Biomarkers for oxidative stress: clinical application in pediatric medicine.

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ABSTRACT

Loads of reactive oxygen species (ROS), including superoxide anion and nitric oxide, that overburden antioxidant systems induce oxidative stress in the body. Major cellular targets of ROS are membrane lipids, proteins, nucleic acids, and carbohydrates. Circumstantial evidence suggests that ROS play a crucial role in the initiation and progression of various diseases in children and adolescents. The involvement of ROS and oxidative stress in pediatric diseases is an important concern, but oxidative stress status in young subjects and appropriate methods for its measurement remain to be defined. Recently, specific biomarkers for oxidative damage and antioxidant defense have been introduced into the field of pediatric medicine. This review is intended to provide an overview of clinical applications of oxidative stress biomarkers in the field of pediatric medicine. First, this review presents the biochemistry and pathophysiology of ROS and antioxidant defense systems. Second, it presents a list of clinically applicable biomarkers, along with pediatric diseases in which enhanced oxidative stress might be involved. The discussion emphasizes that several reliable biomarkers are easily measurable using enzyme-linked immunosorbent assay. Third, this review presents age-related reference normal ranges of oxidative stress biomarkers, including urinary acrolein-lysine, 8-hydroxy-2'-deoxyguanosine, nitrite/nitrate, and pentosidine, and the changes of the parameters in several clinical conditions, including atopic dermatitis and diabetes mellitus. New and interesting data on oxidative stress and antioxidant defenses in neonatal biology are also presented. Fourth, this review discusses the ever-accumulating body of data linking oxidative stress to disturbances of the nitric oxide system and vascular endothelial activation/dysfunction. Finally, this review describes the reported clinical trials that have evaluated the efficacy of antioxidants for oxidative-stress related diseases. Suggestions are advanced for the direction of future trials using antioxidant therapies. Repeated measurement of appropriate parameters will enable us to discern the pathophysiological patterns of pediatric diseases and guide our therapies appropriately.

Keywords: antioxidants, biomarkers, enzyme-linked immunosorbent assay, nitric oxide, oxidative stress, pediatric medicine, reactive oxygen species, urine samples

(1) INTRODUCTION - REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS

Free radicals and reactive oxygen species

Free radicals are defined as "any chemical species capable of independent existence that contains one or more unpaired electrons". Oxygen (O_2) and nitrogen (N_2) together constitute over 98% of the air we breathe. All animals require O_2 for efficient production of energy. By definition, O_2 itself is a free radical. Reactive oxygen species (ROS) are free radicals that are associated with the oxygen atom (O) or their equivalents and have stronger reactivity with other molecules, rather than with O_2 [1, 2].

Typically, ROS are generated as byproducts of cellular metabolism and ionizing radiation, usually indicating the following four species: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen ($^1O_2$). Of those, H_2O_2 and $^1O_2$ are not free radicals by definition, but these species behave similarly to free radicals. Reactivity of either O_2^- or H_2O_2 by itself with other molecules is rather low, but in the presence of trace amounts of a transition metal, they are converted to OH via the Fenton or Haber-Weiss reaction.

Other biologically important free radicals or their equivalents exist: lipid hydroperoxide (ROOH), lipid peroxyl radical (ROO), and lipid alkoxyl radical (RO), which are associated with membrane lipids; nitric oxide (NO), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which are reactive nitrogen species; and thiyl radical (RS), which has an unpaired electron on the sulfur atom. It should be noted here that one major mechanism by which excess NO can injure tissues is by its rapid reaction with O_2^- to give $ONOO^-$. Sections (2) and (8) of this paper include descriptions of the biological chemistry and pathophysiology of the NO system.

Antioxidant systems

The ROS serve in cell signaling as messenger molecules of the autocrine and paracrine systems and in host defense (biocidal effects against microbial and tumor cells) [1, 2]. However, their excess production might engender tissue injury and inflammation. Any excess production of ROS is strictly limited through electron transfer, enzymatic removal, scavenging, and by keeping transition metals tightly sequestered using a well-managed and efficient endogenous antioxidant defense mechanism. Under normal physiological conditions, the balance between production and elimination of ROS is maintained by enzymes and antioxidants.

Biologically active enzymes and antioxidants are categorized into three groups: enzymes, such as super oxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase (GST), thioredoxin reductase, and heme oxygenase (HO); proteins, which include albumin, ferritin, transferrin, lactoferrin, ceruloplasmin, and thioredoxin (TRX); and low molecular weight molecules, including bilirubin, tocopherols, carotenoids, ubiquinol/ubiquinone, ascorbate, glutathione, cysteine, and urate. Any imbalance between oxidant and antioxidant activities results in a state of oxidant-antioxidant disequilibrium. Any excess of the former creates a condition of "oxidative stress".

It is now well established that ROS and oxidative stress are involved in various pathological phenomena including aging, atherosclerosis, hypertension, renal failure, immune alterations, neurodegeneration, reperfusion injury, radiation damage, carcinogenesis, and many other inflammatory and degenerative conditions [1, 2].

(2) NITRIC OXIDE SYSTEM AND OXIDATIVE STRESS

Enzymatic and nonenzymatic production of nitric oxide

Oxidative stress and NO formation are inextricably intertwined. For that reason, this discussion also addresses the L-arginine-NO system in the body. An uncharged, diatomic, free radical gas, NO is diffusible and its biological effects are determined by its chemical reactivity [1]. It is produced enzymatically by nitric oxide synthases (NOSs; 130-160 kDa molecular weight) from L-arginine and molecular oxygen using NADPH as an electron donor, and using heme, FAD, FMN and tetrahydrobiopterin (BH_4) as cofactors through a reaction that consumes five electrons.

In mammals, NOSs exist in three isoforms, which differ in the way their activity is controlled. They are neuronal (NOS1), inducible (NOS2) and endothelial (NOS3). Of them, NOS1 and NOS2 are soluble, whereas NOS3 is membrane bound, with its myristoylated N-terminal. The NOS1 and NOS3 are constitutively present in various types of cells and are activated by transient increases in intracellular calcium. Upon demand by a signal molecule, NO is synthesized in low concentrations by constitutive NOSs. It binds to heme iron of soluble guanylate cyclase to yield the second messenger cGMP, which in turn modulates an array of mediators, including ion channels, phosphodiesterases and protein kinases, which thereby decrease intracellular calcium concentrations, and allow smooth muscle relaxation while inhibiting platelet aggregation. The third isoform, NOS2, is induced in response to inflammatory and immunological stimuli in myriad cells, including vascular endothelial cells, smooth muscle cells and activated immune cells. That isoform generates NO independently of intracellular calcium levels. The output of NO from NOS2 is about 1,000 times that of the other constitutive isoforms.

