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AVALIAÇÃO DO ESTRESSE OXIDATIVO E DA ATIVIDADE DA ENZIMA δ-AMINOLEVULINATO DESIDRATASE EM PACIENTES TRANSPLANTADOS DE MEDULA ÓSSEA

TESE DE DOUTORADO

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Santa Maria, RS, Brasil

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por

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Ciências Biológicas: Bioquímica Toxicológica.**

Orientador: Prof. Dr.João Batista Teixeira da Rocha

Santa Maria, RS, Brasil

2008

Universidade Federal de Santa Maria
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A Comissão Examinadora, abaixo assinalada, aprova a tese de Doutorado

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elaborada por
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Como requisito parcial para obtenção do grau de
Doutor em Ciências Biológicas: Bioquímica Toxicológica

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

AVALIAÇÃO DO ESTRESSE OXIDATIVO E DA ATIVIDADE DA ENZIMA δ-AMINOLEVULINATO DESIDRATASE EM PACIENTES TRANSPLANTADOS DE MEDULA ÓSSEA

AUTORA: Thissiane de Lima Gonçalves
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Data e Local da Defesa: Santa Maria, 19 de Dezembro de 2008.

O transplante de medula óssea (TMO) vem sendo usado no tratamento de várias doenças. Antes do TMO, os pacientes são submetidos a um regime de condicionamento (RC), que consiste na administração de altas doses de quimioterapia ou radioquimioterapia. Este RC tem a finalidade de erradicar a doença residual do paciente e induzir uma imunossupressão que permita a "pega" das células infundidas no TMO. A ação das drogas citostáticas, da radioterapia e do próprio procedimento TMO está associada à produção de grandes quantidades de espécies reativas de oxigênio (EROs), que com uma defesa antioxidante inadequada pode resultar em estresse oxidativo, que por sua vez, tem sido implicado na etiologia de várias doenças e complicações pós-TMO. Os objetivos deste estudo foram avaliar o estatus oxidativo e também analisar a atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D) como possível marcador de estresse oxidativo em pacientes transplantados de medula óssea, alogênico ou autólogo, comparando essas duas modalidades de TMO. Comparar diferentes RCs, visando determinar qual deles poderia ser menos tóxico para os pacientes transplantados de medula do tipo alogênico ou autólogo, em termos de estresse oxidativo. E também analisar a atividade da enzima δ-ALA-D nos diferentes RCs. Para atingir tais objetivos, alguns indicadores de estresse oxidativo como, espécies reativas ao ácido tiobarbitúrico (TBARS), vitamina C, catalase, superóxido dismutase, grupos tióis protéicos (P-SH), e não protéicos (NP-SH) e a enzima δ-ALA-D, foram avaliados em pacientes submetidos à TMO no Hospital Universitário de Santa Maria, no período de Março de 2007 à Março de 2008. Os resultados obtidos revelaram que os pacientes apresentaram sinais de estresse oxidativo antes do TMO, durante o RC e até 20 dias após o TMO. Ocorreu uma diminuição nas defesas antioxidantes e na atividade da enzima δ-ALA-D e um aumento na peroxidação lipídica no sangue dos pacientes alogênicos e autólogos. Com relação aos RCs, nos pacientes alogênicos e também nos autólogos, ocorreu uma diminuição nos antioxidantes enzimáticos e não enzimáticos e na atividade da enzima δ-ALA-D em todos os RCs analisados (fludarabine+ciclofosfamida – FluCy, bussulfan+ciclofosfamida – BuCy e ciclofosfamida+irradiação corporal – CyTBI, para pacientes alogênicos e ciclofosfamida+BCNU+etoposide – CBV e melfalan, para pacientes autólogos), ocorreu também um aumento na peroxidação lipídica no sangue com todos os RCs, porém mais pronunciadamente com o uso de CyTBI nos pacientes alogênicos e melfalan nos autólogos. Concluindo, esse estudo demonstrou estresse oxidativo nos pacientes com TMO alogênico e autólogo. Todos os RCs analisados promoveram estresse oxidativo, porém este foi mais evidente com o uso de CyTBI (pacientes alogênicos) e melfalan (pacientes autólogos) e ainda a avaliação da atividade da enzima δ-ALA-D, parece ser um biomarcador de estresse oxidativo em pacientes TMO, já que além de encontrar-se diminuída nestes pacientes, esta ainda mostrou uma correlação negativa com os níveis de TBARS e positiva com os antioxidantes.

Palavras-chave: estresse oxidativo; antioxidantes; transplante de medula óssea.

ABSTRACT

Doctoral Thesis
Post-Graduate Program in Biological Science: Toxicological Biochemistry
Universidade Federal de Santa Maria, RS, Brazil

EVALUATION OF OXIDATIVE STRESS AND δ-AMINOLEVULINIC ACID DEHYDRATASE IN BONE MARROW TRANSPLANTATION PATIENTS

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Bone marrow transplantation (BMT) has been used in the treatment of a number of diseases. Before BMT, patients are submitted to a conditioning regime (CR) that consists of administration of high doses of chemo- or radiotherapy. This CR has the objective of eradicating the residual disease and inducing an immunosuppression that will allow the engraftment of cells infused in the BMT. The action of the cytostatic drugs, of radiotherapy and of the BMT itself is associated with the production of a great quantity of reactive oxygen species (ROS), which, if anti-oxidant defenses are inadequate, can lead to oxidative stress, which in turn has been implied in the etiology of several diseases and post-BMT complications. The objectives of this study were: to evaluate the oxidative status and to analyze aminolevulinic acid dehydratase activity (δ-ALA-D) as a possible marker of oxidative stress in allogeneic or autologous BMT patients and compare these two modalities of BMT; to compare different CRs to determine which is less toxic in terms of oxidative stress; and to analyze δ-ALA-D in the different CRs. In order to achieve these objectives, some indicators of oxidative stress, such as thiobarbituric acid reactive species (TBARS), vitamin C, catalase, superoxide dismutase, protein thiol groups (P-SH), non-protein thiol groups (NP-SH) and δ-ALA-D, were evaluated in patients undergoing BMT at the University Hospital of the Universidade Federal de Santa Maria, between March 2007 and March 2008. The results obtained showed that patients presented signs of oxidative stress before BMT, during the CR and up to 20 days after BMT. A decrease in antioxidant defenses and in δ-ALA-D activity was verified and an increase in lipid peroxidation in the blood of allogeneic and autologous patients was observed. In addition, there was a decrease in enzymatic and non-enzymatic antioxidants and in δ-ALA-D activity in all CRs analyzed (fludarabine + cyclophosphamide – FluCy, busulfan + cyclophosphamide – BuCy and cyclophosphamide + total body irradiation – CyTBI, for allogeneic patients and cyclophosphamide + BCNU+ etoposide – CBV and melphalan, for autologous patients). There was also an increase in lipid peroxidation in the blood in all CRs, however, it was more pronounced with the use of CyTBI in allogeneic patients and with melphalan in autologous patients. In conclusion, this study demonstrated oxidative stress in allogeneic and autologous BMT patients. All CRs analyzed brought about an elevation of oxidative stress, however, this increase was greater with CyTBI (allogeneic patients) and melphalan (autologous patients). Finally, evaluation of δ-ALA-D seems to be an efficient biomarker of oxidative stress in BMT patients, as well as to be reduced in these patients, also showed a negative correlation with the levels of TBARS and positive with antioxidants.

Keywords: oxidative stress; antioxidants; bone marrow transplantation

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LISTA DE ABREVIATURAS

AAG: anemia aplástica grave

ANOVA: análise de variância

δ-ALA-D: delta aminolevulinato desidratase

BuCy: bussulfan+ciclofosfamida

CAT: catalase

CBV: ciclofosfamida+BCNU+etoposide

CyTBI: ciclofosfamida+irradiação corporal total

DECH: doença do enxerto contra o hospedeiro

EROs: espécies reativas de oxigênio

GSH: glutationa reduzida

LH: linfoma Hodgkin

LLA: leucemia linfocítica aguda

LLC: leucemia linfocítica crônica

LMA: leucemia mielóide aguda

LMC: leucemia mielóide crônica

MDA: malondialdeído

NP-SH: grupos tióis não protéicos

P-SH: grupos tióis protéicos

RC/CR: regime de condicionamento

SOD: superóxido dismutase

TBA: ácido tiobarbitúrico

TBARS: espécies reativas ao ácido tiobarbitúrico

TMO/BMT: transplante de medula óssea

VIT C: vitamina C

SUMÁRIO

1 INTRODUÇÃO

O transplante de medula óssea (TMO) ou transplante de células progenitoras hematopoéticas é uma terapia utilizada para uma variedade de doenças hematológicas, anormalidades genéticas e neoplasias. O TMO tem a finalidade de restabelecer a função medular danificada ou defeituosa em pacientes que receberam quimioterapia citorredutora de altas doses e radioterapia (regime de condicionamento), por meio de infusão intravenosa de células progenitoras ou células tronco (stem cells), com capacidade de multiplicação e diferenciação em todos os tipos de células sangüíneas maduras: eritrócitos, leucócitos e plaquetas (SERBER, 1999).

