botanical extracts either by drying or perhaps by another technique may result in a freely flowing extract suitable for direct compression with minimal additional excipients. Further investigation is warranted.

Table 2. Deformation properties of various Ginkgo biloba extracts

Ginkgo Biloba Extract	Mean Yield Pressure (MPa) ± Coefficient of Variation			Strain Rate Sensitivity (%)	Moisture Content (%)	
	58 tabs/min. As Received	10 tabs/min. As received	Punch Speed 10 tabs/min., Dried Samples		As Received	Dried
T	110±6	96±4	110±3	12.7	2.203	1.381
B	113±4	99±0.6	115±3	12.3	2.761	0.583
I	117±8	106±6	121±2	9.4	1.639	0.879
P	125±6	113±3	160±4	9.6	2.759	1.305
D	52±3	50±3	57±3	3.8	3.115	0.807
Avicel PH-101®	135±13	122±5		9.6		
Emcompress®	217±23	227±13		-4.6 (≅ 0)		

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ORIGINAL RESEARCH

Delineation of Beneficial Characteristics of Effective Probiotics

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INTRODUCTION

Researchers have been producing literature on the nutritional and therapeutic benefits of lactic cultures from as early as 1908. Today evidence supported by clinical data documents that consumption of lactic cultures controls gastrointestinal infections,[1] reduces serum cholesterol,[2] helps in digesting lactose in lactose-intolerant individuals,[3] and reduces the incidence of colon cancer[4] and yeast infections by immunomodulation.[5] Recent studies also indicate that lactic cultures are useful in the management of food allergy.[6]

However, to be nutritionally and therapeutically beneficial to the host, lactic cultures should not only tolerate and pass through high stomach acidity (low pH), but also grow and proliferate at physiological levels of bile salts and adhere to intestinal epithelial cells.[7] Further, they should improve mineral absorption, and be good producers of ß-galactosidase and vitamins.[8] Therefore, efforts are being made to isolate strains with the aforementioned properties for maximum health benefits.

In the present study, we screened different lactic cultures, primarily *Lactobacillus acidophilus* and *Bifidobacterium longum*, for their acid/alkaline tolerance, bile tolerance, acid production, antimicrobial property, and ß-galactosidase activity, and for their ability to adhere to the intestinal mucosa.

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MATERIALS AND METHODS

Bacterial cultures: *Lactobacillus acidophilus* NRRL 629, NRRL 4495, NRRL 1910 were obtained from the National Center for Agricultural Utilization Research (Peoria, Ill). *L acidophilus* DDS-1 was obtained from the University of Nebraska, Department of Dairy Science Culture Collection. *Bifidobacterium longum* ATCC 15707 and 15708 are from American Type Culture Collection (Maryland). All cultures were maintained in 10% reconstituted non-fat dry milk (NFDM) supplemented with 0.5% yeast extract from Difco (Detroit, Michigan). Prior to each experiment, the cultures were transferred three times in NFDM at 1% innoculation level and incubated anaerobically at 37°C for 24 h.

Determination of pH and Titratable Acidity: The pH of the fermented bacterial media was determined with a Fisher pH meter model 291 (Fisher Scientific Co, USA). The titratable acidity of the broth was determined before and after fermentation by titrating 10 ml of the broth against 0.1 N sodium hydroxide to a final pH of 7.0 and reported as milliequivalents of sodium hydroxide. The difference in the titratable acidity before and after fermentation was reported as "developed acidity."

Determination of D and L lactic acid: A Boehringer Mannheim Corporation (Indianapolis, Ind) enzyme kit was used to determine D and L lactic acid in the media. Manufacturer's protocol was followed without deviation.

Determination of Antimicrobial activity: The antimicrobial activity of the fermented broths was determined by the disc assay method of Shahani et al.[9] To 95 ml of sterile antibiotic medium #4 (Difco) tempered at 40°C, actively growing *Bacillus subtilis* ATCC 6633 (18 h culture) was added at 5% v/v (10^7 CFU/ml) and mixed well.

The medium was immediately poured into sterile petri plates and allowed to solidify. Duplicate sterile 0.5-inch diameter antibiotic assay discs (Schliecher and Schuell, Inc) were picked up aseptically by sterile forceps and touched to the fermented broth so that they absorbed it via capillary action. The discs were immediately placed on the surface of the solidified agar medium containing *B subtilis*. The plates were inverted and incubated aerobically at 37°C for 6 h. The inhibition of *B subtilis* growth (antimicrobial activity) was determined by measuring the diameter of the antimicrobial zone.

Determination of ß-galactosidase activity: The ß-galactosidase activity of *L acidophilus* and *B longum* strains was determined by the method of Fisher et al.[10] On the day of each experiment, fresh solutions of ONPG containing 100 mg ONPG in 50 ml of 0.1M sodium phosphate buffer (pH 7.0) were prepared and used as the substrate solution. To a suitable volume of the test mixture (0.25 ml to 3 ml), 1.25 ml of ONPG solution was added, and the final volume was made up to 6 ml using 0.1M phosphate buffer. Subsequently, the mixture was incubated at 37°C for 15 minutes in a water bath. At the end of 15 minutes, the reaction was stopped by adding 2 ml of 1.0M cold sodium carbonate solution, and the optical density of the O-nitrophenol produced was read at OD420 using a blank for each sample in a spectrophotometer (Beckman model 25). ß-galactosidase activity was reported in micrograms of lactase/ml of fermented broth using a previously generated standard curve for pure *E coli* lactase (Sigma Chemicals, St. Louis, Mo).

Acid and alkaline tolerance: Ten ml of overnight culture of *L acidophilus* DDS-1 was centrifuged and washed thrice with phosphate buffered saline (PBS, pH 7.0) and resuspended in 10 ml of PBS. A volume of 200 µl of this cell suspension was added to 2 ml of sterile 0.2 M glycine-HCl buffer (pH 2.2) for acid tolerance or 2 ml of sterile 0.2 M Tris-HCl buffer (pH 8.0) for alkaline tolerance. The contents were mixed and were incubated at 37°C. Aliquots of 100 µl of the sample were taken every 15 min from 0 time to 2 h and total viable count was determined in MRS agar (Difco) at each sampling time.

Bile tolerance: MRS agar-containing bile salts with a concentration ranging from 0.05% to 0.2% were prepared. To the MRS agar-containing bile salts, 100 µl of overnight culture was added and the contents were pour-plated and incubated at 37°C anaerobically. After 48 h of incubation, the total viable count was determined.

In vitro binding to HT-29 cells: HT-29 colonic carcinoma cells (ATCC) were grown in McCoy's 5 A media (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotic solution (Life Technologies). The cultures were grown at 37°C in humidified atmosphere with 5% carbon dioxide, and passages were accomplished using trypsin. For assay, 10^5 HT-29 cells were grown to confluence in 12-well polystyrene plates. After 48 h, the wells containing confluent cells were washed thrice at 37°C with Earle's Balanced Salt Solution (EBSS). McCoy 5 A medium containing 10% FBS and no antibiotic was added to each cell. Overnight-grown *L acidophilus* and *B longum* strains, each standardized to 10^6 CFU/ml, were added to the wells separately, in triplicates. The contents were incubated at 37°C. After 1 h incubation, cells were washed six times with Dulbecco's Phosphate Buffered Saline (D-PBS) and were released from wells by adding trypsin. Following trypsinization, 1 ml of sterile cold D-PBS was added to each well and agitated to dissociate bound bacteria from HT-29 cells. The suspension was serially diluted and pour-plated with MRS agar to determine the counts of bound bacteria. For competitive binding studies, a mixture of *L acidophilus* DDS-1 and *E coli* ATCC 25922 was added to confluent HT-29 cells at different times. The ten different time-sequential treatments are described in detail in Table 3.

Statistical analysis: T test for dependent (correlated) variable was done using the computer software *Statistica* (StatSoft, Inc, Tulsa, Okla).

RESULTS AND DISCUSSION

Selection based on acid production: *L acidophilus* and *B longum* cultures showed a time-dependent decrease and increase in pH and titratable acidity, respectively, when grown on MRS medium. As evident from Table 1, the pH for all strains gradually dropped from 6.5 at zero hour to 3.7 after 48 h, except for *L acidophilus* NRRL 1910, where it dropped to 3.2. The production of titratable acid varied widely among the different strains at different times of incubation. After 48h of incubation, the strains *L acidophilus* NRRL 1910 and *L acidophilus* DDS-1 showed the highest acid production of 119.7 and 110 milliequivalents of 0.1N sodium hydroxide, respectively, whereas the other *L acidophilus* and *B longum* strains produced less than 100 mEq of acidity (Table 1). Although all the cultures tested in the present study produced a good amount of acid, a putative probiotic should produce more of L-lactic acid than any other acid.[11] Excepting *L acidophilus* NRRL 1910, the other three *L acidophilus* and two *B longum* strains, produced 2.3 to 2.5 g of L-lactic acid and a negligible amount of D-lactic acid per liter of medium after 48 h of incubation (Table 2). L. *acidophilus* NRRL 1910 produced relatively low levels of L and D lactic acid (less than 1 g/L), with approximately similar quantities of D and L lactic acid.

Selection based on ß-galactosidase activity: It has been suggested that most of the *L acidophilus* and *B longum* cultures are not efficient probiotics to alleviate the symptoms of lactose maldigestion due to their low produc-

Time (h)	*Lactobacillus acidophilus*								*Bifidobacterium longum*			
	NRRL 629		NRRL 4495		NRRL 1910		DDS-1		15707		15708	
	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH	Titratable acid	pH
0	10.5	6.5	11.3	6.5	10.8	6.5	10	6.5	9.8	6.5	10.7	6.5
12	10.5	6.5	14.5	6.4	45.0	5.7	18	6.5	14.9	6.3	10.7	6.5
18	16.8	6.3	32.4	5.8	69.0	4.9	35	5.3	16.2	6.3	16.4	6.3
24	29.6	5.9	67.6	4.7	99.0	3.9	70	4.1	32.2	5.8	29.8	5.9
30	77.4	4.4	83.6	4.2	111.0	3.5	92	3.9	70.6	4.6	64.9	4.8
36	83.8	4.2	90.0	4.0	112.2	3.5	110	3.8	86.6	4.1	87.2	4.1
48	96.6	3.8	96.4	3.8	119.7	3.2	110	3.7	93.0	3.9	92.9	3.9

Table 1. Changes in pH and titratable acidity during growth of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in MRS broth

Time (h)	*Lactobacillus acidophilus*								*Bifidobacterium longum*			
	NRRL 629		NRRL 4495		NRRL 1910		DDS-1		15707		15708	
	LACTIC ACID (g/L)											
	D-	L-	D-	L-	D-	L-	D-	L-	D-	L-	D-	L-
0	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0.3	0.0	0.5	0.29	0.27	0	0.75	0	0.46	0	0.45
18	0	0.5	0.1	0.9	0.50	0.43	0.02	1.45	0	0.49	0	0.48
24	0	0.8	0.2	1.8	0.76	0.62	0.14	2.38	0.04	0.89	0.04	0.87
30	0.2	2.0	0.3	2.2	0.86	0.69	0.3	2.3	0.21	1.84	0.21	1.79
36	0.3	2.2	0.3	2.3	0.88	0.71	0.35	2.25	0.28	2.26	0.28	2.21
48	0.3	2.5	0.3	2.5	0.95	0.76	0.38	2.5	0.31	2.41	0.31	2.35

Table 2. Production of D- and L- lactic acid during growth of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in MRS broth

tion of ß-galactosidase.[12] However, while screening lactic cultures for probiotic use, it is always beneficial to select a strain that produces high levels of ß-galactosidase for maximum therapeutic effects. We observed that *B longum* strains produced less than 1 unit of ß-galactosidase, whereas *L acidophilus* cultures produced activity up to 6 units (Figure 1). Amongst the strains studied, *L acidophilus* DDS-1 and *L acidophilus* NRRL 629 produced significant higher levels ($P < 0.05$) of 6 and 5.5 units of ß-galactosidase activity/ml of broth, respectively, at 30h of fermentation compared to other cultures. Although the activity remained the same for DDS-1 even after 48h of incubation, it dropped to 4 units by 48 h in the case of *L acidophilus* NRRL629.