Not only enzymatically, NO can also be synthesized nonenzymatically from nitrite (NO_2^-) at low pH under reducing conditions. Nonenzymatic NO production might play a role in similar biological events as when NO is generated by NOS enzymes, in the stomach, on the skin surface, in the ischemic heart, and in infected nitrite-containing urine. Nitrite is now being recognized as a “hypoxic buffer” that potentially contributes to regulation of hypoxic vasodilation and to the modulation of ischemia-reperfusion tissue injury and infarction [3,

4].

Formation of peroxynitrite

A free radical, NO reacts with O_2^- at nearly diffusion-controlled rates (6.7×10^9 $M^{-1}s^{-1}$) to give $ONOO^-$ [1]. This rate is three times higher than that of the reaction of O_2^- with SOD (2×10^9 $M^{-1}s^{-1}$). For that reason, NO can compete with SOD for reaction with O_2^- when produced in concentrations that approach those of SOD. Normal physiological concentrations of NO and O_2^- are much lower than those of SOD. Therefore, little $ONOO^-$ is formed under normal conditions. Pathological situations involving simultaneous increases in NO and O_2^-, especially those occurring in regions of low SOD activity, would markedly increase the formation of $ONOO^-$, which is a potent biological oxidant. The interaction between NO and O_2^- might represent a major pathway for NO toxicity, as well as an inactivation route for NO.

(3) BIOMARKERS FOR OXIDATIVE STRESS

Categorization of oxidative stress biomarkers

All biomolecules can be damaged by ROS (including $ONOO^-$). Oxidative damage to lipids, proteins, nucleic acids, and carbohydrates can be deleterious and concomitant [1, 2]. The primary cellular target of oxidative stress depends on the cell type, the nature of the stress imposed, the site of generation, the proximity of ROS to a specific target, and the stress severity. The half-lives of ROS are usually short. Therefore, special techniques are necessary to detect ROS *in vivo*. So-called “oxidative stress biomarkers” can not only determine the extent of oxidative injury, but also indicate the source of the oxidant [5-8]. Oxidative stress biomarkers are important for predicting the consequences of oxidation and for providing a basis for designing appropriate interventions to prevent or alleviate injury.

Briefly, oxidative stress biomarkers are separable into two categories: (a) formation of modified molecules by ROS; and (b) consumption or induction of enzymes or antioxidants [2] (Table 1). Measurement of these biomarkers in body fluids (e.g., blood, urine, cerebrospinal fluid, bronchoalveolar lavage fluid) or breath condensate [6] enables repeated monitoring of the oxidative stress status *in vivo*, which is not possible with invasive tests. It might be appropriate to evaluate levels of oxidative stress markers in addition to routine laboratory assessments. Detection of ROS generation in human live cells might be possible through the use of fluorescent probes [2, 9]. Unfortunately, at present, it is not easy to apply this method to clinical practice.

The first category (a) includes molecules that are generated in a reaction with ROS. Molecules are subjected to either scission, cross-linking or covalent modification in these reactions. Accordingly, the amount of these molecules is increased when ROS are generated. Some are rapidly removed or repaired, but others remain in intracellular or extracellular compartments for a long time. Major targets of ROS in the molecular components of the cells are membrane lipids, proteins, nucleic acids, and carbohydrates. These markers are often measurable using stable adducts that are produced *in vivo* as a result of oxidative processes. Clinically applicable biomarkers include malondialdehyde-lysine, 4-hydroxy-2-nonenal-lysine, acrolein-lysine (markers of lipid peroxidation), 8-hydroxy-2'-deoxyguanosine (8-OHdG) (a marker of oxidative DNA damage), carboxymethyl-lysine, pentosidine (markers of glyco-oxidation), nitrotyrosine, nitrite/nitrate (markers of nitro-oxidation), bilirubin oxidative metabolites (BOM) (a marker of HO activity), and so forth (Table 1).

Here, brief descriptions of these markers are useful. Acrolein-lysine is a sensitive marker of lipid peroxidation and oxidative protein damage [5, 8]. Among the base modifications induced by ROS, 8-OHdG is an abundant oxidative DNA product. Many studies have measured 8-OHdG as a sensitive marker of oxidative DNA damage [2, 8]. Oxidative stress is usually involved in advanced glycation end product (AGE) formation; AGEs induce

oxidative stress. Pentosidine is a major marker of oxidative glycation [7, 8]. Uniquely among ROS, NO might act as a prooxidant as well as an antioxidant, depending on the degree, site, and timing of its generation [1]. Nitrite/nitrate is used as a marker for endogenous NO formation [8].

A major antioxidant enzyme, heme oxygenase (HO), catalyzes the rate-limiting step in heme degradation [10, 11]. Two isoforms, HO-1 (inducible) and HO-2 (constitutive), are classified as HO. The first, HO-1, is induced in various cells by various stimuli that provoke oxidative stress, including heme, heat shock, proinflammatory cytokines, and toxins. The primary product of HO is bilirubin. Bilirubin functions as a potent antioxidant under physiological and pathophysiological conditions. Bilirubin reacts with ROS and, subsequently, becomes oxidized. Hence, BOM, rather than bilirubin *per se*, might be a good marker for evaluating the antioxidant activity of bilirubin under oxidative stress. In other words, elevated levels of BOM reflect up-regulation of HO under stressful conditions. The BOM concentrations in body fluids can be determined easily using a newly developed ELISA system (Biopyrrin ELISA; Dojindo Laboratories, Kumamoto, Japan) [12-14].

The second category (b) consists of antioxidant enzymes and molecules that are associated with ROS metabolism. In most cases, these molecules are destroyed or modified and exhibit decreased activity or quantity after exposure to ROS. Conversely, they often show an overshooting response for a matter of hours, days, or weeks. A variety of antioxidant enzymes, proteins, and low-molecular-weight molecules can be listed (Table 1).

Oxidative stress biomarkers in pediatric medicine

This review outlines recent achievements of oxidative stress marker determination in pediatric medicine. It is important to examine the combination of oxidative stress biomarkers constructively to confirm the participation of oxidative stress in certain pathological conditions. This review includes our recent achievements in this particular

field and emphasizes the emerging applicability of enzyme-linked immunosorbent assay (ELISA) for investigating oxidative stress status in young people. Reference normal ranges of oxidative stress biomarkers and abnormal changes of the biomarkers in certain pathologies, e.g., atopic dermatitis and diabetes mellitus, are described. New and interesting data on oxidative stress and antioxidant defenses in neonatal biology are presented.

(4) POSSIBLE INVOLVEMENT OF OXIDATIVE STRESS IN PEDIATRIC DISEASES

Determination of oxidative stress biomarkers in pediatric diseases

Numerous diseases of children and adolescents appear to be linked to oxidative damage attributable to ROS in their pathogenesis and progression (Table 2) [15-115]. Oxidative stress might also contribute to tissue damage induced by certain drugs (e.g., analgesics, anticancer drugs, immunosuppressive drugs) [116-121]. In most previous studies, oxidative stress biomarkers were determined in samples of blood (i.e., serum, plasma, erythrocytes, granulocytes, or lymphocytes) or urine. In several studies, the parameters were measured using other body fluids (e.g., cerebrospinal fluid [17, 34, 36], bronchoalveolar lavage fluid [67, 115], joint fluid [85], nasal lavage fluid [74], middle-ear fluid [87]), tissues [24, 31, 32, 56, 60, 62, 78, 82, 87, 105], or exhaled breath [40, 61, 65], either alone or in combination with samples of blood or urine.

