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Cancer chemotherapy reduces plasma total antioxidant capacity in children with malignancies

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Abstract

The sum of endogenous and food-derived antioxidants provides an estimate of the total antioxidant capacity (TAC) of the extracellular fluids, while corrected TAC (cTAC) is an estimation of the exogenously provided antioxidants. Similar values for TAC and cTAC were observed between cancer free children and children with malignancy at diagnosis. Antineoplastic treatment induced a significant decrease of TAC and cTAC during chemotherapy. Additionally to the dietary factors, this might be attributed to the antineoplastic drugs as shown by the significant increase of ROS after administration of chemotherapeutic agents both in vitro and in vivo. According to our preliminary results TAC and cTAC returned to normal after the end of therapy.
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Keywords: Antioxidant capacity; Childhood malignancy; Chemotherapy; Reactive oxygen species

1. Introduction

Antioxidants allow aerobic organisms to withstand daily episodes of oxidative stress by counteracting the adverse effect of free radicals, which are produced by metabolic activities within the body. In addition, antioxidants delay or inhibit oxidation of molecules such as carbohydrates, fat, proteins and DNA [1]. The levels of antioxidants not only provide protection against oxidation but also reflect their consumption during acute oxidative stress. Whenever antioxidant capacity is outflanked by oxidative stress, tissue lesion may occur [2].

Antioxidants constitute a highly heterogeneous group. They include low molecular weight substances either water- (e.g. ascorbic acid) or lipid-soluble (e.g. Vitamin E), incorporated in the body through nutrition. In addition, a number of endogenous metabolites (uric acid, lipids, albumin, bilirubin) possess antioxidant activities.

The sum of endogenous and food-derived antioxidants represents the total antioxidant capacity (TAC) of extracellular fluids, which can be assessed by various methods [3–5]. TAC integrates the cumulative effect of all antioxidants present in the plasma and body fluids and may give more relevant biological information as compared to that obtained by the measurements of individual parameters. TAC represents the number of moles of a given free radical scavenged by a test solution and is independent of the capacity of each and every antioxidant present.

Recently, we have introduced the concept of corrected total antioxidant capacity (cTAC) [3]. In the assay of cTAC, the effect of a number of endogenous metabolites such as uric acid, albumin and bilirubin which either interfere or possess an intrinsic antioxidant activity, is subtracted from the obtained value of TAC. Thus, the cTAC estimates the exogenous part of the TAC in human plasma.

Very few studies have assayed the antioxidant status of children with malignancies. Senturker et al. [6] and Malvy et al. [7] report that endogenous antioxidants are reduced in children with malignant disease as compared

Abbreviations: TAC, total antioxidant capacity; cTAC, corrected total antioxidant capacity; ROS, reactive oxygen species; ALL, acute lymphoblastic leukemia; CNS, central nervous system; AML, acute myeloid leukemia; PMNs, polymorphonuclears

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to normal counterparts. Moreover, decreased levels of alpha-tocopherol/cholesterol ratio in children with bone tumors and of serum zinc in children with bone and central nervous system malignancies during antineoplastic treatment have been detected [7]. In the same study an increase of retinol and selenium was observed during the period of treatment in children with leukemia.

To our knowledge, no data exist up-to-date regarding TAC and cTAC in children with malignancies and their potential fluctuations during chemotherapy. The purpose of this prospective study was to compare TAC and cTAC between cancer free children and children with malignancy at the time of diagnosis and during chemotherapy while a mechanism to elucidate the observed changes is proposed, based upon the assessment of ROS production by human leukocytes after incubation with chemotherapeutics.

2. Materials and methods

2.1. Patient population

Twenty children (13 males) who were diagnosed with various malignant diseases (ALL = 15, CNS tumor = 3, AML = 2) at the Department of Pediatric Hematology/Oncology, University Hospital of Heraklion, from January 2000 to June 2002 were included in the study. As a control group, 80 cancer-free children (39 males), were also enrolled. All children were under a free diet during the time of the study. The age of children with malignancy was similar to that of the control group (5.9 ± 0.5 years versus 6.2 ± 0.9 years, respectively).

