Oxidative Stress in Marasmic Children: Relationships with Leptin

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ABSTRACT

***Aim**: We aimed to investigate the levels and relationships of antioxidants, lipid peroxidation and leptin altogether in marasmic malnutrition.*

***Method**: Thirty marasmic children (age 14.4±10.3 months) and 28 control subjects were included. Erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities, glutathione (GSH) level, and serum malondialdehyde (MDA) and leptin levels were measured.*

***Result**: Malnourished children had significantly lower erythrocyte SOD activity (1583±417 vs. 3403±1901 U/gHb, respectively, P<0.001), CAT activity (1139±92 vs. 1663±302 k/gHb, p<0.001), GSH level (25.9±5.4 vs. 48.1±17.0 µmol/gHb, p<0.001) and leptin levels (3.6±1.1 vs. 11.8±4.5 ng/mL, respectively, p<0.001), compared with control subjects. However mean MDA concentration of marasmic children (11.1±2.5 nmol/mL) was found to be significantly higher than that of the control subjects (6.6±3.9 nmol/mL) (p<0.001). Significant negative correlations were detected between CAT and MDA (r=-0.476, P=0.009), between SOD and MDA (r=-0.534, p=0.004), and GSH and MDA (r=-0.439, p=0.015) in marasmic children. No significant correlation was found between leptin and oxidation markers (p>0.05).*

***Conclusion**: Marasmic children had increased lipid peroxidation and decreased antioxidant enzyme activities and leptin. Lack of associations between leptin, anthropometric measurements and oxidative stress may be due to the excessive loss of adipose tissue and related very low levels of leptin in marasmic children.*

***Key Words**: Leptin, lipid peroxidation, malnutrition, superoxide dismutase, catalase, glutathione*

INTRODUCTION

Malnutrition represents one of the most severe socioeconomic and health problems in the world. Clinically, marasmus is characterized by severe deficit of body mass, results from inadequate energy intake (1-4).

Oxidative stress can occur due to overproduction of reactive oxygen species (ROSs), decrease in antioxidant defenses or a combination of these factors. Free radicals and other reactive species are produced in the body primarily as a result of oxygen consumption. Antioxidants (glutathione, vitamins A, E and C, selenium, zinc etc) and antioxidant enzymes (e.g. superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)) exert synergistic effects in scavenging free radicals. Under physiological conditions antioxidants are in excess or at least in equilibrium with ROSs and free radicals. A pathologic excess of oxidants compared to antioxidants is called as oxidative stress. Such conditions are caused either by enhanced production of ROSs or deficient antioxidants. There has been growing evidence showing that malnutrition (e.g. dietary deficiency of protein, selenium or zinc) gives rise to oxidant stress and cell injury (5-7).

Free radicals are generally unstable and very reactive. Due to lack of any "gold standard" assays to measure activity of reactive oxygen species, three major approaches have been used: 1) Determination of endogenous antioxidant levels, 2) measurement of oxidation by products, and 3) direct detection of free radicals. For assaying antioxidant capacity, most studies have examined the concentration of antioxidants (e.g. vitamins E and C, carotenoids, folat, glutathione and zinc) in plasma and cells, and the cellular activities of antioxidant enzymes (e.g. GPX, SOD and CAT) (9). Among the oxidized by-products malondialdehyde is often used as reliable marker of lipid peroxidation.

Leptin, the Ob gene protein, is an adipocyte-secreted hormone that plays a key role in energy homeostasis of the body by controlling food intake. Leptin concentrations correlate with the amount of fat mass, with higher levels in more obese people. The decrease in leptin after energy restriction is a starvation signal to the brain (10), which probably has a protective effect. Serum leptin levels are low in many forms of malnutrition, including intrauterine growth retardation, untreated anorexia nervosa, and protein-energy malnutrition (11,12). In one of our previous studies, we found positive significant correlation between leptin and malondialdehyde, a lipid peroxidation by product, and negative correlations between leptin and antioxidants. Therefore we suggested a possible relationship between leptin and oxidation processes in a clinical example of children with nephrotic syndrome (13). In studies related to childhood malnutrition, generally very low leptin levels (2,11,14,15) and increased oxidant stress have been reported (5-8), however up to date no previous study has investigated leptin and erythrocyte oxidant stress markers together.