Previous studies measured oxidative stress biomarkers using analytic chemistry techniques, including high-performance liquid chromatography and gas chromatography-mass spectrometry. Recently, specific monoclonal antibodies have been developed and ELISA systems constructed for various oxidative stress biomarkers [8]. Onerous pretreatments and expensive apparatus are virtually obviated by ELISA; it is a labor-saving, cost-saving method that provides results quickly. Examination using a few sample aliquots is possible. Its reproducibility is excellent. This technique is demonstrated as suitable for use in clinical medicine and has spread quickly to analyses of clinical samples. Analyses of oxidative

stress in various pediatric pathologies are now performed predominantly using high-throughput ELISA methods [17, 21, 22, 27, 29, 34, 36, 50, 52, 58, 63, 65, 72, 86, 96, 97, 99, 108, 110, 114].

Importance of oxidative stress research in pediatric medicine

Oxidative tissue injury from pathological conditions might have more serious consequences in young people (especially children) than in older people because of the need for subsequent tissue growth to match somatic growth and because survival is longer in young people than in older people. Primary and secondary prevention of oxidative damage might therefore be important, especially in young people. Furthermore, the use of antioxidants has presented new therapeutic perspectives for diseases that are related to enhanced oxidative stress.

Previous studies of oxidative stress [15–115], although bringing forth interesting results, had limited sample sizes and might not have convincing evidence to prove clear causality between oxidative stress and disease conditions. Other factors might be eminently responsible for disease pathogenesis and progression. It remains difficult to determine the extent to which oxidative stress is a contributory pathogenic mechanism in the etiology of pediatric pathologies. Perhaps it is the right time to pursue intensive research into oxidative stress in pediatric patients with a wide range of diseases.

(5) REFERENCE NORMAL VALUES OF OXIDATIVE STRESS BIOMARKERS

Normal reference ranges in the literature

Investigation of the role of oxidative stress in pediatric diseases requires information about the oxidative stress status of young populations. It will be possible to evaluate the contribution of oxidative stress to various pediatric diseases and establish better approaches

for each disease when we know normal levels of oxidative stress in children and adolescents. However, only a few reports describe oxidative stress status in healthy children and adolescents. The studied subjects in those studies are few or fit a narrow age range [122-126]. In most studies, blood was collected from subjects for analyses of the following: reduced/oxidized glutathione, glutathione peroxidase and glutathione reductase activities, selenium [123]; antioxidant vitamins (including α-tocopherol, γ-tocopherol, α-carotene, and β-carotene) [124]; ubiquinol/ubiquinone [125]; and "thiobarbituric acid reactive substances" and SOD and catalase activities [126]. Schock et al. [122] reported levels of antioxidant vitamins (α-tocopherol, ascorbate), urate, and protein carbonyls in bronchoalveolar lavage fluid of 83 healthy children (age range, 1.6-12.0 years).

Kauffman et al. [124] determined urinary levels of F2-isoprostane in 342 healthy children who were less than 7 years old. To our knowledge, this is the only study that evaluates oxidative stress in a large population of healthy children using urine samples. Other studies used small groups of healthy children and adolescents as age-matched controls, rather than as the focus of those studies [16, 18, 21, 27, 44, 50, 58, 63, 72, 86, 110, 114]. Consequently, normal values for urinary biomarkers of oxidative stress in young people are lacking.

Urine collection is simple, quick, comfortable, non-invasive, and therefore particularly easy to perform in children. Collection of spot urine samples is much more feasible than 24-h collection and standardization by creatinine (Cr) excretion corrects for variation in water intake. The determined urinary levels are thought to reflect the stable condition of the subjects if early-morning urine samples are analyzed. Therefore, urine is inferred to be an appropriate biological fluid sample for evaluating changes of oxidative stress status in various pediatric pathologies.

Our data on normal reference ranges of oxidative stress biomarkers

Our data for evaluation of oxidative stress levels in healthy young subjects were of 100

healthy Japanese subjects (50 males and 50 females) of a broad age range (age range, 1.5-21.0 years). Early-morning void urine samples were obtained for analyses of biomarkers reflecting oxidative damage to lipids, DNA, and carbohydrates. Acrolein-lysine and 8-OHdG were determined using competitive ELISA kits (ACR-Lysine Adduct ELISA; NOF Corp., Tokyo, Japan [34, 58, 63, 86, 114], and 8-OHdG Check; Institute for the Control of Aging, Shizuoka, Japan [17, 21, 58, 63, 86, 96, 108, 114], respectively). Nitrite/nitrate was measured using colorimetric, non-enzymatic assay (Bioxytech Nitric Oxide Non-Enzymatic Assay; Oxis International Inc., Portland, OR, USA) [21, 34, 58, 86, 114]. Pentosidine and pyrraline were determined using high-performance liquid chromatography with fluorometric detection, as detailed previously [63, 77, 127]. Pentosidine is a major marker of oxidative glycation, whereas pyrraline is a marker of pure glycation [6, 8, 127].

The levels of urinary acrolein-lysine, 8-OHdG, nitrite/nitrate and pentosidine showed significant inverse correlations with age: r = -0.54, -0.66, -0.43, -0.56, respectively, by linear regression analysis; $p < 0.001$. More specifically, these urinary biomarkers were highest in the youngest subjects and decreased with age to reach constant levels by early adolescence (Figs. 1 and 2). However, the levels of pyrraline did not correlate significantly with age in the subjects (r = -0.19). No significant differences were found between males and females for any oxidative stress parameters.

Higher levels of oxidative stress biomarkers in young people

The physiological meaning and mechanisms for the high levels of urinary oxidative stress biomarkers in younger subjects are not clarified yet. It is noteworthy that urinary excretion of repair products of oxidative DNA damage (e.g., 8-oxo-7,8-dihydroguanine, 8-OHdG) is several times higher in rapidly growing animals (e.g., mice, rats) than in humans [128]. A high metabolic rate might be responsible for the increased everyday oxidative DNA insults in rapidly growing animals. Younger people, who are growing rapidly, have a higher

metabolic rate than older people. That higher metabolic rate requires a higher level of mitochondrial respiration and subsequent higher production of ROS, which might be responsible for the high levels of urinary oxidative stress biomarkers in younger people. This interpretation of our results is supported by the lack of age-related changes of a non-oxidative glycation marker, pyrraline. It should be emphasized that, under normal physiological conditions, younger people (especially children) are likely to be exposed to higher concentrations of ROS and NO than older people.