In children with malignancy TAC and cTAC were assayed at the time of diagnosis and during the first three monthly cycles of chemotherapy. During the three consecutive chemotherapy cycles, blood samples were withdrawn and processed within 24 h following the completion of the chemotherapy schedule. This consisted of dexamethasone, vincristin, doxorubicine, L-asparaginase, methotrexate, cytarabin, cyclophosphamide for leukemias, and vincristine, etoposide, cyclophosphamide and cisplatin for brain tumors.

The current study was approved by the local ethics committee and an informed signed parental consent was obtained for each child.

2.2. Assay of antioxidant capacity

Blood samples were collected on K3-EDTA, and immediately centrifuged in a refrigerated centrifuge. Plasma was collected, aliquoted, and stored at −80 °C until use. TAC was assayed as previously described [3], using the TAC kit from Medicon SA (Gerakas, Greece). Accuracy and validity of the test have been described previously. Inter- and intraassay CVs were 3.4 and 2.4%, respectively. Corrected TAC (cTAC) was assayed with the same assay as follows: TAC, uric acid, bilirubin and albumin were determined with commercial kits by Medicon/Olympus (Gerakas, Greece) according to the manufacturer's instructions. cTAC was calculated according to Kampa et al [3] using the equation $\text{cTAC} = \text{TAC} - (0.11 \times \text{uric acid} + 0.14 \times \text{bilirubin} + 0.01 \times \text{albumin})$. All assays were performed at an Olympus AU600 biochemical analyzer. TAC and cTAC are expressed as equivalents of the water soluble Vitamin E analog Trolox C and expressed as mmol/L. Blood samples were identified by number only to ensure that evaluation was performed in a blind way in terms of disease status. All samples were analyzed at the same period to avoid technical confounding factors.

2.3. Determination of reactive oxygen species (ROS) by flow cytometry

2.3.1. ROS release after in vitro incubation with chemotherapeutics

Reactive oxygen species (ROS) production (oxidative burst) was assayed in leukocytes from three healthy adult donors by flow cytometry as described by Rothe and Valet [8] before and after in vitro incubation with vincristine, asparaginase and daunorubicin. More specifically undiluted heparinized whole blood was layered over Ficoll–Hypaque (1:1, density 1.077 g/ml). Erythrocytes aggregate at the interface and sediment at room temperature. After letting the tube to stand for 40 min, the upper 800 μl of supernatant plasma were withdrawn, avoiding contact with the plasma layer near the interface with the separation medium and kept on ice. Cells, adjusted at 10^6 cells/ml with Dulbecco's PBS, were incubated with 1 μM dihydroxyrhodamine 123 (Sigma, St. Louis, MO) for 5 min at 37 °C. Thereafter, 10 μl of a solution of each 100 nM phorbol-12 myristate 13-acetate (PMA, Sigma, St. Louis, MO), 1 μg/ml vincristine, 1 μM daunorubicin or 1 U/ml asparaginase in Dulbecco's PBS were added. The above concentrations of vincristine, daunorubicin and asparaginase were selected, as they are close to the expected concentrations in blood after therapeutic administration of these agents. Cells where 10 μl Dulbecco's PBS had been added were used as controls. In the presence of intracellular ROS, dihydroxyrhodamine 123 is transformed to green-fluorescent rhodamine 123, trapped intracellularly. After a 30 min incubation at 4 °C, measurements were conducted using a Coulter Epics flow cytometer (Coulter Electronics, Lutton, Bedfordshire, UK) by acquisition of at least 3000 lymphocytes, 1000 monocytes and 5000 PMNs (polymorphonuclear cells) from each sample, gated according to forward and side scatter. In all our samples the distinction of cell subpopulations was made by morphology with light microscopy and immunophenotyping.

