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REVIEW ARTICLE

The Central Role of Excitotoxicity in Autism Spectrum Disorders

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INTRODUCTION

In this discussion I shall define autism spectrum disorders as a group of disorders of higher cortical function ranging from attention deficit disorder to full blown autism itself. Despite divisions into numerous individually-named disorders – Asperger's, high autism, attention deficit hyperactivity disorder, etc. – many, including myself, feel that they represent a spectrum of related cognitive disorders. I recognize that clinically, several may have characteristics that make them significantly different from the others. Their special physiology and biochemistry will be addressed in this paper as the need arises.

Recent evidence indicates that most neurological disorders, both acute and chronic, have a common set of pathological events despite their varying clinical presentations.[1] At the center of this process is what has become known as excitotoxicity. Named in 1969 by Dr. John Olney, excitotoxicity is a phenomenon characterized by the triggering of neuronal excitation through over-stimulation of susceptible neurons by the excitatory amino acids, primarily glutamate and aspartate.[2]

Using cloning techniques, scientists have characterized five sets of excitatory receptors: NMDA, AMPA, kainate and two metabotrophic-type receptors.[3] We know the most about the NMDA receptor, which controls a voltage-gated calcium channel. Clustered around the calcium channel are various regulator receptors, including the zinc and magnesium sites that modulate the channel, so as to prevent overactivation, and a glycine receptor which enhances the signal during NMDA receptor activation. A phencyclidine receptor powerfully inhibits the opening of the calcium channel.

Glutamate is the most abundant neurotransmitter in the central nervous system, yet it is also the most neurotoxic. It is for this reason that its concentration outside the neuron is so carefully controlled. A family of glutamate transport proteins, which attach to the transmitter soon after its release, maintains this control. Shortly after its release it is transported it to a nearby astrocyte, where it is deposited.[4]

Excess levels of glutamate, or other excitatory molecules, allow the calcium channel to remain open for a relatively long period of time. Calcium excess in the cytosol of the cell triggers the activation of inducible nitric oxide synthase and protein kinase C. The iNOS produces NO in excess, which begins to accumulate within the cell. When NO combines with the superoxide radical it forms the very destructive peroxynitrite radical. This radical is particularly injurious to the mitochondria, the neuron's chief source of energy.[5]

At the same time, protein kinase C then activates phospholipase A2 within the neuron membrane, which brings about the release of arachidonic acid into the cytosol. Here the arachidonic acid is acted on by two enzymes, lipoxygenase and cyclooxygenase, which produce a series of potentially destructive eicosanoids. Of particular concern is the COX II enzyme, which brings about the accumulation of PGE2 and PGD2, both pro-inflammatory molecules. Interestingly, only glutamatergic neurons contain COX II enzymes, which are located on distal dendrites and are concentrated in dendritic spines.[6]

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The accumulation of inflammatory eicosanoids leads to the production of free radicals, including the destructive hydroxyl radical. As the process accelerates, free radicals interact with the neuron's numerous membrane structures, including nuclear, mitochondrial and cellular membranes. Once this process begins, a chain reaction within the membrane's polyunsaturated fatty acids is initiated, a process we call lipid peroxidation (LPO).

Numerous byproducts are produced during lipid peroxidation, including several aldehydic products. While the most abundant of these LPO products is malondialdhyde (MDA), most destructive is a product called 4-hydroxynonenal.[7] Recent research has shown that 4-HNE can produce extensive damage to the cell, including the prevention of dephosphorylation of excessively phosphorylated tau protein, significantly interfering with microtubule function.[8] It has also been shown to inhibit glutathione reductase needed to convert oxidized glutathione to its functional reduced form.[9] It has been demonstrated that children with active autoimmune diseases have significantly higher blood levels of 4-HNE than controls.[10]

It is also known that 4-HNE can conjugate to synaptic proteins, where it impairs the transport of both glucose and glutamate.[11] Several studies have shown that this process is especially dangerous because impaired energy supplies markedly enhance glutamate sensitivity. In fact, under such conditions, even normal levels of glutamate can produce neurotoxicity.[12] Peroxynitrite, by damaging mitochondrial membranes, DNA and electron transport enzymes, can also significantly reduce neuronal energy production.[13]

It is known that numerous pathological events can trigger excitotoxicity, including ischemia, hypoxia, hypoglycemia, viral and bacteriological pathogens, toxic metals, trauma, autoimmune diseases, and free radical excess. It should also be recognized that there is an intimate relationship between excitotoxicity and free radical generation. Free radicals precipitate the release of glutamate in the brain and excitotoxins trigger the production of large numbers of free radicals, both of the oxygen and nitrogen species.

Two events associated with the autism spectrum disorders play a major role in precipitating excitotoxicity, seizure foci and immune activation. It is my contention that these two events are central to the disorder, especially in the face of immune dysfunction.

SEIZURES, AUTISM, AND EXCITOTOXICITY

It has been recognized that seizures are common with several of the autism spectrum disorders. Approximately one-third of autistic children have definable seizures or abnormal EEG seizure foci.[14] Overt seizures are not necessary for regression, and in many such cases, abnormal EEG seizure foci have been found in the absence of clinical seizures.[15] These abnormal seizure foci, with and without clinical seizures, are seen more commonly in autistic children who regress.

Childs and Blair reported dramatic improvements after treatment with valproic acid in a pair of autistic twin boys who were found at age three to have absence seizures.[16] The parents, on reflection, recalled symptoms consistent with seizures occurring at age two. These boys had symptoms characteristic of autism, including perseverative, nonpurposeful and self-stimulatory behavior, a lack of symbolic play, poor eye contact, echolalic and non-communicative speech and a lack of response to discipline.

In some autistic children one finds evidence of tuberous sclerosis, a condition associated with a high incidence of seizure disorders.[17] Approximately 25% of children with tuberous sclerosis will be autistic. When one adds pervasive developmental disorder the incidence increases to 40 to 45%. Among autistic children, 1 to 4% will also have tuberous sclerosis. The incidence increases to 8 to 14% in autistic children with seizures.

There is evidence that seizure foci in autistic children have been grossly underdiagnosed. In a recent study of children with Landeau Kleffner syndrome (LKS) as compared to autistic children with regression, researchers using a highly sensitive magnetoencephalographic technique (MEG), found that out of 50 autistic children examined during stage II sleep, 82% demonstrated epileptiform activity in the same region of the brain as seen in Landeau Kleffner syndrome.[18] The difference in the two groups was that the LKS children demonstrated no epileptiform activity outside the left intraperisylvian area, whereas 75% of the regressive autistic children demonstrated seizure foci with independent activity outside this area. The LKS children demonstrated propagation of the seizure to frontal and parietal regions on occasion, which could explain associated difficulties with socialization and behavior.

During the examinations, standard EEG recordings were done simultaneously with the magnetoencephalographic recordings. While the MEG recordings demonstrated abnormal activity in 85% of cases combined, the standard EEG recordings demonstrated problems in only 68% of cases. This indicates that significant abnormalities are being overlooked during routine examinations. It is also possible that depth electrode recordings would detect even more abnormalities in subcortical areas, such as the amygdala and septal areas.

These findings conform to those of Zilbovicius and coworkers who found marked bilateral perfusion defects on positron emission tomography (PET) scans in 75% of autistic children examined, indicating damage to neuronal structures.[19] These defects were localized to the auditory association area and adjacent muiltimodal temporal cortex. Similar areas of hypoperfusion have been described in cases of infantile spasms.[20] Experimental lesions in the medial temporal lobe of non-human primates strongly resemble autism.[21]

That a persistent seizure focus discharge is pathologically damaging is graphically shown in the case of Landeau Kleffner syndrome. In this disorder, a persistent seizure focus results in a progressive loss of language function and social interaction, both higher cognitive functions. Of particular concern is that the seizures usually occur at nighttime and are very difficult to recognize by parents or doctors, as we have seen. Recovery of language function depends on early seizure control.

Another graphic demonstration of the connection between seizures, glutamate accumulation, and cognitive deterioration is seen in the case of pyridoxine-sensitive seizure in newborns. It has been shown that in the untreated child, CSF glutamate levels are 200X normal and seizures are uncontrollable.[22] When given an intermediate dose of 5 mg/kg/BW/day of pyridoxine, the seizures cease, but mental deterioration continues. Glutamate levels at this dose were still 10X higher than normal. When using pyridoxine at 10 mg/kg/BW/day there were no seizures, no cognitive deterioration, and glutamate levels were normal. It is interesting to note that some reported cases of pyridoxine-dependent seizures also had features of autism.[20]

While most cases of pyridoxine-dependent seizures are present at birth, some individuals have experienced initial onset as late as 14 months.[23] It has been suggested that pyridoxine-dependent seizures are more common than is being reported, and that neurological deterioration can occur in the absence of seizures.[24] A wide array of neurological symptoms can be seen on the basis of excitotoxic lesions produced with this syndrome, including visual agnosia, squint, severe articulatory apraxia, and motor delay. We also know that the excitotoxic process associated with this syndrome can produce physical changes in the brain as seen on MRI and CT scans, usually with cortical and subcortical atrophy and progressive ventricular diliation.[25,26] Another demonstration of the importance of glutamate in seizure pathology comes from the study by Mathern and co-workers who demonstrated increased NMDA receptor content in cases of temporal lobe epilepsy associated with mesial hippocampal sclerosis, indicating dentate granule cell hyperexcitability.[27] Others have shown degeneration of dendritic connections in epileptic hippocampal neurons characteristic of excitotoxicity.[28] Interestingly, a recent study found that the anatomic substrate of the limbic system, which included the subiculum/CA1-CA3 area and the dentate gyrus/CA4 area, was smaller in autistic subjects than matched controls.[29]

It has been observed that a percentage of autistic children improve when supplemented with zinc. It is known that the temporal lobes have the highest zinc content in the brain and that zinc plays a major role in reducing NMDA excitability.[30] Zinc has also been found to reduce dentate granule cell hyperexcitability in epileptic humans.[31]

It is now known from experimental studies that seizures are intimately connected to the excitotoxic process.[32] Not only can glutamate and aspartate precipitate seizures, especially when injected into the brain, but seizures themselves can stimulate the release of excitatory amino acids from the brain, most likely by stimulating free radical generation. Spontaneously-discharging neurons, especially when the process is prolonged, are associated with energy loss, ischemia, and hypoxia, all of which precipitate excessive release of glutamate.

There is considerable evidence that excitotoxicity is responsible for much of the pathological damage produced by prolonged seizures.[33,34] This destructive process has been proposed as the mechanism for both the mirror focus seen with temporal lobe seizures and the cognitive deterioration associated with status epilepticus. Cytopathological changes have been described in the hippocampus following prolonged seizures that closely resemble excitotoxic damage, with destruction of neurons in the CA1 and CA3 areas, and dendritic swelling in the hilus of the fascia dentata, as seen with cases of autism.

Recent studies have shown that ketamine, a powerful NMDA receptor antagonist, can powerfully inhibit seizures, including status epilepticus.[35] Of particular concern is the excitotoxic damage produced during limbic status epilepticus, a common form of epilepsy seen in autism spectrum disorders, and which may explain the above mentioned limbic atrophy in autism.[36]

Another excitotoxic substance associated with seizures is quinolinic acid.[37] This excitotoxin is important for two reasons. First, it is a metabolic product of serotonin breakdown, and second it is released from both astrocytes and microglia when these cells are activated by various stimuli. Quinolinic acid acts at the NMDA receptor and, like glutamate, its activity can be blocked by MK-801.

There is evidence that excessive accumulation of extraneuronal glutamate can inhibit oxidative phosphorylation. Studies using retinal cells have shown that high concentrations of glutamate can reduce complex I, II/III and IV, and that this inhibition can be completely blocked by MK-801.[38] Several studies have shown that neuronal energy deficits dramatically increase excitotoxic sensitivity, even to the point where normal concentrations of glutamate can become excitotoxic.[39,40]

While glycine demonstrates inhibitory actions in the spinal cord, in the cerebrum it is excitatory. This is because it plays a major role in glutamate activation of the NMDA receptor. High concentrations of glycine have been shown to cause marked hyperexcitability and neurotoxicity in hippocampal brain slices.[41]

Kainate can induce kindling, when injected into the cortex or amygdala. The kindling response can occur without initiating seizures. Kindling without clinical seizures

has been observed in autism. Several studies have shown that kindling can produce excitotoxic lesions in the absence of clinical seizures, again, something important to consider in the autistic child.[42]

While neuronal degeneration can result from elevated levels of glutamate, a loss of dendritic connections can occur at much lower concentrations. There is also substantial evidence that elevated levels of glutamate during periods of critical brain formation can result in altered pathway development by overstimulating growth cones.[43] Glutamate levels are carefully regulated during early brain formation and disruptions in glutamate levels can result in alteration leading to either subtle or profound effects on brain function, depending on the timing and dose. Seizures, especially when prolonged, can result in such elevations of glutamate levels.

It is also known that ischemia and hypoxia, not uncommon in prolonged seizures, can produce dramatic increases in glutamate levels for prolonged periods of time. These levels could have a profound effect on pathway formation as well as a loss of neurons, synaptic connections, and stem cells. It is known that after age two years, the developing brain contains more synaptic glutamate receptors than at birth, and that the number slowly declines over the next decade.[44] This makes the infant brain especially vulnerable to excitotoxicity.

