

March 2000 Issue | DHA on Mitochondrial Function

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Welcome to the March 2000 issue of *Functional Medicine Update*. The Seventh International Symposium on Functional Medicine will be held May 23-26 in Scottsdale, Arizona. In preparation for that symposium, I want to focus on mitochondrial function this month in *FMU*. We will address questions about bioenergetics as the theme of the symposium. Better understanding of the energy process in human physiology and the role of the mitochondrion in that process may help us in the amelioration, prevention, and management of age-related chronic diseases.

In this issue of *FMU*, we are fortunate to have a researcher who is an authority in the area of mitochondrial dysfunction. He will speak about fundamental studies that are trying to answer questions about the role of mitochondria in disease, how the environment influences mitochondria, and how potentially to ameliorate mitochondrial dysfunction in people with induced mitochondrial dysfunction.

Let me move to the topic at hand in preparation for this discussion, which is to look at the mitochondrion more as the controlling switch for energy processes in the body. That function interrelates with a variety of symptoms of fatigue and pain in chronically ill patients. These symptoms may be related to miscommunication among the several thousand mitochondria in cells, and among the other organelles and functional components of the cell.

This miscommunication gives rise to the dysfunctional physiology seen as altered membrane electrolyte transport, altered energy gradients, changes in intracellular pH, and the activation of various cell signaling substances through modification of gene expression. These alterations cause changes in cell receptor site activation and, ultimately, the nociceptor activation we see with pain and the low energy potential we see with fatigue. This kind of discussion highlights the focus of the March issue of *FMU*.

A few months ago we talked about the calcium paradox. One theme of any discussion of mitochondria is the role of calcium, not only at the cellular membrane in transport to the intracellular region, but also across the mitochondrial membrane. The mitochondrial membrane is a double membrane; the inner mitochondrial membrane has a very selective transport process that functions to maintain its electrochemical gradient. The energy that resides in this gradient functions like a storage battery and is used to synthesize ATP. Calcium leakage, therefore, creates a change in the energy potential of the mitochondrion, just as it changes the functional dynamics of the cell.

In discussing the calcium paradox, we described work being done at the University of Calgary, Alberta, School of Medicine. This research concerns intracellular calcium uptake as a consequence of the osmotic driving of calcium across the cellular membrane, and the loss of magnesium. (You will recall that calcium

is concentrated in the extracellular environment, and magnesium level is high in the intracellular environment.) As one loses this membrane-pumping dynamic, calcium can leak into the cell, resulting in calciphylaxis. Magnesium can leak out of cells and cause an intracellular hypomagnesium condition.

That simplistic conclusion, according to the University of Calgary research we previously discussed in *FMU*,¹ is actually wrong. Calcium-binding protein (Ca-BP) associated with parathyroid hormone, derived from the parathyroid gland, regulates calcium dynamics. When Ca⁺⁺ is bound to a newly identified protein, parathyroid hypertensive factor (PHF) is unable to enter cells. The production of Ca BP is controlled by calcium concentrations, and too little dietary calcium can increase the influx of calcium across the cellular membrane by reducing the concentration of Ca-BP. This is the calcium paradox. Conversely, increased dietary calcium can reduce calcium uptake across the cell membrane and reduce intracellular calcium accumulation.

A recent paper in *Nature Medicine* provided a follow-up to this concept. In this paper, titled "Is Calcium the 'Cure' for Dilated Cardiomyopathy?",² the authors state that different studies have reported findings about the roles of calcium in myocyte function and the development of dilated cardiomyopathy. Determining whether increasing intracellular calcium is helpful or harmful for heart failure patients, however, is under further investigation. Extracellular calcium concentration does appear to play a role in calcium efflux. When intracellular calcium is low, it can increase electrogenic transport of calcium to the intracellular region. This seems to confirm the previous University of Calgary School of Medicine studies we described, which found that increasing dietary calcium can have an advantage in lowering the uptake of calcium across the cellular membrane. Of course, we want to maintain balance with the magnesium component. In some cases it may be advisable to increase the magnesium-to-calcium ratio to replete intracellular stores of magnesium. The combination of adequate calcium and magnesium, therefore, may play an important role in preventing the increased uptake of calcium across the cellular membrane.

This month, we will focus on the mitochondrion and its energy relationships to health and disease. The first time the potential significance of mitochondrial dysfunction and disease was described in the medical literature was in a review article by Dr. Donald Johns from Beth Israel Hospital in Boston, published in the *New England Journal of Medicine* in 1995.³ This paper, which we reviewed in *PMU*, signaled a new era in the understanding and appreciation for the role mitochondria may play in health and disease.

In that paper Dr. Johns pointed out that a variety of well recognized constitutive mitochondrial dysfunctions are related to mitochondrial deletion mutations. They are associated with conditions called mitochondrial encephalomyopathies, which are a diverse group of disorders resulting from the structural, biochemical, and genetic derangement of mitochondria. Mitochondria, which have their own DNA, are the only place in the cell other than the nucleus in which genetic information is found. Mitochondrial DNA is not so bound up, super-coiled, and coated by histone and non-histone proteins as nuclear DNA. It is more like a bacterial DNA. It seems to be more susceptible to environmental damage by mutagens.

Mitochondria are the principal site where oxygen is used in the process of oxidative phosphorylation. Therefore, oxidants are available, the mitochondrial DNA is more exposed, and the potential for mutational injury to mitochondrial DNA is increased. Individuals who have constitutive mitochondrial deletion mutations may develop potentially severe genetic metabolism diseases of infancy. These diseases are associated with ophthalmoplegia, stroke, seizures, myoclonus, optic neuropathy, myopathy, fatigue and exercise intolerance, ataxia, dementia, dystonia, and basal-ganglia calcification.

Mutation of the DNA in mitochondria of oxygen-rich tissues can be related to a series of dysfunctions across many organs. In his article, Donald Johns talks about skeletal muscle problems, including weakness, fatigue, myopathies, heart conduction velocity disorders like Wolff-Parkinson-White syndrome, ocular problems like retinopathy and ophthalmoplegia, hepatopathies, Fanconi's syndrome, endocrine/pancreas disorders associated with diabetes, Pearson's syndrome with immunological defects, colonic obstructive disorders, and a range of central nervous system problems, including seizures, myoclonus, ataxia, stroke, and dementia.

Mitochondrial function within various tissues plays an important role in the overall performance of that tissue. Conditions associated with genetic metabolism disorders of mitochondrial DNA mutations include MELAS syndrome, seizures and stroke-like events that cause subacute brain dysfunction, myoclonic epilepsy, neuropathies and ataxia, and Leber's hereditary optic neuropathy.

It was only 25 years ago that the first genetic metabolism disorder associated with mitochondrial mutations was identified, and it was believed to be a very uncommon and esoteric part of medicine. In the subsequent 25 years, however, approximately 100 other mitochondrial disorders have been identified. The more one looks, the more one finds. We began by looking at very acute mitochondrial DNA mutation-related dysfunctions. Now we are looking at those with less acute and more chronic implications. The latter have to do with mutations or deletions of mitochondrial DNA components that control the synthesis of proteins that may interrelate with the electron transport system or mitochondrial structure. These defects may cause uncoupling or dysfunction in the way mitochondria engage in electron transport and oxidative phosphorylation. That may increase oxidative stress-induced damage by releasing these oxidant species. It is like taking the insulation off a wire.