Dependendo da origem das células progenitoras, os transplantes podem ser autólogos ou autogênico quando as células são originárias do próprio paciente. Alogênicos quando as células são doadas por um outro indivíduo ou singênico, quando o doador é irmão gêmeo idêntico (CASTRO JR et al., 2001).

Em condições normais nos organismos existe um equilíbrio entre oxidantes como radicais livres e os processos protetores antioxidantes. O aumento na produção de espécies reativas de oxigênio e de outros radicais livres ou a diminuição das defesas antioxidantes podem levar ao estresse oxidativo, que está relacionado com a etiologia ou progressão de uma variedade de doenças (HALLIWELL & GUTETERIDGE, 1990). Enzimas como a superoxido dismutase, glutationa peroxidase e catalase, juntamente com antioxidantes da dieta como α-tocoferol e vitamina C, constituem a principal defesa do organismo contra danos oxidativos (HALLIWELL & GUTTERIDGE, 1999).

A enzima sulfidrílica delta-aminolevulinato desidratase (δ-ALA-D), que faz parte da rota de síntese dos compostos tetrapirrólicos, em situações pró-oxidantes, pode estar inibida resultando em conseqüências patológicas (SASSA et al., 1989; GOERING , 1993; FOLMER et al., 2002). Além da insuficiente produção de heme, a inibição da δ-ALA-D pode resultar no acúmulo do substrato ALA no sangue, que está relacionado com a superprodução de espécies reativas de oxigênio (PEREIRA et al., 1992), e também com a indução de peroxidação lipídica (OTEIZA et al., 1994).

Em todo o procedimento de preparação para o TMO (obtenção de células e regime de condicionamento), durante a internação, os pacientes apresentam toxicidade orgânica intensa

e variável, que está associada a produção de radicais livres e a deficiência de antioxidantes, que podem levar a complicações importantes, como a falência hepática entre outras (WILMORE et al., 1999).

Tendo em vista a grande importância do TMO, e visando contribuir para uma diminuição na morbidade e mortalidade, o presente estudo tem por objetivo avaliar o estatus oxidativo nos pacientes nas diversas fases que envolvem o TMO, principalmente através de novos marcadores, como é o caso da avaliação da atividade da δ-ALA-D nestes pacientes.

2 OBJETIVOS

Os objetivos deste estudo foram avaliar o estatus oxidativo e também analisar a atividade da enzima δ-aminolevulinato desidratase (δ-ALA-D) como possível marcador de estresse oxidativo em pacientes transplantados de medula óssea, alogênico ou autólogo, comparando estas duas modalidades de TMO.

Comparar diferentes RCs, visando determinar qual deles poderia ser menos tóxico para os pacientes transplantados de medula do tipo alogênico ou autólogo, em termos de estresse oxidativo e também analisar a atividade da enzima δ-ALA-D nos diferentes RCs.

3 REVISÃO BIBLIOGRÁFICA

3.1 Transplante de medula óssea

3.1.1 Aspectos gerais

O transplante de medula óssea (TMO) consiste na infusão intravenosa de células progenitoras hematopoéticas com o objetivo de restabelecer a função medular nos pacientes com medula óssea danificada ou defeituosa (ARMITAGE, 1994). Essa terapia é utilizada nas doenças em que existe falência do sistema hematopoético, seja por infiltração de células leucêmicas na medula óssea, ou por doenças que alterem a produção dos constituintes sangüíneos. Também, apresenta indicação nas doenças que comprometem severamente o sistema imunológico e em tumores sólidos em que a dose de quimioterápicos necessária para o tratamento, possa comprometer o sistema hematopoético de maneira irreversível. Basicamente o TMO permite administrar doses elevadas de quimioterápicos e radioterapia, proporcionando o resgate dos sistemas hematológico e imunológico (DULLEY, 1997).

3.1.2 Tipos de transplantes e particularidades

São três as modalidades de TMO:

-Transplante alogênico: o paciente recebe a medula de outra pessoa, que pode ser algum familiar (doador aparentado) ou não (doador não aparentado).

-Transplante autogênico ou autólogo: utiliza as células do próprio paciente, coletadas previamente.

-Transplante singênico: o doador é um irmão gêmeo idêntico (CASTRO JR et al., 2001).

3.1.2.1 Transplante alogênico

No caso do TMO alogênico, a compatibilidade entre o doador e o paciente é de extrema importância para o sucesso do mesmo. O HLA (human leukocyte antigen – antígeno leucocitário humano), que está codificado no braço curto do cromossomo 6 é o responsável pela identidade imunológica. O HLA segue as regras da herança mendeliana simples. Assim quem tem um irmão tem 25% de chance de ter um doador HLA idêntico (ARMITAGE, 1994). Outros fatores que devem ser levados em consideração na procura de um doador são a idade, evitando doadores muito jovens ou idosos, o peso, que preferencialmente deve ser próximo ou superior ao do receptor, o histórico médico, a condição clínica geral. Prefere-se tipagem sanguínea igual doador-receptor, embora possa ser usado grupo sanguíneo diferente. Deve-se ter cuidado também com doenças ligadas à herança genética (CASTRO JR et al., 2001). Caso um doador HLA aparentado compatível não seja encontrado, uma alternativa é procurar um familiar parcialmente compatível, outra alternativa seria a busca de doadores não relacionados de medula óssea ou de cordão umbilical. Nestes casos a chance de sucesso do TMO é menor (BEATTY et al., 1988), principalmente pelo maior risco dos pacientes desenvolverem a Doença do Enxerto contra o Hospedeiro (DECH), (SANDERS, 1997).

3.1.2.1.1 Indicações para TMO alogênico e singênico

Os TMO alogênico e singênico podem ser utilizados para o tratamento de diversas doenças, sendo as principais listadas abaixo:

Doenças não neoplásicas:

- Anemia aplástica grave (AAG)
- Anemia de Fanconi
- Imunodeficiências (Chediaki Higashi, Wiskott-Aldrich, Imunodeficiência combinada severa)
- Osteopetrose
- Doenças de acúmulo (Adenoleucodistrofia, Leucodistrofia metacromática infantil)

- Talassemia maior
- Anemia falciforme com manifestações graves, e com doador aparentado disponível.

Doenças neoplásicas

- Leucemia mielóide crônica (LMC)
- Leucemia mielóide aguda (LMA) em primeira remissão com fatores de mau prognóstico ou em segunda remissão
- Leucemia linfocítica aguda (LLA) em primeira remissão com fatores de mau prognóstico ou em remissões subseqüentes
- Síndromes mielodisplásticas, incluindo a monossomia do cromossomo 7 e leucemia mielomonocítica crônica
- Mielofibrose maligna aguda
- Linfomas não Hodgkin em segunda ou terceira remissão (CASTRO JR et al., 2001)
- Mieloma múltiplo (ARMITAGE, 1994).

3.1.2.2 Transplante autogênico ou autólogo

Este tipo de transplante permite a administração de altas doses de quimioterapia, que jamais poderiam ser utilizadas se não houvesse esse recurso de reinfusão de células progenitoras hematopoéticas, coletadas previamente do próprio paciente. A complexibilidade e as complicações do transplante autogênico são menores, quando comparadas às do TMO alogênico (CASTRO JR et al., 2001). Pode ser realizado em pacientes idosos com maior segurança (ARMITAGE, 1994).