2.3.2. ROS release after in vivo administration of chemotherapeutics

ROS release from PMNs and monocytes of two children with malignancy under chemotherapy before and

30 min after iv administration of vincristine was assessed as described above.

2.4. Statistical analysis

Normally distributed continuous variables (Kolmogorov–Smirnov test with Lilliefors correction) were tested with the *T*-test for independent samples, while not-normally distributed variables with the Mann–Whitney *U*-test. Kruskal–Wallis analysis of variance was used to assess the effect of different regimens on the oxidative burst of human leukocytes. Post hoc comparisons were performed with Kruskal–Wallis *Z*-test. Assessment of TAC and cTAC change during antineoplastic treatment was performed with Friedman's Test (non-parametric repeated measures comparisons). All tests were two-tailed with a confidence level of 95% ($P < 0.05$). Values are expressed as mean ± standard error, unless stated otherwise. Statistical analysis was performed using SPSS v.10 statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Comparison of TAC and cTAC between children with malignancy and cancer free children

3.1.1. At diagnosis

No differences were observed between children with malignancy at the time of diagnosis and cancer free children regarding TAC (median TAC: 1.27, range 0.6–3.1 versus median TAC: 1.25, range 0.7–3.1, respectively, $P = 0.8$) and cTAC (median cTAC: 0.77, range 0.3–2.1 versus median cTAC: 0.76, range 0.3–1.3, respectively, $P = 0.6$). TAC and cTAC assays were age- and sex-independent (Table 1).

Table 1
TAC and cTAC in cancer free children according to age and sex

Age group (years), number of children	TAC[a]	cTAC[a]
0–2, 20		
Median	1.26	0.84
(Range)	(0.7–2.1)	(0.3–1.3)
2–4, 19		
Median	1.18	0.69
(Range)	(1.0–1.7)	(0.5–1.1)
4–9, 22		
Median	1.26	0.75
(Range)	(1.1–1.9)	(0.6–1.1)
>9, 19		
Median	1.27	0.77
(Range)	(1.0–1.7)	(0.5–1.2)
Sex, number of children	**TAC[b]**	**cTAC[b]**
Male, 39		
Median	1.21	0.75
(Range)	(1.0–2.1)	(0.5–1.2)
Female, 41		
Median	1.26	0.77
(Range)	(0.7–1.9)	(0.3–1.3)

[a] Differences among age groups were not statistically significant ($P = 0.13$ and 0.4 for TAC and cTAC, respectively, Kruskal–Wallis analysis of variance).
[b] Differences between male and female cancer free children were not significant ($P = 0.2$ and 0.6 for TAC and cTAC, respectively, Mann–Whitney test).

3.1.2. Under chemotherapy

Treatment of children with malignancy with standard chemotherapeutic protocols resulted in a significant decrease of both TAC ($P = 0.007$) and cTAC ($P = 0.002$) during three consecutive chemotherapy cycles performed at monthly intervals (Fig. 1) compared with the values at diagnosis.

3.2. Preliminary results on TAC and cTAC in six of the above children with malignancy after completion of chemotherapy

3.2.1. One month after completion of the chemotherapy protocol

In four children with malignancy, lower values of TAC and cTAC were recorded as compared with those observed before treatment initiation (median value for TAC: before treatment 1.9, after treatment 0.9; median value for cTAC: before treatment 0.9, after treatment: 0.4).

3.2.2. Six months after completion of the chemotherapy protocol

In two children with malignancy similar values of TAC and cTAC to those observed before treatment initiation were recorded (median value for TAC: before treatment 1.19, after treatment 1.25; median value for cTAC: before treatment 0.77, after treatment: 0.73).