Selection based on antimicrobial activity: All six strains of *L acidophilus* and *B longum* varied significantly in their antimicrobial activity against *B subtilis*. The antimicrobial activity increased with increase in incubation time (Figure 2). Within *L acidophilus*, the strain NRRL 1910 showed significantly ($P<0.05$) higher antimicrobial activity than the strains NRRL 629 and 4495. However, the difference in antimicrobial activity compared to that of *L acidophilus* DDS-1 was insignificant ($P=0.18$). Although both *B longum* strains demonstrated similar antimicrobial activity on *B subtilis*, their magnitudes were significantly lower than those of *L acidophilus* strains ($P<0.05$) (Figure 2). The antimicrobial property of lactic cultures is attributed to the production of natural antibiotics, hydrogen peroxide and organic acids.[13] The high degree of antimicrobial activity of *L acidophilus* 1910 and *L acidophilus* DDS-1 may be partly attributed to their higher acid production.

Treatment No.	Particulars	Harvest time
1	At 0 hours 100 µl of 10^6 CFU of *L acidophilus* DDS-1 was added to the well containing confluent HT-29 cells	4 hours
2	At 0 hours 100 µl of 10^6 CFU of *E coli* ATCC 25922 was added to the well containing confluent HT-29 cells	4 hours
3	At 0 hours 100 µl of 10^6 CFU of *L acidophilus* DDS-1 and 100 µl of 10^6CFU of *E coli* ATCC 25922 were added to the well containing confluent HT-29 cells	4 hours
4	At 0 hours 100 µl of 10^6 CFU of *L acidophilus* DDS-1 was added to the well containing confluent HT-29 cells; after 1 hour medium was aspirated and fresh medium along with 100 µl of 10^6 CFU of *L acidophilus* DDS-1 and 100 µl of 10^6 CFU of *E coli* ATCC 25922 were added	4 hours
5	At 0 hours 100 µl of 10^6 CFU of *L acidophilus* DDS-1 was added to the well containing confluent HT-29 cells; after 1 hour the medium was aspirated and fresh medium along with 100 µl of 10^6 CFU *L acidophilus* DDS-1 was added; and further, after 1 hour the medium was aspirated and fresh medium along with 100 µl of 10^6 CFU of *L acidophilus* DDS-1 and 100 µl of 10^6 CFU of *E coli* ATCC 25922 were added	4 hours
6	At 0 hours 100 µl of 10^6 CFU of *L acidophilus* DDS-1 was added to the well containing confluent HT-29 cells; after the 1st and 2nd hour the medium was aspirated and fresh medium along with 100 µl of 10^6 CFU *L acidophilus* DDS-1 was added; and at the 3rd hour after aspirating the medium, fresh medium along with 100 µl of 10^6 CFU of *L acidophilus* DDS-1 and 100 µl of 10^6 CFU *E coli* ATCC 25922 were added	4 hours
7	At 0 hours 100 µl of 10^6 CFU of *E coli* ATCC 25922 was added to the well containing confluent HT-29 cells; after 1 hour the medium was aspirated and fresh medium and 100 µl of 10^6 CFU *L acidophilus* DDS-1 were added	4 hours
8	At 0 hours 100 µl of 10^6CFU of *E coli* ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st and 2nd hour the medium was aspirated and fresh medium and 10^6 CFU of *L acidophilus* DDS-1 were added	4 hours
9	At 0 hours 100 µl L of 10^6 CFU of *E coli* ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st, 2nd, and 3rd hour the medium was aspirated and fresh medium and 10^6 CFU of *L acidophilus* DDS-1 were added	4 hours
10	At 0 hours 100 µl of 10^6 CFU of *E coli* ATCC 25922 was added to the well containing confluent HT-29 cells; after the 1st, 2nd, 3rd and 4th hour the medium was aspirated and fresh medium and 10^6 CFU of *L acidophilus* DDS-1 were added	4 hours

Table 3: Competitive binding of *L acidophilus* and *E coli* to HT-29 cells: details of the treatments

Selection based on acid, alkali, and bile stability: Due to the secretion of gastric juice, stomach normally has a pH of approximately 2.0. The highly acidic pH acts as a defense mechanism by killing pathogenic microorganisms entering the body with the food we eat.[14] The pH in the small intestine is generally 8.0 due to the secretion of pancreatic juices. To aid in the emulsification of fats ingested with food, a concentration of 0.05 to 0.1% of bile is secreted into the intestine.[15] Thus for any bacterium to survive in the stomach and proliferate in the intestine, it should be stable at low and high pH levels, and be tolerant to bile. Data on acid stability of *L acidophilus* and *B longum* cultures, in the present study, indicated that *L acidophilus* strains DDS-1 and NRRL4495 were fairly stable up to 120 min at pH 2.2. Their viability decreased only by 1 log at the end of 240 min. The strains NRRL 1910 and 629 showed a gradual decrease in viability, a loss of about 3 log CFU/ml within 240 min. Amongst the *B longum* strains, strain 15708 was more acid-tolerant than *B longum* 15707 (Figure 3). The stability of *L acidophilus* DDS-1 was found to be significantly higher ($P<0.05$) than all cultures under study except for LA 4495 which showed no significant ($P=0.4$) difference in stability at acidic pH when compared to LA DDS-1.

In the alkaline buffer (pH 8.0), *L acidophilus* DDS-1 and NRRL 1910 revealed significantly higher stability ($P<0.05$) with no substantial loss of viability up to 240 min; other cultures showed a 3 or 4 log CFU/ml decrease within 240 min (Figure 4). The fairly acid-stable strain *L aci-*

dophilus NRRL 629, was in fact, rather unstable in alkaline pH, whereas *L acidophilus* NRRL 1910 was unstable at acid pH, but stable at alkaline pH. *L acidophilus* DDS-1 was stable under both acid and alkaline conditions. In comparison to *L acidophilus* strains, both *B longum* strains were less stable at alkaline pH with a gradual loss in viability of up to 4 log CFU/ml within 240 minutes.

During exposure to increasing bile salt concentrations, the viability of all cultures decreased gradually, with a total loss at 0.25% bile salt concentration (Figure 5). Though the overall difference in stability was not significant, *L acidophilus* DDS-1 appeared to be the most resistant to bile salts amongst all strains and could withstand even 0.15% bile concentration with a viability of 27% compared to the control. At 0.1% bile salt concentration, more than 80% of *L acidophilus* DDS-1 survived in comparison to the 73, 58 and 42% viability in the case of *L acidophilus* strains NRRL 4495, NRRL 629, and NRRL 1910, respectively. *B longum* strains showed a viability up to 30% at 0.15% bile salt concentration and more than 65% viability at 0.1%.

Selection based on binding to intestinal cell lines: To become established as a successful inhabitant, bacteria must adhere strongly to the intestinal mucosal surface to not be dislodged by the contraction of the gut. The adhesive property of bacteria is generally measured by cell culture studies on intestinal cells, extracellular matrix, or basement membrane material. In the present study, the human colon tumor cell line HT-29 was used.[16] The binding capacity of *L acidophilus* strains DDS-1 to HT-29 cells was significantly higher ($P<0.05$) than all other strains studied. For example, 14 CFU were bound to a single cell of HT-29 cell line, whereas NRRL 1910, NRRL 629, and NRRL 4495 bound at 6, 3, and 2 CFU/cell of HT-29, respectively, after 1h of incubation at 37°C.

Based on the above observations, *L acidophilus* DDS-1 was further studied to determine its ability to scavenge or prevent adhesion of E coli to HT-29 cells, to determine whether DDS-1 could alleviate or reduce the incidence of diarrhea. Competitive studies revealed that *L acidophilus* DDS-1 replaced *E coli* in a time dependent manner. *L acidophilus* DDS-1 and *E coli* bound 14 and 15 CFU/cells of HT-29, respectively, when incubated separately. However, when HT-29 was incubated with approximately the same population of *L acidophilus* DDS-1 and *E coli, L acidophilus* DDS-1 bound in higher proportion than *E coli* on a per cell basis (Figure 6, treatment 3). Continuous-addition of *L acidophilus* DDS-1 to already bound *E coli*, effectively displaced *E coli* from HT-29 cells (Figure 6 treatment 7-10). Addition of *E coli* along with *L acidophilus* DDS-1 did not inhibit or decrease the binding of *L acidophilus* DDS-1 to HT-29 cells nor did it show any higher counts of E coli binding (Figure 6, treatment 4-6). This data indicates *L acidophilus* DDS-1 could potentially reduce binding of E coli to HT-29 cells.

CONCLUSIONS

Our screening studies revealed that *L acidophilus* DDS-1 is a putative probiotic strain. Its acid, bile, and alkaline stability will allow it to survive in the stomach and proliferate in the intestines, producing more of L-lactic acid than D-lactic acid, thus reducing the possibility of development of acidosis in the intestine. *L acidophilus* DDS-1 also demonstrated excellent binding to intestinal cell lines with a capability to displace *E coli*. This corroborates our earlier findings that *L acidophilus* DDS-1 inhibited the initiation of small tumors induced by N-nitrosobis (2-oxopropyl) amine in rats,[17] and that such inhibition may have been immunologically augmented.[18] The strain also possessed high antimicrobial activity, thus might potentially help to alleviate diarrhea and other intestinal infections. It also produced the highest quantity of ß-galactosidase and may be useful in reducing the symptoms of lactose maldigestion.

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LEGENDS TO THE FIGURES

1. ß-galactosidase activity of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures grown in MRS broth

2.Antimicrobial activity of MRS medium fermented with *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures

3. Stability of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in acidic pH (pH 2.2)

4. Stability of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in alkaline pH (pH 8.0)

5. Stability of *Lactobacillus acidophilus* and *Bifidobacterium longum* cultures in the presence of bile salts (o.05% to 0.25%)

6. Competitive binding of *Lactobacillus acidophilus* DDS-1 and *E coli* ATCC 25922 to HT-29 cells

REVIEW ARTICLE

Probiotic Bacteria and the Immune System

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ABSTRACT

A healthy well-functioning protective intestinal flora is of greatest importance in preventing inflammatory, allergic, ulcerogenic, and neoplastic diseases of the gut. The maintenance of a healthy flora involves ingestion of friendly bacteria (probiotics). Thus, probiotic bacteria are dietary requirements with limited or no direct nutritional value. However, their indirect effects include competition with potentially pathogenic bacteria, direct immune stimulation, secretion of vitamins, and secretion of immunomodulatory factors, which act either locally within the gut or in distant anatomical areas such as the spleen. Clinical studies have shown positive results when feeding probiotic bacteria to population groups susceptible to infections, including newborns, elderly, and immunosuppressed patients.