THE ROLE OF IMMUNE STIMULATION

It has been recognized that activation of microglia, as well as astrocytes, during immune stimulation can elicit excitotoxicity.[45] The mechanism involves a complex array of events primarily involving the release of numerous cytokines. It should be appreciated that microglial activation can occur during systemic immune challenge, as with vaccination.[46,47]

Microglial activation elicits the release of several cytokines, including TNF-alpha, IL-1ß, IL-2, IL-6 and INF-gamma.[48] In addition, cytokine activation of inflammatory eicosanoids occurs as well.[49] Closely linked to this process is the generation of numerous species of reactive oxygen and nitrogen intermediates, including superoxide, hydrogen peroxide, hydroxyl radicals, peroxynitrite and 4-hydroxynonenal. These reactive intermediates not only damage synaptic connections, neurons, and cellular components, but also induce the release of glutamate from surrounding astrocytes.[50]

Of particular interest is the recent observation that microglial activation can also elicit the release of glutamate and quinolinic acid, two powerful excitotoxins, from the microglia itself.[51] Interaction with bacterial components, viruses and lipopolysacchrides can increase glutamate release two- to three-fold above basal levels.[52] Likewise, dexamethasone has been shown to reduce glutamate release by 50% following antigen exposure.[53]

It should also be appreciated that glutamate excess, as well as deficiency, interferes with long-termed potentiation, which is critical for learning and memory.[54] In addition, the growth and terminal distribution of developing brain pathways are also adversely affected by excess glutamate, especially when prolonged. Likewise, glutamate deficiency interferes with growth cone function, leading to "miswiring" of the brain's circuitry.

Anything that activates microglia, including viruses, ß-amyloid, mercury, aluminum, oxidized LDL and HDL, homocysteine and excitotoxins, can increase the accumulation of quinolinic acid.[55] This raises concern about the use of L-tryptophan-enhancing supplements and medications. Of particular concern is an imbalance between quinolinic acid and kynurenine formation, since the latter is a neuroprotectant.

Another area of concern is the ability of immune microglial activation products to interfere with glutamate re-uptake. The glutamate transport family of proteins is particularly sensitive to inactivation by IL-1ß, TNF-alpha, mercury, peroxynitrite and 4-hydroxynonenal.[56,57,58] Such interference with glutamate disposal has been associated with amyotrophic lateral sclerosis and possibly Alzheimer's syndrome.[59,60] All of these inhibitory factors can be seen in cases of over-vaccination and autoimmunity.

Mercury is a very powerful inhibitor of GLT-1, the glutamate transport protein, even in very small concentrations.[61] Several studies have shown that children with autism frequently have significantly elevated mercury levels, with vaccines often being the only source of the mercury (as the preservative thimerosal). Mercury exposure from dental amalgam in rats produced significantly elevated levels of immune complexes in the renal glomeruli and vessel walls of numerous organs, including the brain.[62] Based on what we know about overstimulation of the immune system, with concomitant prolonged microglial activation, removing the mercury from vaccines, while helpful, most likely will not eliminate the problem.

Vijendra Singh and co-workers have found that 84% of autistic children examined demonstrated antibodies to myelin basic protein (MBP), suggesting that a state of autoimmunity to brain has occurred in the autistic child.[63] It is known that autoimmune states are associated with high levels of cytokines and inflammatory mediators such as leukotrienes and prostaglandins.[64] These inflammatory mediators increase both brain oxidative stress and excitotoxicity. It is interesting to note that autoimmunity is also found in many of the adult neurodegenerative disorders, such as Alzheimer's disease, ALS and Parkinson's disease.[65,66,67]

Confusing the issue is the observation that autoimmunty against BMP by regulatory T-cells can infer with neuroprotection as well.[68] It appears that whether neurodestructive reactions or neuroprotection results depends on the immune process being tightly regulated, so as to pre-

vent a predominance of the former. With the autism spectrum disorders these regulatory T-cells may be either dysfunctional or reduced in number.

Another possibility is the presence of a persistent virus or a stealth virus. When the immune system has been impaired, either genetically or by exhaustion, viruses can persist in tissues for long periods of time.[69] Because the immune system is impaired, instead of killing the virus, the activated microglia continuously release neurotoxic mediators and a stream of free radicals.[70] Viruses mostly produce their damage in the nervous system by stimulating the release of glutamate and other excitotoxins, which further increases the production of destructive reactive intermediates.[71] The first casualties are the synaptic connections, followed by injury to immature pathways forming during the brain growth spurt.

That the measles virus enters the brain in cases of measles encephalomyelitis has been shown by protein sequencing.[72] Viral entry into the brain can induce either a demyelinating syndrome (subacute sclerosing panencephalitis) or a non-demyelinating syndrome as characterized above. Giving children live measles viruses can possibly lead to invasion of the brain by these persistent viruses.

Singh and co-workers have shown that autistic children have measles and HHV-6 titers slightly higher than normal controls.[73] More importantly, the autistic children had anti-myelin basic protein antibodies in 90% of the measles positive children and 84% of the HHV-6 positive children. They were also found to have anti-neuron-axon fibrillary protein antibodies in 73% of measles positive and 72% of HHV-6 positive children, whereas none of the controls had these antibodies. The higher the antibody titers the greater the chance of autoimmunity to their brain.

Antibodies to brain serotonin receptors, along with hyperserotoninemia, has been demonstrated in a significant number of autistic children.[74] To my knowledge no one has tested for antibodies to the glutamate receptor. This would be important in light of the recent demonstration that antibodies to the glutamate receptor can elicit excitotoxicity.[75]

In one study, using mice infected with hamster neurotropic measles virus, researchers found that after seven days post-innoculation, hippocampal brain slices produced 18 times more quinolinic acid as compared to controls.[76] Three-hydroxyanthranilic acid oxygenase, an astrocytic enzyme responsible for the production of quinolinic acid, increased its activity 3.3-fold on the seventh post-inoculation day. Quinolinic acid accumulation has been associated with HIV dementia as well, secondary to its release from activated microglia. The HIV viral envelope, gp 120 and tau proteins are neurotoxic by an excitotoxic mechanism. Blocking the NMDA receptor prevents quinolinic acid neurotoxicity. In mice, measles virus-induced encephalopathy associated neurotoxicity is also prevented by MK-801, an NMDA antagonist.[77]

Self-limited incidences of mild acute encephalopathy probably occur more often than are reported.[78] Many pediatricians either do not recognize subtle neurological signs or dismiss them as the result of an overanxious mother. Chronic viral infections of the CNS, especially by stealth viruses, with waxing and waning symptoms, are frequently overlooked by those not trained in neurological care.

Another study raises even more concern for atypical presentations of measles infections of the brain.[79] One study found that hamster neurotropic virus could cause a non-inflammatory encephalopathy with degeneration of the hippocampal CA1 and CA3 regions. The excitotoxic reaction increased several days after the inoculation. In humans this could lead to varying degrees of memory loss and learning difficulties, since excitotoxin damage has been shown to interfere with long-term potentiation (LTP). In addition, limbic connections to the amygdala, orbitofrontal cortex, anterior cingulate gyrus and prefrontal cortex would account for defects in processing complex sensory information as well as emotional enrichment of ideas.[80]

A case of postvaccinal parkinsonism has been reported following inoculation for measles. The disorder occurred in a five-year-old boy who developed fever and a rigid-akinetic syndrome beginning 15 days after the vaccine.[81] A follow-up report at age seven found that he was still suffering from parkinsonian symptoms. From these reports one must conclude that the virus localized within the striatum, eliciting an excitotoxic reaction of sufficient degree to produce parkinsonian symptoms.[82,83] The fact that methamphetamine induces nigrostriatal dopaminergic toxicity by an excitotoxic mechanism in this same region questions the wisdom of placing children with autism spectrum disorders on such medications.[84]

By grouping vaccines together, especially live viral vaccines, one increases the stress on the immune system as well as increasing microglial activation within the brain. Not infrequently, very small children are given three to nine vaccines during a single doctor's visit. This not only constitutes a heavy bacterial and/or viral antigen load, but vaccines contain powerful adjuvants to boost immunity so as to increase the likelihood of successful immunization.

This has two effects. First, it overstimulates a dysfunctional immune system, leading to immune-directed damage to the nervous system. Measles virus is known to induce autoimmune reactions to myelin basic protein.[85] Second, it eventually exhausts the immune system, leading to increased susceptibility to subsequent microbial infections or chronic viral infections. This scenario is more likely in the malnourished child, especially with vitamin A deficiencies. Experimentally, retinoids have been shown to significantly reduce the clinical severity of experimental allergic encephalomyelitis.[86] Early nutrition has been shown to play a major role in immune function, not only during the neonatal period, but also throughout life.[87]

Experimentally, using guinea pigs and rats, excitotoxic lesions within the hypothalamus have been shown to suppress both humoral and cell-mediated immunity.[88] These excitotoxic-induced lesions in the hypothalamus have been shown to produce immune dysfunctions that persist throughout life.

It has been observed that autistic children are frequently deficient in zinc, and zinc is known to play a role in neuroprotection.[89,90] Part of the protection arises from the zinc portion of the NMDA receptor, which inhibits receptor activation by glutamate. Zinc is also involved in metallothionein, a protective molecule that increases with brain inflammation and intoxications with heavy metals, especially mercury.[91] Under such conditions, zinc levels in the blood are seen to fall. Interestingly, prenatal exposure to caffeine from maternal consumption induces decreased fetal levels of brain zinc.[92]

Magnesium levels have also been reported to be low in autistic children. Magnesium plays a major role in neuroprotection, primarily by inhibiting NMDA activation. Magnesium also acts as an antioxidant with low levels being associated with a doubling of free radical generation in both epithelial cells and neurons.[93] Low magnesium also lowers cellular glutathione levels and increases excitotoxic neuronal death. Several studies have shown that low magnesium levels dramatically increase excitotoxicity.[94]

It has been shown that low magnesium plays a major role in encephalopathy associated with deficiency of thiamin and other B vitamins.[95] In this study, rats made deficient only in thiamine or the other B vitamins developed mild cytotoxic changes in their pontine tegmentum. Yet, when made hypomagnesmic the lesions were profoundly worsened. Hypomagnesmia has also been shown to inhibit GABA responses as well, which would increase cortical excitability.[96]

One of the principal cytokines released with microglial activation is tumor necrosis factor alpha. While under normal conditions TNF-alpha acts as a neuroprotectant, it can also enhance excitotoxicity both by increasing reactive oxygen and nitrogen intermediates and by inhibiting glutamate re-uptake.[97] In addition, TNF-alpha has also been shown to produce neural degeneration by inducing metalloprotenase-disintegrin (ADAM8).[98] TNF-alpha has been shown to be elevated in several of the neurodegenerative disorders and with experimental allergic encephalopathy (EAE).[99] Cytokines have also been shown to play a major role in neurodevelpment. For example, IL-1ß, IL-6 and TNF-alpha at physiological concentrations can affect the survival of both dopaminergic and sertonergic neurons in the embryo.[100] At higher concentrations these cytokines significantly reduce the survival of the dopaminergic neurons, but not the sertonergic neurons.

Recently Petitto and co-workers demonstrated, by using IL-2 knockout mice, that IL-2 was essential for the development and regulation of hippocampal neurons involved in spatial memory and learning.[101] Likewise, IL-1 has been shown to have tropic functions within the brain.[102,103] At higher concentrations, both IL-2 and IL-1ß have been shown to be cytodestructive, primarily by increasing free radical generation and blocking glutamate re-uptake.[104]

Besides increasing neuronal destruction through immune enhancement of excitotoxicity, viruses can also enhance excitotoxicity by inhibiting mitochondrial enzyme function. The polio virus, for example, has been shown to impair oxidative phosphorylation by inhibiting complex II of the electron transport chain.[105] As stated, reductions in mitochondrial function significantly increase excitotoxity.

Systemic cytokines can also have effects on the nervous system, since they may enter by way of the circumventricular organs and through the impaired BBB.[106] Cytokines can also interact with endothelial cells triggering the release of neuroactive substances within the brain and by altering the permeability of the blood-brain barrier. Interleukin-2 has been shown to cause leaking of brain capillaries, leading to cerebral edema in cases of glioma patients treated with this cytokine.

Cognitive impairments have been attributed to IL-2 and TNF infusions in humans. SPECT scans (functional brain scans) have demonstrated frontal lobe perfusion defects in these patients, which were suspected to be caused by changes in hypothalamic and/or frontal subcortical function.[107]

Treatment of patients with a variety of cytokines has been shown to produce a two-phase effect, acute and chronic. The chronic phase, occurring after two weeks, is often characterized by psychomotor, cognitive and psychiatric abnormalities. Interferon-alpha infusions, even at low doses, are also associated with numerous cognitive and psychological effects, including decreased attention span, an inability to concentrate, impaired short-term memory, and hesitation of speech. Such patients often suddenly stop speaking and stare out into space. On rare occasions patients will progress to dementia. Many of these reactions are reminiscent of autism behavior.

One set of symptoms associated with interferon-alpha use, that are also similar to that seen in autism, include uncontrollable overreaction to minor frustration, marked irritability, and a short temper.[108] Even months later, such patients may become severely agitated, abusive, and withdrawn.

Both interleukin-1 and -2 infusions are associated with mental changes, including delusions, disorientation, and seizures.[109,110] There is evidence that IFN-alpha can enhance spontaneous activity in neurons in the cerebral, hippocampal and cerebellar cortices that can last several hours following a single exposure.[111] It is not clear if this is a direct effect of interferon or if it is acting through enhanced glutamate release.

Most of these clinical studies were on adult patients receiving therapeutic doses of cytokines to treat either viral illnesses or cancer. They demonstrate that peripherally

administered cytokines can have a profound effect on CNS function. In the infant with an immature brain undergoing rapid developmental changes, the neurotoxic effects of the cytokines would be expected to be more profound. Also, because most of the cytokines would be derived from activated microglia within the brain, smaller concentrations would be expected to have a greater effect than systemically administered cytokines.

Finally, one problem frequently found in autistic children is an overgrowth of various fungal species, most often *Candidia albicans*, secondary to either the frequent use of broad-spectrum antibiotics or associated with immune depression. While concern with several of the organic acids released by the yeast organism is legitimate, and have been shown to have a profound effect on neurological function, of equal concern is immune activation of microglia in the brain secondary to systemic Candidia infection, or even infiltration of the brain itself. A recent study has shown that the Candidia organism can penetrate the blood-brain barrier (BBB) by budding and developing pseudohyphae inside human microvascular endothelial cells.[112]

CONCLUSION

Epidemiological studies have shown that from 1960 until 1978, the incidence of autism was fairly stable nationwide, at about 100 to 200 new cases per year. Following the introduction of the measles, mumps, rubella (MMR) vaccine for the widespread inoculation of young children, the incidence of autism increased dramatically, and has continued to increase, with 1944 cases being reported in 1999 alone. In California there has been a 273% increase in severe autism cases over the past eleven years.