3.3. Variations of endogenous metabolites at diagnosis and during consecutive chemotherapy cycles in children with malignancy

cTAC determination is based upon the subtraction of the antioxidant capacity or interfering values of a number of endogenous metabolites, namely uric acid, bilirubin and albumin [3]. Variation of these parameters, during chemotherapy, could therefore influence drastically the obtained values of cTAC. Mean values of uric acid, albumin and bilirubin, the subtraction of which from TAC is used to determine cTAC, were as follows at diagnosis and three times thereafter during chemotherapy treatment: uric acid (mg/dl) 3.7 ± 0.1, 3.1 ± 0.2, 3.4 ± 0.2, 3.3 ± 0.2; bilirubin (mg/dl) 0.37 ± 0.1, 0.54 ± 0.1, 0.56 ± 0.1, 0.55 ± 0.1 and albumin (mg/dl) 3.8 ± 0.1, 3.6 ± 0.1, 3.9 ± 0.1, 4.1 ± 0.1, respectively. The observed fluctuations of these parameters were not statistically significant.

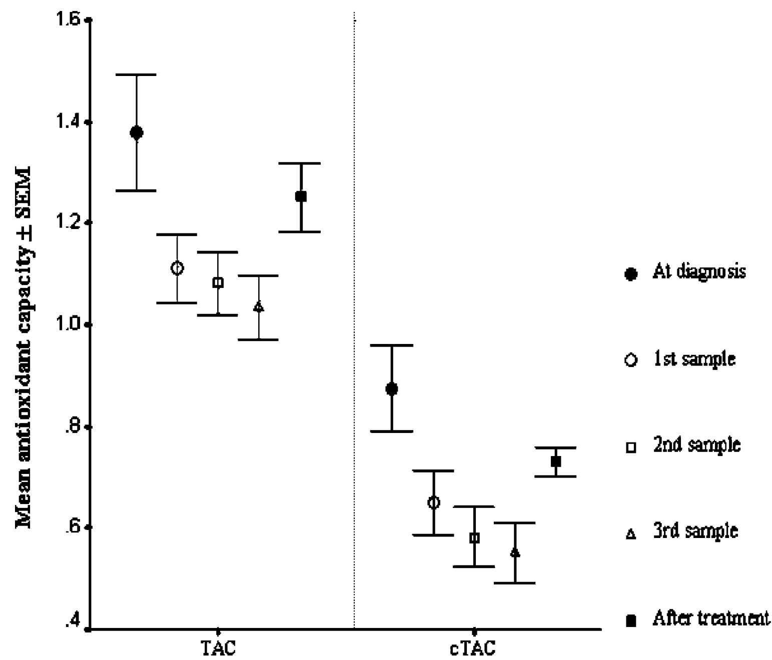

Fig. 1. TAC and cTAC in 20 children with malignancy at the time of diagnosis and during three consecutive chemotherapy cycles (performed at monthly intervals). The decrease observed in both TAC and cTAC is statistically significant ($P = 0.007$ for TAC, $P = 0.002$ for cTAC). The "after treatment" values refers to TAC and cTAC in two of the above children 6 months after completion of the chemotherapy protocol.

3.4. In vitro and in vivo production of ROS in the presence of chemotherapeutic agents

3.4.1. ROS release after in vitro incubation with chemotherapeutics

As mentioned earlier, one of the main effects of chemotherapeutics used in cancer therapy is the increase of the oxidative functions of the cell [9]. In order to verify whether the observed changes in TAC and cTAC during chemotherapy might be partially attributed to this effect, we have assayed the oxidative reactive species generation by the administered drugs of healthy donors' leukocytes in vitro. vincristine, daunorubicin and asparaginase induced increased ROS production from PMNs of healthy adult