INTRODUCTION

The twentieth century has seen a significant increase in human life span, due in part to the control of infectious disease and improved health care. Along with these advances has come an enormous increase in easily prevented human disease related to lifestyle. Modern lifestyle choices, including reduced physical activity and the consumption of processed foods, chemicals, and drugs, contribute to the decrease in resistance to disease. The past century has seen changes in lifestyle to which the human genetic capability, echoing that of our distant ancestors, has been unable to fully adapt. This is especially true with regard to dietary habits.[1] Today's Western diet has been shown to predispose humans to inflammatory,[2] infectious,[3,4] ulcerative,[5,6] degenerative,[7] and neoplastic[8,9] diseases. Such diet includes an average consumption of 100 pounds of refined sugar per person per year; a ten-fold increase in sodium consumption; a four-fold increase in consumption of saturated fat; a doubled consumption of cholesterol; a reduced consumption of vegetable fibers, and of minerals such as potassium, magnesium, calcium, and chromium; as well as a considerable reduction in consumption of n-3 fatty acids, membrane lipids, vitamins, and antioxidants.[10]

Another element of the modern Western diet that differs from our ancestral diet is the virtual absence of dietary beneficial bacteria. Also called probiotic bacteria, these beneficial intestinal bacteria are part of naturally-preserved foods, such as sauerkraut, yogurt, and kefir. Research has shown that they play an essential role in health. A probiotic can be best defined as a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance.[11]

Most organisms studied in this respect are lactic acid producers, namely the lactobacilli and bifidobacteria. Some of the criteria proposed in selecting and evaluating the effectiveness of a probiotic bacteria are: the organism must have a beneficial effect on the host, must be non-pathogenic and non-toxic, must be able to survive transit through the GI tract and adhere to intestinal epithelial lining, and must have a good shelf life in food or powdered preparations.[12]

Today, influenced by the notion that bacteria are a cause of disease, it is a popular belief that any bacterial growth must be prevented through refrigeration, ultrafiltration, and other methods of preservation. As a consequence, our diet is deprived of friendly bacteria, and the only bacteria we encounter are those whose growth was not suc-

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cessfully prevented and which are often pathogenic, such as S*almonella*, *E coli,* and *Clostridium*. With regard to the pediatric population, for example, it is believed that children in Western societies may have difficulty developing a solid indigenous gut flora. Given the immunoenhancing properties of a good intestinal flora, this phenomenon may be linked to the increasing incidence of allergy and infection seen among Western children.[13,14]

Good intestinal flora contribute to health by secreting various compounds, including vitamins and natural antibiotics, by digesting certain foods and providing nutrients for the body, by supporting the body's immune function, by maintaining an intestinal environment that promotes healthy intestinal mucosa, and by preventing the growth of potentially pathogenic microorganisms (PPM). Unfortunately, in Western modern society, the widespread use of antibiotics is deleterious to the intestinal flora, and antibiotic therapeutic regimens frequently cause side effects linked to interference with the physiological microflora, as in an antibiotic kill of bacteria leading to an overgrowth of the fungus *Candida albicans.* It is now known that the physiological microflora play essential roles in the adequate functioning of organs such as the GI tract, skin, and those of the immune system.[15] This paper reviews the role of probiotic bacteria in promoting health through various mechanisms.

CHEMICAL CONTRIBUTION FROM THE PROBIOTIC FLORA

The gastrointestinal tract's primary role is the digestion and absorption of nutrients. However, an equally important role is to act as a reservoir with an internal surface area of ~200 m^2, protecting the human body from the external world and separating it from the nearly 10^{14} bacterial cells[16] and more than 500 bacterial species inhabiting the gut. These bacteria, which vary widely in physiology and biochemistry, exist in a multitude of different microhabitats in the lumen of the small and large intestines. From an anatomical point of view, the distant part of the GI tract traditionally has been seen as an appendage of the digestive tract, whose principal purpose was the conservation of salt and water, and the disposal of waste materials. However, it is now recognized that the metabolic potential of the human colonic microflora is impressive in terms of the number of biochemical reactions and transformations in which it participates. Bacteria play a key role in numerous processes in the large bowel, including carbohydrate and protein fermentation, bile acid and steroid transformations, metabolism of xenobiotic substances, development of the immune system, as well as the activation and destruction of potential mutagenic metabolites.[17] Healthy microbiota also play an important role in stimulating colonic motility and decreasing transit time—and therefore the opportunity for fermentation and the production of pathogenic catabolites by potentially pathogenic microrganisms.

Indeed, the GI bacterial flora exert their effects mainly through catabolic pathways. For example, in exchange for the supply of complex carbohydrates (starches and non-starch polysaccharides) from the host, intestinal bacteria produce butyrate, a bacterial fermentation product and principal source of energy for epithelial cells in the distal bowel. In certain conditions, butyrate can have anticarcinogenic properties.[18] The amounts and types of fermentation products (acetate, propionate, pyruvate) depend on the relative amounts of each substrate available, their chemical structures and compositions, and the dominant bacterial species inhabiting the gut.

Unlike carbohydrate metabolism, many products of protein fermentation (putrefaction) are toxic to the host. The production of these substances is often inhibited or repressed in the case of many intestinal bacteria by a fermentable source of carbohydrate, so putrefactive processes become more important in the distal bowel, where carbohydrate is more limiting.

In addition to producing nutrients for the mucosa, probiotic bacteria substantially contribute to the body's production of micronutrients such as vitamins of the B group, nicotinic acid, biotin, antioxidants, and polyamines.[19] Probiotic bacteria can also produce bioactive compounds such as histamine, 5-hydroxytryptamine, piperidine, and tyramine.[20]

PROBIOTIC FLORA AFFECTS THE IMMUNE SYSTEM

Several studies implicate GI tract microflora as mediators in the development of immune responses. In mice, certain antimicrobial drugs (mezlocillin in particular) were shown to modulate host defense mechanisms resulting in substantial suppression of cellular and humoral immune responses.[21] These immunosuppressive effects were a direct result of changes in GI tract microflora, from interactions between immune cells and antibiotics. In another study, sterilization of the GI tract resulted in the reduction of the chemotactic, bactericidal, and antitumor activities of peritoneal macrophages harvested 24 hours after antimicrobial treatment.[22] In addition to the aforementioned immunosuppressive effects, sterilization of the GI tracts of mice induced a significant atrophy of the thymus and spleen, and significantly decreased lymphocyte function in vitro and in vivo.

Conversely, Yasui et al.[23] found that oral administration of *Bifidobacterium breve* to mice activated the humoral immune system, augmented production of antirotavirus IgA and anti-influenza virus IgG, and protected against rotavirus and influenza. Consumption of fermented milk containing a mixture of *L casei* and *L acidophilus* was also shown to modulate systemic immune response.[24] Thus, human and animal studies in vivo, as well as numerous

clinical studies, indicate that intestinal flora affect many organ systems distant from the gut. The exact mechanisms of proximal and distal immune activation need to be extensively documented. However, the experimental models must be carefully chosen to produce meaningful conclusions. Several recent publications reported on the in vitro effects of mixing probiotic bacteria with purified subsets of peripheral blood lymphocytes. Haller et al.[25] showed that certain strains of lactobacilli were capable of stimulating the proliferation of natural killer cells (NK cells) as well as the expression of CD25 antigen and secretion of IFN-gamma from NK cells. It remains to be shown whether or not intestinal NK cells will respond in a similar manner to the peripheral blood NK cells.

Although the exact mechanism by which the indigenous GI tract microflora trigger basic immune responses is not fully known, one group has shown that certain species of probiotic bacteria liberate low molecular weight peptides (MW<6.5D) that appear to have immunomodulating effects.[26,27] In one model, the intestinal flora of mice was eliminated using antibiotic treatment, resulting in immunosuppression and modification of antitumor immunity. Similarly, Rangavajhyala et al.[28] showed that a non-lipopolysaccharide component of *L acidophilus* strain DDS-1 (LA1) had the ability to induce the production of interleukin-1 alpha (IL-1 alpha) and tumor necrosis factor-alpha (TNF-alpha), which have potent cytotoxic and cytostatic effects on tumor cells. The effect was observed whether living or heat-killed cells of *L acidophilus* were used. There could be a theoretical concern for the therapeutic needs in inflammatory bowel disease, as TNF-alpha levels appear to correspond to disease severity, and anti-TNF-alpha therapy is used for symptomatic treatment of inflammatory bowel disease. However, the TNF-alpha levels secreted in response to probiotics may, in the context of the overall immune modulation induced by probiotics, not have the adverse effect on inflammatory bowel disease that one could immediately suspect. Alternatively, different medical problems may require different strains of probiotic bacteria. In this context, it is interesting that the oral administration of *Lactobacillus* leads to strain-specific cytokine profiles in vivo.[28A] This study used an excellent experimental model, where *Lactobacillus* strains were fed to mice, and the in vivo cytokine induction was evaluated using immunohistochemical analysis of the gut villi. Further investigation into immune stimulation in vivo is much needed, and may lead to the discovery of a wide range of novel medical and biological applications of probiotic bacteria.

BACTERIAL COMPETITION

One such application involves the use of probiotic bacteria to control the overgrowth of potentially pathogenic microorganisms of bacterial, viral, and fungal origin. Such an application may be referred to as microbial interference treatment (MIT). The idea that probiotic bacteria can be effective weapons for preventing and treating many microbial infections is not a new one. In fact, by 1877 Pasteur and Joubert had already observed the antagonistic interaction between some bacterial strains, and by the turn of the century Metchnikoff had discussed the possibility of bacterial replacement therapy. Since then, a small group of scientists has been promoting the benefits of probiotic therapy for preventing infections and some other diseases. During the past 50 years mainstream science has intensely focused on the development of chemotherapeutics and antibiotics to combat disease. However, the past decade has brought renewed interest in infection control through microbial interference treatment for a number of reasons, including the following:

1. Increased awareness of the fact that antibiotics alter and often eradicate the protective flora, and therefore predispose the host to later infections.

2. The emergence of antibiotic-resistant strains of bacteria, a result of misuse and over-prescription of antibiotics.

3. Fear that industry will be unable to develop new drugs fast enough to compete with the development of microbial resistance to old drugs.

4. Public interest in more ecological methods of disease treatment.

The threat of antibiotic resistance is indeed a real one. The World Health Organization (WHO) considers it "a major public health problem in both developed and developing countries throughout the world," and "a significant economic threat as well."[29] Consequently, the WHO has recommended the creation of global programs to reduce the use of antibiotics in both commercial and medical settings. The realization that there is little hope for further treatment

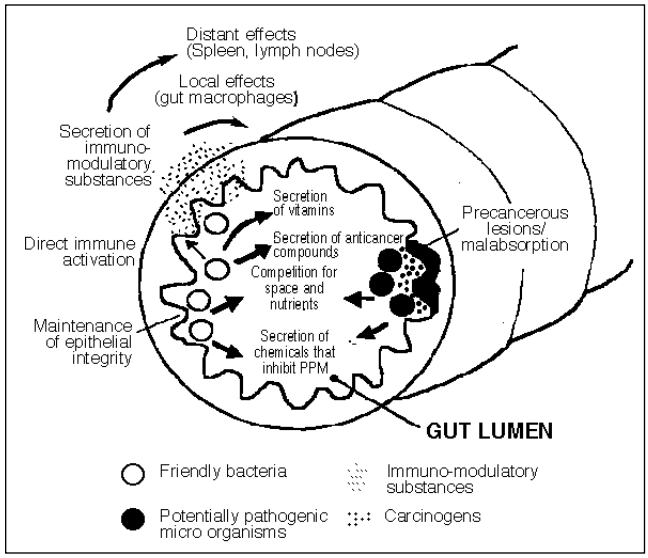
Table 1. Disorders moderated by probiotics

Intestinal disorders
Diarrhea
—Antibiotic-induced
—Travelers'
—Infantile
Constipation
Colitis
Salmonella and *Shigella* infections
Flatulence
Other disorders
Vaginitis
Alcohol-induced liver disease
Precancerous conditions (stomach, small bowel, colon)
Hypercholesterolemia
Skin problem (psoriasis, eczema)
Migraine
Encephalopathies

developments along the current paradigms of antibiotic therapy emphasizes the need for the discovery of newer and the rediscovery of older forms of treatments.

