Evaluation of oxidative stress and antioxidant capacity in healthy children

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Abstract

Background: Measurement of the levels of the derivatives of reactive oxygen metabolites (d-ROMs) and of the biological antioxidant potential (BAP) enables simultaneous assessment of oxidation degree and antioxidant capacity, using the same sample and testing equipment. At present, reference values of healthy adults are clarified, but the reference value of healthy children is unknown. This study was undertaken to clarify the age-related changes and the reference values of d-ROMs and BAP in healthy children.

Methods: The study population consisted of 77 children, ranging in age from 2 to 15 years, in normal mental and physical health as examined by a pediatrician, and seven healthy adult volunteers. Serum samples were obtained from the subjects for assay. Using these samples, d-ROMs and BAP values were measured, and the relationship with age was analyzed.

Results: The d-ROMs level decreased as the age increased, while the BAP showed no correlation with the age. The d-ROMs level was significantly higher in 2-6 years group than in 7-11 years group, 12-15 years group, or healthy adults group. The BAP/d-ROMs ratio, an index of antioxidant capacity, increased significantly with higher age.

Conclusion: This study was carried out for the first time in healthy children in oxidative stress assessment using d-ROMs and BAP. In the infancy 2-6 years, the d-ROMs value was significantly higher and the BAP/d-ROMs ratio was significantly lower. From this, it was suggested that age should be considered when performing oxidative stress assessment using d-ROMs and BAP in children.

Keywords: Antioxidants; Biomarker; Children; Oxidative stress; Pediatrics

1. INTRODUCTION

Oxidative stress is defined as a disturbance in the balance between oxidative and antioxidant reactions, with the balance tilting toward the former, to yield an undesirable physical condition.1 Oxidative stress has been reported to be enhanced in conditions such as tobacco smoking, hemodialysis, rheumatoid arthritis, and various mental illnesses; it is implicated in the pathogenesis, at the molecular level, of many diseases involving cellular injuries.2 Various indicators of oxidative stress are used (eg, acrolein-lysine of lipid oxide, protein carbonyls of protein oxide, 8-hydroxy-deoxy-guanosine (8-OHdG) of nucleic acid oxide, and pentosidine of carbohydrate oxide). Recently, these oxidative stress markers have become easily measurable by popularization of assay kits. And various oxidative stress studies have been conducted. However, since humans have intrinsic antioxidant capacity, the level of oxidative stress in the body should be evaluated comprehensively by measuring both oxidation and antioxidant capacity1 but it is difficult to evaluate antioxidant capacity with frequently used markers (especially 8-OHdG). Evaluation of the oxidative stress level by measuring the plasma levels of derivatives of reactive oxygen metabolites (d-ROMs) and of the biological antioxidant potential (BAP) enables evaluation of both the oxidation degree and antioxidant capacity, using the same sample and testing equipment. Furthermore, the measurement method is a simple procedure in a short time. For these reasons we focused on oxidative stress markers called d-ROMs and BAP.

At present reference values for healthy adult d-ROMs and BAP have been reported.4 In addition there are reports documenting urine 8-OHdG assay data in healthy children too.5,6 However, measurement data of d-ROM and BAP of healthy children has not been reported yet. This makes direct comparison of the relevant data in age-stratified groups of children impracticable. This study was undertaken to clarify the growth/development-related changes in and the reference values of d-ROMs and BAP in healthy children.

2. METHODS

2.1. Subjects

The study population consisted of 77 Japanese children (39 male and 38 female), ranging in age from 2 to 15 years, who/whose parents provided informed consent for participation in this study. A reference group consisting of seven healthy adults aged 23 to 33 years (three male and four female) was also included in the study. The criteria for conformity of the subjects were children who were judged mentally and physically healthy by examination by a pediatrician. The exclusion criteria were as follows: subjects...
(1) with an underlying disease, (2) currently suffering from some disease, (3) receiving drug(s) for treatment or prophylaxis, (4) with a history of some health disorder in the 1-month period before the start of this study, and (5) those with an abnormal value of C-reactive protein (CRP), which is an inflammatory marker.

2.2. Assay parameters

2.2.1. Oxidative stress marker (d-ROMs)
The blood levels of hydroperoxides, which are by-products of free radicals, were measured. Since hydroperoxides reflect peroxidized lipids, proteins, amino acids, and nucleic acids, their blood levels are evaluated in terms of the “blood level of hydroperoxides = amount of free radicals occurring in the body”.

2.2.2. Antioxidant capacity (BAP)
The reducing power from iron (III) oxide to iron (II) oxide was determined as the capacity to arrest peroxidative chain reactions induced by the free radicals. The greater the reducing power from iron (III) oxide to iron (II) oxide, the higher the capacity for eliminating free radicals from the body; thus, the antioxidant potential is evaluated in terms of the “reducing power of blood = biological antioxidant potential.”