In this electrical analogy, if you had a lamp cord going to the wall, and you had low insulation, you get a short circuit. Electrons can spark and travel off the wire under your curtains and burn your house down. That is comparable to what happens with some mitochondrial mutational injuries that create increased oxidative damage to the mitochondria. The cell is on fire, so to speak, to use a term that was coined by Sen in an article on Parkinsonism, which may be a mitochondria-related dysfunction, as well.

There are many systemic manifestations related to mitochondrial DNA mutations. We now know more about constitutive, inborn errors of mitochondrial DNA mutations. This spectrum in disease is much more prevalent than we previously recognized.

Induced Mitochondrial Mutations

An interesting part of this story is the emerging understanding that mitochondrial mutation may be related not merely to constitutive mitochondrial dysfunction, but also to cumulative mitochondrial mutations over the course of living, or induced mitochondrial mutations. This possibility was first brought to light in 1996 with Greg LeMond, the elite bicycle racer who retired following what had been diagnosed as induced mitochondrial myopathy. A Letter to the Editor of the *New England Journal of Medicine* suggested it was fortunate that someone of that prominence had developed this condition. It drew medical attention to the disorder more effectively than would have been the case with someone who did not enjoy that degree of prominence in the media.⁴

The induction of mitochondrial mutational injury in Mr. LeMond may have resulted from a variety of

factors. Genetic susceptibility certainly may have played a role, but it may be also accompanied by his training at high levels for many years in competitive bicycle racing at the edge of anaerobic debt producing ischemic events that increase oxidative stress within mitochondria. It may have been a consequence of an earlier hunting injury, which left lead shot inside his body. (Lead is a known free radical catalyst.) It may have been a consequence of unbalanced diet and lifestyle habits in regard to certain mitochondrially active nutrients that help protect against oxidative damage.

The Lesson of Greg LeMond

Whatever the cause, it appears that Greg LeMond suffered from an induced mitochondrial injury, not a constitutive injury. The question, then, is how broad are the problems associated with dysfunctional mitochondria? Are they beyond those of the very rare constitutive, inborn genetic mutational deletion situations? Do they occur more frequently in older people as a consequence of the induction of mutational injuries throughout the course of living? Chronic exposures may have increased oxidative stress and damage to their mitochondrial genome.

A recent paper in *Science* magazine raises this question to the next level. The paper is titled "Aging-Dependent Large Accumulation of Point Mutations in the Human mtDNA Control Region for Replication."⁵ It is the work of Attardi and his colleagues at the Division of Biology, California Institute of Technology. In this paper, the investigators found progressive damage to mitochondrial DNA throughout a life. They postulate that progressive damage may contribute to accelerated biological aging. This particular paper contains no evidence that one can correlate cumulative mutational injuries to mitochondria with clinical signs of aging. It was not a clinical paper. It contains a discussion of human fibroblasts from normal-younger and normal-older individuals, looking at mutational injuries to mitochondrial DNA. The authors did not correlate this with any of the clinical signs of aging. Other studies that I will describe have at least suggested that as mitochondrial injuries increase, signs may accumulate of what might be commonly called biological aging—fatigue, low energy, and chronic pain-related symptoms.

The editorial that follows this paper in *Science* magazine asks and answers the question, "Do Mitochondrial Mutations Dim the Fire of Life?"⁶ The author suggests that, according to expert researchers in this field, like Dr. George Martin, a gerontologist at the University of Washington in Seattle, there really is no evidence that the mutations functionally impair the cell or change the rate of mitochondrial replication and therefore have specific clinical implications. We have not answered that question yet. The association is interesting, however, and worthy of further inspection, because it does suggest that we could accumulate, at differing rates, induced mitochondrial injuries as a consequence of lifestyle, environmental, dietary, and genetic factors. Those injuries may be associated with running out of energy or energy dysfunctions. Protection of mitochondria may be the theme that emerges.

In a broader context, one might ask about alcoholics with various types of myopathies and certain drugs or medications that produce certain types of myopathies—fatigue and pain-related symptoms. Are they related to mitochondrial injury that we can use as a model? I will discuss that in greater detail, because accumulating evidence seems to suggest that certain environmental factors that increase mitochondrial mutational injury and induce interruption with appropriate mitochondrial function can, over time, increase dysfunction we might associate with biological aging in the individual.

This research is related to the concepts of free radical aging Dr. Denham Harman advanced in the early 1950s. I had the pleasure of meeting Dr. Harman and his wife recently at the airport in Frankfurt. He continues to pursue the research he started over 40 years ago. He has seen support for this concept of free radical aging broaden in the scientific literature. He continues to feel it is an integrating theme around the mechanism of aging that will lead us to new therapies and abilities to remediate accelerated aging associated with these oxidative stress processes.

Uncoupling of oxidative phosphorylation and increased oxidative damage may be a component of the molecular injuries associated with this process, and dietary or other factors may be associated with the amelioration or retardation of this process. These are all exciting frontiers in explore in 21st Century medicine.

Affecting the Krebs Cycle

With regard to the constitutive mitochondrial defects that appear in infants as a consequence of genetically inborn mutational deletions of mitochondrial DNA, the clinical trials that have been done to date have empirically used high doses of Krebs cycle intermediates like sodium succinate or malate, along with Krebs cycle-active antioxidant nutrients like lipoic acid, coenzyme Q10, and vitamin E. They have attempted to demonstrate that by using these substances they can ameliorate either the progression or the severity of the symptoms of these conditions.

The published results have sometimes used 200 mg daily doses of coenzyme Q10 and 3000 mg doses of sodium succinate in young children with inborn errors of mitochondrial function. They have demonstrated modest improvement in some children. This research indicates it may be possible to modify phenotypic expression. In the less extreme cases, with chronic lower levels of mutational injury, it may be much more valuable than in extreme cases of frank deletion mutation of mitochondrial DNA that is found in all cells as a consequence of an inborn error. This area of research provides a model that can be studied and a way to look at variables that could modify mitochondrial dysfunction, many of which are nutrition- and lifestyle-related.

What about individuals with chronic myopathies like fibromyalgia, which is a very difficult-to-define condition? The origin of that condition is not well recognized. Some people have suggested autoimmune origin. Others suggest it occurs as consequence of vascular abnormalities. Some individuals have suggested it is an energy deficit disorder in the muscle, related to the accumulation of lactate and localized nociceptor activation that produce pain.

A recent article in the *New England Journal of Medicine* ties back to the emerging mitochondrial investigation we will discuss further with Dr. Kristal, our Researcher of the Month. This paper is titled "Exercise Intolerance Due to Mutations in the Cytochrome *b* Gene of Mitochondrial DNA."² Exercise intolerance is a common symptom associated with various types of myopathies. Chronic fatigue syndrome, fibromyalgia, and Desert Storm syndrome are three conditions associated with intolerance to previously well-tolerated exercise.

In the case of the encephalomyopathies, or mitochondrial genetic mutational defects, exercise intolerance symptoms are always found as hallmarks of the condition. These usually multi-symptom disorders cut across a variety of different mitochondrial functions. We have often assumed in medicine that you either

have this condition as a consequence of a constitutive defect, or you do not have it. This is the differential diagnostic model. The question that is being raised in this particular paper is, could there be induced forms of mitochondrial injury that are recognizable in the young child but may appear later in life as exercise intolerance and myalgias?