While purely genetic disorders can explain a small subset of cases, most appear to involve children who are healthy until they receive their vaccination. Several of the vaccines are suspect, especially the MMR, DPT and HepB vaccines. Dr. Bernard Rimland has pointed out that before the introduction of the MMR vaccine, most autism cases occurred at birth. Yet, after the introduction of the MMR vaccine, most new cases were occurring around age 15 months, when the MMR vaccine was usually given. This does not exclude the possibility of pre-existing, genetic-related immune defects triggered by the immunizations.

Today, children are being given up to 22 doses of 6 types of vaccines before the age of five years. This represents a tremendous antigenic load for an immature immune system to deal with, especially when given so close together. Until recently, children were not only receiving a massive antigenic load, they were also exposed to high concentrations of mercury. A child receiving all of their vaccinations often received as much as 62.5 ug of mercury per visit, 100 times the exposure allowed by the EPA as safe for an infant.

Another problem is the use of live virus vaccines. The oral polio vaccine and the measles vaccine were found to contain contaminant live viruses, that have been shown to disseminate to other organs, including the nervous system.[113] The oral live polio vaccine has been suspected of containing numerous pathogenic viruses, including HHV-6, SV-40 and possibly SIV. There is serious concern that stealth viruses may have infected millions of unsuspecting people due to contaminated vaccines.

Recently, Guillot and co-workers reported a high incidence (over 50%) of viruses with a recombinant genome, mostly intertypic/vaccine recombinants.[114] In this study they report the finding of recombinant wild polio virus with that isolated from vaccine-related paralytic poliomyelitis patients. They suggest that other combinations could contain non-polio type viruses. This suggests that attenuated viruses from vaccines may mutate by a process of recombination of genetic material with other viruses, with the possibility of transforming to more virulent forms.

Another frightening discovery was the finding that increased oxidative stress associated with antioxidant nutrient deficiencies could cause viruses to mutate from a non-virulent form to a highly virulent form.[115] With the high degree of oxidative stress and low antioxidant defenses in the autistic child, the risk of such an event would be greatly enhanced.

The mechanism by which vaccinations and/or other antigenic loads can precipitate the autistic syndrome is unknown. But we know that immune activation of the brain, especially when intense and prolonged, can precipitate the release of excitotoxins from astrocytes and microglia.[116] Excitotoxicity is now known to be a major mechanism of neural destruction in cases of viral infections of the brain. Even without direct viral invasion, as we see in AIDS and infection with the Maloney Murine leukemia virus, immune activation can trigger the release of the excitotoxins quinolinic acid and glutamate, leading to neurodegeneration.

Chronic elevations of glutamate during critical brain growth periods can result in the development of faulty neural pathway circuitry, which can have profound effects on complex higher cortical functions as well as hypothalamic functions. Even transient interference during the period of rapid brain growth can result in the apoptotic death of millions of developing neurons and the loss of millions of synaptic connections.[117] It should be appreciated that destruction of synaptic connection and dendrites can occur in the absence of neuron death itself, which means that it can occur at much lower levels of glutamate and aspartate, especially when antioxidant levels, cellular energy generation, and/or magnesium levels are low.[118]

Intimately connected with excitotoxicity is free radical generation, including numerous oxygen and nitrogen intermediates. Peroxynitrite, a nitrogen intermediate derived from a union of nitric oxide and superoxide, is especially damaging to the mitochondria, leading to a loss of energy produc-

tion. Low brain energy levels, no matter the cause, result in a dramatic increase in sensitivity to excitotoxicity. Both glutamate and reactive intermediates can induce microglial activation, leading to a release of inflammatory cytokines, lipid peroxidation products, inhibition of glutamate re-uptake, and eventual apoptosis and necrosis reactions.

Glutamate excess has been shown to lead to glutathione depletion secondary to inhibition of cysteine entry into the astrocyte (by way of its effects on the cysteine transport xc system).[119] A recent study indicates that glutathione may not only function as an antioxidant, but may act as a neuromodulator and neurotransmitter as well.[120] As a neuromodulator, glutathione has been shown to down-regulate the excitotoxic NMDA receptor, thus blocking excitotoxicity.[121]

In addition, as stated, clinical seizures occur in approximately one third of autistic children. Excitotoxicity is intimately connected to seizures and explains the neural damage seen when they are prolonged or repeated. Less well appreciated is the fact that chronic seizure foci, even in the absence of clinical seizures, can produce significant neural damage by an excitotoxic mechanism. While the immature brain is less susceptible to neuron death than the mature brain, seizures in the developing brain result in irreversible changes in neuronal connectivity.[122] A recent study found that repeated seizures during early life resulted in persistent changes in the CA1 pyramidal neurons in the hippocampus, which is related to observed behavioral changes.[123]

Mercury exposure is also intimately related to neonatal seizures. A recent study found that maternal exposure to mercury during pregnancy significantly increases epileptogenecity in the offspring.[124] This is of special importance in women having dental amalgam, particularly if this amalgam is disturbed during the pregnancy.

Of special concern as well is the recent discovery that glutamate, by activating the NMDA receptors on the blood–brain barrier can disrupt the barrier, leading to free access of blood-borne toxins to the CNS.[125] In addition, free radicals themselves have been shown to open the BBB.[126] Gupta and co-workers have shown that the developing BBB is highly vulnerable to single or repeated exposure of certain pesticides, and that the effect persists even after the offending agent is removed.[127] Seizures can open the BBB as well.[128] It has been demonstrated that by blocking the NMDA receptor, one can significantly reduce neurovascular dysfunction seen with experimental allergic encephalomyelitis.[129]

It has been shown that humans develop the highest blood levels of glutamate of all known animals tested following MSG exposure.[130] The immature brain is especially vulnerable to food-borne excitotoxins, being 4X more sensitive than the adult brain.[131] An explanation for hypersensitivity of the immature brain lies in the observation that during brain development the NMDA receptor is more sensitive to glutamate and less responsive to magnesium protection.[132] Food additive excitotoxins are found in virtually all processed foods, with very high levels in many junk foods and diet foods.[133] These foods are often eaten in large quantities by children, but especially autistic children.

With knowledge of the central role played by excitotoxicity in the autistic syndrome, numerous options will be available for treatment. Many of the diets now being proposed for autistic children emphasize elimination of foods known to be exceedingly high in excitotoxin additives, even though they are being eliminated for other reasons. They are also low in sugar.

Autistic children have a high incidence of reactive hypoglycemia, which increases their risk of seizures and excitotoxicity. There is some evidence that Candida infections may also increase the incidence and severity of hypoglycemia in autistic children.[134] It has been shown that children respond to glucose challenges with a hypoglycemic response at higher levels of blood glucose, and have a more profound adrenal response than adults.[135]

Many of the vitamins used to treat autism are antioxidants, which, as we have seen, can significantly reduce excitotoxicity as well as protect against the harmful effects of free radicals. Experimentally, vitamin E can completely abolish glutamate excitotoxicity *in vitro*. Metabolic stimulants also greatly reduce excitotoxicity. Thiamine, pyridoxine and nicotinamide have been shown to significantly reduce glutamate toxicity in vitro.[136]

Vitamin B_6 can dramatically lower blood and tissue glutamate levels and raise seizure thresholds. In addition, along with folate and vitamin B_{12}, it reduces homocysteine levels. While homocysteine is a marker for deficiencies of methionine metabolism, it is also metabolized into two very powerful excitotoxins, homocysteic acid and homocysteine sulfinic acid. Methylcobalamin is a glutamate receptor blocker as well.[137] Pyridoxine's ability to powerfully inhibit excitotoxity at least partially explains the often dramatic results reported by Bernard Rimland in treating autistic children with high dose pyridoxine/magnesium combinations.[138]

Magnesium and zinc also powerfully inhibit excitotoxicity as well as act as co-factors in numerous enzyme systems, including energy generation. Low magnesium is associated with dramatic increases in free radical generation as well as glutathione depletion. High glutamate levels have also been shown to deplete cellular glutathione. Glutathione is vital since it is one of the few antioxidant molecules known to neutralize 4-hydroxynonenal and mercury. In addition, both malate and pyruvate protect against glutamate-mediated excitotoxicity.[139]

Of great interest is the use of selected flavonoids as antioxidants, anti-inflammatories and antimicrobials. The flavonoids are more powerful and versatile as antioxidants than are the vitamins.[140] In addition, flavonoids have been shown to have effects on multiple enzyme systems, includ-

ing protein kinase C, phospholipase A2, COX and LOX enzymes, iNOS, Na+/K+ ATPase, mitochondrial energy production, as well as cytokine production, all of which may be beneficial in protecting the brain.

It should be pointed out that enrichment of the autistic child's environment is also critical. Saari and co-workers have shown that enriched environments can override some of the problems produced by neonatal exposure to monosodium glutamate.[141]

Despite the central role played by excitotoxicity, it should be remembered that numerous other mechanisms are at play as well. As a multifaceted disorder, autism requires a multifaceted approach, one that should include protection against excitotoxicity.

Prevention and Treatment of Autism

- Have vaccines separated and spaced by 6 months
- Have your child tested for titers to measles, mumps and rubella: If the titers are sufficient, vaccination is unnecessary. This can be done for other vaccines as well.
- Use allowed exemption for such controversial vaccines as chickenpox, hepatitis, diphtheria and pertussus
- Give your child a daily multivitamin/mineral
- Avoid all excitotoxin food additives
- Avoid excess sugar
- Regularly include fruits and especially vegetables in diet
- Avoid fluoride (fluoridated drinking water, fluoride dental treatments and toothpaste.
- Avoid all mercury-containing vaccines
- **Special supplements:**
  - ◊ Methylcobalamin (vitamin B_{12})
  - ◊ Pyrodoxal-5 phosphate (vitamin B_6)
  - ◊ Vitamin E (mixed tocopherols)
  - ◊ Vitamin C (buffered)
  - ◊ CoQ_{10}
  - ◊ Acetyl-L-carnitine
  - ◊ Alpha-lipoic acid
  - ◊ Zinc
  - ◊ Magnesium glycinate or lactate
  - ◊ Selenium (as selenomethionine)
- Removal of existing mercury by DMSA or garlic extract

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ORIGINAL RESEARCH

Pilot Study: Reduction of Fatigue by Use of a Dietary Supplement Containing Glycophospholipids

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ABSTRACT

Objective: To determine if fatigue, as defined by the Piper Fatigue Scale (PFS), can be significantly relieved by use of a glycophospholipid-rich dietary supplement in a targeted sampling of the general population (mean age=50.3 years).

Methods: Adult listeners of a Los Angeles-based radio talk show on health were invited to participate in a fatigue intervention pilot study. A survey form was mailed to those participants who described a condition consistent with the definition of fatigue as defined by the self-reported Piper Fatigue Scale (PFS). The PFS has been shown to accurately reflect the multifactorial nature of fatigue through statistical factor analysis and clinical studies. Sixty-four (64) respondents were admitted to the study when their self-reported sign/symptom severity scores were converted to fatigue scores and rated as high-moderate to severe. The requirements of the study were fulfilled by thirty four (34) respondents (mean age=50.3±10 years, range= 33-79) completing three PFS reports each, at the fourth and eighth weeks of consuming an open label study product.

Results: The Piper Fatigue Scale scores indicated a 33% reduction in fatigue after eight weeks on the supplementation product. The PFS rates fatigue from a score of 0 (no fatigue) to 10 (severe fatigue). The average initial fatigue score for the group before treatment was reported as severe (mean score=7.9± 0.82 SD, range=6.4-9.9), after four weeks rated moderate (mean=6.1±1.66 SD, range =2.6-9.5) ($p<0.0001$), and at eight weeks rated as moderate (mean=4.7±2.01 SD, range=1.5-9.4) ($p<0.0001$).

Conclusion: In this self-reported study, dietary supplementation significantly reduced fatigue as measured by the Piper Fatigue Scale. The response generated in the initial survey was significant enough to warrant further investigation in an expanded, controlled study utilizing a placebo.

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INTRODUCTION

Most researchers view fatigue as a multidimensional sensation with many possible causes. There is, however, no universally accepted definition of fatigue. Instead researchers have assessed components associated with the complaint of fatigue, such as behavioral, affective, sensory,

and cognitive changes. Piper *et al.*[1] were among the first to propose a multifactorial measurement model for fatigue that combined multiple fatigue-associated elements into an overall fatigue score.

Disease and the complaint of fatigue or loss of energy often precede clinical diagnosis by a considerable length of time. Bland states, "In a sense, fatigue is the body's statement that it cannot manufacture and control adequate energy to meet its needs. Fatigue of the brain leads to confusion, while muscle fatigue leads to exhaustion. Fatigue of the immune system results in an increased susceptibility to infection and/or increased autoimmunity."[2]

A cell's functional capacity can be measured, in part, by the fluidity of its membranes and its cell-to-cell communication capacity. One of the most common forms of cell damage is created by free radicals, which reduce membrane fluidity, cell communication, and cell to cell function. Protecting cell membrane integrity is thought to enhance cellular health, energy and efficient metabolism.