The aim of this study was to evaluate the activities of erythrocyte SOD and CAT as anti-oxidant enzymes, erythrocyte GSH as an antioxidant, MDA level as a marker of lipid peroxidation in childhood energy malnutrition in order to assess the oxidant/antioxidant status; and to investigate leptin variations and possible correlations with oxidant/antioxidants.

MATERIALS AND METHODS

Patients

Thirty patients (16 female, 14 male; mean age 14.4±10.3 months) were included into the study. All patients were severely malnourished, had minimal subcutaneous tissue, had no edema, and were defined as marasmus according to Wellcome criteria of classification (16). Their weight for age was below 60% and weight for height was below 70%. Most of the children had not received sufficient breast milk or proper supplemental food before and after weaning, respectively. A mixed protein- and calorie-balanced diet was introduced in our patients only after hospitalization, when the mean age of patients was approximately 15 months. There were 28 well-nourished children (13 female, 15 male; mean age 17.5±9.2 months) in the control group, who had come to hospital for routine check-up or routine laboratory evaluation before elective surgery including repair of inguinal hernia and hypospadias or undecendent testes, and had no clinical signs of any systemic illness. Malnourished children did not have clinical or laboratory evidence of any infectious or organic diseases at the time of blood collection. Infection screening including throat and urinary cultures were done by standard methods. Chest X-ray was performed to detect a co-incident lung infection. Blood CRP level, peripheral blood white blood count and erythrocyte sedimentation rate (ESR) of all children were found to be within normal reference values in patients and control group. A total of 5

marasmic children and 4 control subjects were excluded due to presence of acute infection, dehydration or an additional disorder on admission; thus, remained 30 marasmic and 28 control children were included. Body weight and height of control group were between 50th and 90th percentiles according to the Turkish Standards (17). All of the marasmic children had normal serum levels of albumin, urea and creatinine.

Nutritional and health history of all subjects were obtained. Anthropometric measurements including body weight, height, mid-arm circumference, triceps skinfold thickness and thorough physical examination were performed and recorded on admission. Body fatness was estimated by measuring subscapular skinfold thickness to the nearest 0.1 mm using a skinfold caliper (Harpenden, British Indicators LTD, London, UK). Recumbent length was measured with length board with a foot sliding board (precision 1 mm) and weight was estimated with precision of 10 g. All anthropometric measurements were recorded as the mean of three measurements. The values were compared with the median age-related Turkish standards (17) and expressed as Z scores compared with the standard deviation (SD) of the reference value. The body mass index (BMI, weight (kg)/height (m^2) was used as a measure of weight for height. Examinations of the children and anthropometric measurements were made by the same physician. Informed consent was obtained from the parents of all children. After anthropometric measurements and blood samples were obtained, an energy- and protein-rich diet for nutritional rehabilitation was given to marasmic children. The patients were followed up at least for a year. All recovered during follow-up and neither of them showed sign and symptoms of an infection, cancer, AIDS or any other serious medical conditions.

Blood samples

Blood samples with and without heparin were obtained before initiation of the feeding. Blood collection was performed between 08-09 hours in the morning from all the subjects studied, in order to prevent the effect of circadian variation of leptin concentration. Erythrocytes were separated from plasma by centrifugation, washed three times with buffered normal saline, and hemolyzed. Serum and plasma samples were stored at −20°C until analysis. Baseline laboratory investigations were carried out in all marasmic children and the controls, including complete blood count, ESR, C-reactive protein (CRP), urea, creatinine, uric acid, bilirubin, glucose, triglyceride, cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and serum albumin. Biochemical measurements including serum glucose, urea and creatinine were determined by autoanalyzer (Aeroset Autoanalyzer, Abbott Laboratories, Illinois, USA).