3.1.2.2.1 Indicações para transplante autogênico

O TMO autogênico pode ser utilizado no tratamento das seguintes doenças:

- Linfoma Hodgkin em segunda remissão
- Linfoma não Hodgkin em segunda remissão

- Neuroblastoma avançado
- Sarcoma de Ewing em segunda remissão
- LMA
- Tumor de Wilms em segunda remissão
- Tumor de células germinativas em segunda remissão
- Meduloblastoma de alto risco ou em segunda remissão (CASTRO JR et al., 2001)
- Mieloma múltiplo (ARMITAGE, 1994).

3.1.3 Fontes de células

Classicamente para o TMO, a procedência das células progenitoras hematopoéticas é da crista ilíaca, através de múltiplas punções e aspirações da medula óssea, ou do sangue periférico e coletadas em máquinas de aférese. E mais recentemente podem ser obtidas do sangue do cordão umbilical (CASTRO JR et al., 2001).

A quantidade de medula óssea necessária para o transplante, geralmente é 10 mL/kg de peso do receptor, que corresponde a um número adequado de células progenitoras suficientes para permitir a pega (engraftment) do enxerto (THOMAS & STORB, 1970). A maioria dos transplantes alogênicos ainda é realizada utilizando a coleta da crista ilíaca da medula óssea do doador (HOROWITZ & KEATING, 2000).

As células progenitoras hematopoéticas periféricas são coletadas com máquinas de aférese, após a mobilização das mesmas da medula óssea utilizando fatores estimuladores de colônias de granulócitos, associados ou não a quimioterapia. Este tipo de coleta vem sendo utilizado em mais de 90% dos transplantes autológos e em cerca de 20% dos alogênicos (HOROWITZ & KEATING, 2000).

O sangue do cordão umbilical (SCU) é coletado logo após o nascimento da criança, sendo posteriormente processado e mantido congelado até a infusão (RUBINSTEIN et al., 1998). O SCU possui algumas vantagens como menor probabilidade de induzir doença do enxerto contra o hospedeiro (DECH) aguda e crônica, mesmo quando a tipagem HLA não é totalmente compatível com a do receptor (ROCHA et al., 2000). Porém apresenta o problema

do limitado número de células por unidade o que leva a um retardo na pega do enxerto, tornando o TMO mais arriscado (CASTRO JR et al., 2001).

3.1.4 Regimes de condicionamento pré-transplante

Os regimes de condicionamento (RCs) pré-TMO tem a finalidade de erradicar a doença residual do paciente e induzir uma imunossupressão que permita a "pega" das células infundidas. A escolha do regime de condicionamento é feita de acordo com a doença de base do paciente, consistindo em altas doses de quimioterápicos associados ou não à radioterapia corporal total. A tabela 1 mostra os principais regimes de condicionamento utilizados atualmente.

Tabela 1- Principais regimes de condicionamento pré-transplante de medula óssea.

Regime de condicionamento (RC)	Doenças freqüentemente tratadas com o RC
TBI (12 Gy) + Cy (120mg/kg)	LLA, LMA, LMC, AAG
TBI (12 Gy) + VP-16 (60mg/kg)	LLA
Bu (16 mg/kg) + Cy (120mg/kg)	LMA, LMC
Bu (4mg/kg) + Cy (200mg/kg)	AAG
Bu (16 mg/kg) + Melfalan (140mg/kg)	LMA, LMC, tumors sólidos
Cy (200mg/kg)	AAG
TBI (12 Gy) + Ara-C (36g/m²)	LLA
BEAM-BCNU (300mg/m²) + VP-16 (800 mg/m² + Ara-C (800 mg/m²) + Melfalan (140mg/m²)	Linfomas Hodgkin e não Hodgkin

Fonte: CASTRO JR et al., 2001.

3.1.5 Complicações pós-TMO

3.1.5.1 Aplasia da medula óssea

O dia da infusão da medula óssea é denominado dia 0. Os dias anteriores, quando é realizado o condicionamento, são denominados como negativos (-2,-1) e os posteriores como positivos (+1, +2).

Após a infusão, a medula óssea de um transplantado fica em aplasia por um período aproximado de 2 a 3 semanas. Nesse período, é maior o risco de ocorrerem infecções, anemias e sangramentos. Geralmente as contagens de leucócitos caem abaixo de 100 células/mm³ por volta do dia +4, dependendo do tipo de condicionamento utilizado e da doença de base. Considera-se que a medula "pegou" quando as contagens mantêm-se acima de 500 células/mm³ por 3 dias consecutivos, o que ocorre em média entre os dias +15 a +19 após o TMO. O número de plaquetas também costuma cair abaixo de 10.000 células/mm³, considera-se sua recuperação quando contagens acima de 20.000 células/mm³ são atingidas sem a necessidade de transfusões por mais de 7 dias, isso ocorre em torno dos dias +19 a + 25. A hemoglobina é mantida acima de 9-10g/dL também com auxilio de transfusões. Concentrados de hemácias e plaquetas devem ser irradiados para inativar os linfócitos, e filtrados para diminuir o número de leucócitos, visando diminuir a incidência de DECH transfusional, infecção por citomegalovírus (CMV) e reações anafiláticas (CASTRO JR et al., 2001).

3.1.5.2 Doença do enxerto contra o hospedeiro (DECH)

A DECH ocorre mais freqüentemente em pacientes submetidos à TMO alogênico, embora seja possível a ocorrência nos transplantados singênicos e mais raramente nos autogênicos (CASTRO JR et al., 2001).

A DECH aguda manifesta-se a partir da “pega” do enxerto, podendo ocorrer até o dia + 100 pós-transplante. Os órgãos mais afetados são pele, sistema gastrintestinal e fígado. A severidade dessa condição é graduada de acordo com o envolvimento desses órgãos (THOMAS et al., 1975).

A DECH crônica é uma doença auto-imune, que envolve vários órgãos e sistemas. Geralmente envolve pele, fígado, olhos e mucosa oral, porém trato gastrintestinal, pulmão e sistema neuromuscular podem estar envolvidos (SHULMAN et al., 1980).

3.1.5.3 Infecções

Quase todos os pacientes transplantados apresentam febre após o período de condicionamento e são muito susceptíveis a apresentar infecções graves. As infecções bacterianas são as mais freqüentes, acometendo sítios como pulmão, seios da face e cateter (WINGARD, 1990). Os fungos também causam infecções freqüentemente em transplantados. O uso de fluconazol profilático durante o período de neutropenia teve um impacto positivo nestes pacientes, diminuindo o número de infecções por *Candida albicans* (UZUN et al., 2000).

A infecção por citomegalovírus (CMV) é comum em transplantados alogênicos, sendo secundária a reativação de vírus latente, primo-infecção ou reinfecção, e pouco freqüente em transplantados autogênicos. A manifestação mais importante é a pneumonia intersticial, que pode ser fatal em até 75% dos casos (SABOYA, 1998). O vírus sincicial respiratório pode causar uma pneumonia intersticial grave e muitas vezes fatal (UZUN et al., 2000). Outros vírus como influenza e parainfluenza também podem produzir complicações pulmonares em TMO.

O adenovírus é associado a diarréias e cistite hemorrágica tardia nos transplantados (BALDWIN et al., 2000). A reativação dos vírus *Herpes simples* e *Herpes zooster* é prevenida com aciclovir profilático (GLUCKMAN et al., 1983).

3.2 Estresse oxidativo

Durante o metabolismo basal das células aeróbicas normais existe uma produção constante de EROS acompanhada pela sua contínua inativação, através da ação de antioxidantes, de forma a manter a integridade estrutural e funcional das biomoléculas. A extensão e os tipos de dano causados pelas EROS depende tanto da quantidade como da qualidade ou natureza dos mesmos a que as células estão expostas, bem como das suas defesas antioxidantes (DAVIES, 1991).

O desequilíbrio entre os mecanismos que causam condições oxidativas e das defesas antioxidantes celulares presentes nos organismos vivos, provoca uma variedade de mudanças fisiológicas, chamadas coletivamente de estresse oxidativo (CROFT, 1998), onde as EROS podem estar aumentadas sem o concomitante aumento das defesas; as proteções podem estar reduzidas sem o aumento daquelas; ou a situação mais crítica, onde o aumento da concentração das EROS vem acompanhado de uma redução paralela das defesas correspondentes (SIES, 1986; AMSTAD & CERUTTI, 1990).

O estresse oxidativo esta relacionado com vários processos tais como: mutagênese, carcinogênese, lipoperoxidação, oxidação e fragmentação de proteínas e carboidratos (SIES, 1986).