Many therapeutic and beneficial claims, included those listed in Table 1, have been studied and discussed extensively.[12,30] Several mechanisms have been postulated to explain the moderating effects of probiotics: 1) secretion of vitamins and anticancer compounds, 2) direct antagonistic effects on pathogens, 3) maintenance of epithelial integrity, 4) improved immune function and stimulation of appropriate immunomodulatory cells, and 5) competition for space as well as available nutrients and other growth factors (see Figure 1).

Figure 1



Despite the fact that the concept of microbial interference treatment has been known for more than a century, this field of research remains in its infancy. Although hundreds of publications on the topic exist, there is still little application of probiotic therapy in mainstream medicine. In a critical review of the role of lactic acid bacteria as a promoter of human health, Sanders stated that "this research area has suffered from a lack of coordinated efforts between the clinicians and the microbiologists, and that differences in strains, levels, model system, and stringency of data interpretation lead to apparent inconsistencies in conclusions from published research."[31] For these reasons microbial interference treatment has never received full acceptance by the medical community.

It is interesting to note that in the first half of the twentieth century, before the discovery of antibiotics, intestinal toxemia, characterized by the growth of pathogenic bacteria in the gut, was considered the primary cause and etiology of many diseases and disorders.[32] One of the main treatments for intestinal toxemia was the reintroduction of probiotic bacteria in the gut.

SPECIFIC ROLES OF VARIOUS PROBIOTICS IN HEALTH

For a long time the idea prevailed that adherence to mucosal surfaces was essential for probiotics to colonize, exert interference with potentially pathogenic microrganisms, and promote health. Therefore, the fact that very few strains of commercially available *Lactobacillus* have the ability of colonizing and adhering to the GI tract mucosa (or even adhering in vitro to Caco-2 and HT-29 cell lines) over a period of a few days contributed to the unpopularity of probiotic therapy. However, more recent studies have shown that several species of probiotics, sometimes referred as "transient bacteria", bring their health-promoting effect as they pass through the GI tract, without necessarily adhering to the mucosa.[33] For example, common commercial dairy strains promoted as health-enhancing substances such as *L bulgaricus* and *S thermophilus* are neither adhesive to Caco-2 cells nor in vivo in humans. Nevertheless, they have been shown to promote health by contributing to maintaining an intestinal environment friendly to probiotics and hostile to PPM. In a double-blind study on travelers', a mixture including *L bulgaricus* and *S thermophilus* was shown most effective at preventing travelers' diarrhea.[34]

On the contrary, other species such as *L acidophilus*, *Lsalivarius*, and *L casei* have been shown to adhere to the intestinal mucosa.[35,36] As a consequence, various species of probiotics are now known to promote health in species- and strain-specific ways. One strain of *Lactobacillus* that received much attention in terms of positive mucosal adherence, and which has been clinically tried in the context of MIT, is *Lactobacillus rhamnosus* GG (ATCC 53103). *L rhamnosus* GG has shown a lot of promise for the prevention and treatment of diarrhea in premature infants,[37] newborns,[38] including those in intensive care,[39] and travelers'.[40] In particular, *L rhamnosus* GG has been shown to shorten acute rotavirus diarrhea in a group of 42 well-nourished children given two doses of 10^{10} lactobacilli every day for five days.[41,42] It has also been shown to be effective against severe intestinal infections such as *Clostridium difficile.*[43]

A very important and often neglected aspect regarding GI health is the permeability of the intestinal mucosa. A pathogenic increase in intestinal permeability, often referred as leaky gut syndrome, may sometimes be the etiology of several disorders, including immune and nervous disorders. For example, leaky gut syndrome has been associated with chronic fatigue, allergies, enteritis, and rheumatism. It was shown in the rat that many types of food common in Western diets, such as cow milk, damaged the integrity of the intestinal mucosa and led to increased intestinal permeability. Consumption of *L rhamnosus* GG was shown to rapidly restore a normal permeability of the intestinal mucosa.[44] In addition, *L rhamnosus* GG was also shown to provide prophylaxis and a potential to act as a therapeutic

agent against *Candida albicans*, by a variety of immunologic and nonimmunologic mechanisms.[45] Oral administration of *Lactobacillus casei* strain Shirota (LcS) has also been found to enhance murine innate immunity by stimulating the activity of splenic NK cells.[46] *L rhamnosus* GG is also effective in the prevention and treatment of travelers' diarrhea and antibiotic-associated diarrhea in children.[40,47]

L salivarius, commonly available on the market for less than a decade, is another probiotic bacteria with promising application. *L salivarius* can prevent and treat infection with the ulcer- and colitis-inducing *Helicobacter pylori*.[48] *L salivarius*, along with *L bulgaricus*, is able to inactivate carcinogens and prevent DNA damage in the colon of rats, possibly by producing thiol-containing breakdown products of proteins through bacterial digestion.[49]

The probiotic bacterium *L plantarum* has been shown to increase nutrient absorption in children with human immunodeficiency virus (HIV) infections.[50] *L plantarum* was also able to increase the production of immunoglobulin and to stimulate the circulation of specific immune cells.[51] It produces plantarin, a wide-spectrum natural antibiotic.[52,53] This probiotic bacterium is widely used in the fermentation of foods, eg, sausages, sauerkraut, and pickles.

Nutritional support for vulnerable population groups, including premature babies and the elderly, should include oral probiotics.[54] Clinical studies have shown beneficial effects on the immune system in these groups. In one study, the effect of *Lactobacillus* and *Bifidobacterium* was investigated on both the intestinal and the peripheral immune system. Ingestion of probiotics (*Lactobacillus* and *Bifidobacterium*) in the elderly resulted in reduced colonic inflammation and an increase in the frequency of B lymphocytes in the peripheral blood.[55] High doses of *Lactobacilli* have documented positive clinical effect on diarrhea in children.[56] Recently, data from a double-blinded, placebo-controlled, European multi-center trial was published; it involved 287 young children with acute diarrhea, and demonstrated that microbial interference treatments resulted in shorter hospital stays and fewer complications.[57] Further studies are needed to develop clinical applications in children, and examine the outcome when microbial interference treatment is used in conjunction with antibiotic therapy.

Probiotic bacteria therapy seems clearly promising in the treatment of colitis and irritable bowel diseases,[58,59] certain cancers of the GI tract,[28,49,60] autoimmune diseases,[61] cardiovascular problems,[62] and food allergies.[63]

CONCLUSION

The study of probiotic bacteria remains of high priority for further clinical studies to establish many unsolved questions regarding the mechanisms of direct and indirect immune stimulation. The profound effects of a healthy gut flora on a wide spectrum of clinical conditions deserve serious attention for integration into existing therapeutic strategies.

ABBREVIATIONS

GI Gastrointestinal
MIT Microbial interference treatment
PPM Potentially pathogenic microorganisms

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REVIEW ARTICLE

The Role of Homocysteine in Atherogenesis

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ABSTRACT

The atherogenic effect of homocysteine was discovered by study of children with homocystinuria caused by different enzymatic disorders of homocysteine metabolism. The atherogenic effect of homocysteine is attributed to a direct effect of the amino acid on the cells and tissues of the arteries. Hyperhomocysteinemia is an independent risk factor for arteriosclerosis, and other established risk factors act synergistically with hyperhomocysteinemia to increase vascular disease risk. The most important cause of hyperhomocysteinemia in populations susceptible to arteriosclerosis is deficiency of vitamin B6 and folate caused by significant losses of these unstable vitamins through food processing. The significant decline in mortality from coronary heart disease mortality in the U.S. since the 1960s is explained by the addition of vitamin B6 and folic acid to the food supply through voluntary fortification of cereals and supplements. Dietary improvement by consumption of fresh whole foods and fortification of processed foods by vitamin B6 and folic acid will assure continued decline in mortality from heart disease in the future.

DISCOVERY OF THE ATHEROGENIC EFFECT OF HOMOCYSTEINE

A nine-year-old mentally retarded girl with dislocated ocular lenses was examined in the pediatric clinic of the Massachusetts General Hospital in 1965. The pediatricians demonstrated the presence of homocystine in urine and blood and diagnosed the newly discovered disease, homocystinuria. The mother of the girl told the pediatricians that an uncle of the girl had died of a similar disease in the 1930s and that the case had been published in the medical literature. The uncle was an eight-year-old mentally retarded boy with dislocated ocular lenses who was treated at the Massachusetts General Hospital in 1933 for hemiplegia, coma, and stroke. In the discussion of the case the pathologist commented that the thickening of the wall of the carotid artery was similar to the changes of arteriosclerosis found in elderly men.[1]

Study of children with homocystinuria had shown that the enzyme cystathionine synthase was abnormal in these cases.[2] This enzyme catalyzes the formation of cystathionine from homocysteine and serine with the participation of the coenzyme pyridoxal phosphate. Many early cases of homocystinuria were found to have thrombosis of arteries and veins throughout the body, and abnormally reactive platelets were considered to explain this finding in some cases. Vascular lesions in these cases were attributed to the effects of thrombosis or to a hypothetical lathyrogenic effect of homocysteine. There was little evidence of hypercholesterolemia or deposition of cholesterol and lipids in arterial plaques, and none of the early reports described the arterial lesions as arteriosclerotic.

Because I was working in the department from which the 1933 index case of homocystinuria had been published, I was able to restudy the findings in this case. My study disclosed scattered arteriosclerotic plaques in arteries throughout the body, in addition to the carotid arteriosclerosis that was described in the original case report. It was not possible to draw conclusions about the possible role of homocysteine in the pathogenesis of these arteriosclerotic plaques because other metabolic abnormalities caused by cystathionine synthase deficiency could have been responsible for the findings.

By chance in 1968 a second case of homocystinuria with unusual features was studied at Massachusetts General Hospital, National Institutes of Health, and Brandeis University. A two-month-old infant was found to have both homocysteine and cystathionine in urine and blood, clearly demonstrating a different etiology of homocystinuria from earlier cases. Detailed metabolic and enzymatic studies demonstrated a deficiency of methionine synthase (methyltetrahydrofolate homocysteine methyl transferase), causing the metabolic disturbance which was titled cobalamin C disease. This enzyme is dependent upon folate and cobalamin (vitamin B12) for activity. In previous cases of homocystinuria the deficient enzyme was cystathionine synthase, which is dependent upon pyridoxine (vitamin B6) for activity.