2.2.3. Inflammatory reactant, C-Reactive protein
There have been reports of existence of a correlation between the CRP value and d-ROMs levels. By measuring CRP and eliminating potential inflammation, we added not only the doctor’s examination or subjective symptoms but also objective indicators to “diagnose health”, excluding factors that affect oxidative stress levels.

2.3. Assay methods
Blood samples were collected during the study period from May 2017 to April 2018 from 10 o’clock to 17 o’clock. The collected samples were immediately centrifuged at 3000 rpm for 10 minutes to separate the serum and the separated serum samples were stored in a freezer at −30°C until assay. Measurement of the oxidative stress marker was carried out by thawing the stored serum sample and analyzing immediately for d-ROMs and BAP with an automatic free radical analyzer “FREE CARRIO DUO” (Diacon International, Grosseto, Italy). For measurement of CRP, the collected blood samples were immediately tested without freezing with an immunoanalyzer (Celltac Chemi CRP 3100; Nihon Kohden Corp., Tokyo, Japan).

2.3.1. d-ROMs test
Each sample (20 μL) was placed in a cuvette filled with a buffer (pH 4.8). The cuvette was then placed upside down and agitated (to induce the release of Fe²⁺ and Fe³⁺ from the blood proteins). Fe²⁺ and Fe³⁺ serve as catalyzers, resulting in the degradation of blood hydroperoxide into an alkoxo radical and a peroxy radical. Then, a color-developing chromogen (N,N-diethyl-p-phenylenediamine) was placed in a volume of 20 μL, resulting in the oxidation of the chromogen substrate by the free radicals to yield a red-colored radical cation. The cuvette was once again turned upside down and agitated, then placed in a photometer for optical measurement. U.CARR is used for the unit, and 1U.CARR is equivalent to the H₂O₂ of 0.08 mg/dL.

2.3.2. BAP test
A chromogen for BAP (a reagent containing trivalent iron, 50 μL) was added to the cuvette to induce red coloration. The cuvette was placed upside down, and the color-developing concentration was measured using a photometer. Then, each plasma sample (10 μL) was placed into the cuvette and agitated. The cuvette was then placed in a thermostat for 5 minutes. Then, the cuvette was placed into a photometer for optical measurement and measured in μmol/L.

2.4. Statistical assessments
Correlation coefficient r value and p value were calculated, and the relation between age and d-ROMs, BAP and BAP/d-ROMs ratio was statistically tested. With regard to d-ROMs and BAP values, 2 to 6 years were classified as Group A, 7 to 11 years as Group B, 12 to 15 years as Group C, and healthy adults as Group D. IBM SPSS Statistics version 21 (IBM Corp., Armonk, NY, USA) was used as the statistical software, and the Kruskal–Wallis test was used to compare the four groups. Sex difference between each measurement value and age group was compared using Mann–Whitney U test.

2.5. Ethical considerations
Based on the Declaration of Helsinki, it was implemented with the approval of the Red Cross Ethics Committee of the Japanese Red Cross Tokushima Hinomine Rehabilitation Center for People with Disabilities. The research team explained to the target children and their families using a document and got the personal agreement as much as possible in writing. Also, if they did not want to participate in the research, they can quit at any time, and explained that there is no inconvenience. Assay data obtained were anonymized to avoid personal identification of individuals.

3. RESULTS
Table 1 shows the background of pediatric subjects. Average age was 8.49 ± 3.75 years, median was 8 years. There were 39 males and 38 females. In the gender comparison by age group, Group A had 15 males and 13 females, Group B had 15 males and 13 females, and C group had 9 males and 12 females. As a reference group, Group D (23-35 years, adult group) had 3 males and 4 females. The average CRP value for children and adults was 0.08 ± 0.05 mg/dL.

**Table 1**

<table>
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<th>Age, y</th>
<th>Means ± SD</th>
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<th>Minimum</th>
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<tr>
<td>C: 12-15, y</td>
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**Gender comparison by age group (n)**

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<tr>
<td>D</td>
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**Sex (n)**

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<th>Number</th>
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<tr>
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<td>39 (50.6%)</td>
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<tr>
<td>Female</td>
<td>38 (49.4%)</td>
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</tr>
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**Blood test result (CRP), mg/dL**

| Mean ± SD | 0.08 ± 0.05 |

*Classified as group A, 2-6 y; group B, 7-11 y; group C, 12-15 y; and group D, 23-35 y reference adults group.*
Fig. 1A shows the intergroup comparison of d-ROMs among the four age groups. The d-ROMs level was significantly higher in group A (2-6 years) than in group B (7-11 years), group C (12-15 years), or group D (healthy adults) (A vs B: \(p < 0.001\); A vs C: \(p < 0.001\); A vs D: \(p < 0.001\)). No significant difference was noted between group B and group C, between group B and group D, or between group C and group D. As a result of analyzing the correlation between age and d-ROMs, the lower the age, the higher the d-ROMs level and the higher the age the d-ROMs level gradually decrease. There were significant correlations \((r = -0.809, p < 0.001)\). Fig. 1B shows the intergroup comparison of BAP among the four age groups. BAP was a comparison between all age groups, and there was no significant difference. Regarding the correlation between age and BAP, there was no correlation between age and BAP values \((r = -0.189, p = 0.105)\).

