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ORIGINAL ARTICLE

Evaluation of oxidative and antioxidative parameters in pediatric hematopoietic SCT patients

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Conditioning regimens preceding hematopoietic SCT (HSCT) usually consist of high-dose chemotherapy. Chemotherapy and radiation therapy are associated with increased formation of free radicals and depletion of critical plasma and tissue antioxidants. Oxidative stress and antioxidant depletion have been described during the transplantation period in HSCT patients. In a limited number of studies, it was observed that the conditioning regimen resulted in oxidative stress and antioxidant depletion in HSCT patients. The objective of this study was to look for further evidence of oxidative stress and antioxidant status in pediatric HSCT patients. In this study, blood samples were collected from 21 pediatric allo-HSCT patients before and after conditioning therapy. Erythrocyte and plasma malondialdehyde (MDA) levels, erythrocyte reduced and oxidized glutathione (GSH) levels, erythrocyte antioxidant enzymes activities, plasma α-tocopherol and β-carotene levels were determined. After high-dose chemotherapy, erythrocyte and plasma MDA levels increased. Reduced GSH levels decreased whereas oxidized GSH levels increased first and then decreased significantly compared with the values before the chemotherapy regimen. It was also observed that catalase, superoxide dismutase and GSH-*S*-transferase activities decreased, but there was no change in GSH peroxidase activity. On the other hand, plasma α-tocopherol levels increased, but β-carotene levels did not change.
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Introduction

Hematopoietic SCT (HSCT) has been increasingly used to treat malignant and non-malignant hematologic diseases, immunodeficiencies, metabolic and autoimmune diseases in both adults and children.[1,2] Conditioning regimens preceding HSCT usually consist of high-dose chemotherapy and/or TBI. They have severe acute and delayed toxic effects on several tissues, such as mucous membranes, gastrointestinal tract, liver, lung, bladder, central nervous system, and, rarely, other tissues possibly related to peroxidation processes and exhaustion of antioxidants.[3–7]

It is well known that chemotherapy and radiation therapy are associated with the formation of reactive oxygen species and depletion of antioxidants, such as glutathione (GSH), antioxidant enzymes, and antioxidant vitamins such as α-tocopherol, β-carotene and vitamin C.[4,8–11]

A limited number of studies demonstrated that the conditioning therapy given to HSCT patients creates a high oxidative stress, resulting in a measured reduction in antioxidants. In summary, these studies revealed a disturbance of the pro-oxidative/antioxidative balance in the plasma of patients undergoing HSCT. Studies clearly demonstrated that plasma antioxidant status deteriorated after conditioning therapy. Therefore, it has been suggested that high-dose chemotherapy increased the oxidative stress and decreased the antioxidant defence system.[3–11]

The objective of this study was to evaluate the oxidative and antioxidative status in pediatric allo-HSCT patients.

Patients and methods

Patients

The study includes 21 children who underwent allo-HSCT at Hacettepe University, Faculty of Medicine, Pediatric Bone Marrow Transplantation Unit. The clinical and transplantation related characteristics of patients are shown on Table 1. The conditioning regimen was myeloablative in 15 patients, and non-myeloablative in six patients (Table 1). GVHD prophylaxis included CsA ± MTX/methylprednisolone. Sources of stem cells are also shown in Table 1.

All patients were hospitalized in hepafiltered single rooms until discharge. Acyclovir was given for HSV and VZV prophylaxis, fluconazole for fungal infection prophylaxis, trimethoprim-sulfamethaxozole for *Pneumocystis carinii* infection prophylaxis. Monitoring CMV infection was routinely performed with real-time PCR assay.