We recognize that our study and its implications are limited by the modest size and cross-sectional design of the study. Nevertheless, this study's findings support continued investigation into age-related changes in urinary oxidative stress biomarkers and their physiological significance with a higher number of subjects and with other techniques to measure oxidative stress.

Kauffman et al. [124] studied healthy children and found that urinary F2-isoprostane levels were highest in infancy and decreased until 7 years old. The ratio of reduced glutathione to oxidized glutathione and that of ubiquinol to ubiquinone were reported to be higher in children than in adults [123, 125]. Glutathione and ubiquinol might function as protective antioxidants in young people. These results probably represent physiological changes associated with normal aging, but a better understanding of oxidative processes in children and adolescents is necessary. We should devote attention to the age of the subjects when interpreting data of urinary oxidative stress biomarkers in young people, e.g., acrolein-lysine, 8-OHdG, nitrite/nitrate and pentosidine. We should also evaluate whether potential changes in oxidative stress status are attributable to disease progression or merely an effect of aging *per se*.

(6) CHANGES OF OXIDATIVE STRESS BIOMARKERS IN PEDIATRIC DISEASES

Panel of oxidative stress biomarkers

Oxidative damage might take place in a selective manner. For instance, lipid peroxidation and oxidative DNA damage are not always accompanied by overproduction of NO. The detection of more than one marker for oxidative stress is an important key because a single marker might give misleading results. It might also be crucial to determine which particular markers, alone or in combination with others, can serve as a true indicator of the contribution of oxidative stress to a disease, thereby allowing the success (or failure) of the treatment to be monitored. The following are good examples.

Atopic dermatitis

Atopic dermatitis (AD) is a chronic or chronically relapsing inflammatory skin disease that is characterized by typically distributed eczematous skin lesions with accompanying lichenification, pruritic excoriations, dry skin, and susceptibility to skin infections [129]. Although the pathophysiological mechanisms of AD are not completely clear, inflammatory cell activation and dysregulated cytokine production appear to play a critical role in AD pathogenesis. In this setting, alterations in the flux of ROS can exert profound influences on the pathophysiology.

We examined the involvement of oxidative stress and antioxidant defense in children with acute exacerbation of AD [58]. We studied 13 children who were hospitalized for acute exacerbation of AD with purulent skin infection by *Staphylococcus aureus* and 28 age-matched healthy controls. Urine samples obtained from the patients on admission, on the 2nd and 7th-9th hospital days and from control subjects were assayed for acrolein-lysine, 8-OHdG, BOM (using ELISA methods) and nitrite/nitrate (using colorimetry). Of these, urinary levels of acrolein-lysine, 8-OHdG and BOM, but not nitrite/nitrate, were significantly higher in AD children on admission than in control subjects. Response to treatment (with systemic antibiotics and topical antiseptics and corticosteroids) was associated with significant decreases in levels of acrolein-lysine and

8-OHdG. Urinary BOM remained almost constant and was significantly higher in AD children during hospitalization.

These results do not indicate that endogenous NO synthesis in children with acute exacerbation of AD is enhanced in comparison to that in control subjects. As described in Section (3), up-regulation of HO and the consequent increased production of bilirubin constitute physiological responses to oxidative stress. This is probably the major mechanism of increased BOM excretion in children with acute exacerbation of AD.

In another study, we found significantly higher urinary levels of 8-OHdG and significantly lower urinary levels of nitrite/nitrate in children having chronic AD without recent flare-up than those in age-matched control subjects [21]. These findings imply that the activity of constitutive and/or inducible NOSs is limited in patients with chronic AD. Reduced production of NO might augment oxidative stress and exaggerate skin lesions caused by chronic AD [130]. Section (8) also presents discussion of this topic.

Diabetes mellitus

Recent studies have indicated that hyperglycemia-induced overproduction of ROS and AGEs appears to be the first and main event in the activation of all pathways involved in the pathogenesis of vascular complications of diabetes mellitus [7, 131-133]. Augmented oxidative stress is accompanied by increased generation of NO and, consequently, formation of the strong oxidant $ONOO^-$ and by poly(ADP-ribose) polymerase activation, which in turn results in acute endothelial dysfunction and activation of inflammation in blood vessels of patients with diabetes. Therefore, the concentrations of oxidative stress biomarkers in blood or urine might serve well as a marker for later development of vascular complications in diabetes mellitus.

Recently, we examined whether AGE production and oxidative stress were augmented in

young patients with type 1 diabetes at early clinical stages of the disease [63]. Urinary samples of 38 patients with type 1 diabetes and those of 60 age-matched healthy control subjects were assayed, using high-performance liquid chromatography, for AGEs, pentosidine and pyrraline. In addition, using ELISA methods, their samples were assayed for markers of oxidative stress, acrolein-lysine and 8-OHdG. Of these four markers, urinary levels of pentosidine, acrolein-lysine, and 8-OHdG, but not pyrraline, were significantly higher in diabetic patients than in control subjects. For the patient group, urinary pentosidine, acrolein-lysine and 8-OHdG, but not pyrraline, correlated significantly with urinary albumin excretion. These results indicate that, in the course of type 1 diabetes, accumulation of AGEs, whose formation is closely linked to oxidative stress, might start early along with renal microvasculopathy.

(7) OXIDATIVE STRESS AND ANTIOXIDANT DEFENSES IN NEONATAL BIOLOGY

Oxidative stress in fetal-neonatal transition

It has been reported repeatedly that newborns are exposed to conditions of oxidative stress resulting from the change from a low oxygen pressure *in utero* (pO_2 20-25 Torr) to a high oxygen pressure at birth (pO_2 100 Torr) [134, 135]. The energy metabolism efficiency increases rapidly after birth because all aerobic organisms require oxygen for energy production and maintenance of cellular functions. Neonates must also withstand the associated generation of ROS, which can oxidize critical macromolecules. A protective mechanism develops during the third trimester of fetal life: increased antioxidant enzyme levels. This mechanism adequately prepares the neonate to endure higher concentrations of oxygen at birth.

Nevertheless, sick neonates frequently suffer from oxidative injury because of their insufficient ability to protect themselves against oxidative insult [134, 135]. Increased oxidative stress plays an important role in the pathogenesis of many neonatal diseases,

including hypoxic ischemic encephalopathy, neonatal respiratory distress syndrome, chronic lung disease, retinopathy of prematurity, and necrotizing enterocolitis [92].

Determination of oxidative stress biomarkers in neonates

We recently characterized oxidative stress status in one-month-old neonates using specific biomarkers, as measured using ELISA systems [86]. Results showed no significant difference in urinary acrolein-lysine and 8-OHdG between healthy term neonates and clinically stable preterm neonates. This finding suggests that stable preterm neonates, who require no supplemental oxygen, are not exposed to greater oxidation of lipids and DNA than healthy term neonates. In contrast, we noted significant elevations of both markers in sick preterm neonates requiring supplemental oxygen and mechanical ventilation in comparison to those of stable preterm neonates and healthy term neonates. In the sick preterm group, neonates developing active retinopathy showed significantly higher levels of acrolein-lysine than the other neonates without retinopathy did.