Measurements of antioxidant enzyme activities

To determine the activities of erythrocyte (RBC) antioxidant enzymes, RBC lysates were prepared by freezing and thawing three times in dry ice. The lysates were diluted to 1:5 with distilled water and frozen at -4°C until analysis. The suspension of RBC was used to measure superoxide dismutase (SOD) and catalase (CAT) activities. Erythrocyte SOD was measured using the method of Winterbourn et al. (18) which is based on the inhibition of the reduction of nitro blue tetrazolium (Sigma Chemical Company, St. Louis, MO, USA) by O2 -produced via photo reduction of riboflavin (Sigma). Fifty per cent inhibition was defined as one unit of SOD activity. Catalase activity was assayed in hemolysates of RBC by monitoring the consumption of H2O2 at 240 nm as described by Aebi (19). Catalase activity is expressed in terms of k/g haemoglobin (Hb), where k is the velocity constant of the decomposition of H_2O_2 to water. Levels of Hb were measured according to the method of Drabkin, which was improved by Van Kampen and Zijlstra (20).

Measurements of glutathione and malondialdehyde

Erythrocyte glutathione (GSH) was measured using the two nitro benzoic acid method of Beutler et al (21). In brief, the non-protein sulfhydryl groups of RBC are in the form of reduced GSH. 5,5'-Dithiobis (DTNB) is a disulfide chromogen. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration. The GSH concentration was expressed in μmol/gHb of RBC. In lipid peroxidation measurement, MDA released was used as an index. Serum MDA levels was estimated according to Asakawa and Matsushita by the thiobarbituric acid (TBA) method (22). The samples were heated with TBA under acidic conditions and the pink color formed was read at 532 nm. Serum MDA values were calculated using the extinction coefficient of the MDA–thiobarbituric acid complex (532 nm = 1.56×105 mol/cm) and expressed as nmol of MDA per mL serum.

***Table 1**. Demographic characteristics of malnourished children and healthy control subjects (Mean±SD)*

	Malnourished (n:30)	*Controls (n:28)*	*p*
Age (months)	*14.4±10.3*	*17.5±9.2*	*ns*
Male:Female	*14:16*	*13:15*	*ns*
Weight (kg)	*6.0±2.2*	*10.1±1.9*	*<0.001*
Height (cm)	*66.6±9.5*	*79.8±8.1*	*<0.001*
Head circumference (cm)	*41.3±3.6*	*45.5±2.7*	*<0.001*
Mid-arm circumference (cm)	*9.1±1.5*	*13.1±1.8*	*<0.001*
BMI (kg/m²)	*13.1±1.9*	*15.8±2.1*	*<0.001*
Skinfold thickness (mm)	*4.1±1.7*	*9.9±2.2*	*<0.001*
Weight Z score	*-4.30±1.59*	*0.11±0.40*	*<0.001*
Height Z score	*-3.03±1.66*	*0.27±0.58*	*<0.001*

ns: not significant

Serum leptin measurement

Serum leptin was measured by the ELISA kit (Cayman Chemicals, USA). The detection limit was 0.5 ng/mL. The intra- assay coefficients of variation (CVS) were 5.5% (n:8) at 6.6 ng/mL and 3.1% (n:8) at 20.12 ng/mL

Statistical analysis

Results were expressed as the mean±standard deviation. Statistical differences between the patients and control group were estimated using the Chi-squared (for gender distribution), Student's t-test and Mann-Whitney U test, according to the statistical distribution of data. Pearson's correlation analysis was performed to determine the magnitude of correlations. Statistical analyses were performed using SPSS 12.0 software package (SPSS, Inc., Chicago, IL). Differences with a p value of less than 0.05 were accepted as statistically significant.