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In reviewing this critical index case of cobalamin C disease, I discovered rapidly progressive arteriosclerotic plaques in the infant's arteries.[3] This discovery demonstrated that, at least in these rare cases of inherited metabolic disease, elevation of blood homocysteine causes arteriosclerotic plaques by a direct effect on the cells and tissues of the arteries, regardless of the enzymatic abnormality leading to homocystinuria. Identical arteriosclerotic plaques in a child with a third type of homocystinuria caused by deficiency of the enzyme methylenetetrahydrofolate reductase confirmed this conclusion concerning the direct effect of the amino acid homocysteine in causing arteriosclerosis.[4] This interpretation explains two important animal models of experimental arteriosclerosis from the earlier literature, pyridoxine-deficient monkeys and choline-deficient rats.

The discovery led to formulation of the homocysteine theory of arteriosclerosis, suggesting that homocysteine is atherogenic, not only in children with rare inherited diseases of homocysteine metabolism, but also in the general population.[3] Thus hyperhomocysteinemia caused by dietary deficiencies of pyridoxine, folate, or cobalamin, genetic abnormalities of homocysteine metabolism, exposure to drugs and toxins like cigarette smoke, hormonal abnormalities like hypothyroidism, postmenopausal hormonal changes, hypertension, diabetes, and radiation injury is of importance in the pathogenesis of arteriosclerosis in persons without known inherited diseases.

In support of the homocysteine theory of arteriosclerosis, injection of homocysteine or feeding homocysteine in an experimental diet causes arteriosclerotic plaques in rabbits.[5,6] The plaques are primarily of the fibrous and fibrocalcific type, associated with deposition of extracellular sulfated matrix, deposition of calcium salts and collagen fibrils, and destruction of elastica interna. Higher doses of homocysteine or methionine cause thrombosis of iliac veins and pulmonary embolism and infarction in rabbits,[6] complications found in children with homocystinuria or older persons with arteriosclerotic vascular disease.

Subsequent results confirmed these findings by demonstrating vascular injury, arteriosclerotic plaques, and thrombosis in baboons given intravenous homocysteine.[7] The arterial plaques and thrombi associated with hyperhomocysteinemia in animals are of the fibrous and fibrocalcific type found in children with homocystinuria. Deposition of cholesterol and lipids is not observed in these plaques, either in children with homocystinuria or in experimental animals. In older survivors with homocystinuria, however, typical atherosclerotic plaques and aneurysms do contain cholesterol and lipid deposition. Moreover, in animals fed an experimental diet containing fats and cholesterol, the fibrous arterial plaques develop prominent lipid deposition with conversion of the plaques to the typical fibrolipid plaques of the type found in advanced human atherosclerosis.

HOMOCYSTEINE AND THE PATHOGENESIS OF ARTERIOSCLEROSIS

Wherever one looks at the pathophysiological processes by which arteriosclerotic plaques are formed, homocysteine plays a key role.[8] In arteriosclerosis all of the fundamental changes in tissues and in plaques require an important contribution from the amino acid homocysteine.

In children with homocystinuria and in animals with hyperhomocysteinemia, increased synthesis of extracellular sulfated glycosaminoglycan matrix is a key feature of early and developing arteriosclerotic plaques. Study of fibroblasts cultured from the skin of children with homocystinuria and cystathionine synthase deficiency demonstrated that the cells produce an aggregated highly sulfated matrix of reduced solubility, helping to explain the deposition of metachromatic extracellular glycosaminoglycan matrix in early plaques. This change in sulfation and solubility is attributable to a helix-coil transition of proteoglycans caused by binding of homocysteine thiolactone to carboxyl groups. Radiolabelled homocysteine thiolactone and homocysteic acid were found to be precursors of the sulfating coenzyme, phosphoadenosine phosphosulfate, which is responsible for introducing sulfate esters into glycosaminoglycans. A scheme for participation of thioretinamide in synthesis of sulfite, sulfate, and phosphoadenosine phosphosulfate implicates reactive oxygen radicals like superoxide as oxidizing agents to oxidize the sulfur atom of the homocysteine thiolactone moiety of thioretinamide.[9] Thioretinamide is formed from homocysteine thiolactone and retinoic acid.

The smooth muscle cells of arteriosclerotic plaques proliferate abnormally, forming thickened layers and nodules of these cells within the intimal layer of arteries. Studies with cultured endothelial cells have shown that high concentrations of homocysteine in the culture medium cause cellular damage by oxidative stress with formation of hydrogen peroxide. Studies with cultured smooth muscle cells have shown that homocysteine increases the growth of these cells by increasing synthesis of cyclin mRNA, triggering cellular synthesis of DNA and cell division. These hyperplastic smooth muscle cells synthesize increased amounts of collagen both in cell culture and in developing arteriosclerotic plaques.

In cellular metabolism of oxygen, thioretinaco is hypothesized to form a disulfonium active site formed by oxidation with ozone.[9] Thioretinaco is synthesized from homocysteine thiolactone, retinoic acid, and cobalamin. Thioretinaco ozonide and oxygen, bound as an ion cluster, are capable of binding ATP in a biochemical model of oxidative phosphorylation. Loss of thioretinaco ozonide both from malignant cells and from proliferating smooth muscle cells in developing arteriosclerotic plaques leads to loss of control of growth and increased oxidative stress because of accumulation of reactive oxygen radicals. These reactive oxygen species may account for the intimal

damage found in early arteriosclerotic plaques in children with homocystinuria, in plaques associated with hyperhomocysteinemia in experimental animals, and in arteriosclerotic plaques in human arteriosclerosis. Loss of thioretinaco ozonide from cellular membranes is suggested to underlie the change in oxidative metabolism associated with cellular aging, leading to accumulation of oxygen radicals and oxidative degradation of cellular macromolecules.[9]

Arteriosclerotic plaques induced by hyperhomocysteinemia in homocystinuria and in experimental animals are of the fibrous and fibrocalcific type with little evidence of lipid or cholesterol deposition. When lipids are fed in an experimental diet or when cholesterol and lipid levels of plasma become elevated in older individuals with homocystinuria, typical fibrolipid plaques with cholesterol and lipid deposition are observed. High doses of homocysteine cause increased synthesis of triglycerides, cholesterol, and apoB protein of low-density lipoprotein in experimental animals. A possible explanation of this effect is increased availability of acetyl-coenzyme A because of inhibition of oxidative phosphorylation and oxidative metabolism by homocysteine thiolactone, which converts thioretinaco to thioco, a complex composed of cobalamin and homocysteine thiolactone.[9]

Low-density lipoproteins contain appreciable amounts of homocysteine, particularly in persons with high plasma cholesterol and lipoprotein levels. When normal low-density lipoprotein is incubated with homocysteine thiolactone, a highly thiolated form of the lipoprotein particle is produced by homocysteinylation of the amino groups of apoB protein. This highly homocysteinylated low-density lipoprotein becomes small and dense, has increased electrophoretic mobility, and self-aggregates and precipitates spontaneously.[10] These LDL-homocysteine aggregates are taken up by cultured human macrophages to form foam cells. Degradation of LDL-homocysteine aggregates by foam cells leads to deposition of lipids and cholesterol in developing plaques and to changes in oxidative metabolism produced by excess homocysteine. These observations show that homocysteine converts LDL to the form that is known to be highly atherogenic, carrying homocysteine to the cells of artery walls where damage occurs by increased oxidative stress.

Foam cells are recognized as participants in the inflammatory aspects of arteriosclerosis, and the release of homocysteine from LDL-homocysteine aggregates of foam cells provides a means by which the inflammatory response is encouraged. Administration of the freshly synthesized free base form of homocysteine thiolactone to the skin of animals elicits an intense inflammatory response. With prolonged exposure to this compound, the skin develops hyperplasia of stromal and epithelial cells, granulation tissue, angiogenesis, and dysplasia of epithelium, leading to microinvasive squamous cell carcinoma.[11] The inflammatory response triggers release of multiple cytokines, adhesion molecules, and growth factors that participate in the cellular features of developing arteriosclerotic plaques.

In children with homocystinuria and in experimental animals with hyperhomocysteinemia, vacuolization, swelling, hyperplasia, and degeneration of endothelial cells are morphological indicators of endothelial dysfunction. Homocysteine induces endothelial dysfunction in man and animals by counteracting the effect of nitric oxide through formation of S-nitrosohomocysteine. This compound no longer has the relaxing effect of nitric oxide on the smooth muscle of artery wall. This effect also leads to increased vascular permeability and increased flow of plasma through damaged endothelium, carrying LDL and LDL-homocysteine aggregates into the heart of developing plaques.

Some patients with homocystinuria have decreased platelet survival and increased platelet adhesiveness. Addition of low concentrations of freshly synthesized homocysteine thiolactone free base to normal platelets causes intense aggregation. Homocysteine also causes activation of multiple coagulation factors, including factors V, VII, Xa, and XII. In addition homocysteine induces endothelial tissue factor, inhibits thrombomodulin and protein C activation, and inhibits tissue plasminogen activator by binding to annexin II.[12] Homocysteine also enhances binding of lipoprotein(a) to fibrin in low concentrations, inhibits von Willibrand factor expression, increases thromboxane and decreases prostacyclin synthesis, and suppresses heparin sulfate expression. Through these multiple effects, homocysteine is regarded as a potent prothrombotic factor of critical importance in thrombogenesis.

HYPERHOMOCYSTEINEMIA AS A RISK FACTOR FOR VASCULAR DISEASE

Beginning with the first human study in 1976 [13] hyperhomocysteinemia has been demonstrated to be a major independent risk factor for vascular disease in many clinical and epidemiological studies over the past two decades. Individuals with cerebrovascular, peripheral vascular, and coronary artery disease have elevated levels of blood homocysteine, compared with healthy controls. Depending upon the definition and method for determining hyperhomocysteinemia, about 10-40% of vascular disease patients have elevated homocysteine levels, according to metanalysis of multiple studies.[14] Although most studies demonstrate that hyperhomocysteinemia is independent of blood cholesterol and lipid levels, a few of the larger studies show a weak correlation of blood homocysteine with dyslipidemia. Most studies have examined fasting levels of blood homocysteine, but in those studies examining post-methionine loading, increased hyperhomocysteinemia is also demonstrated to be a risk factor for vascular disease.[15]

More detailed examination of known risk factors for vascular disease in population studies demonstrates a correlation with blood homocysteine levels in the expected

direction.[16] Such factors as male sex, premenopausal status, aging, lack of exercise, lack of fruits and vegetables in the diet, excess coffee consumption, hypertension, obesity, and insulin resistance all correlate with hyperhomocysteinemia. Moreover, major risk factors interact synergistically with elevated blood homocysteine levels. For example, hypertensive women with hyperhomocysteinemia have a 25-fold increased risk of vascular disease, compared with a 5 to 10 fold increased risk with either factor alone.

Most prospective studies show a correlation between blood homocysteine and risk of vascular disease events and mortality. For example, among 21,000 men followed for 9 years, the risk of fatal and non-fatal myocardial infarction is proportional to the blood homocysteine level.[17] In 14,000 male physicians, the risk of myocardial infarction is over 3-fold greater in those with elevated blood homocysteine over a 5-year period.[18] In over 500 men with proven coronary arteriosclerosis, the risk of mortality is correlated with blood homocysteine over a 5-year period, but blood cholesterol levels fail to predict mortality.[19] A few negative prospective studies have been attributed to differences in patient selection, definition of hyperhomocysteinemia, short duration of follow-up, genetic predisposition, and other factors.