We also measured urinary nitrite/nitrate concentrations in our subjects, but found no significant difference in urinary nitrite/nitrate among the above-mentioned three groups. These findings suggest that no close relationship exists between the degree of oxidative stress and endogenous NO production in neonates.

Preeclampsia

Preeclampsia is a disorder of human pregnancy and a leading cause of premature birth and fetal growth retardation [136]. In pregnancies that are complicated by preeclampsia, ROS production in the placenta increases in connection with inadequate antioxidant defense. Neonates born after preeclampsia have been exposed to more oxidative stress *in utero* than matched neonates [137].

We recently found that pentosidine concentrations (as measured using high-performance liquid chromatography) in umbilical cord blood were significantly higher in neonates born after preeclampsia [77]. Our findings imply that oxidative stress and AGE formation are augmented *in utero* during preeclampsia. Enhanced oxidative stress is inferred to be a crucial factor in the progression of preeclampsia.

Antioxidant biomarkers in neonates

As described above, several urinary biomarkers for oxidative injury have been used to evaluate oxidative stress status in neonates. However, reliable biomarkers of antioxidant defense have never been reported in neonates. Some novel information related to such markers deserves introduction here.

Fatty acid-binding proteins (FABPs) are a family of 14-15-kDa proteins. They are involved in the transport of free fatty acids from the plasma membrane to sites for oxidation (i.e., mitochondria, peroxisomes) and the nucleus for gene regulation [138]. Liver-type fatty acid-binding protein (L-FABP) is expressed in renal proximal tubules and engaged in free fatty acid metabolism [139]. Free fatty acids are loaded into the proximal tubules in oxidative conditions; thereby, they become cytotoxic. Transcription of the L-FABP gene is promoted by various kinds of stresses, including free fatty acids *per se*. For that reason, L-FABP is considered to serve as an antioxidant enzyme in the kidney. Recent clinical studies suggest that urinary excretion of L-FABP reflects oxidative stress on the proximal tubules and might be a useful biomarker for the progression of chronic kidney disease [139-142].

We measured urinary excretion of L-FABP (using the ELISA system developed by Sugaya, et al., L-FABP ELISA; CMIC Co. Ltd., Tokyo, Japan [139-142]) for the first time in preterm and term neonates [96]. Urinary levels of L-FABP in these neonates were remarkably higher than in healthy adults. Urinary L-FABP levels showed significant

positive correlation with those of 8-OHdG during the late neonatal period. These results attest to the expression of L-FABP in the neonatal kidney. They also suggest the potential effect of oxidative stress on the L-FABP expression.

Detoxifying isoenzymes, GSTs, catalyze the conjugation of glutathione to a range of electrophilic compounds. They also exhibit a non-selenium-dependent glutathione peroxidase activity against organic hydroperoxides [143, 144]. The human renal distal tubules contain the π form of GST in great amounts.

We measured urinary excretion of GST-π (using ELISA method, Urinary GST-π ELISA; Biotrin International, Dublin, Ireland) in preterm neonates for the first time [108]. Urinary levels of GST-π showed significantly positive correlation with urinary 8-OHdG at one and four weeks of age. Sick neonates treated with supplemental oxygen and mechanical ventilation showed significantly higher levels of GST-π as well as 8-OHdG than clinically stable neonates did at four weeks. These results indicate the potential effect of oxidative stress on the GST-π expression in the neonatal kidney. Considering the accumulated evidence described above, neonates appear to have two major antioxidant enzymes in the kidney (i.e., L-FABP and GST-π, respectively, in the proximal and distal tubules) through which many kinds of potentially oxidative endogenous and exogenous substances should be detoxified.

Breast milk as a rich source of antioxidants

Breast-feeding is associated with lower rates of several infantile diseases: respiratory illness, necrotizing enterocolitis, and sepsis. Breast milk consumption offers many advantages over consumption of infant milk formula, including provision of antioxidant protection to infants [145]. Breast milk contains various enzymatic and nonenzymatic antioxidants, including SOD, catalase, tocopherols, ascorbate, and lactoferrin. Shoji et al. [146] reported that urinary 8-OHdG was significantly lower in breast-fed infants than in formula-fed infants at

one month of age. This finding implies that oxidative DNA damage is lower in breast-fed infants. Therefore, it is conceivable that abundance of antioxidants in breast milk might help infants to eliminate ROS.

We determined concentrations of nitrite/nitrate, ubiquinol/ubiquinone, tocopherols, β-carotene, and lycopene in human milk obtained from lactating women during the early postpartum period [147, 148]. The respective mean (SD) concentrations of these antioxidants were 479 (274) μmol/l, 352 (24), 10,760 (950), 153 (131), and 161 (196) ng/ml. Although the concentrations of the first three in Japanese standard infant milk formulas are comparable to those in human milk, concentrations of the latter two are remarkably lower in these formulas.

A ubiquitous protein (12 kD), TRX has a three-dimensional structure containing two redox-active cysteine residues (-Cys-Gly-Pro-Cys-) [149]. The TRX system comprises several related molecules that interact through active-site cysteine residues. Against oxidative stresses of various sorts, TRX plays a potent cytoprotective role. Recently, we observed that breast milk concentrations of TRX (as measured using the ELISA system developed by Nakamura et al. [150], TRX ELISA Kit; Redox Bioscience Inc., Kyoto, Japan) during the early postpartum period (mean (SD) concentration, 268 (23) ng/ml) were seven to eight times higher than those in blood of lactating women [148]. In breasts of lactating women, TRX might be synthesized in high amounts. This abundance of TRX in human milk might be absorbed into the neonatal circulation and exert antioxidant functions in the neonate.

Birth is a complex process that includes the abrupt challenge posed by oxidative stress. Neonates must adapt themselves rapidly and drastically to extrauterine life as the function of gas exchange is transferred from the placenta to the lungs [134, 135]. The above observations engender the contention that high levels of antioxidants in early breast milk provide a unique protective mechanism that allows the maintenance of redox balance

during the fetal-to-neonatal transition.

(8) OXIDATIVE STRESS AND ENDOTHELIAL ACTIVATION/DYSFUNCTION

Vascular endothelium

The vascular endothelium, rather than being a mere barrier between intravascular and interstitial compartments, is a widely distributed organ that is responsible for regulation of hemodynamics, angiogenic vascular remodeling, and metabolic, synthetic, anti-inflammatory, and anti-thrombogenic processes. Understanding of the interrelationship of oxidative stress and endothelial activation/dysfunction [130, 151-154] will allow delineation of a rational therapeutic strategy in conditions that are associated with oxidative damage. Our experimental results on this topic, which are presented below, are illustrative.