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Table 1 General characteristics of the patients and results of the transplantation

Age (years)	8.3 ± 4.8
Gender (M/F)	12/9
Diagnosis	
CML	5
AML	3
Acquired AA	3
FAA	3
Hemophagocytic syndrome	2
MDS	1
Thalassemia major	1
SCID	1
Osteopetrosis	1
Hypereosinophilic syndrome	1
Donors	
HLA identical sibling	17
Other donors	4
Conditioning regimen	
Myeloablative (BU based)	15
Non-myeloablative	6
GVHD prophylaxis	
CsA + MTX	15
CsA	5
CsA + MP	1
Sources of stem cell	
BM	12
PB	5
CB	2
BM + CB	2
Engraftment	18/20
Neutrophil engraftment day	15.9 ± 2.9 day (9–20)
Acute GVHD	6/18
Chronic GVHD	1/18
VOD	5/21
Mild	4/21
Severe	1/21
Hemorrhagic cystitis (grade 2–3)	2/21
Mucositis (grade 3–4)	8/21
Outcome (alive)	16/21

Abbreviations: AA = aplastic anemia; CB = cord blood; F: female; FAA = Fanconi aplastic anemia; M = male; MDS = myelodysplastic syndrome; MP = methylprednisolone; PB = peripheral blood; VOD = veno-occlusive disease.

Broad-spectrum antibiotic coverage was initiated with the first evidence of fever (T max $\geqslant 38\,^{\circ}C$). Iv Ig was administered weekly at a dose of 400 mg/kg from day 1 to discharge and then iv Ig implementation was made according to the Ig level. Standard veno-occlusive disease prophylaxis consisted of enoxaparin, ursodeoxycholic acid, glutamine and vitamin E given at 1–2 mg/kg daily doses.

Definitions. Neutrophil engraftment was defined as the first of three consecutive days when the neutrophil count was higher than 0.5×10^9/L. Platelet engraftment was defined as a platelet count higher than 20×10^9/L with no transfusion at least 7 days. Absence of hematopoietic recovery at day 60 and autologous hematopoietic reconstruction were considered as engraftment failure. Acute and chronic GVHD were diagnosed and graded according to Seattle criteria.[12,13] Poor graft function was diagnosed in patients with two or three cytopenic lines (Hb < 10 g/dL, neutrophil count $< 1.0 \times 10^9$/L, platelet count $< 30 \times 10^9$/L) for at least 2 consecutive weeks beyond day +14 post transplant, with transfusion requirement, associated with hypoplastic-aplastic BM, in the presence of complete donor chimerism and in the absence of severe GVHD and relapse.[14] Patients surviving for more than 14 and 100 days post transplantation were evaluated for acute and chronic GVHD occurrence, respectively. Veno-occlusive disease was diagnosed and staged according to Seattle criteria.[15] The definition of hemorrhagic cystitis was used as follows: painful hematuria with a negative urine culture for bacteria or fungus and without any other explanation such as general bleeding diathesis, urinary tract catheterization for reasons other than hemorrhagic cystitis, urinary calculi or bladder neoplasms.[16]

The study was approved by Hacettepe University Ethical Committee according to the 'Declaration of Helsinki'.

Materials

Chemicals. Folin–Ciocalteu's reagent, pyrogallol, trizma TM base and sodium carbonate were obtained from Sigma (Sigma Chemicals, St Louis, MO, USA). Sodium azide, potassium monohydrogen phosphate, potassium dihydrogen phosphate, hydrogen peroxide solution and sodium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). α-tocopherol and β-carotene were obtained from Sigma.

The *N-(*1-pyrenyl) maleimide, reduced GSH, 1,1,3,3-tetraethoxypropane and aminoguanidine hydrochloride were purchased from Sigma. Thiobarbituric acid and HPLC-grade acetonitrile, methanol and butanol were purchased from Merck and HPLC-grade acetic acid and 85% phosphoric acid were purchased from Riedel (Seelze, Germany).

The HPLC system was Agilent 1200 Series (Agilent, Waldbronn, Germany). The system consists of a G 1311A pump, a G 1313A autoinjector and a G 1321A fluorescence detector. The 100×4.6 mm column is packed with 3-μm particle size C_{18} materials.

Specimen collection. Blood samples were collected on pre-conditioning day (10 days before HSCT day), day 0 (HSCT day), day +7, day +14 and day +28 (7, 14 and 28 days after the HSCT day) in heparinized polypropylene tubes. After immediate centrifugation (2500 r.p.m., 10 min, $+4\,^{\circ}C$), plasma was separated. After removing the buffy coat, RBCs were washed in an equal volume of phosphate buffer (100 mM, pH: 7.4) twice. Following the second washing supernatants were removed and packed RBCs were obtained. All samples were stored at $-80\,^{\circ}C$ until analysis.