Fig. 2 shows the intergroup comparison of BAP/d-ROMs ratio among the four age groups. The BAP/d-ROMs ratio was significantly lower in group A (2-6 years) than in group B (7-11 years), group C (12-15 years), or group D (healthy adults) (A vs B: \(p < 0.001\); A vs C: \(p < 0.001\); A vs D: \(p < 0.001\)). As a result of analyzing the correlation between age and BAP/d-ROMs ratio (antioxidant capacity), the lower the age, the lower the BAP/d-ROMs ratio, and the higher the age the BAP/d-ROMs ratio increases. These were significant correlations \((r = 0.743, p < 0.001)\).

Table 2 shows comparison of gender differences among each measured value and age groups. In the same age groups, there was no significant gender difference of measured values.

### 4. DISCUSSION

Free radicals in vivo have remarkably short half-lives, hence it is almost impossible to quantify those radicals by means of electron paramagnetic resonance (ESR). d-ROMs, on the contrary, can be measured highly stably, inasmuch as this parameter is a quantitative marker of the levels of hydroperoxides (ROOH), which are precursors of peroxyradicals (ROO) and alkoxy radicals (RO). It is also feasible to carry out the test with minute quantities of blood sample (serum, 20 μL) obtained by fingertip puncture. Furthermore, the test requires only 5 minutes for optical measurement; it is minimally invasive and not time consuming. Moreover, d-ROM is a marker of oxidative stress that is highly reproducible, as reported by Nojima et al. A study by Alberti et al., in
which they investigated the validity of measurement of the levels of d-ROMs, demonstrated the existence of a correlation between the d-ROMs test data and the ESR assay data. Therefore, in recent years, measurement of d-ROMs has become a screening tool for a variety of diseases and for the evaluation of therapeutic responses. It is now applied in the clinical setting for a wide variety of disorders, including cardiovascular diseases, renal failure, pulmonary diseases, hypertension and Alzheimer-type dementia.12–14 Apart from sporadic reports of studies in pediatric patients,17–19 there are no reports yet of the d-ROM and BAP values in normal healthy children. In the current study, we demonstrated that the levels of d-ROMs were associated with the age, while there was no correlation between the age and the BAP. Consistent with the results of the present study, Nojima et al.18 also reported that in adults the level of d-ROMs changed with advancing age, while the BAP showed no association with age. These findings suggest the importance of age-related comparisons even in children. Tsumura et al.4 reported that the level of 8-OHdG, an oxidative stress marker, also decreased with advancing age in healthy children. Tsukahara et al.20 have reported that the level of 8-OHdG plateaus by 10 to 12 years of age. The age-associated changes in the levels of d-ROMs observed in the present study are similar to these reports, and compatibility between the results for d-ROMs and 8-OHdG may be correlated in healthy children. In our study, subjects set a number of exclusion criteria, guaranteeing “healthy condition” such as confirming physician’s examination and CRP, which correlates with d-ROMs. However, why does the level of d-ROM decrease with increasing age? An elevated level of d-ROM is presumed to be due to damage of cells, mitochondria, and DNA.21 Therefore, it is speculated that repeated cell death and regeneration and rapid turnover may have led to an increase in d-ROMs, as subjects of low age are in the period of active growth and development. In addition, infants may have influenced the measurement results by the involvement of psychological stress such as fear and anxiety on blood collection. However, these are guesses, and it is a future task to explain this result. These results suggest that the lower the age, the higher the oxidative stress level and the higher the antioxidant capacity. Humans have biological homeostasis trying to adapt to changes in the environment.22 It is possible that elevation of the oxidation degree, as reflected by elevation in the blood levels of d-ROMs and urinary 8-OHdG, may activate antioxidant capacity, with resultant reparation of the oxidation degree to antioxidant capacity may enable more accurate evaluation of the oxidative stress level in vivo. The true oxidative stress status may be best elucidated by assessing two conflicting parameters, oxidation degree and antioxidant capacity, rather than by assessing either of the two parameters alone. Also, when biomarkers are used clinically, judgment taking age into account is necessary especially for children. As for the limitations of this study, a wide spectrum of exclusion criteria was laid down to ascertain the normal health status of the subjects enrolled in this study, and the possible involvement of undetected factor(s) influencing the levels can still not be ruled out. Further investigation with larger sample sizes and collection of assay data for stratified age groups is needed to strengthen the clinical credibility of the study. In this study, the trend of d-ROMs and BAP of healthy children was clarified. The results also suggest that measurement of the levels of d-ROMs and BAP enables comprehensive evaluation of the oxidative stress level via allowing analysis of two conflicting parameters, i.e., the oxidation degree and antioxidant capacity. Also the results of d-ROMs and BAP values of healthy children at each age enables direct comparison by age for children with disease and it may be put as a useful objective index for disease diagnosis/treatment evaluation.

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REFERENCES