Endothelial activation experiments

The adhesion molecules and chemokines expressed in endothelial cells play an important role in regulating recruitment of leukocytes to inflammation sites [151, 152]. Although enhanced expression of adhesion molecules and chemokines have been described for various allergic and immunologic diseases, including AD, bronchial asthma, and Kawasaki disease, the effects of antioxidants and NO on these inflammatory changes in human microvascular endothelial cells have not been clarified.

First, we examined the effects of antioxidant, pyrrolidine dithiocarbamate (PDTC) and NO donor, spermine NONOate (Sper-NO) on adhesion molecule expression and nuclear factor κB (NF-κB) activation induced by tumor necrosis factor-α (TNF-α) in cultured human dermal microvascular endothelial cells (HDMEC) [155]. Treatment of cells with TNF-α significantly induced the surface and gene expression of E-selectin, intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, and activation of NF-κB. The

up-regulation of these adhesion molecules and activation of NF-κB induced by TNF-α were decreased significantly by pretreatment with PDTC or Sper-NO. Next, we examined the effects of PDTC and Sper-NO on the secretion and gene expression of chemokines, interleukin-8, monocyte chemotactic protein-1, and regulated-upon-activation normal T-cell expressed and secreted [156]. Treatment with TNF-α significantly increased secretion and gene expression of these chemokines in cultured HDMEC; all were suppressed significantly by the two pretreatments mentioned above. These findings indicate that both expression of adhesion molecules and secretion of chemokines in HDMEC induced by TNF-α are inhibited significantly by pretreatment with PDTC or Sper-NO, possibly in part through blockage of NF-κB activation.

Our investigations also showed that both expression of adhesion molecules and secretion of chemokines in human pulmonary microvascular endothelial cells induced by TNF-α were inhibited significantly by pretreatment with PDTC or Sper-NO, possibly via blocking of redox-regulated NF-κB activation [157, 158]. These results are consistent with findings of experiments using HDMEC; they provide some evidence that the redox-sensitive NF-κB activation is essential for TNF-α-induced microvascular endothelial cell activation in various human organs.

Endothelial dysfunction experiments

Previously, we established a young rat model of chronic NO deficiency and endothelial dysfunction. We examined the effects of chronic NO blockade on oxidative stress status and renal function in young male SD rats [159, 160]. Two types of NOS inhibitor were used: N^G-nitro-L-arginine methyl ester (L-NAME) as a non-selective inhibitor and aminoguanidine as a selective inhibitor of the inducible isoform (NOS2). Oral administration of L-NAME, but not aminoguanidine, for 4 weeks induced systemic hypertension, a significant reduction in urinary nitrite/nitrate, and a significant increase in urinary 8-OHdG compared with non-treated animals. These rats developed proteinuria and

tubular enzymuria (high excretion of N-acetyl-beta-D-glucosaminidase), with normal serum creatinine levels. Chronic aminoguanidine administration did not significantly alter urinary 8-OHdG, protein, N-acetyl-beta-D-glucosaminidase, or serum creatinine. The effects of L-NAME on blood pressure and urinary parameters were restored by a large dose of L-arginine. These observations highlight the importance of continuous generation of NO by constitutive NOS (especially NOS3) in the control of vascular tone, renal function, and antioxidant capacity in young animals.

Rats receiving chronic non-selective NOS inhibitor treatment exhibit various parenchymal lesions [161]. Enhanced oxidative stress might participate in the development of such organ damage in animals with chronic NO deficiency and endothelial dysfunction.

Imbalance of NO/O_2^- formation

Importantly, ROS act as signal transducers and represent a versatile cellular control mechanism for gene regulation [2, 149, 152]. Related to signaling of several cytokines, e.g., TNF-α and interleukin 1β, ROS themselves might act as an intracellular signaling system. Activation and DNA binding of several transcription factors depend on the cellular redox state. An appropriate amount of ROS generated in the cells appears to be used as a signaling system or as a modulating factor on signal transduction pathways from surface receptors. The generation of oxidants in greater quantities than the buffering capacity might engender excessive signals to the cells, in addition to direct damage, thereby causing cell death or pathological processes in which ROS are involved.

As its role, NO provides cells with a reducing environment and scavenges O_2^- that is formed through metabolism. The balance between NO and O_2^- might be a key determinant in promoting oxidative stress [1, 120, 130, 131, 152-154]. Under normal physiological conditions, when NO is produced in greater amounts than is O_2^-, endogenous NO serves to inhibit oxidative reactions. Maximum oxidant production will occur presumably by

formation of $ONOO^-$ when fluxes of each radical are identical (the flux ratio of each radical is 1.0), especially in regions of low SOD activity. Endothelial activation (as represented by overproduction of O_2^- induced by TNF-α treatment [155-158]) and dysfunction (as represented by diminished NO synthesis [159-161]) might both produce a situation in which a situation of balance of NO and O_2^- in favor of NO is altered in favor of a more oxidative environment.

It is noteworthy that normal function of NOS3 requires the presence of the essential cofactor BH_4 as well as the substrate L-arginine [130, 154]. The cofactor BH_4 is a potent, naturally occurring reducing agent; it is highly sensitive to oxidation by $ONOO^-$. Diminished levels of BH_4 promote O_2^- production by NOS3 (so-called "NOS3 uncoupling"). This transformation of NOS3 from a protective enzyme to a contributor to oxidative stress has been observed in several *in vitro* models, in animal models of cardiovascular diseases, and in patients with cardiovascular risk factors [162-164].

The L-arginine-NO system might maintain a low steady state concentration of ROS that are being constantly formed *in vivo*. Furthermore, constitutively produced NO, by controlling the availability of ROS and the redox state of the cells, might modulate various cellular activities, including nuclear gene regulation. Although the results described above cannot be extrapolated directly to the human situation, they suggest a potential therapeutic approach using antioxidant agents or NO pathway modulators in the treatment of situations that are characterized by endothelial activation or dysfunction.

(9) THERAPEUTIC IMPLICATIONS

As clinicians, prevention and treatment are our ultimate interest. As described in preceding sections, clinical studies suggest that an imbalance between oxidant and antioxidant activities in favor of the former contributes to the pathogeneses of many diseases that are encountered in the field of pediatric medicine [15-121]. Our experimental results and those

of other investigators suggest that restoration of the redox balance using antioxidant agents might offer potential therapeutic interventions. Collectively, therapeutic interventions that decrease exposure to ROS or augment antioxidant defenses might be beneficial as adjunctive therapies for oxidative-stress related diseases.

Malfunction of the NO system is implicated in numerous disease processes, resulting in reduced [21, 55, 64] or excessive NO production [20, 34, 39, 43, 44, 73, 85, 88, 94, 114]. The NOS substrates, i.e., L-arginine and BH_4, can be administered to enhance NO production; increased NO levels can be achieved directly through administration of NO donors. Using NOS inhibitors, NOS activities can be decreased. The effects of NO are widespread. Therefore, development of therapeutic interventions is challenging. The design of organ-specific delivery methods as well as agents targeting specific NOS (especially NOS2) is necessary. Because of the short-lived nature of NO, topical treatments will present few systemic side effects.