3.2.1 Espécies reativas de oxigênio (EROS)

Os radicais livres são espécies químicas capazes de existência independente e que contém um ou mais elétrons não-pareados em sua órbita de valência (HALLIWELL & GUTTERIDGE, 1999). Os radicais livres derivados do oxigênio, conhecidos como espécies reativas de oxigênio (EROS), representam a principal classe de radicais gerados nos sistemas vivos, podendo desempenhar efeitos benéficos ou maléficos nesses organismos (VALKO et al., 2004). Os efeitos benéficos ocorrem em concentrações baixas ou moderadas de EROS envolvidas na resposta celular fisiológica, por exemplo contra agentes infecciosos. Já as EROS em altas concentrações podem mediar o dano causado em estruturas celulares, como os

lipídios, membrana, proteínas e ácidos nucléicos, resultando em estresse oxidativo (POLI et al., 2004).

No processo respiratório tem-se, seja em maior ou em menor escala, a formação de espécies intermediárias instáveis de oxigênio, as EROS. As EROS são compostos altamente reativos, a maioria apresentando um tempo de vida incrivelmente fugaz. Apesar da breve existência, possuem um certo instante de vida livre, no qual podem reagir com a matéria circundante e assim adquirir estabilidade (SIGNORINI & SIGNORINI, 1995).

No processo de transformações químicas, grande parte do oxigênio molecular é reduzido à água através das transferências de elétrons na cadeia respiratória. Estima-se que em torno de 98% do oxigênio consumido em organismos aeróbios é reduzido de forma tetravalente até H_2O (CHANCE et al., 1979), através da ação da citocromo c oxidase mitocondrial, sem a concomitante geração de EROS, permitindo que a principal via de transferência de elétrons e de energia para a síntese de ATP celular participe da citotoxicidade decorrente destas formas deletérias (FRIDOVICH, 1979). Mesmo assim, o principal sítio de formação endógena do ânion superóxido estaria localizado na cadeia respiratória mitocondrial (BOVERIS & CHANCE, 1973), correspondendo a cerca de 2% do oxigênio consumido (BOVERIS & CADENAS, 1982).

O peróxido de hidrogênio (H_2O_2), gerado essencialmente em mitocôndrias e peroxissomos, é uma molécula que, a rigor, não pode ser considerada um radical livre, já que não possui elétrons não-pareados, mas possui atividade oxidante e elevada capacidade de difusão (HALLIWELL & GUTTERIDGE, 1999). As mitocôndrias encontram-se em todas células aeróbicas de organismos multicelulares, e estão localizadas principalmente nos músculos esquelético e cardíaco, sendo, portanto, fisiologicamente o sítio gerador mais importante de peróxido de hidrogênio nos organismos (CHANCE et al., 1979).

A produção de $HO^{\bullet}$ intracelular a partir da reação do $O_2^{\bullet-}$ com o H_2O_2 (reação de Haber-Weiss, postulada em 1934), ou a simples mistura de H_2O_2 com metais de transição como o ferro e o cobre (reação descrita por Fenton em 1894) tem grande significado biológico devido à sua elevada reatividade e nocividade (FRIDOVICH, 1986; PRYOR, 1986; HALLIWELL & GUTTERIDGE, 1999). A elevada reatividade do $HO^{\bullet}$, apesar de suas concentrações intracelulares extremamente baixas e seu reduzidíssimo tempo de vida-média, conferem uma toxicidade tão elevada aos organismos aeróbios, que as proteções enzimáticas correspondentes às moléculas geradoras ($O_2^{\bullet-}$ e H_2O_2) ultrapassam várias vezes as

concentrações intracelulares estimadas para seus substratos (CHANCE et al., 1979). O $HO^{\bullet}$ é o RLO mais reativo, pois, na busca imediata de sua estabilidade, este radical transforma as moléculas circundantes em radicais, que, por sua vez, também precisam estabilizar-se. Esta seqüência de eventos é que dá origem às reações em cadeia com os constituintes celulares, oxidando resíduos de aminoácidos produzindo as bases de Schiff, pode também levar á uma alteração química das bases púricas e pirimídicas quebrando a fita de DNA. Pode ainda atacar os lipídios de membrana celular, além de danos a proteínas e a outras moléculas orgânicas (HALLIWELL & GUTTERIDGE, 1999).

A toxicidade do oxigênio é um fenômeno contínuo e presente mesmo sob normóxia em organismos aeróbios sadios dotados de diferentes e específicas defesas antioxidantes (WILHEM FILHO, 1994). A partir do descobrimento da enzima superóxido dismutase (McCORD & FRIDOVICH, 1969), foi possível verificar mais consistentemente a hipótese formulada por Gershman et al. (1954) de que a principal causa de toxicidade do oxigênio era decorrente da ação das EROS.

O interesse relativo aos danos provocados pelas EROS em nível molecular, celular e de organismo, cresceu enormemente durante as últimas décadas, principalmente pelas evidências ou suspeitas vinculando as EROS a diversas patologias humanas e ao processo de envelhecimento (JENKINS & GOLDFARB, 1993). Destacam-se especialmente aquelas relacionadas com o sistema imunológico (onde as EROS desempenham também papel de defesa), aparelho respiratório, cardiovascular, neurológico e da visão, ademais de processos ligados à carcinogênese e ao processo de envelhecimento (JI, 1993; HALLIWELL & GUTERIDGE, 1999).

Diversos autores citam o envolvimento das EROS em um grande número de patologias, como causa secundária a doenças crônico-degenerativas, ao aparecimento de alguns tipos de câncer e ao processo de envelhecimento humano. Neste sentido, à medida que as pessoas envelhecem, ocorre uma diminuição na eficácia do sistema das enzimas antioxidantes, enquanto que a formação de EROS se mantém ou é aumentada (PAOLISSO et al., 1998).

3.2.2 Antioxidantes

Halliwell & Gutteridge (1999) definem como antioxidante qualquer substância que, quando presente em baixas concentrações, comparadas a de um substrato oxidável, retarda ou inibe significativamente a oxidação deste substrato. Esta definição compreende compostos de natureza enzimática e não enzimática (Tabela 2). Através de diferentes mecanismos, as EROS são inativadas de forma a impedir reações posteriores de propagação (SIES, 1993).

Tabela 2 – Resumo das principais defesas antioxidantes nos sistemas biológicos.

SISTEMA	FUNÇÃO
Não Enzimáticos	
α-Tocoferol	Intercepta reações de lipoperoxidação
β- Caroteno	Quencher de O_2^-
Licopeno	Quencher de O_2^-
Ubiquinol 10	Seqüestrador de radicais
Ácido Ascórbico	Inúmeras funções antioxidantes
Enzimáticos	
Superóxido dismutase	Reações de dismutação de $O_2^{\bullet -}$
Catalase	Catalisa a reação sobre H_2O_2
Glutationa Peroxidase	Catalisa a reação sobre peróxidos
Enzimáticos Auxiliares	
Glutationa-S-Transferase	Conjugação e excreção de xenobióticos
Glutationa Redutase	Colabora no ciclo da GSH

Fonte: SIES, 1993.

Entre as principais enzimas responsáveis pela defesa antioxidante do organismo destacam-se a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), que constituem a primeira defesa endógena de neutralização das EROS. Através delas,

as células tentam manter baixas as quantidades do radical superóxido e de peróxidos de hidrogênio, evitando assim, a formação do radical hidroxil (BOVERIS & CADENAS, 1997).

A SOD, presente na quase totalidade dos organismos eucarióticos, catalisa a dismutação do radical $O_2^{\bullet-}$ em H_2O_2 (MCCORD & FRIDOVICH, 1969): $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$. O H_2O_2 por sua vez é degradado pela ação da CAT ou GPx, resultando em O_2. e H_2O, respectivamente (FARBER, 1990): $2\ H_2O_2 \rightarrow H_2O + O_2$.

Entre os antioxidantes não enzimáticos destaca-se a vitamina C (ácido ascórbico), que tem sido mostrado como um eficiente seqüestrador de $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$ e do radical peroxil ($^{\bullet}OOL$) (ROSE, 1987). O ascorbato age protegendo biomembranas contra a peroxidação, perpetuando, desta forma, a atividade do α-tocoferol, um antioxidante não enzimático lipossolúvel. O ácido ascórbico é um dos antioxidantes mais importantes em tecidos de mamíferos (BANHEGYI et al., 1997), tendo sido demonstrado que ele é eficiente na redução da toxicidade de vários xenobióticos, tais como chumbo (FOX, 1995), organofosforados (CHAKRABORTY et al., 1978) e cloreto de mercúrio (CHATTERJEE & RUDRA PAL, 1975).