Preventing loss of membrane integrity due to damaged components and the resultant loss of cellular energy may be accomplished, in part, by replacement of damaged lipids. Among the important cell membrane lipids, polyunsaturated phosphatidylcholine (PPC) molecules are essential for the structure, function, and regeneration of all biological membranes.[3,4] As cell membranes will incorporate exogenous PPC (derived from soybean semen), dietary supplementation is a logical first step in the restoration process. Such processes may play an important part in preventing disease, in general, and certainly fatigue.[5-6]

Several clinical and pharmacological trials have shown the relationship between loss of membrane phospholipids, membrane damage, and disease. PPC administration as a dietary supplement has resulted in enhancement of cognitive performance of the aging brain, improvement of coronary, peripheral and cerebral blood flow, activation of liver metabolism, and detoxification and gastrointestinal function through mucosal restoration.[8]

The product used in this pilot study, Propax™ with NT Factor™ (PNTF), is a dietary supplement rich in phospholipids, particularly polyunsaturated phosphatidylcholine. PNTF provides moderate doses of multivitamins, minerals, antioxidants and essential fatty acids that the body uses to repair itself, resulting in a substantial positive impact on fatigue, nausea, diarrhea and other quality of life indicators. The effect of PNTF has been documented in a double-blind, crossover placebo-controlled, randomized study on cancer patients undergoing chemotherapy.[17] Seidman[7] conducted a pilot study with rats and found that PNTF elevated mitochondrial membrane activity, as determined by rhodamine 123 metabolic dye uptake and reduction in mitochondria measured by fluorescence flow cytometry, compared to the activity of mitochondria isolated from control animals on an identical diet without the PNTF supplement. The increase in mitochondrial activity of rats fed PTNF was statistically significant ($p<0.05$) compared to control animals, demonstrating its protective effect on mitochondrial membrane function. Additionally, brainstem auditory responses were recorded for the two groups at the onset and conclusion of this six-month study. Preservation of hearing was noted in the treated rats at all frequencies examined, suggesting substantial protection of auditory nerve function. In contrast, the control group showed continuing age-related hearing loss at all examined frequencies. Differences between test and control groups were significant ($p<0.005$).[7] The rats were euthanized to obtain tissue samples from brain and cochlear sites to study mitochondrial DNA deletions associated with aging. Quantitative determination[8] revealed a significantly lower ratio of this common age-associated phenomenon in the experimental group compared to control rats, indicating that PPC as a dietary supplement had a protective effect on mitochondrial DNA damage.

As an important part of membrane structures, phospholipids maintain membrane integrity, and through changes in membrane fluidity regulate mitochondrial enzyme activities and membrane transport processes.[3,5] Phospholipids have other specific functions. The choline portion of PPC may be used in neural tissue for the synthesis of acetylcholine, a neurotransmitter. Oral administration of choline has been shown to increase plasma and neuronal concentrations of PPC, which stimulates the release of acetylcholine in neuro-muscular systems. Physical stress depresses plasma choline concentrations as evidenced by a decline in muscle function.[9-11] This was noted through the examination of individuals in the Boston Marathon where a 40% decrease in plasma choline levels were found during the race.[11] It has been established that providing PPC prior to exercise can compensate for these choline losses.[12-15]

SUBJECTS AND METHODS

Subjects: Participants were prescreened on the basis of an initial phone conversation to determine whether their symptoms were consistent with persistent, intractable fatigue, or merely an intermittent "tiredness" linked to work or lifestyle. Those who described a condition consistent with the definition of fatigue as defined in the Piper Fatigue Scale, "an unusual sense of tiredness that is not usually relieved by either a good night's sleep or by rest", were mailed a survey. The completed surveys were scored as described previously.[1,16] After the initial survey, participants aged 20 years and older with a Piper Fatigue Scale score of 6 to 10 were admitted to the study. This corresponded to having high-moderate to severe fatigue (0 = no fatigue, 1-3 = mild fatigue, 4-6 = moderate fatigue, 7-10 = severe fatigue).

Thirty-four (34) respondents with a mean age of 50.3 completed the study: 21 (61.8%) were women ranging in

age from 33 to 62 at an average of 47.0 years, and 13 men ranging in from 38 to 79, at an average of 54.2 years. Thirty-one were from California, and one each was from Ohio, Connecticut and New York. The majority of Californians resided in the greater Los Angeles metropolitan area.

To gain insight into the type of respondent who was over 21 years old and had a PFS score of 6 or higher, (moderately to severe fatigue) we telephoned 24 of the 34 respondents who completed the study. They were asked what, if any, prescription medications they used, and were asked to confirm the length of their fatigue and to discuss any preexisting medical conditions. Seven (29%) reportedly experienced fatigue, as defined above, lasting over a month, and 17 (71%) experienced fatigue lasting over a year. A variety of diagnoses were represented in the study group (Table 1), and 50% (5 males, 7 females) were on prescription medications. Table 2 presents the category of medications by class and the number of individuals using them. Overlap occurs in Tables 1 and 2, as some respondents reported more than one condition and/or used more than one medication. However, of the 9 subjects in Table 1 who indicated persistent, intractable fatigue, only two used medication. All five who listed depression as a diagnosis were on antidepressants, and the two hypothyroid respondents were on Armour Thyroid supplementation.

Study Design: Subjects admitted into the study with severe fatigue (7-10 on the Piper Fatigue Scale) were given a 4-week supply of PNTF and instructed to use 3 packets daily; those with high-moderate fatigue (6 on the PFS) were instructed to use two packets per day. No one was admitted to the study with a PFS score less than 6. All respondents were told to repeat the PFS self-assessment at the end of three weeks and return the survey to Nutritional Therapeutics, Inc., Hauppauge, NY. All PFS scores were then verified for accuracy and completion. Based on their their new scores participants were instructed to use three packs of PNTF per day for PFS scores of 7-10, two packs per day for scores of 4-6, and one pack daily for scores of 1-3. Subjects were supplied with PNTF for four more weeks, at which time participants completed and mailed in their third and final PFS questionnaire for verification and completion of scoring accuracy.

Table 1. Diagnoses of Participants in the Study

Depression:	5 (all on antidepressants)
Hypothyroid:	2 (all on Armour Thyroid)
Malignancy:	2
Anemia:	3
Asthma/allergies:	4
HTN/CVD:	1
General Fatigue:	9
Paralysis/Spinal Cord Injury:	1
Narcolepsy:	1
Glaucoma:	1
Fibromyalgia:	2
Candida/Viral:	1
Pregnant:	1
Restless Leg Syndrome:	1
DJD/OA:	2

Table 2. Medications used by study participants (n=24)

Medications Represented by Category	
Anti seizure:	2
Asthma/allergies:	2
Anti inflammatory/Non Steroidal:	2
Thyroid replacement:	2
Female hormone replacement:	1
Anti depressant:	5
Anti acids/H2-blocker:	2
Anti hypertensive:	1
Pain meds:	1

Table 3. Factor Loading for Items on the Four Piper Fatigue Scale Subscales

Subscale and Items	Factor Loading
I. Behavioral/Severity	
1. Fatigue distress	0.658
2. Interference with work/school	0.710
3. Interference with socializing with friends	0.775
4. Interference with sexual activity	0.528
5. Overall interference with enjoyable activities	0.840
6. Fatigue intensity/severity	0.730
II. Affective meaning	
1. Pleasant/unpleasant	0.704
2. Agreeable/disagreeable	0.815
3. Protective/destructive	0.838
4. Positive/negative	0.953
5. Normal/abnormal	0.705
III. Sensory	
1. Strong/weak	0.614
2. Awake/sleepy	0.812
3. Lively/listless	0.745
4. Refreshed/tired	0.844
5. Energetic/unenergetic	0.812
IV. Cognitive/mood	
1. Patient/impatient	0.788
2. Relaxed/tense	0.847
3. Exhilarated/depressed	0.552
4. Ability to concentrate	0.648
5. Ability to remember	0.547
6. Ability to think clearly	0.566

Materials and Methods: In its current form, the PFS survey form is composed of 22 questions rated from "0", no fatigue, to "10", the most fatigue. Each question has word anchors that vary from the generic (*none to a great deal*) to the specific (such as: *able to concentrate to unable to concentrate*). These items measure four dimensions of subjective fatigue: behavioral/severity (6 items), affective meaning (5 items), sensory (5 items), and cognitive/mood (6 items). Answers are used to calculate four sub-scale/dimensional scores and total fatigue scores. The Severity Code scoring the degree of fatigue is as follows:

0 = NONE 1-3 = MILD 4-6 = MODERATE 7-10 = SEVERE

Table 3 displays the factors and subscales with their respective factor loading values.[16] The standardized alpha (Cronbach's alpha) did not drop below 0.92 for any subscale, and the standard alpha for the entire scale of 22 questions was 0.97, indicating excellent reliability for an established instrument.[16,17] The study product, (PNTF) is a proprietary nutrient complex (Table 4).

RESULTS

The total PFS group average (mean) score, 7.9, improved 33% from the initial survey before taking PNTF to 6.1 after four weeks, and to 4.7 after eight weeks. By sex, the group mean final score improved 35% for women and 29% for men. Age was not associated with the degree of change in fatigue. Table 5 shows the clustering of values for the degrees of change in the PFS scores, before and after supplement usage.

By PFS subscale, respondent's Behavioral/Severity scores improved an average of 37% for the entire group after PNTF (women 39%, men 33%). The Affective Meaning subscale responses improved by an average of 31% (women 36%, men 24%). The Sensory subscale revealed a 34% average improvement (women 33%, men 35%. The Cognitive/Mood subscale showed an average improvement of 27%, (women 30%, men 23%).

Summary scores on the PFS showed women improving by 35% and men by 29%. The difference in the improvement in for the entire group was significant ($p<0.0001$).

DISCUSSION

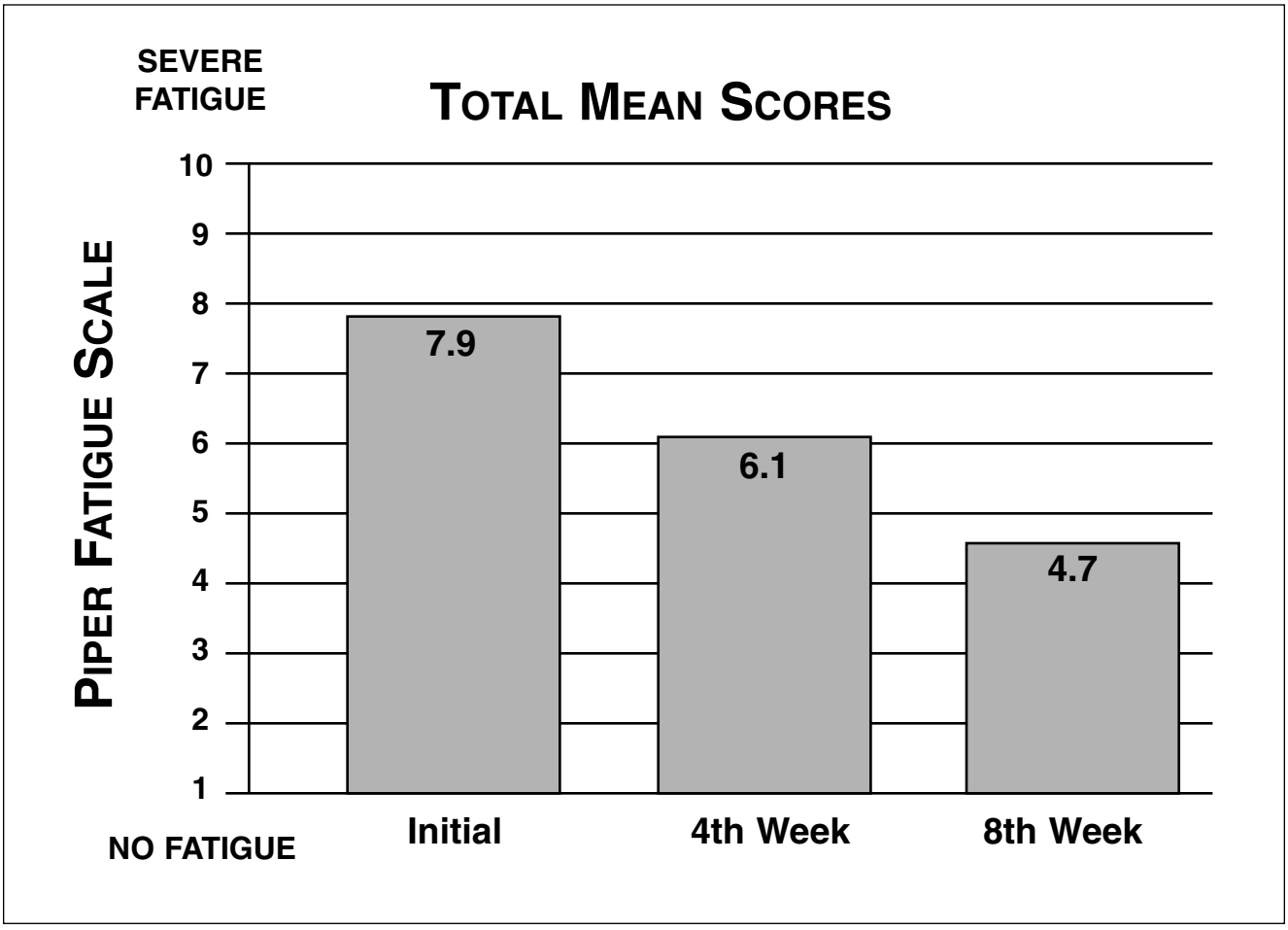
The pilot study sampled a general population of predominantly older subjects from the Los Angeles metropolitan area who were listening to a health talk radio show on the subject of fatigue. The only admission criteria was that subjects had to be 20 years old or more and have moderately high or severe fatigue corresponding to a score of 6-10 on the Piper Fatigue Scale. The only intervention was the use of the phospholipid-rich nutrient complex PNTF. After eight weeks on this dietary supplement product, all but one of the 34 respondents showed improvement in their fatigue. The

Table 4. Contents of Study Product, (PNTF)
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
L-Carnitine Tartrate	160 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

Components of NT Factor™
Defatted rice bran, arginine, beet root fiber, black strap molasses, glycine, magnesium sulfate, enriched polyunsaturated phosphatidylcholine (phospholipids), saponin (glycolipids), para-amino-benzoate, leek, pantethine (bifidus growth factor), taurine, garlic, calcium borogluconate, omega-6 essential fatty acids, omega-03 essential fatty acids, artichoke, 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.