Methods

Reduced and oxidized GSH levels. GSH and oxidized glutathione (GSSG) determinations were performed using the HPLC method developed by Winters *et al.*[17]

Malondialdehyde levels. Malondialdehyde (MDA) levels, an index of lipid peroxidation, were determined by HPLC methods according to Draper *et al.*[18] and Templar *et al.*[19]

with minor modifications. A volume of 250 µL RBC hemolysates (1/5 diluted), plasma samples (2/3 diluted) and MDA standards were incubated with 650 µL TCA (10%), 100 µL butylated hydroxyl toluene (500 p.p.m.) in a boiling water bath for 30 min. After cooling in ice-cold water, they were centrifuged at 3000 r.p.m. for 10 min. Supernatants (500 µL) were incubated with an equal volume of thiobarbituric acid in NaOH (1%) for 30 min. After cooling in ice-cold water, the thiobarbituric acid–MDA complex was extracted into 1 mL of n-butanol phase and the fluorescence intensity of the organic phase was detected at the Ex: 532 nm, Em: 553 nm. The elution buffer was prepared from 65% 50 mM KH_2PO_4–KOH, pH 7.0, and 35% methanol.

Glutathione-S-*transferase.* The measurement of glutathione-*S*-transferase enzyme activity was performed according to Habig *et al*.[20]

Catalase. Erythrocyte catalase (CAT) activity was measured as described by Aebi.[21]

Superoxide dismutase. Erythrocyte superoxide dismutase (SOD) activity was measured spectrophotometrically as described by Marklund *et al*.[22]

Glutathione peroxidase. The measurement of glutathione peroxidase enzyme activity was performed according to Pleban *et al*.[23]

α-tocopherol and β-carotene levels. Plasma α-tocopherol and β-carotene determinations were performed using the HPLC method according to Steghens *et al*.[24]

Protein. The protein content of the samples was determined according to the method of Lowry modified by Lowry *et al*.[25]

Statistical analysis. Statistical analyses were performed using the SPSS computer program (IBM, Chicago, IL, USA). Differences in parameters before and after chemotherapy were analyzed by analysis of variance and LSD tests. $P<0.05$ was considered statistically significant. All results are presented as mean ± s.d.

Results

MDA levels

Plasma and erythrocyte MDA levels were significantly increased in day 0, day 7, day 14 and day 28 samples compared with pre-conditioning day (before chemotherapy) (Figure 1).

GSH and GSSG levels

On day 0, day 7, day 14 and day 28 significant reductions of erythrocyte GSH levels were observed compared with pre-conditioning day. Besides, erythrocyte GSSG levels were increased in day 0 but decreased in day 7 and 14. On day 28 GSSG levels increased again (Figure 2).

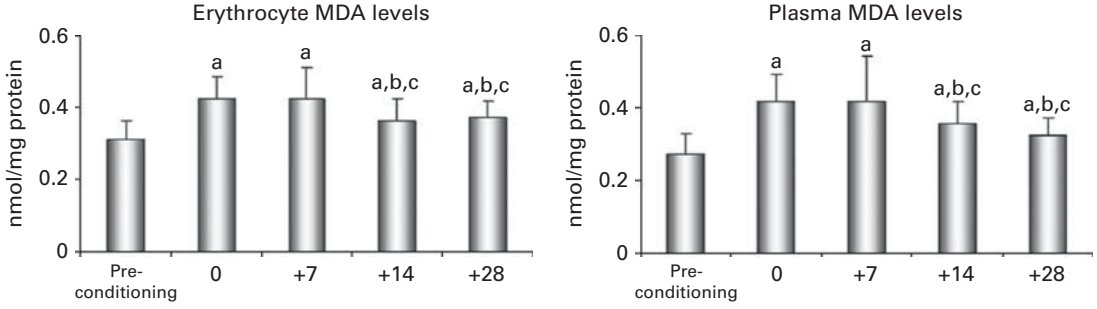

Figure 1 Effect of chemotherapy on erythrocyte and plasma MDA levels. [a]$P<0.01$: significantly different from pre-conditioning day, [b]$P<0.01$: significantly different from day 0, [c]$P<0.01$: significantly different from day 7. Pre-conditioning: 10 days before HCST, 0: transplantation day, +7: 7 days after transplantation, +14: 14 days after transplantation, +28: 28 days after transplantation.