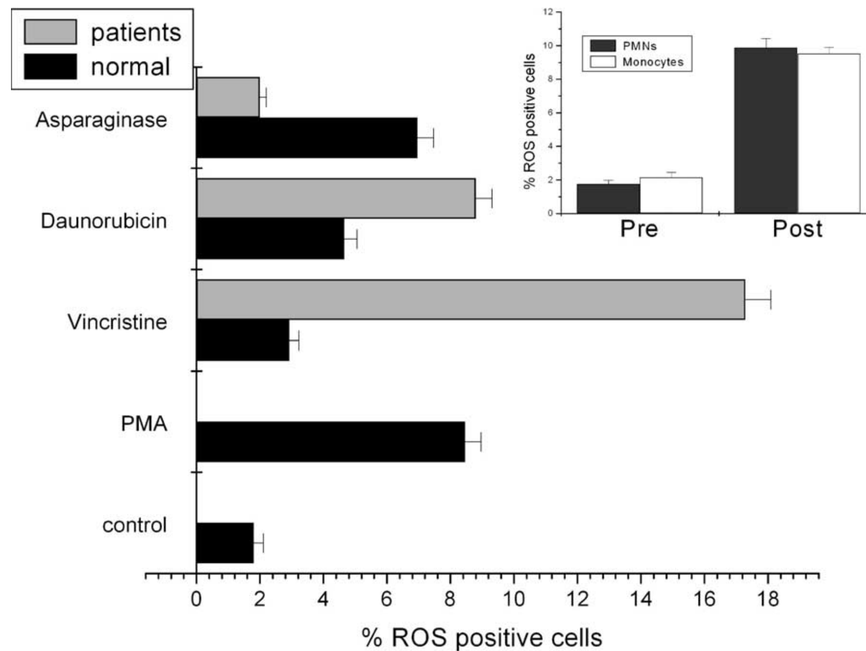

Fig. 2. % ROS release from PMN cells of healthy donors (normal) after in vitro exposure to antineoplastic agents and children under chemotherapy (patients) 30 min after IV (in vivo) administration of the same chemotherapeutic agent ($n = 5$). Inset: % ROS positive cells assessment before (pre) and 30 min after in vivo vincristine administration (post).

individuals after 30 min incubation (2.93, 4.66 and 6.97% positive cells, respectively, compared to 1.8% for controls).

3.4.2. ROS release after in vivo administration of chemotherapeutics

As shown in Fig. 2, ROS production by PMNs and monocytes increased from 1.7 to 9.9% and from 2.1 to 9.5%, respectively, following administration of vincristine in two children with malignancy.

4. Discussion

Reactive oxidant species (ROS) are essential for life and are involved in cell signaling. In addition, they are used as bactericidals by macrophages and they are a component of mitochondrial respiration. However, ROS may result in oxidative stress in the cellular and extracellular environment and have been implicated in the etiology and progression of many diseases mainly chronic. ROS are held under control by an elaborate antioxidant defense system, which may become depleted in conditions of stress. In normal cellular milieu, ROS are essential for life while in cases of antioxidant exhaustion they may become detrimental.

Aggressive chemotherapy is the cornerstone of cancer therapy and extensive research is carried out on the development of more specific and less toxic antineoplastic agents. Some reports suggest that endogenous antioxidants are reduced in children with cancer while others show that administration of antineoplastic agents during cancer chemotherapy results in a much greater degree of oxidative stress than is induced by cancer itself [9]. It has been postulated that chemotherapeutic agents may increase ROS generation by affecting mitochondrial respiration chain [10,11]. It could therefore be considered that nutritional factors could play a key role in enhancing the anti-cancer effect of chemotherapy and reducing or preventing certain chemotherapy-induced side effects.

Recently, an antioxidant "cascade" has been proposed in order to explain the observed differences in antioxidant levels in human peripheral fluids based on the redox potential of discrete antioxidants [5,12]. According to the authors, all exogenous antioxidants are continuously distributed between the water- and lipid-soluble fractions, as a result of their specific redox potentials. If this theory also applies in vivo, it could explain the lack of variation of TAC values in children, with neoplastic diseases at diagnosis.

The results of the present study provide some new insights in cancer chemotherapy, in children with malignancies. TAC and a number of endogenous metabolites (albumin, uric acid, bilirubin), as an integrated measurement of the redox state of the patients were found normal in children with malignancies prior to the initiation of therapy. According to these results the major metabolic burst due to the expected increased metabolism of cancer cells is not reflected in biological fluids.