Over the past decade the fasting level of blood homocysteine associated with increased risk of vascular disease has been addressed in multiple studies. Originally, homocysteine levels of greater than 16 mcM were associated with increased risk. More recently, larger studies have shown that risk is proportional to the homocysteine level even in the normal range of 6-10 mcM. A study of stored plasma samples from the 1970s recently showed that countries such as Japan, France, and Spain with a low mortality rate from cardiovascular disease have homocysteine levels of 7-8 mcM.[20] Countries with a high mortality rate from vascular disease, such as Finland, Scotland, Northern Ireland, and Germany have homocysteine levels of 10-11 mcM. This study suggests that levels of 7-8 mcM are desirable for prevention of arteriosclerosis.

Now that accurate and reproducible testing of plasma homocysteine is available with quick turnaround time and reasonable pricing, wider use of this test is warranted for assessing risk in increased numbers of patients. As many as half to two-thirds of patients with vascular disease have no identifiable risk factors, including dyslipidemia, smoking, hypertension, diabetes, or obesity.[21] The recent Centers for Disease Control and Prevention report on decline in vascular disease in the US comments on the importance of identifying and studying emerging risk factors for vascular disease, including homocysteine.[22] Since homocysteine is an independent and potent risk factor for vascular disease, homocysteine testing will identify many of those patients at risk who have no other identifiable risk factors. Accordingly, in the future homocysteine testing should assume the same importance as testing for dyslipidemia in assessment of risk. Other disease groups besides vascular disease that will benefit from homocysteine testing include diabetes, renal failure, thyroid disorders, Alzheimer's disease and cognitive dysfunction, schizophrenia, cancer, and rheumatoid arthritis. It is safe to predict that homocysteine testing will enter the clinical diagnostic armamentarium in a major way in the next few years.

VITAMINS, HYPERHOMOCYSTEINEMIA, AND VASCULAR DISEASE

The most important factor leading to hyperhomocysteinemia in the general population is deficiency of three B vitamins, vitamin B6 (pyridoxine), folic acid, and vitamin B12 (cobalamin). These three vitamins are precursors of the coenzymes of the enzymes responsible for regulating the synthesis and disposal of homocysteine within the body. Deficiency of either folic acid or vitamin B12 causes elevation of blood homocysteine in the fasting state. Deficiency of vitamin B6 causes elevation of blood homocysteine after an oral dose of methionine or following a meal containing abundant protein.

The only biochemical precursor of homocysteine is the methionine of dietary proteins. Methionine is converted to adenosyl methionine, the methylating coenzyme responsible for the majority of the methylation reactions in metabolism. Adenosyl methionine regulates homocysteine synthesis and disposal by allosteric activation of cystathionine synthase and by inhibition of methylenetetrahydrofolate reductase. Cystathionine synthase and its coenzyme pyridoxal phosphate are responsible for synthesis of cystathionine, which is converted to cysteine, cysteine sulfinic acid, sulfate, taurine, and other related compounds for excretion in the urine or utilization in metabolism. Methionine synthase and its coenzyme methylcobalamin are responsible for transfer of the methyl group from methyltetrahydrofolate to homocysteine to reform methionine. Methyltetrahydrofolate is generated from methylenetetrahydrofolate by the enzyme methylenetetrahydrofolate reductase. In the liver, a separate pathway transfers methyl groups from betaine to homocysteine to form methionine, catalyzed by betaine homocysteine methyl transferase.

Large-scale epidemiological studies demonstrate an increased mortality and morbidity from coronary heart disease among individuals with insufficient dietary intakes of vitamin B6 or folic acid. The Nutrition Canada Study found a 50% increase in death rate from coronary heart disease among individuals with low levels of blood folate in a 15-year follow-up study of 5000 men and women.[23] The Nurses' Health Study found a 30% decrease in mortality and morbidity from coronary heart disease among women with high dietary intakes of either vitamin B6 or folate in a 14-year follow-up study of 80,000 subjects.[24]

A survey of 1100 elderly participants in the Framingham Heart Study found that blood homocysteine levels become elevated when the intake of vitamin B6 is

below 3 mg per day or when the intake of folate is below 350 mcg per day.[25] The intake of vitamin B12 does not correlate with homocysteine levels, but when the blood vitamin B12 level is marginal or deficient because of decreased absorption, the blood homocysteine level is elevated. In a follow-up study of carotid arteriosclerosis using ultrasound assessment of wall thickness, those participants with elevated homocysteine levels were found to have a greater incidence of stenotic lesions.[26] The results of the Framingham survey agree well with the Nurses' Health Study, indicating that the amount of folic acid needed to prevent increased homocysteine levels, 400 mcg per day, is precisely the amount needed to decrease vascular disease risk. Similarly the amount of vitamin B6 needed to prevent increased homocysteine levels, 3 mg per day, is precisely the amount needed to decrease vascular disease risk. In addition, this intake of vitamin B6, adjusted for body weight, is the amount needed to prevent arteriosclerotic plaques in monkeys fed a diet deficient in pyridoxine.

The death rate from coronary heart disease reached a peak in the mid 1960s in the US. In the past three decades the death rate has steadily declined, until now the rate is less than half of the incidence of 30 years ago. Dietary intakes of fats and cholesterol have changed very little since the 1960s, and blood cholesterol levels have only declined about 5%. At a nationwide conference in 1979, none of the accepted risk factors for cardiovascular disease were found to explain this major decline in disease incidence.[27] A factor that explains the large decline in vascular disease mortality is the addition of vitamin B6 and folic acid to the food supply beginning in the late 1960s and early 1970s.[28] These vitamins are provided through voluntary fortification of breakfast cereals and through consumption of vitamin supplements. In the early 1970s the amount of vitamin B6 consumed per capita from these sources reached about 2 mg per day. In 1998 the Food and Drug Administration mandated the addition of folic acid to refined grain foods in an amount sufficient to provide 100 mcg per day to the average person. A recent study showed that this level of fortification doubled the folate content of blood and significantly decreased the blood homocysteine level among Framingham Heart Study participants who were studied in 1996 before fortification and in 1998 after fortification.[29]

FOOD PROCESSING, VITAMIN DEFICIENCY AND HYPERHOMOCYSTEINEMIA

An adequate supply of the three B vitamins, B6, folic acid, and B12, is needed to prevent hyperhomocysteinemia in the population. Why then are dietary intakes of B6 and folic acid so low? The reason is that these two vitamins are sensitive to the chemical and physical conditions imposed by processing of food.[30] Factors such as heat, light, chemical oxidants, and milling of grains cause major losses of these nutrients, when comparing the amounts in fresh whole foods with the amounts in processed foods. In the case of vitamin B12, food processing modifies the molecular structure of the vitamin, but enzymatic processes within cells and tissues are capable of restoring the full vitamin activity from these modified forms. Only with decreased absorption of vitamin B12 in the elderly or in pernicious anemia does the amount of B12 in cells and tissues become limiting.

Two of the most deleterious processes to vitamin B6 and folic acid are canning of foods and milling of grains. In commercial canning foods are heated to the boiling point or autoclaved under pressure in order to sterilize them and to prevent growth of microorganisms. This heat treatment causes the loss of about 50-85% of both vitamin B6 and folic acid from canned foods. In the case of fish or processed meats, canning causes the loss of 50-75% of vitamin B6. Freezing of vegetables and fruits causes a smaller loss of vitamin B6 and folic acid because the heating step to blanch produce destroys only about 15-30% of these vitamins.

Milling of grains to produce white flour, rice flour, or cornstarch typically causes a loss of 80-85% of both vitamin B6 and folic acid from whole grains. Because white flour is used so extensively in commercial food preparation, its use is a major factor in causing inadequate vitamin B6 and folic acid dietary intakes. Vitamin B6 is not added to fortify white flour and other refined grains, and only in 1998 has folic acid been added to fortify these food products. Other factors causing loss of these nutrients are chemical oxidants, ionizing and non-ionizing radiation, a variety of additives and preservatives, and extraction of sugars and oils from vegetables and grains.

In 1989 the Recommended Dietary Allowance for folic acid was lowered from 400 mcg per day to 200 mcg per day. One reason for this action is that the average American eats only about 250 mcg per day. In 1998 the RDA, now called the Recommended Dietary Intake (RDI) was restored to 400 mcg per day, and the Food and Drug Administration approved addition of 140 mcg per 100 g of refined grain foods. The stated reason for this reversal of position and requirement for added folic acid in fortified foods is prevention of neural tube defects in newborn infants. Although prevention of hyperhomocysteinemia and vascular disease was not cited as a reason for this historic reversal of policy by the FDA, addition of folic acid to the food supply by fortification will help to continue the decline in heart disease that has been underway for three decades.

The RDI for vitamin B6 is 1.3-1.7 mg per day, depending upon age, sex, and pregnancy in the case of women. The average American eats about 1.6 mg per day of vitamin B6. Yet the Nurses' Health Study clearly shows that consumption of less than 3 mg per day of vitamin B6 is associated with an increased death rate from coronary heart disease. The same amount of vitamin B6 is needed to prevent arteriosclerosis in monkeys fed an experimental diet, when

adjusted for body weight. The Food and Nutrition Board, which sets the RDI amounts for nutrients, has never acknowledged the role of vitamin B6 or folic acid in prevention of hyperhomocysteinemia and vascular disease. In my opinion, the RDI for vitamin B6 should be raised to 3 mg per day, and this vital nutrient should be added in sufficient amount to refined grains and processed foods to restore the amount removed by food processing. This policy will help to continue the decline in heart disease mortality in the US.

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L-Arginine and Its Role in Cardiovascular Diseases

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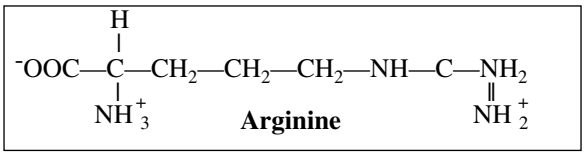
INTRODUCTION

There has been a lot of interest in L-arginine within the last two years simply because of the awarding of the Nobel Prize to doctors Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad for their discovery of nitric oxide (NO) as a signalling molecule in the cardiovascular system.[1]

Found in the endotehlium of blood vessels is the semi-essential amino acid L-arginine, the substrate precursor to nitric oxide (NO). L-arginine is a basic genetically-coded amino acid ($C_6H_{14}N_4O_2$) with a moluecular weight of 174.20 and an isoelectric point (pH) of 11.15, as shown in Figure 1. The enzyme nitric oxide synthase endogenously catalyzes the oxidation of the terminal nitrogen atom of L-arginine to produce the signalling molecule NO and citrulline.[2]

Figure 1



THE ENDOTHELIUM AND ENDOTHELIUM FUNCTION

The endothelium consists of cells that line blood and lymphatic vessels, the heart, and other body cavities. Endothelial cells essentially separate blood from tissue and filter and transport stubstances in and out of blood. Endothelial cells are metabolically active, secreting a host of factors that regulate the local environment, among them: substances that inhibit or promote growth of smooth muscle cells; antithrombotic, anticoagulant, and fibrinolytic factors; and anti-inflammatory and antioxidant agents.

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Capillaries are composed exclusively of endothelial cells. Larger blood vessels have additional layers of connective tissue and smooth muscle, which give tone and substance to blood vessel walls. A major negative outcome of endothelial dysfunction is the production of atherosclerosis. Hypertension, diabetes, heart failure, hypercholesterolemia, and smoking can lead to endothelial dysfunction.