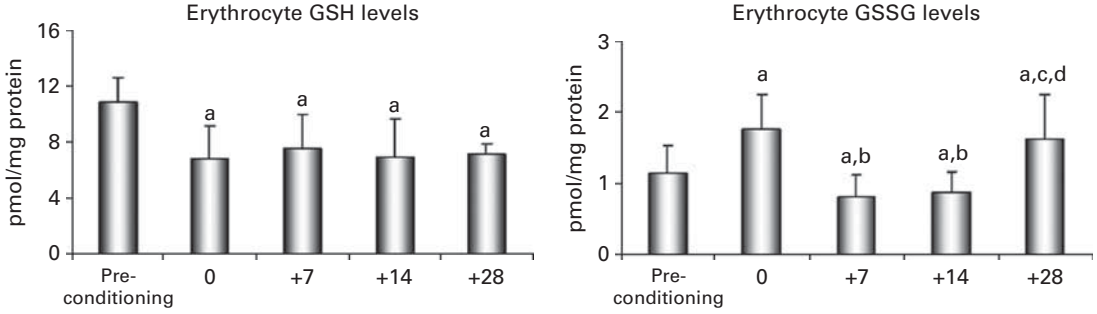

Figure 2 Effect of chemotherapy on erythrocyte GSH and GSSG levels. [a]$P<0.01$: significantly different from pre-conditioning day, [b]$P<0.01$: significantly different from day 0, [c]$P<0.01$: significantly different from day 7, [d]$P<0.01$: significantly different from day 14. Pre-conditioning: 10 days before HCST, 0: transplantation day, +7: 7 days after transplantation, +14: 14 days after transplantation, +28: 28 days after transplantation.