3.3 Estresse oxidativo e transplante de medula óssea

Em todo o procedimento de preparação para o TMO (obtenção de células e regime de condicionamento), após o TMO e durante a internação, os pacientes apresentam toxicidade orgânica intensa e variável, que está associada à produção de radicais livres e a deficiência de antioxidantes, que podem levar a complicações importantes, como a falência hepática entre outras (WILMORE et al., 1999).

3.3.1 Quimioradioterapia e estresse oxidativo

Quimioterapia e radioterapia estão associados com a geração de grandes quantidades de espécies reativas de oxigênio/nitrogênio e depleção de antioxidantes, resultando em

estresse oxidativo (SANGETHA et al., 1990; DURKEN et al., 1995, 2000; CETIN et al., 2004). Dados da literatura indicam que a quimioterapia e a radioterapia provocam um aumento na peroxidação lipídica e uma diminuição na concentração de antioxidantes como vitaminas A, C e E e também no conteúdo de glutationa reduzida (GSH) em pacientes transplantados de medula óssea (HUNNISETT et al., 1995; JONAS et al. 2000).

3.4 A enzima delta-aminolevulinato desidratase (δ-ALA-D)

3.4.1 Histórico e função

A enzima citoplasmática delta-aminolevulinato desidratase (δ-ALA-D, E.C.4.2.1.24), também conhecida como porfobilinogênio sintase ou 5-aminolevulinato hidroliase foi isolada na década de 50 (DRESEL & FALK, 1953). Esta enzima catalisa a condensação assimétrica de duas moléculas de ácido delta-aminolevulinato (ácido 5-aminolevulínico, ALA), com perda de duas moléculas de água, para formar o composto monopirrólico porfobilinogênio (PBF) (JAFFE, 1995).

A reação catalisada pela δ-ALA-D faz parte da rota biossintética dos compostos tetrapirrólicos (corrinas, bilinas, clorofilas e hemes). A grande importância destes compostos reside na sua função como grupos prostéticos de proteínas. O heme (ferroprotoporfirina), faz parte da estrutura de proteínas que participam do transporte de oxigênio (hemoglobina e mioglobina), transporte de elétrons (citocromos a, b e c), biotransformação de xenobióticos (citocromo P_{450}) e do sistema de proteção contra peróxidos (catalases e peroxidases) (JAFFE, 1995).

A via para a biossíntese de porfirinas é semelhante em bactérias, vegetais e animais, favorecendo a ampla distribuição da δ-ALA-D na natureza (RODRIGUES, 1987). Em mamíferos os tecidos que apresentam maior atividade são o hepático, o renal e os tecidos hematopoiéticos (GIBSON et al., 1955). Há alguns anos foi demonstrado que a enzima δ-ALA-D é idêntica ao inibidor de proteossomas de 240-kDa (CF-2) (GUO et al., 1994). Estes achados conferem à δ-ALA-D uma importância adicional, uma vez que os proteossomas

atuam na degradação de proteínas anormais, fatores de transcrição, oncoproteínas, bem como no processamento de antígenos (WLODAWER, 1995).

3.4.2 Características estruturais

Existe uma grande similaridade entre as seqüências do gene da δ-ALA-D isolada de diversas fontes, sugerindo que a enzima apresenta estrutura e mecanismo básico de ação similares em diferentes organismos.

A δ-ALA-D de fígado bovino possui massa molecular de 280 kDa (TIGIER, 1970; WU et al., 1974; SHEMIN, 1976), sendo composta por 8 subunidades de 35 kDa cada uma, arranjada em uma estrutura cúbica octamérica, com simetria diédrica (WU et al., 1974).

Todas as enzimas δ-ALA-D isoladas até o momento requerem um íon metálico bivalente para estarem ativas, sendo em sua maioria inibidas por EDTA. Apesar da similaridade entre os genes da δ-ALA-D proveniente de diversos organismos, a enzima requer metais diferentes para sua ativação, de acordo com a sua fonte. A δ-ALA-D de animais, leveduras e de algumas bactérias é uma enzima dependente de zinco (FINELLI et al., 1974), tendo sido demonstrado o envolvimento de resíduos de cisteína na união deste metal (MITCHELL & JAFFE, 1993).

A δ-ALA-D, independente de sua fonte, é uma enzima de natureza sulfidrílica (SHEMIN, 1976), sendo inibida por agentes bloqueadores de grupos tiólicos, tais como N-etilmaleimida, iodoacetato (BATLLE et al.,1967), paracloromercuriobenzoato, monoiodoacetamida e DTNB (BATLLE et al., 1967; TIGIER et al., 1970; BARNARD et al., 1977) e por metais pesados que possuem afinidade por grupamentos sulfidrílicos, tais como chumbo, cobre e mercúrio (GIBSON et al., 1955; ROCHA et al., 1993; EMANUELLI et al., 1996). Alguns compostos orgânicos e inorgânicos de selênio e telúrio também podem inibir a enzima δ-ALA-D pela oxidação de seus grupamentos sulfidrílicos (BARBOSA et al., 1998).

A enzima é facilmente inativada durante a purificação e a perda de atividade está relacionada com a perda de dois grupos sulfidrila/subunidade. Para a obtenção da atividade catalítica máxima é necessária, geralmente a adição de ativadores tiólicos, como DTT, cisteína, glutationa e ß-mercaptoetanol. Entretanto quando a enzima é isolada na presença de

zinco ou de um agente redutor, ela apresenta atividade máxima mesmo sem a adição de um ativador tiólico no meio de incubação (TSUKAMOTO et al., 1979). Até o momento foram identificadas três isoenzimas diferentes em humanos, designadas δ-ALA-D 1-1, δ-ALA-D 1-2 e δ-ALA-D 2-2 (BATTISTUZZI et al., 1981; PETRUCCI et al., 1982), resultantes da expressão de dois alelos comuns ALA-D¹ e ALA-D². Foi observado que indivíduos portadores do alelo ALA-D² apresentam maior risco de intoxicação por chumbo, possivelmente devido à maior afinidade da enzima pelo chumbo, determinada por este alelo (WETMUR, 1994).

3.4.3 Ação catalítica

O sítio da enzima parece ser composto por resíduos de cisteína, dois átomos de zinco, um resíduo de histidina, um resíduo de lisina e resíduos de aminoácido hidrofóbicos (TSUKAMOTO et al., 1979).

Atualmente sabe-se que três tipos diferentes de aminoácidos são essenciais para a atividade da δ-ALA-D:

- um resíduo de lisina ao qual se liga a primeira molécula de substrato, através de uma base de Schiff (NANDI, 1978);
- um resíduo de histidina, o qual pode sofrer fotoxidação, reduzindo tanto a atividade enzimática quanto a ligação ao zinco (TSUKAMOTO et al., 1979). Este resíduo poderia participar no mecanismo de transferência de prótons do meio aquoso ao sítio ativo hidrofóbico (BATLLE & STELLA, 1976);
- dois resíduos de cisteína, os quais devem estar reduzidos para que a enzima apresente atividade (CHEN & NEILANDS, 1976). Estes resíduos são altamente reativos (GIBBS et al., 1985), podendo formar uma ponte dissulfeto em presença de ar, formar mercaptídios por reação com metais pesados ou ser modificados por agentes químicos. A oxidação desses resíduos leva à inativação com concomitante perda do zinco (TSUKAMOTO et al., 1979).

3.4.4 Importância toxicológica

Devido à sua natureza sulfidrílica, a enzima δ-ALA-D pode ser inibida por uma variedade de metais pesados e não metais que possuem a propriedade química de oxidar grupamentos –SH. Sabe-se que o prognóstico de pacientes que sofreram intoxicação por chumbo pode ser avaliado de acordo com a inibição da δ-ALA-D de eritrócitos e seu índice de reativação por DTT (BONSIGNORE, 1966). A inibição da δ-ALA-D pode prejudicar a rota biossintética do heme, resultando em consequências patológicas (SASSA et al., 1989; GOERING, 1993). Além da insuficiente produção de heme, a inibição da δ-ALA-D pode resultar no acúmulo do substrato ALA no sangue, com conseqüente aumento na excreção urinária do mesmo. O acúmulo do ALA está relacionado com a superprodução de espécies reativas de oxigênio (MONTEIRO et al., 1989; BECHARA et al., 1993), que podem atuar nos mais diferentes órgãos e organelas celulares onde são gerados, podendo acarretar em conseqüências patológicas inespecíficas (SASSA et al., 1989).