Antioxidant strategies, including administration of pharmacological or dietary agents are based on two main mechanisms: the enhancement of ROS elimination and the inhibition of ROS generation. The agents acting according to the former mechanism include antioxidant enzymes catalyzing ROS degradation and scavengers neutralizing ROS. Classic antioxidants include tocopherols, carotenoids, and ascorbate. It has been postulated that the antioxidant potency of these antioxidants may be limited because they work as scavengers of already-formed ROS in a stoichiometric manner. They might therefore be thought of as a more “symptomatic” treatment rather than as a treatment for causes of oxidative stress-associated clinical problems [165]. On the other hand, it has been suggested that angiotensin-converting enzyme inhibitors and angiotensin II type-1 receptor antagonists suppress intracellular overproduction of ROS and work as “causal” antioxidants and that many of their beneficial effects are the result of this property [165-167].

Numerous clinical trials have involved the administration of various antioxidants in the

pediatric field. Previously reported therapeutic antioxidant strategies include the following: corticosteroids for bronchial asthma [40] and bacterial meningitis [34]; L-arginine for endothelial dysfunction in cardiac transplantation [64]; angiotensin-converting enzyme inhibitors and angiotensin II type-1 receptor antagonists for endothelial dysfunction in diabetes mellitus [7, 95]; melatonin for neonatal asphyxia [20] and for epilepsy [76]; folinic acid, betaine and methylcobalamin for autism [66]; tocopherols and ubiquinol/ubiquinone for Friedreich ataxia [90]; selenium for skeletal muscle disorder in selenium deficiency [59]; tocopherols and ascorbate for endothelial dysfunction in hyperlipidemia [42]; glutathione and α-lipoic acid for kwashiorkor [111]; amifostine for total body irradiation [71] and for anticancer drug use [121]; tocopherols, carotenoids and ascorbate for immunosuppressive drug use (cyclosporine A, tacrolimus) [116]; and ubiquinol/ubiquinone for anthracycline use [117].

The authors found some favorable effects of the above antioxidant strategies. However, these results should be interpreted cautiously and confirmed with studies that have been conducted with more numerous patients and with other techniques to measure oxidative stress status because the studies explained in this report analyzed samples using only a few parameters from only a few subjects. Under certain conditions, surprisingly, the antioxidant supplements may exhibit prooxidant properties and even worsen tissue damage. Large-scale, prospective, controlled clinical trials using a multi-parameter set of oxidative stress biomarkers, as well as further fundamental investigations of the basic science are necessary to establish both the efficacy and safety of antioxidant strategies in clinical practice.

(10) CONCLUDING REMARKS

The challenges for the future are to elucidate the molecular mechanisms that engender oxidative stress in pediatric diseases, to develop effective therapeutic antioxidants, and to demonstrate their benefits to patients. However, before antioxidant therapy becomes

accepted in clinical practice, detailed longitudinal studies must be conducted, evaluating panels of oxidative stress biomarkers together with traditional clinical endpoints in patients who are undergoing treatment for diverse acute and chronic diseases.

This review has provided current knowledge about noninvasive means of assessing oxidative stress status in the field of pediatric medicine. The biomarkers presented herein, such as acrolein-lysine, 8-OHdG, BOM, and L-FABP, can be measured easily in urine samples using commercially available ELISA methods. Measurement of these parameters will have far-reaching potential for the monitoring and treatment of oxidative-stress related diseases in pediatric medicine.

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Abbreviations: AD, atopic dermatitis; AGE, advanced glycation end product; BH_4, tetrahydrobiopterin; BOM, bilirubin oxidative metabolites; Cr, creatinine; ELISA, enzyme-linked immunosorbent assay; FABP, fatty acid-binding protein; GST, glutathione-S-transferase; HDMEC, human dermal microvascular endothelial cells; HO, heme oxygenase; IL-8, interleukin-8; L-FABP, Liver-type fatty acid-binding protein; L-NAME, N^G-nitro-L-arginine methyl ester; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; SOD, superoxide dismutase; Sper-NO, spermine NONOate; TNF-α, tumor necrosis factor-α; TRX, thioredoxin

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Table 1. Oxidative stress biomarkers of two categories

(a) Formation of modified molecules by reactive oxygen species
Lipid peroxidation: malondialdehyde-lysine, 4-hydroxy-2-nonenal-lysine, acrolein-lysine*, F2-isoprostane
Oxidative DNA damage: 8-hydroxy-2'-deoxyguanosine*
Glyco-oxidation: carboxymethyl-lysine, pentosidine*, argpyrimidine, methylglyoxal
Nitro-oxidation: nitrotyrosine, nitrite/nitrate*
Others: o,o'-dityrosine, ortho-tyrosine, bilirubin oxidative metabolites*, dehydroascorbate, oxidized glutathione, "thiobarbituric acid reactive substances"
(b) Antioxidant enzymes and molecules
Enzymes: superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase*, thioredoxin reductase, heme oxygenase
Proteins: albumin, ferritin, transferrin, lactoferrin, cerulolasmin, thioredoxin*, L-type fatty acid binding protein*
Low molecular weight molecules: bilirubin, tocopherols, carotenoids, ubiquinol/ubiquinone, ascorbate, glutathione, cysteine, urate, nitrite/nitrate*, selenium
Others: "total antioxidant reactivity", "total radical trapping antioxidant potential"

Only biomarkers that are determined in samples of blood (serum, plasma, erythrocytes, granulocytes or lymphocytes) or urine are shown. Nitric oxide behaves either as a prooxidant or as an antioxidant according to its environment. Therefore, its stable metabolites, nitrite/nitrate, is listed in both categories (a) and (b). *Clinical significance of these biomarkers is described in detail in the following sections: acrolein-lysine, 8-hydroxy-2'-deoxyguanosine, pentosidine, nitrite/nitrate (Sections (5) and (6)), bilirubin oxidative metabolites (Section (6)), glutathione-S-transferase, thioredoxin, L-type fatty acid binding protein (Section (7)).