Delays in Induced Forms of Mitochondrial Injury

Authors of this study examined only five patients, but in each case the onset of exercise intolerance did not occur in infancy. It occurred later, either in childhood or in adulthood, and it was associated with the deletion of specific portions of the mitochondrial genome, the DNA of the mitochondria, although it was not a 100 percent deletion. It ranged from 50 percent deletion to 87 percent deletion, depending upon the patient, which means it was not found in all cells. It was as if some cells had been injured and lost their mitochondrial DNA in a certain region, while other cells had not. Expression was variegated; it was unlike classic genetic metabolism diseases associated with mitochondrial mutations.

As this paper explained, we do not know the exact origin of the mitochondrial mutations in these individuals. We do not fully recognize why certain cells were influenced and others were not, and why certain mitochondria appeared to be mutated and others did not. What we can say from this research, however, is that it moves us ahead another step in our understanding of the variegation of expression of this condition. It is not always just on or off. It is not always present or absent. You may have intermediate forms of mitochondrial dysfunction that are expressed as chronic complaints, rather than as acute complaints. You may end up with chronic muscle pain, like fibromyalgia, or chronic fatigue-like symptoms. We have not yet found the cause(s). Obviously, a variety of agents may induce increased risk of mutational injury of mitochondria and increase oxidative imbalance, producing more than mutational injury to the mitochondria.

Symptoms of Pain and Fatigue

The author of the editorial that follows this paper on exercise intolerance due to induced mutations in the cytochrome *b* gene of mitochondrial DNA states that muscle pain and fatigue affect nearly half of patients who seek medical care. This pain, obviously, is related to fibromyalgia or chronic fatigue. One of the paradoxes in medicine is that patients with these symptoms seldom have a recognizable disorder of muscle that can be identified by objective diagnostic tests, such as muscle biopsy, electrophysiological testing, or even imaging. The inability to arrive at a specific diagnosis is frustrating to both patients and physicians. The case reports related to the cytochrome *b* gene mutation in mitochondrial DNA prompt several questions. How does a clinician recognize a mitochondria-related dysfunction? Are there other manifestations of this dysfunction that may have been overlooked? What are the implications for patients and their siblings and children? Is there any specific treatment for these mitochondrial energy-related dysfunctions?

Patients who report generalized weakness, fatigue, or exhaustion, particularly those with a normal neurologic examination, seldom have myopathy. Fatigue of specific muscle groups is common in patients with neuromuscular junction disorders such as myasthenia gravis. Constant muscular pain unrelated to exercise is rarely due to organic disease. Episodic muscle pain, particularly with exercise, is a common feature of myopathies that affects energy metabolism and those due to ischemia such as dermatomyositis. Muscle cramps and involuntary muscle contractions are painful. Idiopathic cramps are usually confined to

a single muscle and last seconds or minutes.

Pain, Fatigue, and Mitochondrial Dysfunction

A number of conditions can contribute to muscle pain and fatigue. It is premature to conclude that in all cases it is a consequence of a mitochondrial dysfunction. This research and other published papers we have described in *FMU* indicate that a number of individuals sustain some form of mitochondrial DNA injury that reduces the ability of the mitochondria to function at optimal levels. This injury increases the anaerobic byproducts as extra mitochondrial anaerobic glycolysis, and increases oxidative stress as a consequence of the uncoupling of mitochondrial function.

The editorial by Dr. Robert Griggs from the University of Rochester Medical Center concludes that a patient might benefit from knowing he or she has a mitochondria-related dysfunction as a consequence of opening doors to potential remediation that may, in fact, involve modifying the risk of oxidative stress, modifying those factors that lower energy production at the mitochondrial level, and trying to get more out of the nonmutated mitochondria within cells. Fortunately, we have thousands of mitochondria within cells. Therefore, if 50 percent are mutated, we still have 50 percent that are not mutated. They can carry on function if they are properly encouraged to do so.

This situation is analogous to that of sickle cell anemia. If you could increase fetal hemoglobin expression, you could dilute the sickle hemoglobin and prevent sickle crisis. If we can increase the function of the nonmutated mitochondria, we can improve function to compensate in part for the loss of function in those that are mutated. This possibility opens doors in therapy that were previously not available until we understood the origin of chronic fatigue and muscle pain symptoms that may originate in mitochondrial dysfunction.

Factors in Mitochondrial Mutation

A number of factors can increase the potential risk mitochondrial damage. First of all, oxidative stress comes from low oxygen tension. It almost seems a paradox that conditions of ischemia or anoxia have the highest oxidative stress potential. Conditions of higher oxygen delivery have lower oxidation potential. That is because the condition of ischemia, or low oxygen delivery to tissues, creates an environment of catabolism of purines that activate xanthine oxidase, producing more superoxide. The superoxide can convert to hydroxyl radical and increase oxidative stress. Therefore, low oxygen tension is associated with increased oxidation.

Every type of traditional medical therapy, including Ayurvedic Medicine, has involved a way of delivering oxygen to tissue. It may be Yoga exercise, deep breathing, or dance. A number of different therapies can be used to improve oxygen before we get to the technology of intubation. Oxygen can be delivered to tissues by manipulation, movement, improving pulmonary function, or improving red cell function so red cells can transport oxygen better. These are historical uses of oxygen as a therapy. Oxygen is a nutrient, and it may be the limiting nutrient in mitochondrial function where there is ischemic involvement. In low oxygen tension in cells, there is increased oxidative stress, and mitochondrial oxidation—mitochondrial injury to DNA.

Research on Oxidative Stress at High Altitude

Individuals who work at high altitude encounter oxygen tension. Oxidative stress is increased in humans working at moderate altitude, and we have reviewed a number of papers that demonstrate that increased levels of antioxidants can be protective against oxygen dysfunction at high altitude. Research was conducted among elite mountain climbers working at high altitude. The study evaluated various parameters of oxidative stress, lactic acid in the blood as an indicator of diminished respiratory capacity, and breath pentane, an indicator of enhanced lipid peroxidation in well-conditioned athletes working at very high altitude.⁹

The researchers found that when they supplemented the climbers with therapeutic doses of vitamin E their accumulated levels of these oxidant metabolites were much lower than if they just consumed their ad lib diets. An ad lib diet at that altitude would generally be simply high in calories, just trying to keep body mass constant.

An example indicating that working at moderate altitude (2546-3048 above sea level) with low oxygen tension may increase oxidative stress appeared in a recent paper in the *Journal of Nutrition*.¹⁰ It described increased oxidative stress associated with moderate altitude activity. Supplementation with an antioxidant cocktail significantly reduced the production of breath pentane. Other indicators were unaffected. The authors speculate failure to demonstrate profound changes in oxidative stress indicators may be due to the fact that the subjects (US Marines) were already well conditioned.

A person in poor aerobic shape, who consumes a poor-quality diet and quickly transitions to high-altitude skiing from a sea level job undergoes oxidative stress in that transition. He or she has not accommodated the change in altitude and oxygen tension. A person whose diet has not included enough of some of these protective nutrients and has poor tolerance is more likely to be ischemic to begin with. Again, there is an exercise tolerance; there is an oxygen delivery component; any ischemic or anoxic events obviously increase oxidative stress and mitochondrial injury.