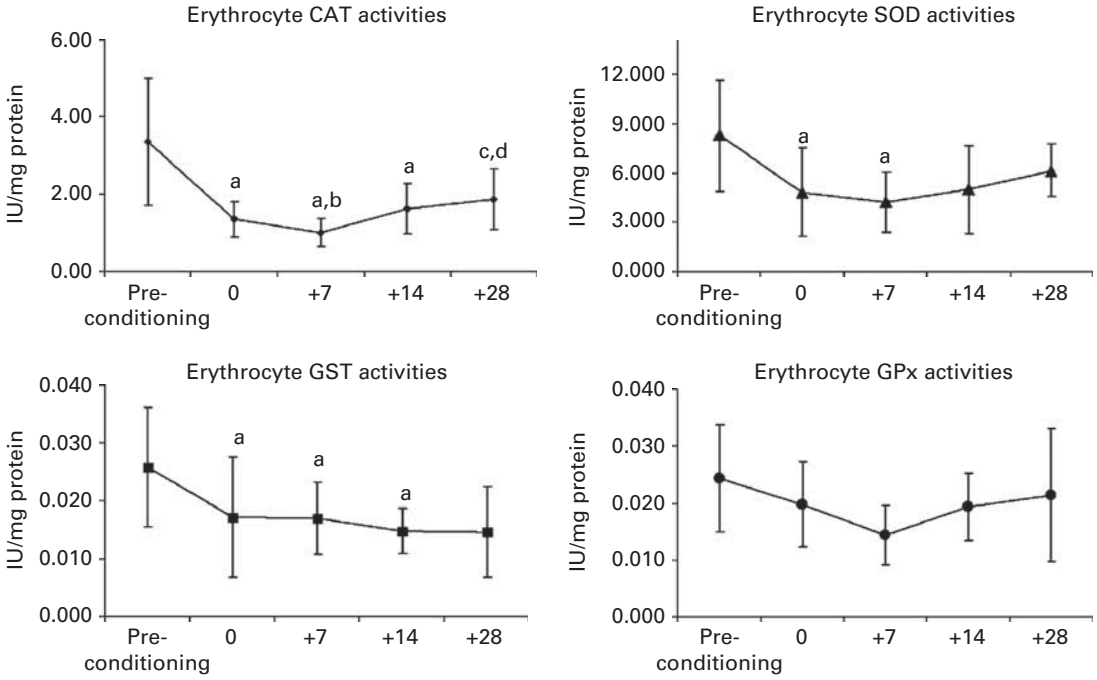

Figure 3 Effect of chemotherapy on erythrocyte antioxidant enzymes (CAT, SOD, glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST)) activities. [a]$P<0.01$: significantly different from pre-conditioning day, [b]$P<0.01$: significantly different from day 0, [c]$P<0.01$: significantly different from day 7, [d]$P<0.01$: significantly different from day 14. Pre-conditioning: 10 days before HCST, 0: transplantation day, +7: 7 days after transplantation, +14: 14 days after transplantation, +28: 28 days after transplantation.

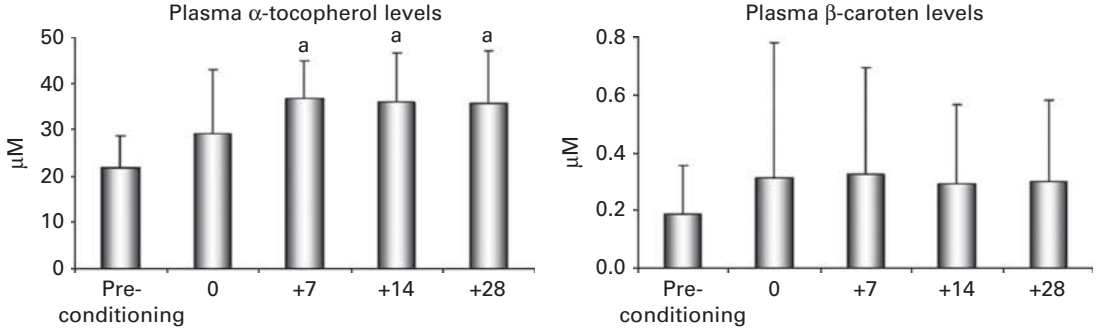

Figure 4 Effect of chemotherapy on plasma α-tocopherol and β-carotene concentrations. [a]$P<0.01$: Significantly different from pre-conditioning day. Pre-conditioning: 10 days before HCST, 0: transplantation day, +7: 7 days after transplantation, +14: 14 days after transplantation, +28: 28 days after transplantation.

Antioxidant enzyme activities

Erythrocyte CAT activity was significantly decreased in day 0, day 7 and day 14 compared with pre-conditioning day. However, this decrease reversed back on day 28. Significant decreases in days 0, 7 and on days 0, 7, 14 were also observed in erythrocyte SOD and glutathione-*S*-transferase activities, respectively, compared with pre-conditioning day. Although not statistically significant, erythrocyte glutathione peroxidase activity tended to decrease after chemotherapy (Figure 3).

α-tocopherol and β-carotene levels

Although plasma β-carotene levels did not change, α-tocopherol levels were increased significantly after high-dose chemotherapy (Figure 4).

Outcome of patients

Engraftment was achieved in 18 patients. One case was excluded from engraftment analysis due to early death (day +6). The mean neutrophil engraftment day was day 15.9 ± 2.9. Acute and chronic GVHD were seen in six and one out of 18 patients who had engrafted, respectively. Veno-occlusive disease developed in five patients, four of whom had mild, and one had severe veno-occlusive disease. Hemorrhagic cystitis (grade 2–3) was observed in two patients and mucositis (grade 3–4) in eight patients. Five patients died and causes of death were sepsis in one patient, pulmonary hypertension in one patient, engraftment failure and sepsis in one patient, poor graft function and pulmonary infection in one patient, pulmonary infection and acute respiratory distress syndrome in one patient.

Discussion

It has been reported that chemotherapy results in increased oxidative stress and depletion of tissue antioxidants. Much debate has focused on whether antioxidants interfere with the efficacy of cancer chemotherapy. However, several studies showed that antioxidants do not interfere with and can actually enhance the killing capabilities of cancer therapeutic modalities and decrease their side effects.[3–7]

Conditioning therapy preceding HSCT usually consists of high-dose chemotherapy. A limited number of studies has shown that high-dose chemotherapy decreased antioxidant enzyme and vitamin levels in patients undergoing HSCT. Plasma MDA levels were found to be increased after high-dose therapy in these patients (1, 3, 7). In erythrocytes, SOD, glutathione peroxidase and CAT activities were reported to be decreased or increased (1, 3, 4, 7, 9). Furthermore, plasma antioxidants such as α-tocopherol, β-carotene and vitamin C were shown to be depleted (3, 8–10).