RESULTS

We detected the origin of malnutrition as primary in our marasmic children reviewing their medical histories. Nineteen (65.2%) of our patients were fully breast-fed and ten were fed with diluted cow's milk in their first six months of infancy. After six months of life, all of malnourished children were found to have received a diet not sufficiently supplemented with necessary nutrients. This means malnutrition was the result of inadequate food intake. There were no other diseases that led to low food ingestion, inadequate nutrient absorption, increased nutritional requirements and/or increased nutrient losses. Low social and economical conditions, maternal illiteracy, poverty, overcrowded and improper child care (early weaning and feeding with over diluted milk or milk formulas) were found nearly in all of our marasmic children. We detected formerly experienced respiratory tract infections in 12 (33.4%) and diarrheal episodes in 14 (46.7%) marasmic children.

The mean age and male to female ratio were not statistically different between marasmic children and healthy controls ($p>0.05$) (Table 1). Marasmic children had significantly lower weight, height, BMI, mid-arm circumference, head circumference and weight and height Z scores compared to control group as expected ($p<0.001$) (Table 1).

Serum albumin, uric acid and bilirubin levels were similar in malnourished and healthy children ($p>0.05$); however, malnourished children had lower total cholesterol, HDL-C and LDL-C, and higher triglyceride levels compared with control group ($p<0.05$). Plasma leptin concentrations of marasmic children was significantly lower than that of the control group ($p<0.001$) (Table 2). C-reactive protein level of marasmic children was found to be higher than that of control subjects ($p=0.003$) (Table 2).

Marasmic children had significantly higher MDA level

***Table 2**. Serum albumin, lipoproteins and leptin levels in malnourished children and healthy controls (mean±SD).*

	Malnourished (n:30)	*Controls (n:28)*	*p*
Serum albumin (g/dL)	*3.6±1.1*	*3.7±1.1*	*ns*
Leptin (ng/mL)	*3.6±1.1*	*11.8±4.5*	*<0.001*
Cholesterol (mg/dL)	*74.7±4.4*	*150.9±54.5*	*<0.001*
HDL-C (mg/dL)	*35.3±9.9*	*55.9±6.6*	*<0.001*
LDL-C (mg/dL)	*13.6±3.7*	*77.6±45.3*	*<0.001*
Triglyceride (mg/dL)	*241±96*	*142±65*	*<0.001*
Uric acid (mg/dL)	*5.4±2.3*	*4.7±2.1*	*ns*
Total bilirubin (mg/dL)	*0.6±0.3*	*0.7±0.2*	*ns*
CRP (mg/dL)	*6.9±3.1*	*4.7±2.2*	*0.003*

HDL-C High-density lipoprotein cholesterol, LDL-C Low-density lipoprotein cholesterol, CRP C-reactive protein, ns: not significant

compared with control subjects (p<0.001) (Table 3). Erythrocyte SOD (p<0.001) and CAT (p<0.001) activities and GSH level (p<0.001) were found to be significantly lower in marasmic children than in control subjects (Table 3). Significantly lower serum leptin level was found in malnourished children compared with healthy controls (p<0.001) (Table 3). Significant negative correlations were detected between CAT and MDA (r=-0.476, p=0.009), between SOD and MDA (r=-0.534, p=0.004), and GSH and MDA (r=-0.439, p=0.015) in marasmic children. However, no significant correlation was found between leptin and anthropometric measurements and between leptin and antioxidants or MDA (data not shown) (p>0.05).