Table 5.



one respondent who did not improve, was contacted by the study's principal investigator to confirm their diagnosis and medications use and this was one of two participants who had a malignancy and was currently on five different medications. Although the number of subjects sampled was small, the statistical difference in the results ($p<0.0001$) were significant. This suggests that further studies need to be conducted that control for disease or diagnosis, medication use, and potential changes in lifestyle or nutrient use.

The integrity of cellular and intracellular membrane structures is critical to cell function and energy production.[3,5] Previous animal studies using the phospholipid nutrient complex in PNTF suggested that maintenance of membrane integrity with appropriate phospholipids improved mitochondrial function. In addition, in clinical trials on chemotherapy patients using the multivitamin and mineral supplement PNTF, the ability to reduce fatigue was demonstrated in this most challenging class of patients.[17] In the pilot study presented here where fatigue alone was analyzed, the results suggest that directing dietary support at the cellular and intracellular membrane level with the phospholipid-rich supplement PNTF may provide the cell membrane structural repair components needed for restoration and maintenance of cell and mitochondrial function. This, in turn, may improve cellular energy and eventually reduce fatigue.

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ORIGINAL RESEARCH

A Proteinase Inhibitor Extract from Potatoes Reduces Post-Prandial Blood Glucose in Human Subjects

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ABSTRACT

Background: Dietary proteinase inhibitors (PIs) can increase the level of the post-prandial peptide hormone cholecystokinin (CCK) leading to delayed gastric emptying, which in turn has been associated with reduced post-prandial glucose levels. Commercial quantities of an extract containing potato proteinase inhibitor II (PI2) are now available to test the hypothesis that PI2 extract reduces post-prandial glycemia which may be beneficial in combating obesity.

Methods: This randomized, placebo-controlled double-blind study enrolled 39 healthy subjects (26 male, 13 female) mean age 35 y (range 23-61 y) with a mean body mass index of 27 (range 23-32). Participants were randomized to receive two of three different active doses of PI2 extract (7.5, 15, or 30 mg) and one placebo, 30 minutes prior to a test meal. Peripheral blood glucose levels were measured every 30 minutes for two hours after the meal.

Results: Oral ingestion of PI2 extract prior to a meal challenge test resulted in significantly reduced 30-minute post-prandial blood glucose levels ($p<0.05$), as well as a reduction in the area under the post-prandial blood glucose curve in the majority of subjects given either of the two higher doses. Gender and differences in body mass index, age, or fasting blood glucose did not predict responsiveness. Some subjects from both placebo and experimental groups noted mild side effects, including headache and slight gastrointestinal disturbance, but neither were statistically significant.

Conclusion: To date, this is the largest trial of a potato-derived proteinase inhibitor in human subjects, and the first to demonstrate that a low dose taken prior to a meal can reduce post-prandial glycemia. PI2 extract has a potentially useful role in modifying the glycemic response to food and could have important implications for the management of some metabolic disorders and obesity.

Key Words: Blood glucose, proteinase inhibitor, obesity.

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INTRODUCTION

Regulation of body weight depends on genetic as well as physiologic and lifestyle factors known to influence energy balance, such as diet, appetite control, metabolism,

and physical activity.[1,2] Despite measures to combat obesity and an increased awareness of the associated co-morbidities, the condition has become an epidemic, with nearly 60% of Americans classified as overweight or obese.[3] Since the gene pool has not changed, researchers believe the extra pounds are primarily due to a combination of environmental and lifestyle influences. A focus on dietary fat as a leading cause of obesity the last several decades has been successful in reducing overall fat intake by Americans (from 40% to just over 30% of total calories, from the 1960s to present), but has done little to stave off the rise in obesity rates.[4]

The rise in obesity incidence parallels a similar rise in the consumption of foods higher in processed and refined carbohydrates[5] along with an increased incidence of type II diabetes.[6,7] These events have led researchers to question the effect of dietary fat on body fat accumulation, and to suggest that dietary factors other than fat consumption play an important role in body weight regulation.[8,9] Evidence now suggests that chronic glycemia can lead to increased fat synthesis and storage, and may contribute significantly to the development of obesity and other chronic diseases such as diabetes and cardiovascular disease.[10-12] Concerns over safety and efficacy of many anti-obesity products have limited their usefulness. Therefore, development of natural, safe, and effective nutraceutical and/or medications that can help treat or prevent obesity are essential to mitigate this public health crisis.

Both soybeans and potatoes are sources of proteinase inhibitors (PIs), proteins that have been hypothesized to enhance the release of cholecystokinin (CCK), one of several gut peptides that regulate gastric emptying and satiety in humans.[13-15] Delayed gastric emptying, in turn, has been shown to result in a decreased rate of glucose absorption, and lower post-prandial glucose levels.[16] Proteinase inhibitor II (PI2) is a naturally occurring 21 kDa dimer and potent trypsin and chymotrypsin inhibitor present in white potatoes.[17,18] Previous studies using large doses of highly pure PI2 demonstrated increased CCK release and satiety in humans.[19-21] In addition, oral administration of PI2 at high doses in a liquid form has been shown to reduce both post-prandial glucose and insulin levels in humans,[21] supporting the use of PI2 as both a promising hunger management tool and an effective agent to reduce post-prandial glycemia experienced by the body.

The development of an efficient proprietary commercial process providing an extract from potatoes containing PI2 has increased the availability of this compound. We hypothesized that administration of PI2 extract as a nutraceutical ingredient in a low-dose, encapsulated form, prior to a meal, might reduce post-prandial glucose levels. This could have important implications for the use of PI2 as part of a diet to help maintain healthy blood sugar levels and reduce the propensity for weight gain.

METHODS

Subjects

Twenty-six men and 13 women, mean age 35 y (range 23-61 y) with a mean body mass index of 27 (range 23-32) participated in the study. Sample size was based on the study by Schwartz *et al*. who showed significant decreases in mean post-prandial glucose in six type II diabetic subjects following ingestion of a glucose/protein shake in the presence and absence of a high dose of PI2 (1.5 g).[21] All subjects gave informed consent before the study began, and could withdraw at any time. The Institutional Review Board of Des Moines University–Osteopathic Medical Center approved the study.

Study Design

Subjects were randomly allocated to receive placebo and two of the three following doses: 7.5, 15, or 30 mg PI2 extract. On each study day subjects arrived at 8.00 AM after a 10-hour fast. They were given breakfast and 500 ml of water to drink throughout the morning, but ate nothing further until the test meal. Height and weight of all subjects were recorded during their first visit. Three and a half hours after breakfast the first blood glucose measurement was made, and subjects were given the experimental capsule of PI2 and 500 ml of water. Thirty minutes later the test lunch was served. As soon as each subject completed the meal, the timing for post-prandial glucose measurements began. Subjects recorded any adverse reactions at 15-minute intervals for 200 minutes after eating the meal.

Test Meal

On each test day subjects were fed a breakfast of granola, skim milk, and orange juice that contained 330 k calories derived from 67 g of carbohydrate, 2.5 g of fat, and 12 g of protein. No other food was permitted until the test meal, which was consumed at noon on the test day. The test meal was Chicken Teriyaki (Boston Market) and contained no potato products (see Table 1 for macronutrient content). All subjects consumed all meals in their entirety.

Glucose Measurements

Finger-prick capillary blood samples were taken 30 minutes before the test meal (Baseline), and 30, 60, 90, and 120 minutes post-prandially. Glucose measurements were made with a Dex glucometer, Model# 3952E (Bayer Pharmaceuticals), in accordance with the manufacturer's instructions.

Proteinase Inhibitor

PI2 extract was provided by Kemin Consumer Care, L.C. (Des Moines, Iowa), and was supplied in 00 gelatin capsules containing 7.5, 15, or 30 mg, respectively. A mixture of dextrose and whey protein was used to bring all capsules to a uniform weight and volume and also served as a placebo. The doses in the present study were chosen based on previous studies demonstrating efficacy at 30 mg,[22,23] and 7.5 mg

(Gary Green, University of Texas, San Antonio, 1996, 1997, unpublished data) administered in liquid form. The active material was produced from a single lot of potatoes (Russet Nuggets; Kemin lot 87289C, approximately 244.39 mg PI2 extract/kg).

Measurement of PI2

RP-HPLC: Formulation of the active doses was based on quantitation by high performance liquid chromatography (HPLC). Briefly, reversed-phase HPLC (RP-HPLC) analyses were performed on a Hewlett Packard Model 1100 equipped with a Diode array detector using a Microsorb C-18, 5 mm particle size, 300 angstrom pore size, 4.6 X 250 mm (Varian Analytical Instruments, Walnut Creek, CA). The chromatographic conditions were as follows: Isocratic elution for five minutes of 80% of 0.% TFA in H_2O (20% of 1% TFA in acetonitrile). Gradient from 80-30% of 0.1% TFA in H_2O (20-70% of % TFA in acetonitrile) for 34 minutes. Gradient from 30-0% of 0.1% TFA in H_2O (30-100% of 1% TFA in acetonitrile) for 4 minutes. Flow rate was 1 ml/min for all gradients, and the column temperature was maintained at 30°C. Integration of the HPLC peak area provided the relative concentration of each sample (mg/g solids).

SDS-PAGE: To further characterize the PI2 extract, samples were analyzed by gel electrophoresis. SDS gels were prepared as 4% stacking, 15% resolving with 1.5 M Tris, 0.5 M Tris, 10% SDS, 30% ammonium persulfate, TEMED, and 40% Acryl/Bis. Wells were loaded with prestained marker, PI2 standard, and PI2 extract. A current of 80 volts was applied for 1.5 hours. Pure PI2 standard was obtained by sequential RP-HPLC followed by gel filtration chromatography. Western blot using a rabbit polyclonal antibody developed by Kemin Foods, L.C., against PI2 protein was used to further determine the identity of the major protein in the potato PI2 extract used in the current study.

Calculations and Statistical Analysis

The difference between the 30-minute post-prandial and baseline blood glucose values was calculated for each subject visit (Δ glucose). The integrated area under the blood glucose-time curve (AUC) after each test meal was calculated using the pre-meal value as the baseline, and integrating the area from 0 to approximately 120 minutes after the meal. Repeated measure analysis of variance was used to test for significant differences between areas. The research design involved repeated measures, so the PROC MIXED function in SAS was used, as this allows a more general specification of the covariance matrix of the dependent variable, and allows random factors of both the model and error terms to be correlated.[24] All subjects received placebo on one visit, but only two of the three possible active treatments during the other visits, so an incomplete block design was used to evaluate the relative effectiveness of the doses. The strategy described by Wolfinger[25] was followed to select an appropriate variance-covariance structure for the ANOVA test. The Akaike's Information Criterion was used to select the appropriate variance-covariance structure for the model. Chi-square analysis was used to evaluate data obtained as discrete variables with $p<0.05$ considered to be significant.

RESULTS

Doses of active PI2 extract were quantified by RP-HPLC. The integrated peak representing the PI2 extract co-eluted with a pure authentic PI2 standard, indicating that PI2 is contained in the extract and that it is the major protein (Figure 1). Results of gel electrophoresis further confirm the findings of the analysis by RP-HPLC and show that the PI2 in the extract is likely present as a monomer with a molecular weight of approximately ~12 kDa (Figure 2). MALDI MS analysis of the purified PI2 protein demonstrated that this protein has a molecular weight of 12 kDa (data not shown). Western blots of the separated proteins using a rabbit polyclonal antibody for PI2 protein demonstrated that the major protein band separated by SDS-PAGE is PI2. The actual amount of PI2 protein present in a given extract could vary and ranges from 17 – 20%. The PI2 extract was also characterized for its trypsin and chymotrypsin inhibition activity using an *in vitro* assay demonstrating both trypsin and chymotrypsin inhibition. PI2 extract product contained a ratio of 0.9 - 1.7:1 units of trypsin: chymotrypsin inhibition activity, respectively.

The volunteers in the present study consumed 120 test meals. Forty placebo doses were administered, along with 27 each of the 7.5 mg and 15 mg doses, and 26 of the 30 mg dose, respectively (one individual declined to provide blood samples and was included in determination of adverse events monitoring). Table 1 shows the nutrient value of the test meal and the mean glucose AUC following placebo. We first examined the effect of PI2 extract on AUC; the repeated measure ANOVA model used for this analysis showed a statistically significant effect of the experimental treatment ($f = 3.3$, $p<0.05$) but no statistically significant difference between the experimental blocks. Subjects given a dose of 7.5 mg PI2 extract before the test meal experienced no significant reduction in post-prandial glucose compared to placebo. The AUC of subjects receiving both 15 and 30 mg PI2 extract prior to the test meal was significantly reduced compared to placebo, but there was no significant difference in post-prandial AUC between the two higher doses (Figure 3). The decrease in AUC for 15 and 30 mg was 29.8% and 24.5% respectively, each compared to placebo. There was a significant reduction in Δ glucose at both the 15 mg and 30 mg dose levels compared to placebo, but there was no significant difference in Δ glucose between the two higher doses (Figure 4). The decrease in Δ glucose for the 15 and 30 mg doses was 25% and 20% respectively, each compared to placebo.

Figure 1.