4 ARTIGOS CIENTÍFICOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigos científicos e manuscrito, os quais se encontram aqui organizados. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se no próprio artigo ou manuscrito. Os artigos e manuscrito estão dispostos da mesma forma que foram submetidos para a publicação nas revistas.

Os itens DISCUSSÃO E CONCLUSÕES, encontrados no final desta tese, apresentam interpretações e comentários gerais sobre o artigo e os manuscritos científicos contidos neste trabalho.

4.1 δ-ALA-D activity is a reliable marker for oxidative stress in bone marrow transplant patients

Manuscrito em fase de correções para publicação na Revista BMC Cancer

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Background: Bone marrow transplantation (BMT) is often used in the treatment of various diseases. Before BMT, patients are submitted to a conditioning regimen (CR), which consists of the administration of high doses of chemotherapy. The action of many cytostatic drugs involves the overproduction of reactive oxygen species, which together with inadequate antioxidant protection can lead to oxidative stress and this has been implicated in the etiology of various diseases. The objectives of this study were to look for evidence of oxidative stress and also to analyze δ-Aminolevulinato dehydratase (δ-ALA-D) activity as a possible marker of oxidative stress in autologous and allogeneic BMT patients.

Methods: Lipid peroxidation, vitamin C and thiol group levels as well as catalase, superoxide dismutase and δ-ALA-D activity were determined in 37 healthy controls, 13 patients undergoing autologous peripheral blood stem cell transplantation and 24 patients undergoing allogeneic BMT.

Results: We found that patients presented signs of oxidative stress before they were submitted to BMT, during CR and up to 20 days after BMT. There was a decrease in enzymatic and non enzymatic antioxidant defenses, in δ-ALA-D activity, and an increase in lipoperoxidation in the blood of both patient groups.

Conclusion: This study has indicated that autologous and allogeneic BMT are associated with oxidative stress. Moreover, blood δ-ALA-D activity seems to be an additional biomarker of oxidative stress in BMT patients.

Background

Bone marrow transplantation (BMT) is a therapeutic method used in various malignant, hematologic, immunologic, and genetic diseases [1]. Allogeneic transplantation involves the transfer of marrow from a donor to another person, and one of the main problems in this procedure is the graft-versus-host disease [2]. Allogeneic transplantations are preferred for patients with leukemias or myeloproliferative diseases [3] whereas autologous transplantations are generally performed in patients with multiple myeloma (MM) or lymphoma [4,5]. Autologous BMT involves the use of the patient's own marrow to reestablish hematopoietic cell function after the administration of high-dose chemotherapy. In this case the stem cells can be taken from the patient's bone marrow or peripheral blood [2]. Peripheral blood stem cell transplantation (PBSCT) with collection by apheresis has

replaced bone marrow as a source of hematopoietic stem cells for autologous transplantation, because this type of collection results in a more rapid hematopoietic recovery [5,6]. In contrast, in allogeneic transplantation, bone marrow continues to be the principal source of cells [7].

Unlike allogeneic transplantation, autologous transplantation is a more simple procedure that can be performed safely in older patients, because there is no risk of graft-versus-host disease as a complication [2]. However, the high incidence of relapse as a consequence of reinfusion of malignant stem cells is an important problem in autologous BMT [5].

BMT is always associated with intense and variable organic toxicity and with severe and prolonged myelosuppression, which give rise to a period of high vulnerability in which patients may develop complications. Administration of high doses of chemotherapy with or without total body irradiation (TBI) is a feature of BMT protocols known as the CR. These regimens are designed to abate the underlying malignant cells in the autologous BMT, to cause immunosuppression in order to avoid destruction of the allograft, and to destroy any residual cancer cells in allogeneic BMT [2].

The production of free radicals and the deficiency of antioxidants can to lead to a condition known as oxidative stress, which can be associated with serious complications in different types of transplantation [8]. Chemotherapy and radiotherapy are associated with the generation of large amounts of reactive oxygen/nitrogen species and depletion of antioxidants (vitamins A, C and E, reduced glutathione-GSH) and with an increase in plasma lipid hydroperoxides and TBARS [9-16]. Sulfhydryl groups (-SH) play important roles in a variety of cell activities and they can be easily modified by oxidants or alkylating agents [17]. In fact, –SH group content can be depleted after disproportional oxidation by free radicals or after formation of adducts with reactive chemicals [18].

δ-Aminolevulinate dehydratase (δ-ALA-D) is a sulfhydryl-containing enzyme [19,20] that catalyses the synthesis of tetrapyrrolic compounds such as billins and hemes. Consequently, δ-ALA-D is essential for aerobic organisms [21,22]. Recently, data from literature and from our laboratory have indicated that δ-ALA-D is sensitive to situations associated with oxidative stress [23-27]. Furthermore, enzyme inhibition can lead to accumulation of its substrate, 5-aminolevulinate (ALA), in the blood, which in turn can

intensify oxidative stress by generating carbon-centered reactive species or by releasing iron from proteins such as ferritin [28].

The BMT procedure is toxic and excessive free radical production has been implicated in the action of many cytostatic drugs [29], but little is known about how cytostatic drugs affect the antioxidative system in human beings and only a few studies have been performed in the clinical setting [10]. In this context, the objective of this study was to look for further evidence of oxidative stress and also to analyze δ-ALA-D activity as a possible marker of oxidative stress in autologous and allogeneic BMT patients as well as to compare these two procedure in order to determine whether either of them could be less toxic to patients, when markers of oxidative stress are used as the end point of toxicity.

Methods

Subjects

Altogether, 37 patients: 13 undergoing autologous PBSCT, 03 female and 10 male, mean age 48.15±12.67 years, 24 undergoing allogeneic BMT, 12 female and 12 male, mean age 34.13±15.41 and 37 controls (matched by age and sex with the patients) were included in the investigation (samples were obtained from March 2007 to March 2008). The patients were under treatment in the 'Hospital Universitário' from the 'Universidade Federal de Santa Maria' (HUSM), RS, Brazil. They were treated with autologous PBSCT or allogeneic BMT which were preceded by CR (see table 1).

The present study was approved by the Human Ethical Committee of the Universidade Federal de Santa Maria, protocol number 0152.0243.000-06. All persons gave their informed consent prior to their inclusion in the study.

The subjects included in the study were classified in three groups: Group I – Control: healthy volunteers of HUSM Blood Bank served as control group and they were receiving no treatment for any diseases; Group II (autol.): autologous PBSCT patients; Group III (allog.): allogeneic BMT patients.

Table 1 shows the characteristics of the patients included in this study.

In our study, the patients were significantly older in group II (autol.) than in group III (allog.), $p<0.05$. This is in agreement with the literature, where 40 to 55 years is considered the oldest age for patients undergoing allogeneic transplantation and autologous

transplantation is reported to be safer in older patients, because there is no risk of graft-versus-host disease as a complication [2]. In our study there were also fewer females in group II (autol.) than in group III (alog.), perhaps because the autologous procedure is utilized for treatment of diseases that are predominant in males, such as lymphomas. Some studies have shown a male predominance in this disease [30-32] as well as in myeloma [33].

Sample collection

Blood (4 mL) was collected during routine examinations by venous arm puncture in EDTA vaccutainer tubes in the Hematology and Oncology Laboratory. The blood used in this study was the leftover of this 4 mL sample. Four samples of each patient were taken, the first before CR, the second during CR (the last day the patient received chemotherapy), the third 10 days after BMT and the fourth 20 days after BMT. Control group samples were the leftover blood of HUSM Blood Bank donors, which was collected on the same day of the matched patient. Only one sample was used from these volunteers (before CR, CR, 10 and 20 days after BMT, consequently, control values for each time point are from different subjects). The plasma and cells were separated by centrifugation at 1000 rpm for 12 min. Then a portion of the collected plasma and erythrocytes were stored for analysis at –20^{o}C for less than 3 weeks. The other analyses were performed on the same day.