DISCUSSION

In the present study, we found very low total cholesterol, HDL-C and LDL-C, but higher triglyceride levels in marasmic children compared with healthy control subjects. Lipid metabolism has been poorly studied in marasmus (23). Although, published studies have shown normal or high triglyceride and low cholesterol concentrations in marasmus (23,24), the mechanisms of hypocholesterolemia and hypertriglyceridemia in marasmus have never been clearly explained. The cause of marasmus in our patients is a diet comprised of very low total energy. Although energy depletion predominates in marasmus, there is also inevitably insufficient protein intake. When there is a severe lack of food, endocrine ad-

***Table 3**. Comparison of oxidant/antioxidants and leptin levels of malnourished children and healthy controls (mean±SD)*

	Malnourished (n:30)	*Control (n:28)*	*p*
MDA (nmol/mL)	*11.1±2.5*	*6.6±3.9*	*<0.001*
SOD (U/gHb)	*1583±417*	*3403±1901*	*<0.001*
CAT (k/gHb)	*1139±92*	*1663±302*	*<0.001*
GSH (µmol/gHb)	*25.9±5.4*	*48.1±17.0*	*<0.001*
Leptin (ng/mL)	*3.6±1.1*	*11.8±4.5*	*<0.001*
Triglyceride (mg/dL)	*241±96*	*142±65*	*<0.001*
Uric acid (mg/dL)	*5.4±2.3*	*4.7±2.1*	*ns*
Total bilirubin (mg/dL)	*0.6±0.3*	*0.7±0.2*	*ns*
CRP (mg/dL)	*6.9±3.1*	*4.7±2.2*	*0.003*

MDA malondialdehyde, SOD superoxide dismutase, CAT catalase, GSH glutathione, *ns: not significant*

justments (increased growth hormone, glucocorticoids and epinephrine) mobilize fatty acids from adipose tissue and amino acids from muscle tissue, plasma protein concentration remains normal and hepatic gluconeogenesis is enhanced (25). With negative energy balance the major fuel to maintain life is free fatty acids drown from adipose tissue. This fat mass catabolism may be partly responsible from the increased triglyceride levels of marasmic children, since fatty acids and triglyceride are released into circulation during lipolysis (25). In marasmus there is also hypoinsulinemia (2,14) and insulin is the most important activator of lipoprotein lipase (LPL) (23). Some authors have shown LPL deficiency in PEM (26,27). These above mentioned factors suggest that the marasmic children are unable to use triglyceride to generate LDL-C and might explain very low LDL-C and the paradox of higher triglyceride in marasmus. Although we did not strictly estimate the definite amount of protein, carbohydrate and fat intakes; we took a detailed history of diet in each child and found that marasmic children had not received sufficient supplementary food reach in oil after 6 months of age. Therefore, one explanation for the very low lipid levels is that these children had very low fat stores and very low intake of dietary oil. Low LDL and HDL levels are seen in children fed by low oil-diet and with wasting (28).

Several mechanisms may lead to oxidative stress in malnourished children. The most important one is the subnormal intake of nutrients such as carbohydrates, proteins and vitamins, leading eventually to accumulation of ROSs. Reduced concentrations of vitamin A and of the anti-oxidant vitamins C and E together with deficiency of trace elements (selenium) were previously reported in children with malnutrition (5-7). The second mechanism for oxidative stress in malnutrition may be a non-specific chronic activation of the immune system due to chronic inflammation. Many conditions leading to malnutrition and wasting may also cause inflammation. Oxidative stress may be a major underlying cause for both conditions (29).

The chronic inflammation may promote an imbalance between oxidant and antioxidant mechanisms in malnutrition, since malnourished children are prone to develop frequent infections. Although a total of 86.7% of our marasmic children experienced either diarrheal episodes or respiratory infections, we could not prove continuous inflammation in our patients. However, moderately elevated CRP levels of our marasmic children can be taken as a clue for inflammation.

Studies concerning serum leptin concentrations in malnourished patients are limited. In these studies on protein-calorie malnourished children, serum leptin levels were found to be lower than healthy subjects (2,11, 12,14,15). Serum leptin concentrations fall rapidly in response to complete fasting and out of proportion to changes in fat mass. Since, loss of adipose tissue is more prominent in marasmus compared with protein malnutrition, decreased leptin levels of marasmic children may be due to diminished subcutaneous adipose tissue (2). Low leptin levels help to increase appetite and food intake during malnutrition, and stimulate the hypothalamic-pituitary-adrenal axis for effective catabolism, so that ensure the diversion of substrate away from growth to keep on metabolic homeostasis (11).