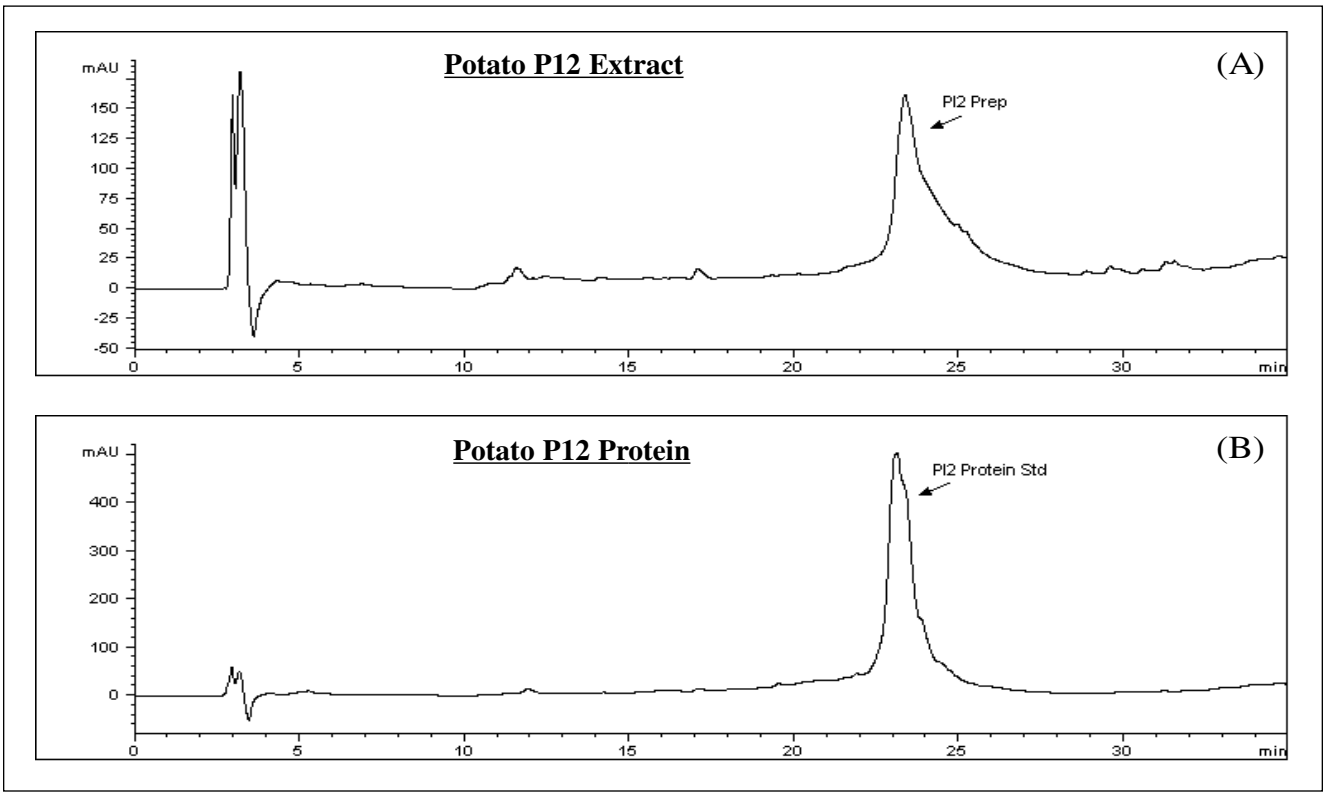


Figure 1. *HPLC chromatograms of potato PI2 extract and authentic PI2 standard*
PI2 extract (PI2 prep.) used to formulate the active doses in the present study was quantitated by HPLC as described in the Methods section.

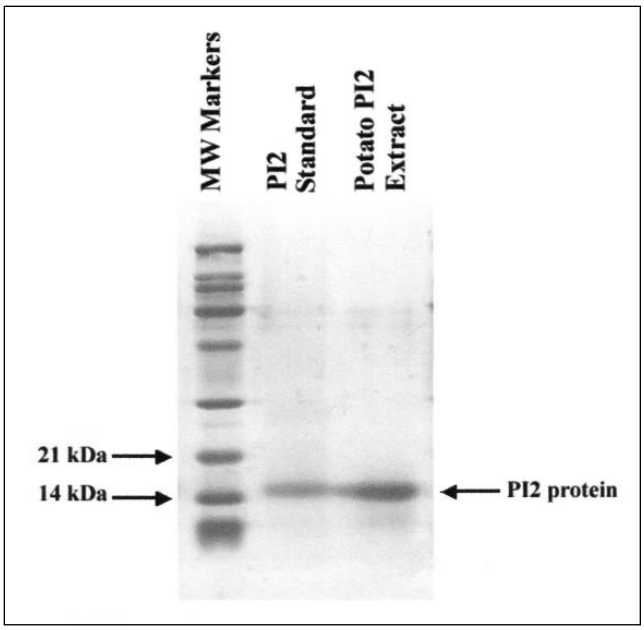
Feeding 120 test meals resulted in 14 adverse action reports summarized in Table 2. Gastrointestinal symptoms included nausea, cramping and diarrhea. Differences in occurrence rates of adverse reactions between the treatments and the placebo were not significant ($p>0.05$, Chi square). Subjects experiencing symptoms rated them as mild, and frequently they were noted at only one of the recording times.

DISCUSSION

The drastic rise in obesity rates over the past 10 years has been accompanied by diets resulting in chronic glycemia and hypersecretion of insulin[5,26] This, in turn, initiates a cascade of metabolic and physiologic events resulting in decreased lipolysis, increased *de novo* lipogenesis, and faster onset of hunger and subsequent food intake.[10,11] Rapid and drastic excursions in blood sugar may not only contribute to obesity but other chronic diseases, including diabetes and cardiovascular disease (summarized in Figure 5 and in Ludwig's *JAMA* article).[11]

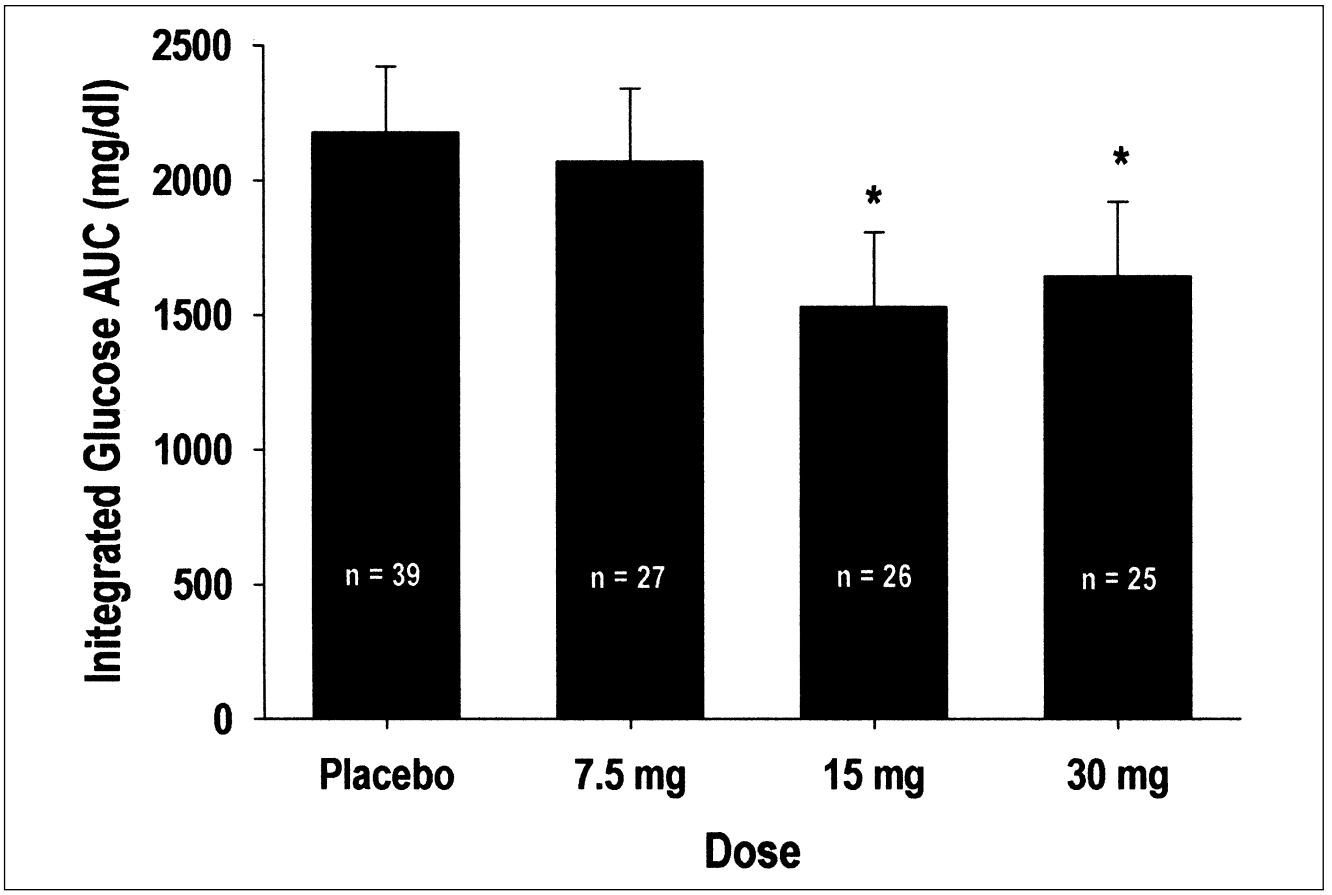
Accordingly, lowering the glycemic load experienced by the body by diet or other means may be an effective way

Figure 2.



SDS-PAGE of Potato PI2 extract and an authentic PI2 standard.

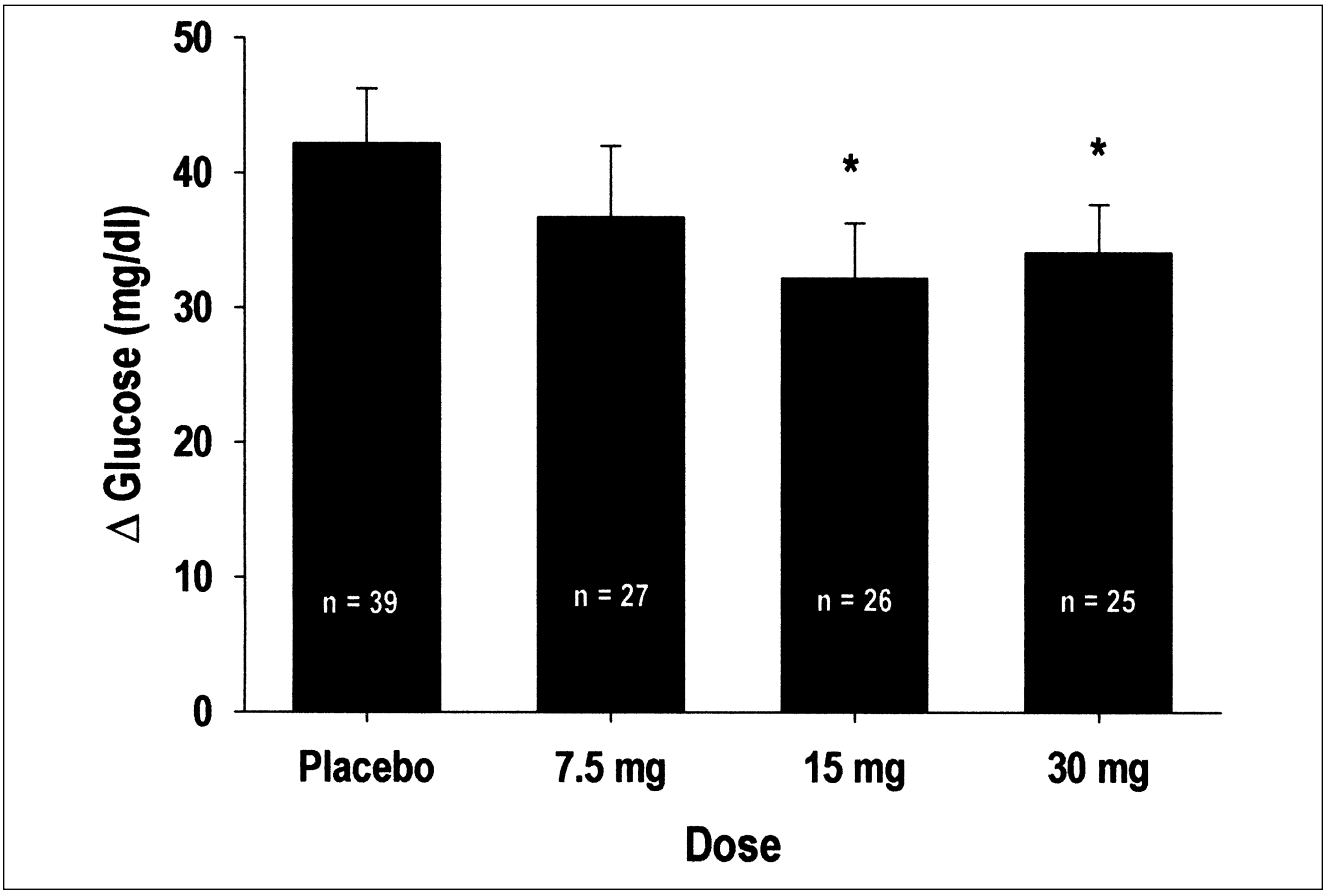
Figure 3.