In this study, the blood samples were collected before the preparative regimen (10 days before transplantation, preconditioning day), before transplantation (day 0) and after transplantation (day 7, 14 and 28) from pediatric HSCT patients and oxidative/antioxidative parameters were determined.

The results of the present study showed that MDA and GSSG levels were increased while CAT, SOD and glutathione-*S*-transferase activities and GSH levels are decreased after high-dose chemotherapy in HSCT patients. There was no correlation between some clinical (blood urea nitrogen, alanine aminotransferase, aspartate aminotransferase, creatinine, uric acid) and oxidative stress parameters.

Our results are in accordance with the limited number of previous studies indicating that the conditioning regimen results in oxidative stress and depletion of antioxidants in HSCT patients.[3–5] On average, engraftment takes 15–20 days for new BM to engraft or to begin making new stem cells. Interestingly, MDA levels were found to be increased whereas enzyme activities (such as SOD and CAT) decreased at the engraftment period. On the other hand, statistical analyses revealed significant negative correlations between MDA levels and both CAT and SOD activities (MDA-CAT, $r=-0.47$, MDA-SOD, $r=-0.51$). A negative correlation was also found between MDA and GSH levels ($r=-0.34$). These findings may be interpreted as indirect evidence of the contribution of oxidative stress to antioxidant enzyme depletion. Contrary to previous results, we found that plasma β-carotene levels did not change whereas α-tocopherol levels were increased significantly after the conditioning regimen. Vitamin E supplementation may be the cause of the increase in α-tocopherol concentration in HSCT patients. Similarly, multivitamin supplementation may have contributed to maintenance of β-carotene levels closer to baseline values. Furthermore, differences between the conditioning regimens may also have had a role. Radiotherapy was not administered to any of the patients in this group. It seems that vitamin supplementation was sufficient to maintain plasma α-tocopherol and β-carotene vitamin levels after conditioning therapy in this group of patients. However, our data indicate that conditioning therapy is associated with increased oxidative stress and depletion of antioxidant enzymes.

Although the diagnosis and conditioning therapy was heterogenous in this pediatric HSCT patient population, significant changes in oxidant and antioxidant mechanisms were observed after initiation of conditioning regimen. Further studies with a larger number of patients may allow comparison of myeloablative vs non-myeloablative regimens, and manipulation of supportive regimens accordingly.

As far as we know, this is the first study that determines oxidative stress and antioxidant parameters in pediatric HSCT patients, who may be more susceptible to the negative effects of high-dose chemotherapy. The present study confirmed that conditioning therapy results in increased oxidative stress and depletion of antioxidants in pediatric HSCT patients.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

1 Gonçalves TL, Benvegnú DM, Bonfanti G, Frediani AV, Pereira DV, Rocha JB. Oxidative stress and delta-ALA-D activity in different conditioning regimens in allogeneic bone marrow transplantation patients. *Clin Biochem* 2009; **42**: 602–610.
2 Atkinson K, Champlin R, Ritz J, Fibbe WE, Ljungman P, Brenner MK eds. *Clinical bone marrow and blood stem cell transplantation*. Cambridge University Press: Cambridge, 2004.
3 Clemens MR, Waladkhani AR, Bublitz K, Ehninger G, Gey KF. Supplementation with antioxidants prior to bone marrow transplantation. *Wien Klin Wochenschr* 1997; **109**: 771–776.
4 Sari I, Cetin A, Kaynar L, Saraymen R, Hacioglu SK, Ozturk A *et al*. Disturbance of pro-oxidative/antioxidative balance in allogeneic peripheral blood stem cell transplantation. *Ann Clin Lab Sci* 2008; **38**: 120–125.
5 Dürken M, Agbenu J, Finckh B, Hübner C, Pichlmeier U, Zeller W *et al*. Deteriorating free radical-trapping capacity and antioxidant status in plasma during bone marrow transplantation. *Bone Marrow Transplant* 1995; **15**: 757–762.
6 Hunnisett A, Davies S, McLaren-Howard J, Gravett P, Finn M, Gueret-Wardle D. Lipoperoxides as an index of free radical activity in bone marrow transplant recipients. Preliminary observations. *Biol Trace Elem Res* 1995; **47**: 125–132.
7 Lin JQ. Effect of nutrition intervention on antioxidant capacity and lipid peroxide in patients with bone marrow transplantation (abstract). *Di Yi Jun Yi Da Xue Xue Bao* 2002; **22**: 530–532.
8 Jonas CR, Puckett AB, Jones DP, Griffith DP, Szeszycki EE, Bergman GF *et al*. Plasma antioxidant status after high-dose chemotherapy: a randomized trial of parenteral nutrition in bone marrow transplantation patients. *Am J Clin Nutr* 2000; **72**: 181–189.