Biochemical estimations

All biochemical assays were made in duplicates or triplicates, depending on the availability of samples.

Thiobarbituric acid–reactive substances

Thiobarbituric acid-reactive substances (TBARS) assay measures the peroxidative damage to lipids that occurs by excessive ROS generation. Lipid peroxidation was estimated in plasma according to the method of Lappena et al. (2001), using 1% phosphoric acid and 0.6% thiobarbituric acid (TBA). The pink chromogen produced by reaction of TBA with malondialdehyde (MDA), was measured spectrophotometrically at 532 nm. The results were expressed as nmol TBARS/mL plasma, using MDA as standard [34].

Catalase enzyme activity

Catalase (CAT) enzyme activity was measured by the method of Aebi (1984). Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water, then, 20 µL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 µL of freshly prepared 300 mM H2O2 in phosphate buffer (50 mM, pH 7.0) to give a final volume of 1 mL. The rate of H2O2 decomposition was measured spectrophotometrically at 240 nm during 120 s. The catalase activity was expressed as µmol H2O2/mL erythrocytes/min [35].

Superoxide dismutase enzyme activity

Superoxide dismutase (SOD) activity was performed according to the method of Misra and Fridovich (1972). Briefly, epinephrine rapidly auto oxidizes at pH 10.5 producing adrenochrome, a pink colored product that can be detected at 480 nm. The addition of samples containing SOD inhibits epinephrine auto-oxidation. The inhibition rate was monitored during 150 s at intervals of 10 s. The amount of enzyme required to produce 50% inhibition at 25 °C was defined as one unit of enzyme activity. The SOD activity was expressed as U/mL erythrocytes [36].

Vitamin C

Plasma vitamin C (VIT C) was estimated as described by Galley et al. (1996) with some modifications by Jacqes-Silva et al. (2001). Plasma was precipitated with 1 volume of a cold 5% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 µL of the supernatants were mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL) and 13.3% trichloroacetic acid and incubated for 3h at 37° C. Then, 1 mL 65% sulfuric acid was added to the medium and the orange red compound was measured at 520 nm. The content of ascorbic acid was calculated using a standard curve (1.5 – 4.5 µmol/L ascorbic acid freshly prepared in sulfuric acid) and expressed as µg vit C/mL plasma [37,38].

Protein thiol groups

Protein thiol groups (P-SH) were assayed in plasma by the method of Boyne and Ellman (1972) modified by Jacques-Silva et al. (2001), which consisted of the reduction of 5.5´-dithio(bis-nithrobenzoic) acid (DTNB) in 0.3 M phosphate buffer, pH 7.0, measured at

412 nm. Quantification of total protein –SH groups may indicate thiol status and may also indicate the general state of thiol-containing proteins and, indirectly, the redox state of the blood cells. A standard curve using glutathione was constructed in order to calculate the protein thiol groups. The results were expressed as nmol P-SH/mL plasma [39,38].

Non protein thiol groups

Erythrocyte non protein thiol groups (NP-SH) were determined as described by Boyne and Ellman (1972) modified by Jacques-Silva et al. (2001). Red blood cell pellets (300 μL) obtained after centrifugation of whole blood were hemolyzed with 10% triton solution (100 μL) for 10 min. Then, the protein fraction was precipitated with 200 μL of 20% trichloroacetic acid followed by centrifugation. Quantification of non-protein thiols (NP-SH) may indicate 90% of GSH content in the blood [40], which is one of the most important reducing agents in different mammal cells [16]. The colorimetric assay was carried out in 1 M phosphate buffer, pH 7.4. A standard curve using glutathione was constructed in order to calculate non protein thiol groups. The NP-SH level was measured at 412 nm and expressed as nmol NP-SH/mL erythrocytes. [39,38].

δ-Aminolevulinate dehydratase (δ-ALA-D) activity

δ-Aminolevulinate dehydratase (δ-ALA-D) activity was assayed in whole blood by the method of Berlin and Schaller [41] by measuring the rate of porphobilinogen (PBG) formation in 1 h at 37°C. The enzyme reaction was initiated after 10 min of pre-incubation of blood with 1mM ZnCl2. The reaction was started by adding δ-aminolevulinic acid (ALA) to a final concentration of 4 mM in a phosphate buffered solution, and incubation was carried out for 1 h at 37° C and the reaction product was measured at 555 nm and expressed as nmol PBG/mL blood/h.

Statistical analysis

Biochemical assay results were expressed as median (lower/ upper quartiles) and ages were expressed as mean ± SD.

Since data had no homogeneity of variance, statistical analysis was performed using Kruskal-Wallis ANOVA followed by Mann-Whitney U test to compare the difference among the groups, Friedman ANOVA followed by Wilcoxon test to analyze changes in blood indices

over time and Spearman correlation to analyze correlations between biochemical estimations. Test T was used to compare the difference in the ages and sex among the groups. A value of $p<0.05$ was considered statistically significant.

Results

Lipid peroxidation

Plasma TBARS levels were significantly higher in group II (autol.) than group I (control), before CR, during CR and on days 10 and 20 after BMT, ($p<0.05$). TBARS levels were higher in group III (allog.) than in group I (control), during CR and on days 10 and 20 after BMT, ($p<0.0001$). Groups II and III were not significantly different from each other in TBARS levels. TBARS levels increased from before CR and during CR to on days 10 and 20 after BMT in group III ($p<0.05$), (table 2).

Vitamin C

Plasma vitamin C (VIT C) concentrations were significantly decreased in groups II (autol.) and III (allog.), before CR ($p<0.05$), during CR and on days 10 and 20 after BMT ($p<0.005$), when compared with group I (control). Groups II and III were not significantly different from each other in VIT C levels. In group II, VIT C levels decreased significantly from before CR to during CR and in group III from before CR to during CR and on days 10 and 20 after BMT ($p<0.05$), (table 3).

Catalase

Erythrocyte catalase (CAT) activity was significantly lower in group II (autol.) than in group I (control) and on day 10 after BMT, ($p<0.05$) and was also lower in group III (allog.) than in group I (control) during CR ($p<0.01$). Groups II and III were not significantly different from each other in erythrocyte CAT activity. In group II, CAT activity decreased from during CR to day 10 after BMT ($p<0.05$), (Fig. 1).

Superoxide dismutase

Erythrocyte superoxide dismutase (SOD) activity was significantly lower in group II (autol.) than in group I (control), on day 10 after BMT ($p<0.0005$) and was also lower in

group III (allog.) than in group I (control) during CR ($p<0.05$) and on day 10 after BMT ($p<0.01$). Groups II and III were not significantly different from each other in erythrocyte SOD activity and enzyme activity did not change over time in these groups (Fig. 2).

Protein thiol groups

Plasma protein thiol (P-SH) group levels were significantly lower in groups II (autol.) and III (allog.), before CR ($p<0.01$), during CR and on days 10 and 20 after BMT, ($p<0.00001$) when compared with group I (control). P-SH levels in groups II and III were not significantly different from each other. P-SH levels decreased from before CR to day 20 after BMT in the patients of group III and decreased from during CR to day 20 after BMT in group II ($p<0.05$), (table 4).

Non protein thiol groups

Erythrocyte non protein thiol (NP-SH) group levels were significantly higher in group II (autol.) during CR than in group III (allog.) ($p<0.05$). NP- SH levels in groups II and III were not significantly different from group I (control). Erythrocyte NP-SH levels decreased from before CR to during CR in group III (allog.) ($p<0.01$), (table 5).

δ-Aminolevulinate dehydratase

Blood δ-ALA-D activity was significantly lower in groups II (autol.) and III (allog.) than in group I (control) during CR and on days 10 and 20 after BMT ($p<0.005$). δ-ALA-D activity decreased from before CR to during CR and on days 10 and 20 after BMT in group III (allog.) and also in group II (autol.), where δ-ALA-D increased from during CR to day 20 after BMT ($p<0.05$). Blood δ-ALA-D activities in groups II and III were not significantly different from each other (table 6).