Additionally it has been shown that during chemotherapy TAC as well as cTAC decrease progressively during the cycles of chemotherapy. The observed decrease could be due to the direct effect of chemotherapy. Indeed the chemotherapeutics used throughout this study can induce an increased production of ROS in vitro as well as in vivo which has been found in the few cases studied. This is directly reflected in biological fluids of children by a substantial decrease of both TAC and cTAC.

This is the first study in which TAC and cTAC are assessed in children with cancer and their levels have been followed during antineoplastic treatment. The only two studies performed in children with acute lymphoblastic leukemia before [6,7] refer to specific antioxidants and not to the total antioxidant capacity and in addition to limited evaluated samples during the study period. The current study suggests that there is a decrease of TAC in children undergoing antineoplastic treatment. This might be due to the tumoricidal action of anti-cancer drugs which has been suggested to be mediated through a free-radical dependent mechanism where it was shown that many anti-cancer drugs increase free radical generation [13]. This is further supported by the results of the present study in which increased oxidative burst was observed following incubation of human leukocyte populations with chemotherapeutic agents in vitro.

The decrease of TAC in children undergoing antineoplastic treatment could be also attributed to insufficient dietary intake of exogenous antioxidants during the treatment period.

Under chemotherapy, there is increase of lipid peroxidation products and marked reduction of specific antioxidant plasma levels of substances such as Vitamin E, Vitamin C and carotenes [14]. The high level of oxidative stress during chemotherapy may overcome the oxidative defense mechanisms of the cancer cell which has specific systems to reduce lipid peroxidation. Increased lipid peroxidation reduces or inhibits cancer cell proliferation and interferes with the activity of chemotherapy. This has important implications because the antioxidant status of cancer patients may play an important role in response to chemotherapy. However, supportive nutritional therapy with antioxidants during chemotherapy which reduces the generation of lipid peroxides, may overcome the inhibition effects of oxidative stress and maintain response to chemotherapy [14]. Furthermore, it is now recognized that chemotherapy kills tumor cells not by damaging essential biological functions but by initiating programmed cellular death [9,15]. On the other hand, mutations that interfere with apoptosis may produce tumor chemotherapy resistance [9,15].

The progressive decrease of TAC and cTAC during the first three cycles indicate that the effect of these agents might be additive during the study period. While at the completion of chemotherapy the TAC and cTAC levels continued to be low, this finding has been reversed by the 6th month off treatment. Further studies evaluating many chemotherapy courses may be necessary for precise determination of the

fluctuations of the antioxidant capacity and their correlation with the kind and aggressiveness of treatment protocols.

The above results provide another insight in the possible role of antioxidants in childhood cancer but further study is necessary to elucidate the clinical significance of the observed decrease in total antioxidant capacity. It also remains to be shown if a dietary intervention aiming to increase the exogenous component of total antioxidant capacity is related to an identifiable effect in serum levels of TAC and in response to treatment, disease course and prognosis. Although it is known that antioxidants are useful in the reduction of adverse effects of chemotherapy, there is a reluctance in their wider use since they can reduce the effect of chemotherapy [16–20]. There is a theoretical concern that antioxidant therapies interfere with chemotherapy and radiation by lowering oxidative damage. Evidence though supporting this mechanism is currently lacking [21]. Despite these contradictory data antioxidants may be a choice for therapeutic intervention along with chemotherapy with obvious benefit in tumor size reduction and/or increased survival [16,20,22–29] since they exert their action as biologic response modifiers inducing apoptosis [9,15,17,30,31] additionally to their ability to enhance antitumor effects of chemotherapy both in vitro and in vivo [9,22,27,29,31].

In conclusion, our results offer another insight in the possible role of antioxidants in childhood malignancies during antineoplastic treatment. Further investigation is required in order to fully elucidate the mechanism involved and the clinical significance of the observed decrease in TAC and determine a possible role of any supportive nutritional intervention.

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