Glucose intolerance is second to oxygen delivery as a contributor to mitochondrial DNA injury. Diabetes and dysglycemia can potentiate injury to mitochondrial DNA. Many papers have been published on this topic. One comes from the work of Burton Sobel and his colleagues at the University of Vermont School of Medicine and the University of Alberta. The authors reported that high concentrations of glucose potentiate injury to mitochondrial DNA in *ex vivo* experiments with vascular smooth muscle cells from rats. This may, once again, help us understand the association between diabetes and biological aging as they go on to point out. I refer to a paper titled "Aging and High Concentrations of Glucose Potentiate Injury to Mitochondrial DNA."¹¹

A patient with dysinsulinism and dysglycemia who has increased glycosylated hemoglobin levels and increased risk to protein glycation may also be at higher risk for mitochondrial DNA damage and oxidative stress. By the model we have been describing, this is associated with increasing prevalence of age-related diseases, i.e. biological aging. Again, normalization of glucose and insulin improved regulation of glycolysis, glucose control, and insulin dynamics. I believe this is a very important part of this mitochondrial story.

Sleep Debt

The third item on my list is sleep. We may not think of sleep as being related to mitochondrial oxidative

stress or lack of stress, but the evidence indicates that sleep, which is very important for releasing melatonin into the nervous system from the pineal gland, plays an important role in the central nervous system's elimination of oxidants produced in the mitochondria during the day. It is as if sleep allows us to perform neurological and perhaps other physiological system garbage collecting repair work.

Melatonin plays an important role as a CNS antioxidant that can soak up or quench oxidant radicals that have been formed, or the byproducts of those radicals. Sleep debt has a significant impact on metabolic and endocrine function. By virtue of its impact on melatonin production, it is possible that sleep debt increases oxidative damage to mitochondria and increased free radical aging problems. A discussion of the physiological impact of sleep debt occurs in a recent *Lancet* review titled, "Impact of Sleep Debt on Metabolic and Endocrine Function."¹² Depletion of melatonin and alteration in ACTH, cortisol, DHEA, insulin-like growth factor 1 are impacted by sleep debt. Therefore, sleep is likely to be an important part of an overall mitochondrial health program to provide reserves we to balance against the inevitable stressors in our lives.

Dietary Factors

Fourth on the list are dietary factors, including macronutrients like dietary fats. High-fat diets may put more of a load on the mitochondrial wire, so to speak. It is like taking a wire that is supposed to handle so many amps and then quadrupling the number of amps that you try to put through the wire. Suddenly, it becomes a toaster element and starts glowing red hot. This may explain why animal studies show that calorie restriction lowers the incidence of mitochondrial injury and mutation. Lowering the flux of specific substances through the wire, if the "wire" is the electron transport chain, slows the loss of these free radical agents that can damage the system.

An interesting paper just published in *Carcinogenesis* talks about what happens in animals fed high levels of corn oil.¹³ This treatment rapidly activates the expression of nuclear factor- κ B (NF- κ B) in the liver Kupffer cells, a response to increased oxidative stress. Diets high in fat, even polyunsaturated fatty acids of the omega 6 family, may increase the risk of injury to mitochondria as a consequence of this oxidant mechanism that is induced through nuclear factors of transcription like NF- κ B. High corn oil intake increases NF- κ B, a modulator of oxidative stress-induced reactions.

What about the highly unsaturated omega 3 fatty acids like eicosapentaenoic (EPA) and docosahexaenoic (DHA)? These fatty acids affect the function and activity of mitochondria and on another organelle in the cell, the peroxisome, which we will be discussing in greater detail this year in *FMU*.

EPA and DHA may be preferential substrates for certain types of oxidative processes in the peroxisome. The peroxisome communicates with the mitochondria and influences oxidative stress reactions in a way that is different from omega 6 linoleic acid-rich triglycerides like corn oil.

The omega 3 fatty acids might also play a role in constructing appropriate mitochondrial membranes, in which the specific *sn*-2-positions of their phospholipids are occupied by these omega 3 fatty acids. In terms of their effects on the composition of the mitochondrial membrane and on intercellular communication through the peroxisome mitochondrial interrelationship, the effect of EPA and DHA on mitochondrial oxidative stress may be different from omega 3 linoleic acid. I am basing these hypotheses on a recent paper titled, "Eicosapentaenoic and Docosahexaenoic Acid Affect Mitochondrial and

Peroxisomal Fatty Acid Oxidation in Relation to Substrate Preference," which appeared in the journal *Lipids*.

Many of the cytochromes involved in detoxification, the monooxygenases that produce excited oxygen or molecular oxygen species that can be involved in oxidative damage, are associated with upregulation of specific aspects of mitochondrial function. A person who is exposed to a high level of xenobiotics may have increased risk of mitochondrial oxidative injury. That appears to be well documented in animal studies. A review paper on this topic appeared in a recent issue of *Antioxidants & Redox Signaling*" The author of this paper, titled "Redox-Mediated Gene Therapies for Environmental Injury: Approaches and Concepts,"¹⁵ explains that exposure to pesticides, herbicides, and other biocides relates to increased oxidative damage within mitochondrial-rich areas of the cell. That increase causes compensatory upregulation of antioxidant enzymes like superoxide dismutase. If that exposure is either acute or chronic, it may overwhelm the ability of the natural antioxidant systems to accommodate the exposure and cause cellular damage.

The fifth factor on our list of things that can influence oxidative stress in the mitochondria are environmental xenobiotics. I would also include gut endotoxins in that category, things that are produced as a consequence of dysbiosis in the gut that then can create the need for upregulation of these toxins and pathways and increased oxidative damage. Hepatic oxidative injuries frequently occur after exposure to xenobiotics or endotoxins.

Next on the list are medications. Various medications can also increase mitochondrial oxidative injury. Two extensively studied areas are antiretroviral nucleoside analogues like AZT, which can cause mitochondrial dysfunction, and specific antibiotics. The persistent mitochondrial dysfunction associated with exposure to antiretroviral nucleoside analogues is now being documented and recognized, particularly in infants who received perinatal mother-to-child exposure *in utero*. A recent article in the *Lancet*¹⁶ explained that when the fetus is exposed to the medications and mitochondrial injury is induced before it is born, it will be born with low-grade, persistent mitochondrial dysfunction that may be expressed as neurological or muscle-related dysfunctions.

Another paper on the same theme is titled "Mitochondrial Toxicity Induced by Nucleoside-Analogue Reverse-Transcriptase Inhibitors Is a Key Factor in the Pathogenesis of Antiretroviral-Therapy-Related Lipodystrophy."¹⁷ This article suggests how the uncoupling of mitochondria and the mitochondrial injury seems to be associated with that process.

Among the medications on this list I have discussed the reverse transcriptase inhibitors, nucleoside analogues, and certain antibiotics. I should also add the fibrate drugs such as clofibrate, which appear to have some effect on mitochondrial oxidative stress. The authors of a recent paper in *Free Radical Biology & Medicine*, titled "Mitochondrial Damage by the 'Pro-Oxidant' Peroxisomal Proliferator Clofibrate,"¹⁸ discuss mitochondrial damage as a consequence of the prooxidant effect of the fibrate drugs through peroxisomal proliferated activated metabolism. This damage increases mitochondrial oxidative injury, as revealed by the byproducts of that damage, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the plasma, which is an indirect measurement for the amount of DNA damage that is occurring as a consequence of induced mitochondrial oxidative stress.

Chronic inflammation is also associated with increased release of cell messengers that have mitochondrial

effects and should be included on the list. These cell messengers, like tumor necrosis factor *alpha* (TNF-*a*), or interleukin-1 and interleukin-6, can alter mitochondrial function, create uncoupling of mitochondrial electron transport, and increase the potential for mitochondrial injury. A person who has a chronic infection like herpes, *Chlamydia pneumoniae*, Giardia, or *Helicobacter pylori* may sustain increased mitochondrial oxidative injury. That injury can have other untoward effects, depending upon the tissue or type of cell in which it occurs. It may even relate to the association between inflammation and heart disease.