Endothelium-produced nitric oxide is a potent vasodilator. It also inhibits adhesion of platelets and leukocytes to the endothelium and inhibits proliferation of smooth muscle cells.[3] Hence, we explore known nitric oxide therapueutic effects in cardiovascular disease.

It has been demonstrated that coronary blood flow response to acetylcholine, an endothelium-dependent vasodilator, decreases significantly with aging. A 1996 study done by Chauhan et al. demonstrated that L-arginine restored the endothelial function of coronary vessel microcirculation in older subjects.[4] It has also been shown that endothelium-dependent vasodilatation is impaired in hypercholesterolemic humans and that this abnormality can be improved acutely by administration of L-arginine.[5] In 1996 Dubois-Rande et al demonstrated that intravenous L-arginine improved endothelial vasodilator function in atheromatous left anterior descending arteries.[6] Several other studies confirmed these findings with use of both intravenous and oral L-arginine supplementation. These studies have shown improvement in endothelial and vascular function in young hypercholesterolemic adults and on exercise capacity in patients with stable angina pectoris.[7] It has been demonstrated that coronary artery stenosis dilatation can be induced by L-arginine. These positive effects of L-arginine are probably based upon providing the endothelial NO synthase with adequate substrate to enhance NO synthesis.

It is also believed that L-arginine has an antioxidant effect by either neutralizing superoxide anions produced in the endothelium or preventing their release.[8] While the effects of L-arginine have been found to enhance endothelial function, a May 2000 study by Blum showed that oral L-arginine therapy does not improve NO bioavailability in

coronary artery disease patients on appropriate medical management and thus may not benefit this group of patients.[9] In his double-blind study, 30 coronary artery disease patients on appropriate medical management were randomly assigned to L-arginine (9 g) or placebo daily for one month with crossover to alternate therapy after one month off therapy.

At the end of each treatment period, nitrogen oxides in serum (as an index of endothelial NO release), flow-mediated brachial artery dilatation (as an index of vascular NO bioactivity), and serum cell adhesion molecules (an index of NO - regulated markers of inflammation) were measured. L-arginine significantly increased arginine levels in plasma compared with placebo. However, there was no effect of L-arginine on nitrogen oxides, on flow-mediated dilatation of the brachial artery, nor on intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. In Blum's study, most patients were taking Statin lipid-lowering therapy, which can enhance endothelial-dependent vasodilator response. Had these individuals not been on Statin drugs, or had they been more hypercholesterolemic, a positive effect might have been shown.

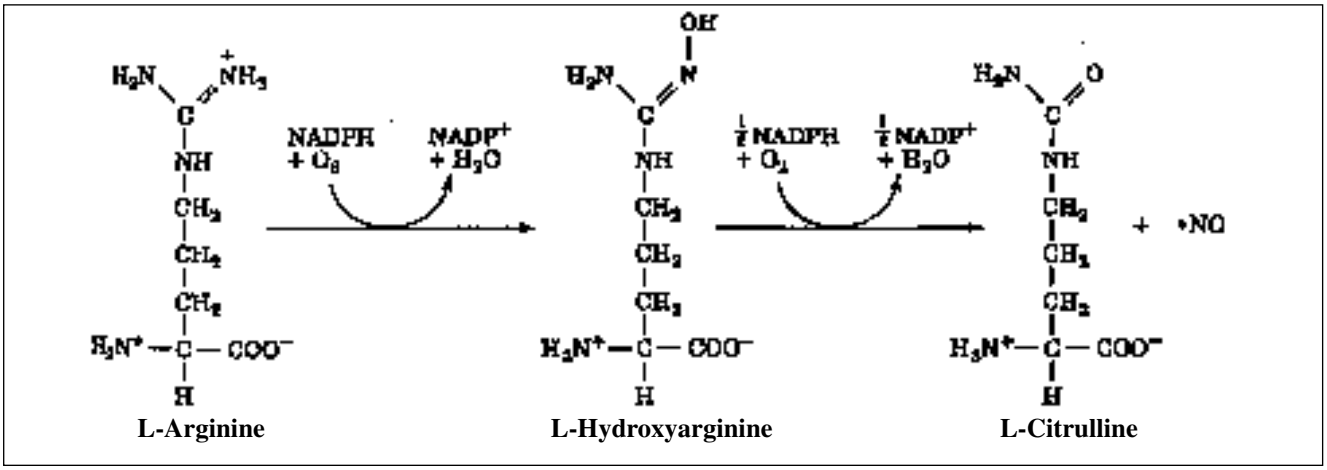
The major effect of L-arginine is in the enhancement of nitric oxide synthesis, a major benefit to patients with stable angina pectoris and coronary artery disease and patients with hypercholesterolemia. However, L-arginine has been shown to act as an antioxidant by inhibiting the release from the endothelium of the powerful free radical superoxide anion.[10] It is interesting to note that intracellular levels of L-arginine are in the millimolar range[11] whereas the endothelial NO synthase K_m is in the micromolar range.[12] K_m is the substrate concentration at which the reaction velocity is half maximal. When an enzyme has a small value of K_m, it achieves maximal catalytic efficiency at low substrate concentrations. It was shown by Miyazaki et al, that patients with atherothrombotic disease showed increased endogenous NO synthase inhibitor asymmetric dimethyl arginine (ADMA).[13] Infusion of L-arginine can decrease the competitive inhibition by ADMA of NO synthase (Figure 2). L-arginine is first converted to L-hydroxyarginine in the reaction that converts L-arginine to NO and citrulline. Arginase activity happens to be increased in patients with abnormal vascular biology. Arginine use by this patient group can decrease arginase activity, but the intermediate product L-hydroxyarginine can also inhibit arginase and, by so doing, increase the intracellular levels of L-arginine.[15]

Numerous interactions with L-arginine and other substances have been reported in the medical literature including inhibition and impaired transport of L-arginine into endothelial cells by lysophosphatidyl choline and oxidized LDL.[16,17] L-arginine has been shown to increase insulin secretion and histamine release from mast cells.[18] Both can promote vasodilatation. L-arginine has been shown to promote an acidic environment, extracellularly and intracellularly. Acidic environments can enhance the reduction of nitrite to nitric oxide.[19] In addition, L-arginine can cause attenuation of the vasoconstrictor norepinephrine and subsequently exert a positive influence on vasodilatation.[20]

While the use of coronary artery stents in association with angioplasty has become commonplace throughout the world, the restenosis rate is about 15-25 percent. The cause of restenosis may be due to proliferating smooth muscle cells and monocyte adhesion and infiltration into the endothelium, which contribute to the neointimal formation. It has been shown that intramural delivery of L-arginine immediately after angioplasty causes a sustained increase in tissue L-arginine levels associated with enhancement of local NO synthesis. This increase is associated with an attenuation of monocyte binding and increased apoptosis (programmed cell death) of resident macrophages,[21] a treatment strategy that could be valuable for the prevention and management of restenosis.

Endothelium-dependent vasodilatation is impaired in hypercholesterolemia, even prior to development of athero-

Figure 2. The reaction that converts arginine to NO and citrulline is catalyzed by **nitric oxide synthase (NOS):**



sclerosis. A 1992 study done by Creager et al revealed that this abnormality of impaired vasodilatation can be improved acutely by administration of L-arginine.[22] Intravenous L-arginine augmented the forearm blood flow response to methacholine in the hypercholesterolemic individuals, but not in normal subjects.

Cigarette smoking is associated with increased monocyte endothelial cell adhesion when endothelial cells are exposed to serum from healthy young adults. This abnormality has been shown to be reversible. A study by Adams et al demonstrates by biochemical measurement that the intracellular adhesion molecule (ICAM-1) was increased in smokers. After administration of oral L-arginine, the endothelial cell expression of ICAM-1 was reduced.[23]

CONGESTIVE HEART FAILURE

Exercise-induced vasodilatation of the extremities is attenuated in patients with heart failure. In addition, patients who are in heart failure have an attenuated endothelium-dependent vasodilatation evoked by acetylcholine. Kubota et al have shown that the intra-arterial infusion of L-arginine increases not only endothelium-dependent vasodilatation response to acetylcholine, but also ischemic vasodilatation during reactive hyperemia in patients with heart failure.[24] It has also been reported that cardiac performance can be improved by intravenous infusion of L-arginine in patients with moderate congestive heart failure. Additionally, a 1996 double-blind placebo-controlled study reported by Rector et al showed improvement in cardiac performance in heart failure patients after supplemental oral L-arginine administration.[25]

PERIPHERAL VASCULAR DISEASE

In a 1996 study done by Gryglewski et al., 22 patients with peripheral arterial obstructive disease received intravenous L-arginine infusions, for three hours daily for seven consecutive days.[26] This treatment resulted in elongation of the pain-free and maximum walking distances as well as shortening of the period of time required for pain relief after walking the maximum distance. A rise in the ankle-arm pressure ratio was associated with an increase of arterial blood flow in both calves, and the transcutaneous oxygen tension in the ischemic foot was also increased. In addition, ADP and collagen-induced platelet aggregation was suppressed, the plasma level of platelet-activated inhibitor decreased, and cGMP levels increased.

The above data certainly indicate beneficial effects of L-arginine as a positive therapeutic agent in patients with peripheral arterial obstructive disease. The presumption in these patients is that the administration of L-arginine is most likely converted to NO, enhancing endothelial function. Platelet aggregation is an important physiopathologic mechanism in both coronary artery disease and peripheral vascular disease. Marietta et al showed in 1997 that the in vivo administration of L-arginine induced vasodilatation in humans and, when orally supplemented, reduced platelet aggregability both in hypercholesterolemic rabbits and healthy men.[27] It has been demonstrated that platelets contain a constitutive, calcium-dependent NO synthase activated by collagen-induced platelet aggregation. This leads to a nitric oxide synthesis from L-arginine, which in turn increases cyclic GMP and down-regulates platelet aggregation. A study by Bode-Böger et al shows that urinary excretion of cyclic GMP increased 65.4 percent after L-arginine and 25.1 percent after placebo, and that aggregation was inhibited 32.7 percent after L-arginine while unchanged after placebo.[28] The conclusion here is that intravenous L-arginine decreased peripheral arterial tone and inhibited platelet aggregation in healthy human subjects by enhancing NO formation and concomitantly cyclic GMP formation.