Effect of increasing PI2 dose on post-prandial integrated area under the blood glucose curve (AUC) after a test meal. Subjects ingested placebo, 7.5, 15 or 30 mg PI2 30 minutes prior to a standardized test meal. Blood sugar was measured at time 0 (pre-meal), and at 30, 60, 90, and 120 minutes post-meal. All subjects received placebo, and were randomly assigned to two of the three other doses. Shown is the Mean±SEM for area under the curve (AUC). *p<0.05 *vs.* placebo.

to reduce the post-prandial glycemia that can lead to weight gain and obesity. Findings of the present study suggest that it is possible to lower the glycemic load experienced by the body by ingesting a supplement containing a low dose of PI2 extract prior to a meal. Doses of either 15 mg or 30 mg taken 30 minutes before a test meal significantly reduced the subsequent rise in blood glucose (Figures 3 and 4). A dose of 7.5 mg had no significant effect, indicating that under these test conditions the lowest effective dose lies between 7.5 and 15 mg. This study was limited to acute observations, and the effect of chronic oral administration of PI2 extract on blood sugar levels remains to be studied. However, this study is unique because PI2 has not previously been administered in solid form in an encapsulated supplement prior to the meal, and because a solid mixed meal was used for the first time. In addition, the dose used was substantially lower and less pure than that previously reported, and a larger cohort of subjects was studied. A dose of 1.5 g PI2 (90 - 100% pure) by column chromatography (Clarence Ryan, Washington State University, Pullman, WA), administered in liquid form was used in two previous studies; in one study PI2 was added to soup and fed 8 minutes before a test meal, and in the other it was incorporated in a test beverage.[20, 21] In neither case was it encapsulated. Other differences include the size and glycemic index of the test meals and potential variations in the PI2 dose bioactivity. We found a mean reduction in post-prandial blood glucose AUC of 29.8% with a 15 mg dose of PI2 extract and 24.5% decrease with a 30 mg active dose (Figure 3). Schwartz *et al.* reported a comparable 24.5% reduction in AUC after feeding 1.5 g PI2 with a liquid glucose and protein beverage administered to diabetics.[21] While the dose of PI2 administered in that study was apparently 100-fold larger, we cannot be sure that it was of the same specific activity as used in our current study. Therefore, it is unclear whether larger doses of PI2 extract would evoke a greater response.

Figure 4.



Effect of increasing PI2 dose on the initial rise in blood glucose above baseline (Δ Glucose) 30 minutes after a test meal. Subjects ingested placebo, 7.5, 15, or 30 mg PI2 30 minutes prior to a standardized test meal. Blood sugar was measured at time 0 (premeal), and at 30, 60, 90, and 120 minutes post-meal. All subjects received placebo, and were randomly assigned to two of the three other doses. Shown is the Mean±SEM for the initial increase in blood sugar from time 0 to 30 minutes (Δ Glucose); *p<0.05 *vs.* placebo.

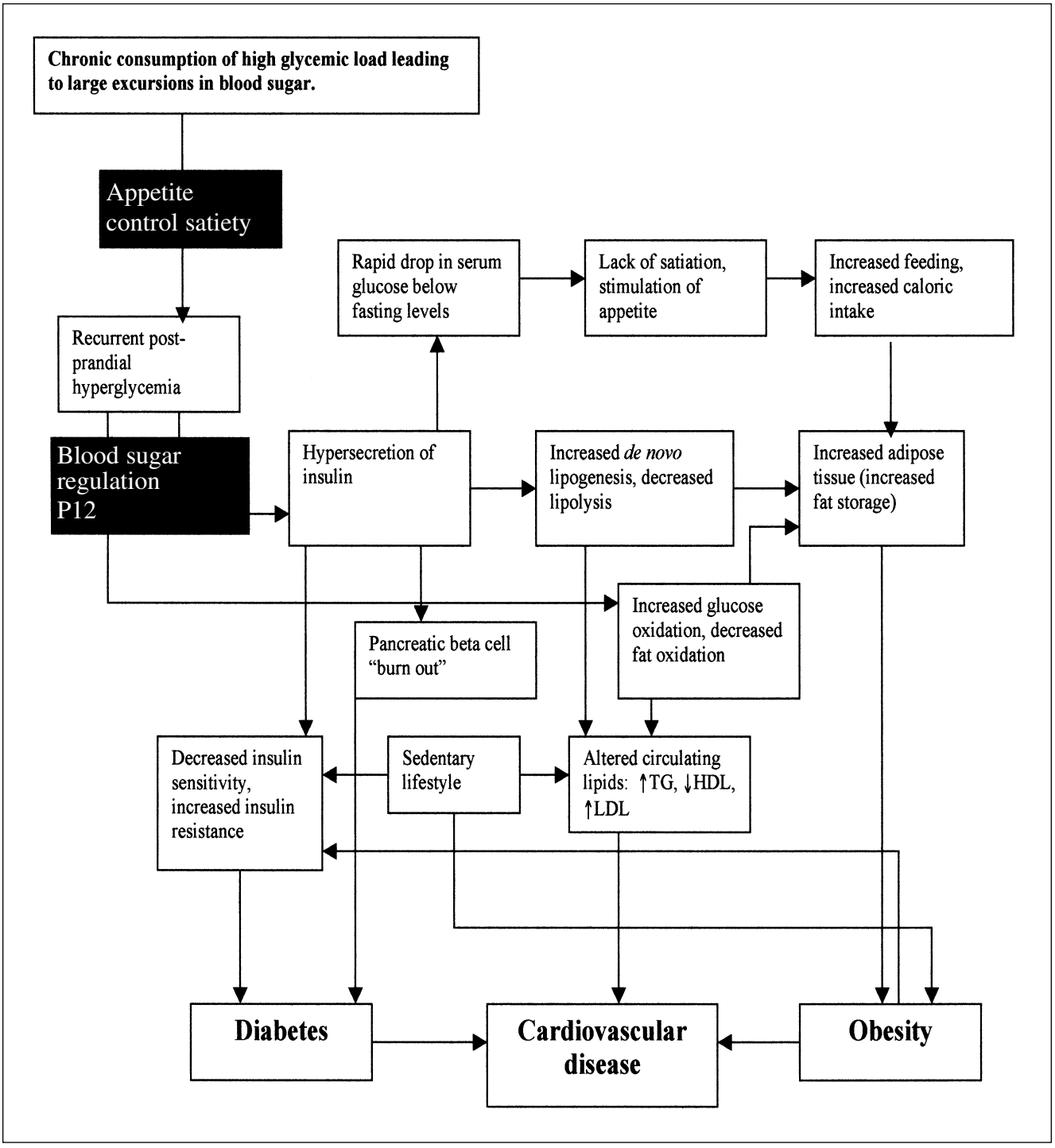
Inspection of the responses of individual subjects to placebo or active dose reveals that 9 subjects experienced no reduction in glycemia with either of the two dose levels of PI2 extract administered (non-responders). There was no significant effect of BMI, age, or fasting blood glucose on responsiveness. Among the 9 non-responders from the initial study, 8 were male and one was female, although this difference was not significant (p=0.07, Chi-square).

The notion of lowering the glycemic load to reduce or maintain weight is supported by both animal and human studies. Normal rats fed isocaloric diets differing dramatically in terms of glycemic load, experience large differences in post-prandial glycemia and insulin response.[27,28] Maintaining rats on these diets for weeks at a time results in drastic differences in glucose and lipid metabolism. The level of *fatty acid synthase* and *de novo* lipogenesis, as well as adipocyte size are higher in rats consuming a high *vs.* low glycemic load diet.[27,28] These data provide evidence at the cellular and metabolic level, that drastic elevations in blood sugar caused by exposing the body to a high glycemic load results in increased fat accumulation over a relatively short window of time. Consistent with this are results from long term research showing that adult rats fed isocaloric diets evoking chronic hyperglycemia gain a significant amount of weight while those fed a diet with moderate glycemia maintain their weight.[29]

Weight loss studies in humans suggest that reducing the glycemia experienced by the body is an effective means to reduce and maintain weight. Subjects consuming isocaloric diets consisting of low glycemic index foods lose more weight or maintain their weight relative to those consuming high glycemic index foods.[30-32] These findings suggest that manipulation of the glycemic load, in these cases by consuming low glycemic load diets, can effectively stimulate weight loss and/or prevent weight gain. Combined with the results from the present study, these support the hypothesis that PI2

continued on page 37

Figure 5.



Schematic of the effects of chronic consumption of a high glycemic load.
Bold black font (bottom) represents potential chronic diseases. Black boxes with white font (top left) represent two possible approaches to reduce recurrent large excursions in blood sugar that can lead to weight gain and chronic disease. Derived from information provided in Ludwig 2002.

Table 1.

Characteristics of the test meal	
	Lunch Test Meal
Energy (kcal)	460
Fat (g)	11
Carbohydrate (g)	53
Protein (g)	27
Number of participants taking meal challenge after placebo	39
Average AUC for placebo participants (SD)	2196.6 ± 1567.2

Table 2.

Subjects recording adverse effects after eating a test meal preceded by PI2 extract				
PI2 Extract Dose (mg)	**Gastrointestinal Symptoms**	**Headache**	**Total**	**Chi-square (*vs.* Placebo)**
0	3	---	3	---
7.5	5	1	6	0.19
15	---	2	2	1.76
30	3	---	3	0.23

Table 3.

Summary of PI2 clinical trials				
Study	**Institution**	**Dose**	**Form**	**Outcome**
Spiegel *et al.* 1999 (22)	Columbia University	30 mg PI2 extract	Liquid (pre-meal shake)	Significant decrease in hunger ratings; increase in fullness ratings; 2 kg weight loss
Vasselli *et al.* 1999 (23)	Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey	30 mg PI2 extract	Liquid (pre-meal shake)	Significant decrease in hunger ratings; increase in fullness ratings
Schwartz *et al.* 1994 (21)	University of Texas, San Antonio	1500 mg PI2	Liquid (shake)	Significant increase in plasma CCK; delayed gastric emptying; decreased blood sugar
Hill *et al.* 1990 (20)	University of Leeds, U.K	1500 mg PI2	Liquid (pre-meal soup)	Significant decrease in food consumption
Peikin *et al.* 1987(19)	Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey	1000 mg PI2	Liquid (shake)	Significant increase in plasma CCK levels
Green, 1996 – 1997^	University of Texas, San Antonio	7.5 – 100 mg PI2	Liquid (shake)	Doses as low as 7.5 mg delayed gastric emptying and reduced blood sugar level

^Unpublished data

extract can serve as an effective nutraceutical to lower the glycemia experienced by the body, and may help promote weight loss and reduce the propensity for weight gain.

PI2 extract is proposed to exert its effect on post-prandial glucose by enhancing the release of a well characterized peptide hormone, CCK, which is naturally secreted into the bloodstream by enteroendocrine cells in response to a meal.[33] CCK acts on various target tissues throughout the body including the gastrointestinal tract, where it delays gastric emptying leading to feelings of fullness, and the brain leading to feelings of satiety. Although not measured in the present study, previous studies in the late 1980s and 1990s demonstrated that large doses of purified PI2 enhance the release of CCK,[19,21] delay gastric emptying time,[21] and decrease energy intake[20] in humans. These were followed by studies using a lower dose of less pure PI2 extract which demonstrated reduced hunger and increased fullness ratings [22,23] (summarized in Table 3). These findings are consistent with the well-established fact that PIs are potent stimulators of CCK release in rats.[13]

PI2 is a pH, heat, and salt-stable protein,[18] allowing it to be effective when administered orally, and making it unique among plant PIs. The extract used in the present study contains PI2 (Figures 1 and 2), and is derived from white potatoes using proprietary technology (Kemin Consumer Care, L.C. Des Moines, IA). Although normally present in potatoes as a dimer, the PI2 separated from our extract appears to be in the monomeric form. Both the pure PI2 and PI2 extract possess comparable trypsin and chymotrypsin inhibition activities. The added beneficial effect of reducing post-prandial glycemia make PI2 extract a unique and promising nutraceutical.

Some studies involving the direct infusion of CCK have reported minor adverse side effects such as headache, nausea, and diarrhea.[33,34] For this reason we questioned participants specifically about these effects which may ultimately have prompted reporting of events that would otherwise have gone un-noticed. Although there are a number of reports in the literature demonstrating morphological changes in the pancreas as a result of long-term exposure to extremely high doses of natural and synthetic PIs in rodents, similar studies in pigs and primates are not associated with such effects.[35-38] Such effects also have yet to be observed in humans using PIs from natural sources. Furthermore, previous studies using PI2 have not demonstrated any side effects with doses many times that used in this study.[19,21] Side effects noted by our subjects were mild and inconsistent, and caused no withdrawals from the study. No increasing dose response was noted for any of these effects and the rate of occurrence was not different between placebo and treatment. If persistent use of PI2 extract were contemplated we might see additional mild side effects, although it is equally possible that tolerance to undesired effects would develop over time.

In conclusion, we have demonstrated in the largest randomized controlled clinical trial to date that a low dose of PI2 extract prior to a standardized meal reduces significantly post-prandial glycemia in the majority of healthy subjects. Additional studies will be required to ascertain long-term effects of this supplement on blood glucose, appetite and body weight. While a mechanism of action has been proposed, it will be important to confirm this hypothesis in future studies addressing changes in serum CCK, insulin, etc... Such studies could be instrumental in applying PI2 to the clinical problems of obesity and diabetes.

ACKNOWLEDGEMENTS

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PERSPECTIVE

Policosanol – A New Nutraceutical Tool for Reducing Cholesterol

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Coronary artery disease is the most common cause of death and disability in the United States and many countries worldwide. Elevated levels of total cholesterol and LDL and low levels of HDL are important risk factors for its development and subsequent morbid events. The statin drugs have made a major impact in reducing morbidity and mortality from coronary artery disease.[1] However, the statin drugs can and do cause serious side effects such as myalgias, myopathy, liver dysfunction, neuropathy, and rhabdomyolysis with subsequent kidney failure. The side effect profile is dose-related: with increased doses, one is more likely to see serious side effects. In addition, a variety of drugs which, when used concomitantly with the statin drugs, cause interactions which may also trigger the above side effects.

Sixty percent of the world's refined sugar is derived from sugar cane, a coarse grass that grows in semitropical and tropical climates. Most cultivated sugar cane today is an ancestor of *Saccharum officinarum,* which originally came from New Guinea. Cuba is one of the world's leading producers of sugar cane. The nutraceutical Policosanol is derived from sugar cane wax. Because of the high cost of statin drugs and the fact that there is an embargo on Cuba, Cuban researchers in their own inventive way have shown that sugar-cane-derived Policosanol reduces total cholesterol and LDL, and raises HDL levels. They also found Policosanol to be safe and well tolerated, without the side effect profile reported with statin drugs. Policosanol is a mixture of the aliphatic alcohols, which includes octacosanol, triacontanol, and hexacosanol, plus five other fatty alcohols as the minor components. Ingested, Policosanol is mainly distributed in the liver; its rapid hepatic consumption is advantageous for a cholesterol-lowering drug as the liver is the main organ for synthesizing and regulating cholesterol metabolism.