This information is from a paper that was published recently in the *Antioxidants & Redox Signaling* journal. The article, titled "Regulation of Tumor Necrosis Factor-Induced, Mitochondria- and Reactive Oxygen Species-Dependent Cell Death by the Electron Flux through the Electron Transport Chain Complex I," describes how TNF-*a* impacts induced mitochondrial influences and increased reactive oxygen species-induced cell death.¹⁹

These reports show the wide variety of factors that can contribute to increased risk of mitochondrial injury. Obviously, people with specific genotypes may be at higher risk for agents creating induced mitochondrial injury. On side II we will discuss what may help prevent mitochondrial injury.

INTERVIEW TRANSCRIPT

Researcher of the Month

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JB: This month on *FMU* we are fortunate to have a researcher as our Clinician of the Month. Dr. Bruce Kristal, for whom I have tremendous respect, is a researcher at the Burke Medical Research Institute in New York and a professor at the Department of Biochemistry, Cornell University Medical College. I have gotten to know Dr. Kristal in relation to his expertise in the area of mitochondrial biochemistry and the potential medical implications of mitochondrial function in health. The importance of this area is emerging, and Dr. Kristal is an investigator in mitochondrial function. He will tell us about how the mitochondria is influenced by its environment, including nutrition.

Welcome to *Functional Medicine Update*, Dr. Kristal. I usually start the interview by asking what brought you to your professional focus—the mitochondrial research and biochemistry area?

BK: I started as a pure molecular biologist looking at gene expression. I went from there to trying to look at how some of these systems played out—what was actually going on as we went more from the genes to the animal, and as we went from more basic research, not all the way to applied research, but to that midpoint where you start to apply the understanding of the basic research.

I became interested in aging. When you begin to ask what causes biological or biochemical aging, there are several theories, one of which I am sure will be familiar to most of your listeners. That is the free radical theory of aging—that aging occurs because of wear-and-tear damage from reactive species. While that may or may not be all of aging, it most likely plays a role. If you look at that and then ask where free radicals come from, most of the oxygen in the body (probably close to 90 percent) is used by mitochondria in the process we call oxidative phosphorylation, which is how you produce ATP. A small percentage of that, which is classically considered 1 to 4 percent, but based on more recent understanding, may be as little as .1 to .4 percent, is lost in that process we call electron leak, or uncoupled oxygen utilization. That, in turn, turns into free radicals.

That's probably the major source of free radicals from most of the cells in the mammalian body, including, for example, the neurons. That now becomes a possible causative factor in aging. That's where I got interested in mitochondria. First of all, mitochondria play a role as a target of free radical attack. Second, mitochondria play a role as a cause of free radical generation. Finally, there's the interaction in which mitochondria essentially try to defend themselves from this problem. That's the basis of how I got started

JB: About six years ago in *FMU*, we reviewed work on Greg LeMond, the elite endurance bicycle racer with mitochondrial myopathy. For the first time in the experience of many health practitioners, that introduced the concept that mitochondria had something to do with function beyond being just the nascent powerhouse of the cell. Do you see the origin of this interest in mitochondria in medicine as an emerging crescendo? Do you see it growing from what you've been able to view from your own work and that of others?

BK: It's growing tremendously, not so much because of the mitochondrial myopathy work. That started growing perhaps 15 years ago, with the understanding that the mitochondrial genome might actually be the cause of disease. That was the first time people had looked at the mitochondria from anything other than the pure energetic standpoint, with the exception of a couple of people who did some work on other aspects of the biochemistry of mitochondria.

What's really made the field of mitochondria research take off in the last couple of years is the understanding that came to light about three or four years ago, that mitochondria play roles in cell death. When it comes to cell death, the first thing that people think of is the idea that mitochondria are there, and the cell dies, and the mitochondria go bad. But about three or four years ago, we realized that mitochondria actually play very active roles in this process. A factor that's released from mitochondria—cytochrome C—propagates some of these death cascades. That's where a lot of the interest in mitochondria has come from.

JB: You are alluding to a term we've used frequently—apoptosis, a mechanism of cell death different from necrosis. How does that interrelate with the mitochondrial genome, mitochondrial DNA, and is there an emerging theme that comes from the translation of the message between nuclear DNA and mitochondrial DNA?

BK: That's probably less important in the aspects of apoptosis cell death. What appears to happen in the mitochondrial genome problem, for example, is what Greg LeMond probably has. You can no longer make the proteins you need for the mitochondria to function, or you make a protein that doesn't function

as well as one of the other proteins. For that reason, you may end up with a progressive loss of function or a progressively decreased level of function. In his case, for example, at least when he was diagnosed, they didn't ever expect him to have functional problems other than at the level of an elite cyclist.

When we're talking about the cell death cascades, we don't talk about a chronic problem, although maybe you have apoptotic cell death occurring chronically in Alzheimer's. What we're talking about is an acute problem within a cell in which that cell sees a challenge. It may be a response to a ligand at a receptor on the surface of the cell. It may be a response to ischemia reperfusion in which the mitochondrion sees a stress which it responds to by either completely shutting down or essentially blowing up, in a process we call the permeability transition that I work on, or by a very subtle release of cytochrome C or other mediators that go on to induce what we call caspases, these proteases, which then chew up the cell.

When you talk about apoptosis, you're talking about a relatively neat form of cell death in which the cell decides it is going to kill itself and do it in the most convenient way possible for the body, as opposed to necrosis, where the cell simply dies.

JB: When we look at the model you are describing and try to map it against people who have chronic illness and start to lose function, I'm reminded of an article in the October 22, 1999 issue of *Science* magazine. It talks about age-dependent accumulations of point mutations in human mitochondrial DNA as if the mutations that occur due to injury may accumulate over time, creating less and less efficient energy production.²⁰ Is that an emerging theme in chronic mitochondrial dysfunction?

BK: It's a theme that goes back to mitochondrial DNA problems. People who develop mitochondrial myopathies when they're children often have 90 percent deleted genomes. And they often have something called the common deletion, which knocks out a specific part of the genome. Or, they have specific point mutations, or specific small deletions or larger deletions. Each of those is characteristic of certain phenotypes, with some overlap between them.

People started to ask if these could play a role in aging, not because you have a person with 90 percent, but because this person gradually accumulates these deleted genomes. There was evidence that these deleted genomes increase with age. What was criticized about this, and it still is a big problem, is that nobody can ever map these problems back to function. That's one of the problems people have to solve—how do you prove that even a 50, 60 or 70 percent increase in abnormal mitochondrial genomes is functionally related to a deficit we see?

One approach that has been taken by Judd Aiken, among others, is to look at single fibers in muscles and show that it's actually specific fibers that are losing all of their mitochondrial DNA. It's not that a certain percentage, let's say 5 percent deleted across the cell, which wouldn't be predicted to have any effect, but rather, you're looking at 100 percent deleted in a very small segment of the cell. That may, of course, cause that fiber to become useless and then may weaken the muscle because now some fibers are inactive.

JB: In clinical medicine, there is often a leap from an observation that appears to may have cause and effect, to the assumption that the effect is related to the cause. I think you're saying we need to be very cautious in leaping to a cause-and-effect assumption or conclusion about these mitochondrial mutations and how they could impair function in an aging individual.

BK: Yes, I think that's true.

JB: What mechanism does a biochemist focused on mitochondrial function employ to assist a clinician in making decisions down the road?