It has been reported that leptin levels are deeply influenced by BMI, gender and age in normal subjects (30) and leptin has also been shown to increase generation of reactive oxygen species (31). In the present study, we did not find significant relationships between serum leptin concentrations and anthropometric measurements (mid-arm circumference, triceps skinfold thickness, BMI, and weight and height Z-scores) or between serum leptin and total protein or albumin. We suggest that the decrease of serum leptin concentrations in marasmic children is relatively independent of the severity of energy malnutrition. The clinically low caloric intake leads primarily to the exhaustion of body fat stores that is accompanied by the lower leptin concentrations. The fact that serum leptin levels in patients with protein and energy malnutrition do not significantly correlate with BMI as found by Cederholm et al. (32) and Haluzik et al. (15) was supported by our study results in energy deficient marasmic malnutrition. The most likely explanation for the lack of significant associations between leptin and BMI and between leptin and oxidant/antioxidant parameters in our marasmic group may be the presence of a threshold beyond which leptin can not decrease physiologically, since excessive loss of adipose tissue is the main characteristics of marasmus. In previous studies, leptin was reported to be positively correlated with oxidant markers in children with nephrotic syndrome or chronic renal failure (33, 34). In those studies elevated leptin levels had been found to be related to increased oxidative stress. In contrast to those studies, our marasmic children had very low leptin level and high oxidative stress markers. These results lead us to

think that lack of association between leptin and oxidative stress markers in the present study may be due to very low leptin levels of our malnourished children.

Increased ROS leads to disintegration of polyunsaturated fatty acids on the cell membrane and formation of MDA. This process is called as lipid peroxidation (8). In present study a significant increase in serum MDA and decrease in erythrocyte SOD, CAT and GSH were observed in the marasmic group which suggests that energy deficient state might result in enhanced lipid peroxidation and decreased antioxidant enzyme activities. These alterations could be attributed to the insufficient intake of micronutrients (zinc, selenium, copper etc.) and antioxidant vitamins (vitamins E, C and A). Previously three studies have investigated oxidant stress in marasmus (5-7). In the first study, lower antioxidant vitamins E, C and A, and cofactors copper and selenium levels but higher SOD activity have been found in marasmic children compared with control subjects (5). In the second study (6), similar erythrocyte SOD activity together with lower GSH and higher MDA levels have been reported in marasmic children compared with the controls. In that study, selenium and copper -which we did not measure in present study- (as cofactors for antioxidant enzymes) have been found to be lower in malnourished children than in healthy controls. In the third study lower antioxidant potential and higher plasma MDA levels were found in marasmic children (7). Our results are parallel to the latter two studies in the view point of lower GSH and higher MDA levels of malnourished children. However lower SOD activity of our marasmic children is more reasonable together with low CAT activity and higher MDA levels of the patient group. We suggest that activities of these antioxidant enzymes are depleted as a compensation for protection from hazardous effects of increased ROSs that indicated by increased MDA level in malnourished children. Negative correlations between GSH and MDA, between CAT activity and MDA, and between SOD and MDA indicate that the magnitude of initial oxidant stress was so high, it exceeded the compensatory capacity of antioxidants.

In conclusion, marasmic children had an increased oxidative stress and decreased antioxidant defense compared with healthy controls. Increased oxidative stress may result from some deleterious effects of deficient caloric and micronutrient intake. In view of the reduced anti-oxidant defense capacity and the presence of increased oxidant stress, strategies should be developed to support the anti-oxidant system of children with energy malnutrition. Further investigations are required to explain the mechanisms involved in the oxidant/antioxidant imbalance in malnourished children.

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