The mechanism by which Policosanol reduces cholesterol is not entirely known; however, it has been shown that it inhibits cholesterol biosynthesis from $_{14}$C acetate or tritiated water, but not from $_{14}$C mevalonic acid.[2] In addition to reducing cholesterol levels,[3] Policosanol has also been shown to decrease platelet aggregation[4] and inhibit the development of atherosclerotic plaques.[5] Numerous human trials have been done, mostly in Cuba, to evaluate Policosanol in hypercholesterolemic patients and patients with non-insulin-dependent diabetes mellitus.[6] The results from these studies show significant reductions in LDL and total cholesterol and significant increases in HDL. In 1999,

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Mas *et al.* [7] published a double-blind, randomized, placebo-controlled study on the effects of Policosanol in 437 patients with type II hypercholesterolemia and other coronary risk factors. After three months of using both 5 and 10 mg per day, there was a significant reduction in LDL cholesterol of 18.2% and 25.6% respectively and total cholesterol levels of 13% and 17.4% respectively. There was also a significant increase in HDL levels of 15.5% and 28.4% respectively. A surprising finding was that triglycerides decreased by 5.2% with the 10 mg per day dose. It is also interesting to note that the side effect profile was lower in the Policosanol group than in the placebo group. There were no serious adverse events seen in the patients receiving Policosanol.

There is a high incidence of peripheral arterial disease in patients with coronary artery disease and the distressing symptom that occurs with patients who have significant peripheral arterial disease in intermittent claudication, that is, pain in the calves and legs when walking. In a double-blind placebo-controlled study that evaluated Policosanol in 56 patients with moderately severe intermittent claudication,[8] it was found that with the use of 20 mg per day over a period of two years, the patients who received Policosanol had significant improvement in their walking distance and reduction of their intermittent claudication symptoms compared to the placebo group. Some animal studies and *in vitro* and human studies suggest that Policosanol also reduces the pro-inflammatory substances thromboxane A_2 and B_2.[9] Also, studies have shown that in experimental models and in hypercholesterolemic humans, serum prostacyclin levels have increased with Policosanol consumption.[10] With increased serum prostacyclin levels, smooth muscle cell proliferation is inhibited, as is peroxidation of LDL.[11] If indeed this is the case, then Policosanol may have a protective effect on the development of atherosclerosis.

The statin drugs lower cholesterol by inhibiting HMG-CoA reductase, thereby inhibiting cholesterol synthesis. Investigative studies have shown that Policosanol does not directly inhibit HMG-Co2 reductates, but does have a modulatory effect on the activity of reductase.[12] From our search of the literature to date, it appears that numerous studies involved about 3,000 patients in randomized placebo-controlled and comparative studies versus statins. Most of these studies were done by Cuban investigators and published in peer-reviewed journals.

The authors feel that Policosanol, being a natural compound, provides an exciting approach to combat cholesterol as an agent that can be used alone or in combination with the statin drugs. We feel that further randomized double-blind placebo-controlled studies need to be conducted in the United States. The buzz on Policosanol has already started in this country. Several companies produce Policosanol and health food stores and some drugstores are selling it under brand names that include Policosanol Advantage™, Lesstanol™ and Cholestin™. Some companies produce Policosanol from beeswax; whatever their distribution of fatty alcohols, it would certainly behoove anybody who manufactures this compound to keep the same distribution of fatty alcohols as seen in the studies done using Cuban Policosanol.

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BOOK REVIEW

The Paleo Diet

By Loren Cordain, PhD
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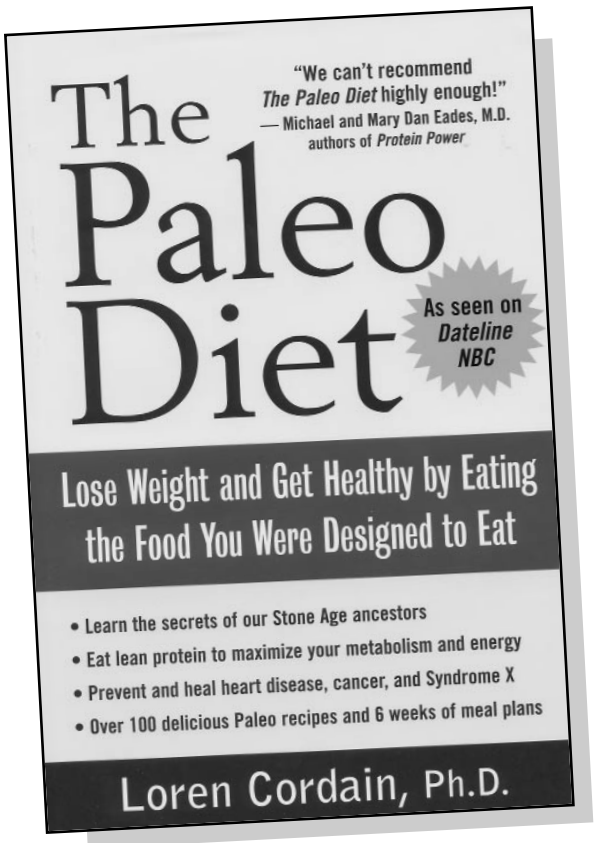

Loren Cordain, PhD, world renowned scientist and leading expert in his field has for the past 20 years been involved in research on the original human diet, that is the paleolithic or stone age diet. He is a professor in the Health and Exercise Science Department, Colorado State University, and lectures throughout the world. Dr. Cordain's latest book, *The Paleo Diet,* is revolutionary, intuitive, logical and compelling. It is based on enormous accumulated evolutionary, anthropologic and modern nutritional concepts. Utilizing fossil records, contemporary hunter-gatherers diets, chimpanzee diets, nutrient analysis of wild animals and plants, and computer analysis, Dr. Cordain has compiled exhaustive scientific data to support his hypothesis about genetics, nutrition, and health in the paleolithic societies as compared with modern day societies. The paleolithic age began 2.5 million years ago in Africa (stone tools), and ended 10,000 years ago in the Middle East with the advent of ancient farming and agriculture. Over 13 different species of ancient humans inhabited the earth at that time.

The basic premise of the *Paleo Diet* is that diets of the paleolithic and modern day hunter-gatherers may represent an optimal reference standard for modern human nutrition as well as one for prevention and treatment of numerous diseases of affluence or "civilization" in agricultural societies. When ecologically possible, the majority of worldwide hunter-gatherer societies derived about 55 to 65% of energy requirements from animal foods, with the remainder supplied by low-carbohyrdrate nutrient-dense, high-fiber,wild plant foods, berries, and fruits.

This easy to read and understand book provides chapters on the paleolithic diet basics, the paleolithic ancestor's diet, the impact of civilization and straying from the original diet, and the resultant illnesses, diseases and obesity. The final chapters discuss the role of the paleo diet in weight loss and prevention and treatment of disease, and provide detailed paleo diet meal plans and recipes. There are excellent tables and appendices comparing the micronutrient and macronutrient content of the paleolithic diet versus modern nutrition, and there are discussions on exercise and the practical implementation of the paleo diet on a global scale.

The human genome today is over 99.9% identical to that of our stone age ancestors 40,000 years ago. However, the hunter-gatherer lifestyle was supplanted by the agricultural revolution 10,000 years ago, followed later by diets of processed, refrigerated, fast, and "industrialized" foods incompatible with our genetic makeup ("nutritional missteps"). Our genes determine our nutritional needs, and

selective environmental and nutritional pressures determine our genes (theory of evolution by natural selection to diet and health). Modern foods are at odds with our genetics. This nutrient-gene interaction may result in disease depending on the makeup of each.

Our stone age ancestors, as well as some remaining modern day hunter-gatherer societies in South America and the Andaman Islands, had (and have) diets consisting of lean wild meat, fresh berries, and other fruits, and vegetables. These societies were (and are) healthy, fit, strong, vivacious, and free from signs and symptoms of chronic modern diseases. They have low body fat, more lean muscle, better aerobic fitness and no hypertension, hyperlipidemia, insulin resistance or diabetes mellitus. This is in stark contrast to the U.S. population, where 63% of men over 25 years of age and 55% of women over 25 are obese. Cardiovascular disease accounts for 41% of all deaths and remains the number one cause of mortality in the U.S. In addition, there are 50 million adults with hypertension, 40 million with hyperlipidemia, and 10 million with diabetes mellitus.

The human genome provides the "blueprint" for optimal nutrition. When bad nutrition is provided, bad health ensues. This is analogous to using diesel fuel in a gasoline engine. The paleolithic diet consisted of no dairy, no processed food, no cereal grains, no salt, refined sugar (some honey), and was rich in lean animal meat and seafood with high protein, low fat, and low carbohydrate content. There were numerous carbohydrates from non-starchy wild fruits and vegetables with high fiber content and beneficial fats with monounsaturated fats (MUFA), polyunsaturated fats (PUFA), and plenty of omega 3 fatty acids, but no saturated (SFA) or trans-fatty acids (TFA). A comparison of the paleolithic and modern diet in general terms is shown below:

	Paleolithic	**Modern**
Protein	19 – 35%	15 – 16%
Carbohydrate	22 – 40%	49%
	(low glycemic foods)	
Fat	28 – 47%	34%
	good fats MUFA, PUFA	excessive SFA and TFA
	Omega 3/6 ratio ~ 1:1	

High protein content in the form of lean wild meat has numerous health benefits as it is 80% protein and only 20% fat, compared to "modern meat" which has significantly more fat and less protein. Protein increases the thermic effect (dietary-induced thermogenesis) which is twice that of fat or carbohydrate and which increases the basal metabolic rate. In addition, protein has a satiating effect greater than carbohydrate or fat, both at mealtime and in-between meals. Protein has been demonstrated to improve the lipid profile, reduce blood pressure, lower homocysteine, improve thyroid hormone levels, decrease insulin resistance and type II diabetes, and to reduce weight, stroke and breast cancer risk. High protein intake does not change renal function in the absence of renal insufficiency. Overeating protein is unlikely to occur as it results in nausea, vomiting, diarrhea, and weight loss (called protein toxicity or "rabbit starvation") when one consumes over 200-300 grams of protein per day. Dr. Cordain advises that 55% of total calories should be from lean meats. He notes that red meat (protein and fat) became the scapegoat for the saturated fatty acid and cholesterol scare in the 1950s when it was really about the relative percent of fat in some modern meats.

The increased salt intake (NaCl) in modern versus paleolithic diets increases blood pressure, stroke, calcium loss, osteoporosis, renal insufficiency, proteinuria, left ventricular hypertrophy, congestive heart failure, nephrolithiasis, asthma, insomnia, motion sickness, Meniere's disease and eclampsia. The high chloride load increases the acid load presented to the kidneys.

A higher content of acid foods such as dairy, cereals, legumes, certain meats, fish, and eggs may alter calcium balance and increase osteoporosis incidence. Consumption of more alkaline foods such as fruits and vegetables will improve calcium balance and reduce osteoporosis.

The paleo diet advocates seven key features, but there is no calorie counting and one can eat until full:

1. Increase lean protein
2. Increase fruits and vegetables with low glycemic index
3. Increase fiber
4. Increase MUFA and PUFA, with omega 3/6 ratio = 1.0
5. Increase potassium, but reduce sodium
6. Increase alkaline food load and decrease acid food load (avoid dairy, cereals, legumes)
7. Increase plant phytochemicals, vitamins, minerals, and antioxidants

This nutritional plan provides 100% of the RDA nutrient requirements except for vitamin D, which can be taken as a supplement or provided by periodic sun exposure. The correct combination of fruits, vegetables, and protein optimizes calcium balance and prevents osteoporosis.

The present U.S. diet is very unhealthy. Protein content is only 50% of the needed amount. Carbohydrates are too much of the wrong type, providing "anti-nutrients", poor nutritional value, and over 50% of calories with a high glycemic index. By the very consumption of carbohydrates, potein intake is reduced. The sugar content (sucrose) of the U.S. diet is enormous and when metabo-

lized to glucose (GI = 97) and fructose (GI = 23), obesity, insulin resistance and other health problems such as Syndrome X (metabolic syndrome) result. For example, one 12-ounce soda contains 10 teaspoons of fructose. The fiber content of the U.S. diet is too low, which would be easily corrected by eating more fruits and non-starch vegetables, which respectively contain two to eight times more fiber than whole grains. The fat content is also the wrong type with twice the amount of SFA, too much TFA, and not enough MUFA and PUFA, as well as an omega 3 to 6 ratio of 1:10, not the optimal 1:1. The sodium/potassium ratio is too high, acid foods are too numerous, and the phytochemical vitamin, mineral and antioxidant content is too low. Furthermore, grains contain phytates which reduce iron, zinc, copper, and calcium absorption; as well, they contain pyridoxine glucosides, which decrease vitamin B_6 bioavailability.

The diseases that result from this poor nutrition are, in part, related to an imbalance of IGF-1 (insulin growth factor-1) and IGF-BP-3 (insulin growth factor-binding protein-3), and include carcinomas, myopia, acne, polycystic ovarian syndrome, metabolic syndrome with insulin resistance, diabetes mellitus, hypertension, dyslipidemia (dense LDL, high TG, low HDL), obesity, gout, clotting disorders, cardiovascular disease, and many other nutritionally-related diseases including gastrointestinal diseases, psychiatric diseases, dental caries and skin cancers.

Although it is no longer possible or practical for contemporary men and women in western industrialized countries to adopt and follow the exact dietary patterns of humans living during the paleolithic age, it is certainly possible to emulate the essential characteristics of historically-studied hunter-gatherer diets, as suggested by Dr. Cordain in his book, with common foods and food groups available in most supermarkets. This could have a major impact on nutritionally-related disease mediated through the vital nutrient-gene interaction.

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