BK: The one that has been done is to look at energy production. The reason for that is the myopathies, which, in terms of clinical medicine, have received almost all of the attention, show up as problems in energetics. You simply can't make ATP as fast as you'd like. For that reason, a lot of it has focused on getting a muscle biopsy, looking at what happens to a muscle when you make mitochondria from it, how well can they produce energy, and how well can they produce energy under certain biochemical conditions. The failure under certain conditions but not others is diagnostic for some of these. When they can, they do a DNA analysis. That allows them to look and, if they're lucky, avoid a biopsy, although in the end, they may need one anyway.

But mitochondria are involved in a lot more than energetics. They're involved in gene expression. They're involved in reactive oxygen species formation. They're involved in regenerating antioxidants. They're involved in calcium transport and regulation of calcium signaling. That's an area we haven't gotten to yet in terms of any sort of clinical application. I'm not sure we would know how to recognize a defect in calcium transport in the clinic. We can recognize it biochemically because we can put the mitochondria in a test tube and determine if they transport calcium. But we don't yet know exactly what that phenotype would look like in an individual, and we're certainly nowhere near knowing what to do about it if we did recognize it.

JB: I'd like to share a selected list of titles of some of your recent publications, to give listeners a sense as to how broad this field is, and the degree of your impact on it. Papers are titled "Oxidant-Mediated Repression of Mitochondrial Transcription in Diabetic Rats,"²¹ and "Oxidant-Mediated Repression of mtDNA Transcription."²² This is obviously a big area of what you are talking about. Another paper is titled "Defects at Center P Underlie Diabetes-Associated Mitochondrial Dysfunction."²³ A more recent paper is "Simultaneous Analysis of the Majority of Low-Molecular-Weight, Redox-Active Compounds from Mitochondria."²⁴ A 1999 article is titled "Purine Catabolism: Links to Mitochondrial Respiration and Antioxidant Defenses?"²⁵

Clearly, in your research you have been looking at ways of addressing these questions. We have to have the tools in science before we can address the broader issues.

BK: That's what we did, especially in the diabetes papers you mentioned. We started out seven or eight years ago when I was in Texas working with Byung P. Yu and J.J. Chen. We showed that mitochondrial gene expression is very sensitive to oxidants. We were looking at this because we thought there might be two very different situations, one in which a cell or organism is exposed to a very low level of chronic oxidative stress, such as you might see in aging or diabetes, versus a high, sudden oxidative stress such as you might see in ischemia reperfusion. If that's true, in ischemia reperfusion, the most critical thing is to protect ATP, because if you don't transport calcium, you'll live, but if you don't make ATP, you're not going to live very long. If you fail in that sudden attack, you propagate cell death

In the case of a chronic, low-level stress, the critical system is the one that fails at the lowest level of a specific stress. Eventually, whatever cards you remove from a house of cards will bring the whole thing

down. The mitochondria must transcribe their genome. Their genome encodes 13 polypeptides that are involved in energy production. If you remove the genome, the mitochondria will eventually fail. That may take a day, a week, or a month, but eventually it will fail.

Gene expression involves transcription and translation basically. It also involves regulation. If we just look at transcription, it involves DNA integrity, RNA integrity, protein function, and the DNA when it's at its most sensitive, which is when it's unrolled, unwrapped. We asked whether or not this type of defect could be seen, this type of loss of function when you hit mitochondria with an oxidant. We showed that indeed you could and, in fact, you lost function before you ever affected the lipids. So, this is a very sensitive system.

We also found it was sensitive to specific types of radicals, but not others. We then tried to go a step further which is to say we now have a biochemical phenomenon. We characterized that a little bit biochemically. We showed there are some tissue-specific differences. We showed there were some antioxidant-specific defects.

Then we asked if we could see this in a situation in which we know long-term you lose function. We know from work done in the 50s that severely diabetic animals will eventually lose liver mitochondrial function. We looked early and late and we focused mostly on a stage at which the animals had not yet lost the ability to produce energy, but had lost the ability to start to transcribe. We observed defects in transcription.

Then we showed those were associated with a series of changes in oxidants. We went a step further in the paper on center P and asked if there was a mitochondrial defect that might produce the reactive species. We found, indeed, that there was a specific site called center P which appears to be the cause of these reactive species. In the process of doing this, though, we realized that at least seven or eight different, low-molecular weight oxidants were involved in defense. If this was the case, now we needed a way to characterize these. We decided to go back toward working out more techniques. We've done a lot of that with Wayne Matson and Karen Vigneau-Callahan. That's the paper dealing with low-molecular weight that you mentioned. We developed a technique that lets us look at 500 or 1000 compounds simultaneously. This gives us the analytical power to go back and ask this question, either in terms of known subsets, such as the purine catabolites we looked at, or in terms of unknown subsets like a marker we found for mitochondrial dysfunction that we are trying to characterize.

JB: In that description of your work, you said something I think might pique the listeners' interest. You alluded to the fact that in the sequence of events that create damaged mitochondria and, ultimately, maybe even mitochondrial loss, the loss of ATP-generating capability may occur later in the sequence, and other functional changes may occur in the mitochondria prior to the loss of ATP. Could you discuss that? I think many people might say that if a mitochondrion loses function, it loses its ATP-producing-ability first.

BK: Mitochondria protect ATP. They're tremendously redundant. They probably have 75 percent more genome than they need, probably 75 percent more expression than they need, and more pure respiratory capacity than they need. They use this essentially as a buffer, as a protective shield. If they lose part of that shield, the first thing they lose is something called proton leak, which is essentially a capacitance across the membrane. If they lose that, there may not be any great harm. They may just lose a little of the

speed responsiveness of the system.

As you continue to go down, however, you lose calcium transport. A small change in membrane potential will have a huge change in transport capacity, but a much smaller change in ATP production. You lose the ability to regenerate antioxidants, because you essentially speed the waiting time through parts of the chain that are involved in antioxidant production. You'll no longer be able to siphon off some of the reducing compounds that are used for antioxidants.

So, each of these systems will go down possibly before you lose ATP. That will depend on the conditions of the experiment or the conditions *in vivo* in a specific disease. It will depend on the specific defect that you see. Some defects will actually impair ATP production earlier, whereas others may not impair ATP production until very late.

JB: Do we know if nutrition, nutrients, or substances in a complex diet influence this control of mitochondrial function? I'm thinking of antioxidants, purines, glucose control, cytokines—all the things that might influence mitochondrial function.

BK: In the late 1950s, I believe, there were a couple of papers in which it was suggested that there is an interaction between mitochondria and nutrients. We'd expect that because we know the lipids of the mitochondria come from the diet to some extent. As you change those lipid compositions, you change mitochondrial function, which is highly dependent on the lipids.

We know from work I've done that low-calorie diets, which prolong life expectancy and maximum life span and reduce disease, have tremendous protective effects, at least in some strains of rats maintained on these low-calorie diets. People who have fed animals certain antioxidants have looked at mitochondrial function. They've seen some changes, but the problem with that is feeding an antioxidant doesn't mean it gets to the mitochondria. Even if it gets to liver mitochondria, for example, it may not get to brain mitochondria.

This is an area essentially for the future. We've developed the analytical tools in the field of mitochondrial biology. We have developed the tools we need to look at that, but we haven't really yet done it. One reason is that a lot of the mitochondrial work is technically very difficult. The people who did a lot of this work in the 1970s and 1980s, because of the technical difficulty of the work they did, focused on very small parts of the mitochondria. They focused on single electron transfers or two electron transfers or proton transfers. That's how we understand what's going on.