Table 2. Possible oxidative stress involvement in pediatric diseases

Allergic/Immunologic: atopic dermatitis [21*, 58*], bronchial asthma [40, 51, 74], chronic arthritis [15, 85], Henoch-Schonlein purpura [37], Kawasaki disease [48], systemic lupus erythematosus [68], vasculitis syndrome [73]
Cardiovascular: cardiac surgery [56], cardiac transplantation [64], cardiopulmonary bypass [98], essential hypertension [55]
Endocrinologic/Metabolic: diabetes mellitus [39, 63*, 95, 100, 114*], glutathione synthetase deficiency [33], hyperthyroidism [79], iodine-deficient goiter [26], mitochondrial disorder [30, 105], multimetabolic syndrome [81], phenylketonuria [91], X-linked adrenoleukodystrophy [69]
Environmental/Toxicologic: carcinogenic metal (chromium, arsenic) exposure [97], ozone exposure [74], passive smoking [101], urban residence [89]
Gastrointestinal/Hepatologic: autoimmune hepatitis [38], chronic constipation [112], inflammatory bowel disease [47], nonalcoholic fatty liver disease [75, 106], viral hepatitis [19], Wilson disease [78]
Genetic: Cockayne syndrome [49], Down syndrome [54, 57], Zellweger syndrome [44]
Hematologic: acute leukemia [52, 53, 88], β-thalassemia [45], erythropoietic protoporphyria [104], Fanconi anemia [72], sickle cell anemia [93]
Infectious: acute bronchiolitis [70], acute infectious mononucleosis [48], acute otitis media [102], acute tonsillitis [102], adenovirus infection [48], chronic nail candidiasis [80], chronic otitis media [87], chronic tonsillitis [62, 82], cutaneous leishmaniasis [94], HIV infection [28, 99], measles encephalitis [31], meningitis [17*, 34*], septic shock [18]
Neonatal: asphyxia [20, 41], maternal preeclampsia [77*], neonatal respiratory distress syndrome [86*, 96*, 108*], premature birth [27, 29, 92], retinopathy [86*, 92]
Neurologic/Muscular: ataxia telangiectasia [23], attention deficit hyperactivity disorder [61], autism [43, 66, 110], cerebral organic acid disorder [35], cerebral palsy [107], congenital muscular dystrophy [32, 50, 60], epilepsy [30, 76, 84], Friedreich ataxia [90], inflammatory myopathy [60], selenium-deficient skeletal muscle disorder [59], spinal

muscular atrophy [24], traumatic brain injury [36, 46]
Nutritional: hyperlipidemia [42], kwashiorkor [16, 111], obesity [83, 103]
Pharmacologic/Therapeutic: analgesics [120], anticancer drugs [117-121], immunosuppressive drugs [116], total body irradiation [71]
Renal: glomerulonephritis [109], nephrotic syndrome [25], renal insufficiency/failure [22, 109, 113], urinary tract infection [109]
Respiratory: chronic pulmonary disease [115], cystic fibrosis [65, 67]

*The authors' works.

Figure 1. Age-related changes of urinary levels of acrolein-lysine, 8-hydroxy-2'-deoxyguanosine (8-OHdG) and nitrite/nitrate.

Data are presented as mean (SD). One-hundred healthy young people (50 males and 50 females) are grouped into the following four groups: 1-6 years (n = 33); 6-11 years (n = 34); 11-16 years (n = 20); 16-21 years (n = 13). Statistically significant inter-group difference: *$p < 0.05$ vs. any of the older age groups; **$p < 0.05$ vs. any of the older age groups (ANOVA using Scheffe's method).

Figure 2. Age-related changes of urinary levels of pentosidine and pyrraline.

Data are presented as mean (SD). Ninety-six healthy young people (50 males and 46 females) are grouped into the following four groups: 1-6 years (n = 30); 6-11 years (n = 33); 11-16 years (n = 20); 16-21 years (n = 13). Statistically significant inter-group difference: *$p < 0.05$ vs. any of the older age groups (ANOVA using Scheffe's method).

Figure 1.

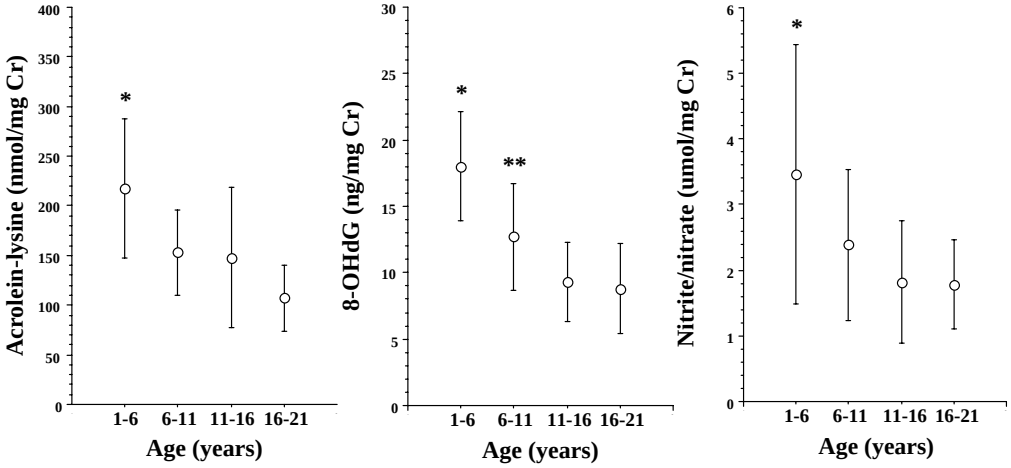
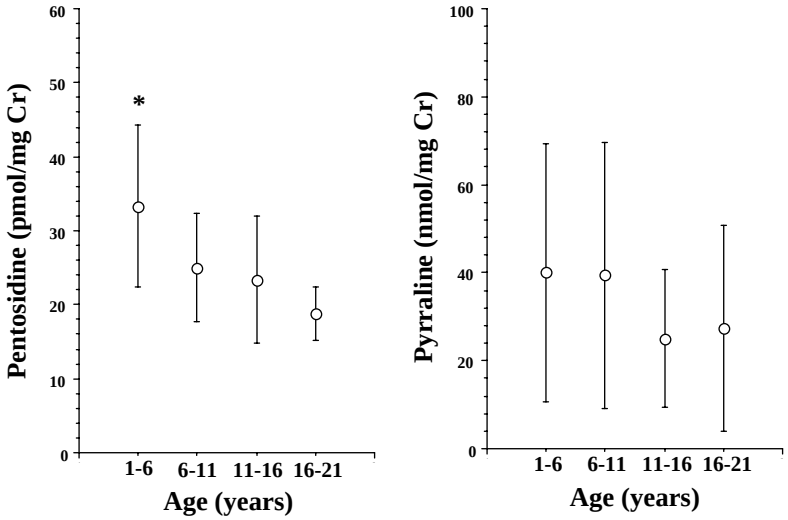

Acrolein-lysine (nmol/mg Cr)
400
350
300
250
200
150
100
50
0
*
1-6
6-11
11-16
16-21
Age (years)
8-OHdG (ng/mg Cr)
30
25
20
15
10
5
0
*
**
1-6
6-11
11-16
16-21
Age (years)
Nitrite/nitrate (umol/mg Cr)
6
5
4
3
2
1
0
*
1-6
6-11
11-16
16-21
Age (years)

Figure 2.


Pentosidine (pmol/mg Cr)
60
50
40
30
20
10
0
*
1-6
6-11
11-16
16-21
Age (years)
Pyrraline (nmol/mg Cr)
100
80
60
40
20
0
1-6
6-11
11-16
16-21
Age (years)