9 Clemens MR, Ladner C, Ehninger G, Einsele H, Renn W, Bühler E *et al*. Plasma vitamin E and beta-carotene concentrations during radiochemotherapy preceding bone marrow transplantation. *Am J Clin Nutr* 1990; **51**: 216–219.
10 Clemens MR, Ladner C, Schmidt H, Ehninger G, Einsele H, Gey KF *et al*. Decrease of alpha tocopherol and beta-carotene by high-dose radiochemotherapy preceding bone marrow transplantation. *Ann NY Acad Sci* 1989; **570**: 431–434.
11 Ladner C, Ehninger G, Gey KF, Clemens MR. Effect of etoposide (VP16-213) on lipid peroxidation and antioxidant status in a high-dose radiochemotherapy regimen. *Cancer Chem Pharmacol* 1989; **25**: 210–212.
12 Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA *et al*. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation* 1974; **18**: 295–304.
13 Lee SJ, Vogelsang G, Flowers MED. Chronic graft-versus-host disease. *Biol Blood Marrow Transplant* 2003; **9**: 215–233.
14 Larocca A, Piaggio G, Podestà M, Pitto A, Bruno B, Di Grazia C *et al*. Boost of CD34 + -selected peripheral blood cells without further conditioning in patients with poor graft function following allogeneic stem cell transplantation. *Haematologica* 2006; **91**: 935–940.
15 Jones RJ, Lee KS, Beschorner WE, Vogel VG, Grochow LB, Braine HG. Venoocclusive disease of the liver following bone marrow transplantation. *Transplantation* 1987; **44**: 778–783.
16 Hassan Z, Remberger M, Svenberg P, Elbander M, Omazic B, Mattsson J *et al*. Hemorrhagic cystitis: a retrospective single-center survey. *Clin Transplant* 2007; **21**: 659–667.
17 Winters RA, Ukowski J, Ercal N, Matthews RH, Spitz DR. Analysis of glutathione, glutathione disulfide, cysteine, homocysteine, and other biological thiols by high performance liquid chromotoghraphy following derivatization by N-(1-pyrenyl)-maleimide. *Anal Biochem* 1995; **227**: 14–21.
18 Draper HH, Squires EJ, Mamoodi H, Wu J, Agarwal M, Hadley MA. Comperative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. *Free Radic Biol Med* 1993; **15**: 353–363.
19 Templar J, Kon SP, Milligan TP, Newman DJ, Raftery MJ. Increased plasma malondialdehyde levels in glomerular disease as determined by a fully validated HPLC method. *Nephrol Dial Transplant* 1999; **14**: 946–951.
20 Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**: 7130–7139.
21 Aebi H. Catalase *in vitro*. *Method Enzymol* 1984; **105**: 121–126.
22 Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; **47**: 469–474.
23 Pleban PA, Munyani A, Beachum J. Determination of selenium concentration and glutathione peroxidase activity in plasma and erythrocyte. *Clin Chem* 1962; **28**: 311–316.
24 Steghens JP, Van Kappel AL, Riboli E, Collombel C. Simultaneous measurement of seven carotenoids, retinol and alpha-tocopherol in serum by high-performance liquid chromatography. *J Chrom B Biomed Sci Appl* 1997; **694**: 71–81.
25 Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.