GENERAL

The literature is replete with scientific studies showing the beneficial effects of L-arginine and endothelial function as it is converted to NO, which enhances endothelial dilator function, decreases platelet aggregation, and prevents adhesion of leukocytes and monocytes to the endothelium. Other effects that are beneficial that have been shown in the literature: When L-arginine is administered in pharmacologic doses to rodents, it improves their nitrogen balance after injury,[29] enhances wound healing,[30] and effects a number of T lymphocyte functional parameters including increases in enzymic weight, total lymphocyte count, and blastogenic response of thymic lymphocytes to mitogens.[31] Similar wound healing and immune-enhancing properties have been noted in humans supplemented with arginine.[32] L-arginine possesses strong secretatagogue properties, stimulating the release of growth hormone and prolactin from the pituitary, and insulin and glucagon from the pancreas.[33-35] Because of the numerous beneficial effects, arginine is being supplemented alone or as part of a complete nutritional regimen in clinical practice. However, I believe we need more placebo-controlled double-blind randomized studies with oral L-arginine in larger number of patients who have endothelial dysfunction, coronary artery disease, angina pectoris, hypertension, congestive heart failure, and peripheral vascular disease to solidify the previous data that have been reported in this review article. In a May 2000 editorial, Loscalzo states that L-arginine continue to be investigated as a beneficial agent in the treatment of endothelial function: such research may shed some light on the complex mechanisms of its action.[36]

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REVIEW ARTICLE

Nutritional Supplements for the Pet with Heart Disease

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Major advances have been made in the creation of diagnostic tools for assessment and in the evolution of new treatment therapies for heart disease in the last two decades. Increased availability of noninvasive procedures such as echocardiography, electrocardiography, radiology and electronic blood pressure measurement have made it possible for the veterinarian to make precise measurements of cardiac function. New generations of pharmaceutical agents have provided therapies to improve cardiac function and quality of life for the veterinary patient. Specific nutrients have also been shown to be of benefit as adjuncts to conventional drug therapies. In this article, we will examine the more relevant supplements and what we know about them. Some have proven to be of limited benefit; others, such as the amino acid taurine, have proven to be an important nutritional discovery for feline health.

In 1987, published research demonstrated a cause and effect relationship between dietary taurine deficiency and dilated cardiomyopathy in cats.[1] This condition is characterized by the development of an enlarged heart with very thin walls that results in poor contractility and eventual death. Taurine supplementation was shown to actually reverse this condition. Cats lack the ability to manufacture sufficient quantities of taurine from the precursor amino acids cysteine and methionine. They also use taurine, rather than glycine, for bile acid conjugation; the loss of taurine through bile excretion adds to the demand for dietary sources of this essential amino acid. Taurine is essential for the regulation of calcium uptake and movement in the heart muscle cells.

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Prior to this research, consistently adequate amounts of taurine were not found in commercial cat foods. Quality pet food manufacturers quickly responded by supplementing their cat foods with taurine, and the incidence of this deadly disease (as well as blindness caused by taurine deficiency) dramatically decreased. This is still an important consideration since some cats have access to dog food, which is not supplemented, and others are fed homemade diets. Taurine is available in supplement form by itself, in combination with other vitamin/mineral/nutrient formulations, and is also found in some products such as clam juice. The recommended daily supplementation of taurine for cats is 500 to 1,000 mg per day given in divided dose.[2] Cats that develop dilated cardiomyopathy and are eating a good quality commercial cat food should still be supplemented with taurine as some of these will still show a response.[3]

Dogs are normally able to synthesize adequate levels of taurine, and they, too, use taurine for bile acid conjugation. There have, however, been reports of taurine-responsive dilated cardiomyopathy. One study revealed low plasma taurine levels in 17% of dogs with dilated cardiomyopathy. Of these 13 dogs, seven were Cocker Spaniels or Golden Retrievers.[4] Another study found that 14 Cocker Spaniels with dilated cardiomyopathy had low plasma concentrations of taurine.[5] These findings have led researchers to suspect a genetic defect in the manufacture or homeostasis of taurine.[6] The dose used in the above Cocker Spaniel study was 500 mg taurine given orally three times daily.[5] Other researchers reported using 500 mg taurine orally three times a day for dogs between 23 and 30 pounds and 1000 mg orally three times daily for those between 50-60 pounds.[3]

The quaternary amine L-carnitine, produced in the liver, is found in high concentrations in the heart and skeletal muscle, where it serves to transport long-chain free fatty acids into the mitochondria. The primary source of energy for the heart is through the metabolism of fatty acids. An association has been found between L-carnitine deficiencies in the myocardium and cardiomyopathies in certain

breeds, such as Cocker Spaniels, Boxers, Doberman Pinschers, Great Danes, Irish Wolfhounds, Salukis and Newfoundlands.[7] Forty percent of dogs with dilated cardiomyopathy have low myocardial levels of L-carnitine; however, plasma L-carnitine levels may not actually correlate with levels found in the heart muscle.[8] While endomyocardial biopsy is the definitive method of determining myocardial levels of L-carnitine[8], physical or economic constraints may limit the use of this tool. It is not known if all cases of dilated cardiomyopathy in dogs with carnitine deficiency are deficient prior to or as a result of this condition. There have been reports, however, of benefit from L-carnitine supplementation in American Cocker Spaniels, Boxers and Dalmatians.[3] One study noted improvement in myocardial function in two dogs with dilated cardiomyopathy with low myocardial L-carnitine levels when the dogs were supplemented with L-carnitine; myocardial function subsequently worsened when supplementation was discontinued.[9] Some dogs have benefited from combined taurine and L-carnitine supplementation.[5] The d-form and d,l-carnitine formulations should not be used as the d-form is nonfunctional as a transport compound and competes with the available l-form, resulting in a worsening of cardiac function. While taurine is relatively inexpensive, the major drawback to routine L-carnitine supplementation is its cost.

Coenzyme Q_{10} (ubiquinone) is a lipid-soluble vitamin-like compound synthesized in the liver and found in the mitochondria of all cells in the body where it functions in the formation of ATP (adenosine triphosphate), the energy source for the cell. Because of the high energy demands of the heart, myocardial cells have the highest number of mitochondria. While there is not a cause-and-effect relationship, studies have shown a decrease in CoQ_{10} levels in patients with chronic heart disease. A number of human clinical trials have shown benefit from CoQ_{10} supplementation. CoQ_{10} was determined to be of benefit in cardiac disease states such as hypertrophic cardiomyopathy (a condition in which ventricular walls are thickened causing a decrease in ventricular capacity and contractility),[10] idiopathic dilated cardiomyopathy,[11-14] and other cases of heart disease which resulted in heart failure.[15-17] Veterinary cardiologists have reported clinical improvement in canine and feline patients with severe heart disease; dog breeds treated included the Doberman Pinscher, Great Dane, St. Bernard, and Saluki.[18] Improvement was reported in a case of mitral regurgitation. Another cardiologist reported improvement in some cases of cardiomyopathy in dogs. In cases where CoQ_{10} supplementation was discontinued, clinical signs worsened; improvement occurred when therapy was reinstituted.[18] Doses range from 30 mg per day for cats to 120 mg per day for giant-breed dogs.[19] Coenzyme Q_{10} is highly lipophilic and research has shown better absorption of oil-based products.[18,20] No adverse effects have been seen with the use of coenzyme Q_{10}.

Intake of long chain n-3 polyunsaturated fatty acids (omega-3 fatty acids) primarily found in cold water marine fish, such as salmon, has been associated with improved cardiac function. Docosahexaenoic acid, eicosapentaenoic acid from fish oil, and alpha-linolenic acid from plant sources have been studied in humans and animals with positive results.[21,22] Substitution with these fish oils or other oils in the diet of human patients reduced the incidence and severity of arrhythmias that resulted from ischemia.[22] Fatal ventricular fibrillation was also significantly reduced,[22] and as little as one fatty fish meal per week has been associated with a 50% reduction in the risk of primary cardiac arrest.[23] Marmosets fed a diet rich in these oils had a higher threshold for ventricular fibrillation when ischemia was induced by coronary artery occlusion.[24] A study of Doberman Pinschers with dilated cardiomyopathy revealed that sudden death occurred in 20% of these dogs, and atrial fibrillation occurred in 30% with resulting death in about 4 weeks.[25] The ability to reduce the occurrence of these events would definitely be beneficial! In Chongqing, Sichuan, The People's Republic of China, an association was found between low omega-3 fatty acid levels and an increased incidence of dilated cardiomyopathy in the population.[26] The evidence suggests that incorporation of these omega-3 fatty acids into the phospholipid membrane of cardiac cells inhibits sodium ion channels by increasing membrane fluidity, that is, to increase its resistance to electrical disturbance.[27,28] Dogs with congestive heart failure secondary to dilated cardiomyopathy that were supplemented with omega-3 fish oil showed a trend toward improved fractional shortening (the amount the heart is able to contract to pump blood) and an 80% reduction in the level of tumor necrosis factor, a cytokine that leads to further degenerative changes.[29] In healthy dogs, omega-3 oils were shown to decrease platelet aggregation, but not to affect other clotting factors or cause clinically significant changes in bleeding times.[30] This is beneficial as it helps to prevent thrombus (clot) formation resulting in a myocardial infarction. In a small study, cats fed the omega-3 fish oils did not demonstrate this effect.[31] Since one of the complications of hypertrophic cardiomyopathy in cats is the formation of thromboemboli, further studies are warranted to verify or refute this finding. Feeding these oils to cats with heart disease will have no detrimental effect, so they may be given without harm pending the results of further studies. It would be wise to be sure of adequate vitamin E supplementation for those cats given fish oil to prevent oxidation of the fatty acids leading to "steatitis", a painful inflammation of the fatty tissues. The daily dose of omega-3 fatty acids, depending on the size of the pet, ranges from 250 to 1,000 mg per day.[32]

Magnesium supplementation in such forms as magnesium citrate, magnesium aspartate hydrochloride, and magnesium chloride has also been evaluated for its effects on

cardiac function in human clinical trials. Magnesium deficiencies have been seen in patients with congestive heart failure, likely due to the use of loop diuretics, such as furosemide, ethacrynic acid and bumetamide[33] and may lead to arrhythmias, which may be fatal.[34] An epidemiologic study showed a correlation between low dietary magnesium and incidence of death in patients with ischemic heart disease.[35] One study showed a decreased rate of ventricular arrhythmias and a decrease in mean arterial pressure in those receiving magnesium supplementation.[34] Other studies evaluating effects on blood pressure (in patients not specifically having low serum magnesium levels) showed mixed results, however.[36-39] It stands to reason that patients having a low baseline serum magnesium level when starting supplementation are more likely to benefit than those that have normal levels before starting supplementation. One animal study showed that the high blood pressure and harmful effects on the kidneys of rats receiving cyclosporin A in conjunction with a high sodium diet were ameliorated by supplementing with magnesium.[40] Mechanisms by which magnesium supplementation may affect blood pressure are via a lowering of intracellular sodium levels,[37] an increase in intracellular levels of magnesium and potassium,[37,41] and possibly an effect on intracellular levels of calcium.[42] The conclusion is that magnesium may counteract the electrolyte imbalances caused by loop diuretics.[41] Many cats are currently fed a magnesium-restricted commercial diet to reduce the incidence of struvite urolithiasis; however, urine pH appears to be a more significant factor in the formation of these crystals.[43] Reduced dietary magnesium was shown to lower serum total and ionized magnesium levels.[44] Cats with heart disease are commonly treated with loop diuretics. The combination of a loop diuretic with a magnesium-restricted diet has the potential to create a hypomagnesemic condition; therefore, serum magnesium levels in these cats should be closely monitored. Hypermagnesemia has been known to occur in animals and people with compromised kidney function. Serum magnesium levels should be checked prior to and monitored during administration of magnesium.

All or some of the above supplements may play a role in managing different types of heart disease; however, more clinical trials in animals are needed to determine the exact benefits and which animals would benefit most. On the whole, these supplements have been shown to be safe, and there are no known contraindications to their use, with the exception of magnesium in the face of compromised kidney function. When formulating a treatment plan including the use of cardiac drugs and dietary modifications, the use of one or more of these supplements may be indicated. As with all dietary supplements, care must be taken to select quality products from companies that will provide validated assays. Select them as if your pet's life depends on it, because when it comes to the heart, it just may.

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