Those people laid the groundwork for us to build on, and even the people who looked at the whole chain tended to look at it only as a couple of modules. It is not that they didn't understand the greater context, but what they were trying to do in many cases was too difficult for them to jump immediately to the whole animal.

JB: One class of nutrients or bioactive molecules you have looked at is the purine area—xanthine and hypoxanthine. Would you tell us about how purine catabolism leading to these compounds is linked to mitochondrial function and perhaps antioxidant defense systems?

BK: The short answer is that we know very little about it. We looked at the purines because, first of all,

Wayne (Matson) was very interested in them when we were looking at this. Second of all, uric acid, which is the downstream component, is a tremendous antioxidant as urate. The low-molecular weight antioxidants in the mitochondria are not well understood, especially the hydrophilic ones, which urate would serve. We know that mitochondria had a couple of stages of purine catabolism. They had a couple of the enzymes involved.

It makes sense to look at the catabolites of this pathway. Indeed, when we looked, we saw they go way up in the stages of diabetes where function is still protected, or at least we believe function is relatively well protected. But that increase is lost as the mitochondria get sicker and the disease progresses.

We have to go back and do this very rigorously. We have to go back with long-term studies with larger end's essentially, so we can prove that point. We also will have to show that it has a function. What we have right now is an association, not a function. The other part of that paper is we showed that if you simply ask mitochondria to function, they change purine levels. Again, we don't know the functional significance of that change. We have to prove that.

JB: I'd like to shift from there to the clinical conditions we know with constitutive mitochondrial DNA mutations and deletions, things like MELAS or Leber's optic neuropathy. Some clinical studies have been published showing some amelioration of both the progression and severity of symptoms, giving things to the children like sodium succinate as a Krebs cycle intermediate, or coenzyme Q10, lipoic acid, vitamin E, or a combination of those supposedly mitochondrially active nutrients. Do you feel that mechanistically we can't say much about that? How would you put that in the context of the work researchers are doing in mitochondrial biochemistry?

BK: I guess I would have to say that right now we don't know. A lot of this work was done as people took educated guesses. You have compounds that are fairly safe, or at least we believe they are fairly safe. We know the defect is in a certain part of the chain in a certain disease, and they said, okay, maybe this will help. Right now, that's where we are, with more rational interventions we really don't know much about yet. Can we design from scratch something that will cure the MELAS patient? I don't think we can do that yet. It is my understanding that a lot of those interventions do not work in all patients. Again, remember, I'm not a clinician.

JB: A fascinating chapter in the evolving story of oxidants and antioxidants relates to a number of your papers, certainly the one on the low-molecular weight redox-active compounds associated with mitochondria. That is the concept of redox buffering. Most clinicians learn about blood buffer biochemistry and its role in maintaining physiological homeostasis, but they don't learn about redox buffering. How is redox buffering research progressing?

BK: Define redox buffering a little bit more specifically first, if you would. There are a couple of ways we could use it in the mitochondria, and I'm not sure of the context to which you are referring.

JB: The context I'm alluding to relates to having a reserve of redox-active compounds available to create electron transport to provide a source for that single electron as a free radical to be quenched before it has an adverse effect.

BK: Let me give you one example. When an electron enters the mitochondria (electron transport chain),

it can go through complex I or complex II. Either way, it then goes into complex III. When it goes into complex III, it takes ubiquinone or the ubisemiquinone to ubiquinol, which is the more powerful antioxidant. Ubiquinol can regenerate the alpha-tocopheroxyl radical (the spent form of vitamin E) back to alpha-tocopherol. By doing this, electrons in the mitochondria essentially become a nearly limitless source of the ability to regenerate tocopherol in the mitochondrial membrane. That is a type of redox buffer you're talking about.

There are a lot of these systems. Mitochondria can generate NADH, which can be converted into NADPH and be used to regenerate glutathione. These are examples of where that type of regeneration can occur. The proton gradient may actually be used as an antioxidant to help protect the cell from superoxide generated in the mitochondria. That's been proposed by a man named Shu Shen Lu.

JB: That's precisely what I hoped listeners would get from you—the concept that we have moved beyond the "antioxidant of the month club." From the clinical perspective, in the early days in this field, we saw these trials of beta-carotene, smoking, and lung cancer. Next we saw trials on vitamin E and heart disease. Then we saw trials on vitamin C and viral infections, suggesting these bioactive materials work singularly like drugs. But the emerging research, as you have explained, suggests they work in teams, combinations, or redox-coupled mechanisms, which sense that research must take different directions from single agent/single effect research.

BK: One of the things that bears on that goes back to the diabetes work. It looks as though each of the stressors we apply, the stress on transcription, the stress on lipids, has different defenses. Those different defenses defend against different attackers. So there may be a primary lipid defense against lipophilic oxidants, and a primary lipid defense against hydrophilic oxidants. We are certainly not the first people to show that. The transcription defenses are fundamentally different between a hydrophilic peroxide radical generator—the one we use is an artificial one called AAPH—or a hydrophobic peroxide radical generator, again an artificial one we use called ANVN. These, while they're artificial, mimic, for example, lipid peroxidation byproducts that are going to be found in the lipid membranes.

JB: You have helped us see that it is a simplistic leap of faith to believe that certain antioxidants will deliver a certain effect when administered orally at the mitochondria. These effects require much more exhaustive evaluation through the kind of work you and your colleagues are doing. We will be privileged to have you as a plenary presenter at our Seventh International Symposium on Functional Medicine in Arizona in May. Then we will have more opportunity to get an update and more education in this area of tremendous clinical importance as we move forward in the 21st Century.

I thank Dr. Kristal for his lucid and articulate description of a complex topic—research into the biochemistry of the mitochondrion. We can all appreciate the sophistication and the level of questions that must be answered to fully understand the mitochondrion, health, and disease, and how we might modify its function and prevent the dysfunction associated with early-stage, age-related diseases.

Dr. Kristal pointed out that we do not yet have empirical evidence indicating that specific antioxidants may help ameliorate mitochondrial injury. An increasing body of clinical evidence and animal work is moving us forward in examining the role antioxidants may play in preventing oxidative injury, not just to the mitochondria, but to other regions of cells, tissues, and organs.

The question whether antioxidants work singularly or in combination as redox-coupled substances arose in the discussion with Dr. Kristal. Dr. Kristal's eloquent description of how these are coupled systems helps us understand why studies using a single antioxidant against a single outcome or variable are frequently unsuccessful. These studies did not use antioxidants in the way they operate within the body as part of a redox-coupled balanced system.

The way in which antioxidants interact is described in a recent article titled "Is the Biological Antioxidant System Integrated and Regulated?".²⁶ The authors of this paper, who are from the Department of Human Nutrition and Metabolism, Department of Pharmacy, at the Faculty of Medicine in Jerusalem, Israel, describe the two principal classes of biological antioxidants, which are helpful in systems to provide the buffering to oxidants. The first class are the enzymes, encoded for on the nuclear genome, include catalase, peroxidase, and superoxide dismutase. The low-molecular-weight antioxidants, the second class, include tocopherols, vitamin E, ascorbic acid, glutathione, coenzyme Q10, lipoic acid, and polyphenols.

Epidemiological studies suggest that ischemic heart disease and some cancers are inversely related to antioxidant status, while intervention trials with single antioxidants have not been proven successful. The epidemiological trials are done on people who eat complex diets which include multiple antioxidants, while the intervention trials typically add a single antioxidant to a standard diet, which in no way mimics neither the epidemiological work nor the way the body has evolved to utilize these redox substances.

From the discussion on side I, we know that many variables can increase the risk of mitochondrial oxidative injury. Those variables include metabolic, lifestyle, and environmental considerations, which can uncouple mitochondria and increase the level of superoxide, hydroxyl radical, singlet oxygen, hydrogen peroxide, or lipid peroxides, all of which may engage in their own cell-specific mechanisms of damage. Controlling the release of these substances is, in part, related to the sufficiency of the reduction/oxidation (redox) system and how much redox buffering is present in terms of the ability to diffuse, soak up, or neutralize oxidants when they are formed by the appropriate redox-balanced couple. Therefore, antioxidants taken singly may not provide full protection against a specific oxidant that depends on that specific tissue's function.

The authors of this paper explain that dietary recommendations should be to take antioxidants as families, not as single agents. Studies should look at the role of antioxidants taken in combination, not singly. That type of thought process that will lead to a better outcome in the protection against untoward oxidative stress, according to these authors.

There are many to introduce these small molecular weight antioxidants into the diet. We are not sure, however, how many of them are absorbed across the gastrointestinal tract, delivered to tissues from the plasma, taken up into various cells, and concentrated in the organelles. A number of questions remain regarding how to get the most "bang for the buck" in nutrient supplementation.

Studies demonstrate that specific types of supplemental antioxidant nutrients are absorbed across the GI tract, transported in plasma proteins, and delivered to tissues where they are taken up. The role they play in reducing or influencing oxidative stress reactions at cellular organelles is still, as Dr. Kristal pointed out, a subject of contemporary research. Clinical studies looking at lipid peroxide markers like malonaldehyde or DNA damage markers like 8-OHdG demonstrate that supplementation with complex arrays of antioxidants can lower the concentration of these markers, the secondary indicators of oxidative

stress. *In vitro* data gives encouraging indications that relate to their ability to modify oxidative reactions.

New substances are being developed in an attempt to mimic or activate the enzyme-related antioxidant pathways—superoxide dismutase, catalase and peroxidase. Selenium is the central mineral in glutathione peroxidase, so selenium deficiency can increase the risk of oxidative stress. As a result of reduced enzymatic activity, the consequences of super oxide anion, i.e. H₂O₂, hydroxyl radical, and the lipid peroxides are magnified. Superoxide dismutase requires zinc, manganese, and copper, so those trace minerals also play a very important role in nutrition. The mitochondrial isoform of superoxide dismutase contains manganese.

Mimics of SOD are being synthesized which can be administered as drugs to help bolster antioxidant defense. One of these mimics, which was recently described in *Sciencemagazine*,²⁷ is a manganese chelate of a pyridine porphorin-like molecule with SOD-like activity in animals. This study indicates the research that is attempting to try to develop active substances that will prevent superoxide damage or its dismuted form called hydroxyl radical.

Foods are the most important place to look, however, because that is where these bioactive antioxidants and redox-active substances reside. Red wine, for example, is central to the so-called French paradox we have heard about for years. People have asked why it is that French people who eat a traditional fresh diet that is high in fat have such low incidence of heart disease. Their heart disease incidence increases when they move from their traditional diet to adopt a more Americanized diet with food of commerce and shelf-stable foods that may be even lower in fat.

The suggestion has been made that red wine consumption explains this seeming paradox. Red wine contains polyphenolics like resveratrol. Some people believe the alcohol in the red wine may provide additional benefit, because alcohol increases the cardioprotective HDLC isoform and the transport of these flavonoid compounds in the plasma by the increasing level of plasma proteins. Some research indicates wine provides more benefit than grape juice.

A recent paper in *Human and Experimental Toxicology* examined resveratrol, one of the polyphenolic compounds in red wine, from the French paradox perspective.²⁸ Resveratrol is just one of hundreds of flavonoids that may have different redox potential.

The phenolic compounds in a diet can help to protect LDL against oxidation. According to Daniel Steinberg, LDL oxidation is one of the steps toward atherogenesis by foam cell conversion and potential formation of an atherogenic lesion. Therefore, dietary intake of appropriate antioxidant phenolic compounds, the gallate and catechin compounds, may help protect against oxidative processes associated with chronic degenerative disease origin. This information is included in a paper published in *Clinical Chemistry*, "Detection of Dietary Antioxidant Phenolic Compounds in Human LDL."

Dietary sulfur-containing compounds also play a role in maintaining the redox state in tissues, cells, and extracellular fluids. These sulfhydryl groups include not only cysteinal proteins, or cysteine itself as a sulfur-containing amino acid, but also molecules like lipoic acid, or its disulfide dihydrolipoate, which helps trap oxidants and protect mitochondria against injury. Lipoic acid may play an important role in establishing intracellular redox potential, which influences gene expression. It may also lower the expression of nuclear regulatory substances like NF *Kappa B*, which starts the cycle moving forward in

oxidative stress.

A paper titled "Gene Expression and the Thiol Redox State," which appeared in *Free Radical Biology & Medicine*,³⁰ shows that dietary substances like glutathione, lipoate, cysteine, and the allicin compounds and thiols in garlic and other sulfur-containing vegetable products provide the body with parts of the redox buffering system that, through the sulfur transport of electrons, play a role in oxidation/reduction control. A complex diet, not just a single antioxidant, plays an important role in providing the raw materials that influence redox potential in the body.

Although glutathione plays an important role in cellular function, we should not jump to the conclusion that dietary glutathione itself will necessarily increase intracellular glutathione levels. Dr. Helmut Sies, who originated the term "oxidative stress," discussed this topic in a paper recently published in *Free Radical Biology & Medicine*.³¹ He discusses the important role of glutathione as a molecule that is readily oxidized to glutathione disulfide and regenerated by glutathione reductase to form a redox couple.

The result is a kind of molecular machine that traps free radical species and then defuses their oxidative potential. The removal of H₂O₂ by glutathione peroxidase also produces glutathione sulfide which is reduced to glutathione by glutathione reductase. The latter reaction requires proper selenium and vitamin B2 (riboflavin) and plays an important role in maintaining redox potential.

N-acetyl-cysteine is an important precursor to glutathione biosynthesis. Glutathione is a tripeptide of glutamic acid, cysteine, and glycine. The amino acid cysteine is rate limiting and thus controls glutathione biosynthesis. The sulfhydryl group of cysteine is essential in the redox function of glutathione.

Deficiency of the amino acids, usually cysteine, that make up that tripeptide can impair glutathione synthesis. N-acetyl-cysteine (NAC) provides the cysteinyl moiety necessary for the stimulation of glutathione synthesis. It also delivers directly of a thiol through the cysteinyl residue, thereby increasing the sulfur-buffering effect for redox control. Topically administered NAC has proven useful for reducing inflammatory skin conditions, because inflammation is associated with oxidative reactions in the upregulation of various functions of the oxidative pathway.

If you expose an animal to high levels of some pesticides, glutathione is oxidized to its disulfide. Normally, the reduced-to-oxidized form (GSH:GSSG) ratio is 100:1. If you expose an animal to the pesticide lindane, for instance, the oxidized form of glutathione in the animal's liver increase at the expense of the reduced form of glutathione. The redox potential of the hepatocyte changes as a consequence of that oxidative stress imbalance. That puts pressure on the antioxidant reserve. Under higher conditions of oxidative stress, more antioxidant reserve may be required to defend against that stress.

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