Correlations of biochemical estimations

Statistical analysis (Spearman correlation) revealed a significant negative correlation between TBARS and P-SH and a positive correlation between SOD and δ-ALA-D before CR for all groups (Panel 1, A). There was significant negative correlation between TBARS and CAT, P-SH and δ-ALA-D and a positive correlation between VIT C and P-SH and δ-ALA-D

as well as between CAT and SOD and δ-ALA-D and also between δ-ALA-D and SOD and P-SH during CR for all groups (Panel 1, B). There was a significant negative correlation between TBARS and VIT C, SOD, P-SH and δ-ALA-D and a positive correlation between VIT C and SOD, P-SH and δ-ALA-D as well as between CAT and SOD and between SOD and P-SH and δ-ALA-D and also between P-SH with δ-ALA-D on day 10 after BMT for all groups (Panel 1, C). There was a significant negative correlation between TBARS and VIT C, P-SH and δ-ALA-D and a positive correlation between VIT C and P-SH and δ-ALA-D as well as between CAT and SOD and also between P-SH and δ-ALA-D on day 20 after BMT for all groups (Panel 1, D).

Discussion

Lipid peroxidation can cause a profound alteration in the structural integrity and functions of cell membranes and free radical-induced lipid peroxidation has been implicated in the pathogenesis of several disorders, including cancer [42]. Treatment with radiotherapy and chemotherapy has been shown to cause peroxide accumulation [9,13,43-45]. In our study, both procedures, autologous PBSCT and allogeneic BMT, were associated with higher TBARS levels during CR, and after BMT when compared with group I (control) and TBARS also showed a negative correlation with the antioxidants. However, in group II (autol.), this increase was already present before CR and there was no further increase in lipid peroxidation, probably because of the disease itself and procedures carried out previously, such as chemotherapy that precedes the collection of the patient's own bone marrow for posterior use in BMT. However, in group III (allog.), there was an increase in TBARS levels over time. This indicates that, principally in group III, both the CR and the procedure itself (BMT) were associated with an increase in lipid peroxidation.

Vitamin C is a powerful reducing agent and an important water-soluble vitamin for humans and it can scavenge $O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$, aqueous peroxy radicals and singlet oxygen. Vitamin C also protects plasma lipids against lipid peroxidation and has an important role in the regeneration of α-tocopherol [46]. VIT C levels were lower in the plasma of patients from groups II (autol.) and III (allog.) than in group I (control), at all time points analyzed and VIT C also showed a negative correlation with TBARS. The CR in both procedures, autologous PBSCT and allogeneic BMT, caused a decrease in VIT C, indicating that this

antioxidant defense was impaired in both groups. These results are in agreement with data published in literature [13].

Thiols fulfill important antioxidant functions in cells and biological fluids. The changes in the content of -SH groups, may indicate membrane protein damage [47]. Alkylating agents, a common group of drugs utilized in the CR, produce free radicals that can directly interact with thiol groups of proteins [17], oxidizing them to disulfides [18]. In our study, autologous and allogeneic patients presented plasma P-SH levels lower than those of healthy volunteers and also a negative correlation with TBARS, at all time points analyzed. There was also a decrease in plasma P-SH levels after the CR and BMT, demonstrating that the protein thiol groups were oxidized by cytostatic drugs utilized in the CR preceding BMT. Erythrocyte NP-SH group levels in the patient groups were not significantly different from those of the control group, however, NP-SH levels were lower in allogeneic patients (group III) than in autologous patients (group II). In group III, there was also a decrease in NP-SH levels from before CR to during CR, demonstrating that the allogeneic procedure triggered a major imbalance in this antioxidant.

As demonstrated, CAT and SOD enzymatic antioxidants were similar in the patient groups and control group before the initiation of chemotherapy or radiochemotherapy. SOD is the key enzyme required for the removal of $O_2^{\cdot-}$ by converting it to hydrogen peroxide (H_2O_2), which can be eliminated by CAT and peroxidases. Catalase helps in neutralizing the toxic effect of H_2O_2 converting it to water and non-reactive oxygen species, thus it prevents the generation of hydroxyl radicals and protects cells from oxidative damage [48]. Our study demonstrated that after the transplant, SOD and CAT activity were lower in both groups II and III, when compared with the control group, at different times, and also demonstrated a positive correlation between these enzymes. This shows an imbalance in enzymatic antioxidants caused by autologous and allogeneic procedures, which together with lipid peroxidation indicates a state of oxidative stress in both situations. Thus, it is plausible to suppose that oxidative stress can exacerbate the complications in patients undergoing bone marrow transplantation.

Blood δ-ALA-D activity before the CR in both patient groups was similar to that of the control group and chemotherapy or radiochemotherapy (CR) led to a decrease in δ-ALA-D activity over time in the patient groups. δ-ALA-D is a zinc metalloenzyme, essential for all aerobic organisms, that requires reduced thiol groups for its activity [49]. Reactive oxygen

species, produced during the CR [9-12] can oxidize thiol groups located inside the active site of mammalian δ-ALA-D [50,51], decreasing its activity as observed here. In fact, the decrease in δ-ALA-D activity after the CR was extremely accentuated and this occurred in all patient groups, and δ-ALA-D also showed a significant positive correlation with important antioxidants such as VIT C, CAT, SOD and P-SH and a negative correlation with TBARS, indicating that δ-ALA-D activity is a reliable marker for oxidative stress in BMT. Finally, δ-ALA-D inhibition can result in ALA accumulation, which can have pro-oxidant effects [52] and contribute to oxidative stress caused by the CR and also by the bone marrow transplantation itself. In fact, an increase of ALA is typically associated with porphyria, hereditary tyrosine and leads to poisoning and its accumulation causes oxidative stress and may lead to cancer [53]. Thus, an inhibition of δ-ALA-D after the CR may contribute to cancer relapse or secondary malignancies that may arise after chemotherapy [11]. However, a decrease in δ-ALA-D could be due to the general decline of the heme route, which does not implicate ALA accumulation. To answer this question, it is necessary to study the activity of ALA-S (δ-aminolevulinic acid synthase), the rate-limiting step of heme synthesis, which could indicate whether there is, in fact, ALA accumulation.

In conclusion, the results presented here indicate that some patients undergoing BMT present signs of oxidative stress even before transplantation, which are possibly a consequence of their disease or previous procedures, as is probably the case in autologous patients, which already presented increased lipid peroxidation even before the transplantation. However, in allogeneic patients this increase occurred during the CR. We also observed that the CR and the autologous or allogeneic transplantation themseles promoted modifications in the levels of non-enzymatic and enzymatic antioxidant defenses and in δ-ALA-D activity. Moreover, blood δ-ALA-D activity seems to be an additional biomarker of oxidative stress in BMT patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TLG performed the study, carried out the assays, performed the statistical analysis and drafted the manuscript. DMB and GB carried out the assays and helped in the acquisition of data. AVF carried out the assay. JBTR helped in the statistical analysis and participated in the design and the editing of the manuscript. All authors read and approved the final manuscript.

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Legends of figures

Figure 1. Erythrocyte catalase (CAT) activity over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).[a] Significantly different from group I (control), [d] significantly different from CR.

Figure 2. Erythrocyte superoxide dismutase (SOD) activity over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).[a] Significantly different from group I (control).

Table 1. Characteristics of patients

Number of patients (n=37)	Autologous PBSCT group II (n=13)	Allogeneic BMT group III (n=24)
Mean age (years)	48.15±12.67[b]	36.00 ±16.08
Male	10	12
Female	03[b]	12
Diagnosis		
Multiple myeloma	03	01
Lymphoma Hodgkin	05	03
Lymphoma No Hodgkin	02	02
Leukemia		
Leukemia Acute myeloid	01	02
Leukemia Chronic myeloid	00	07
Leukemia Acute lymphoblastic	01	01
Leukemia Chronic lymphoid	00	01
Myelodysplasia	00	05
Aplastic anemia	00	02
Tumor Ewing	01	00
Conditioning regimen		
BuCy 120	01	13
BuCy 200	01	00
M-200	04	01
CBV	04	00
FluCy	00	05
BEAM	02	00
CyTBI	01	03
another	00	02

The CR patients were divided according to diseases. M-200: melphalan (200 mg/m2) given only 1 day (day minus 3) before the transplantation for multiple myeloma in autologous peripheral blood stem cell transplantation (PBSCT) or allogeneic BMT. CBV: cyclophosphamide (1500 mg/m2) given 4 consecutive days starting 6 days before the transplantation (days minus 6 to minus 3) + carmustine (BCNU - 450 mg/m2) given only 1 day before the transplantation (day minus 6) + etoposide (VP-16 – 250 mg/m2) given 3 consecutive days two times a day, starting 6 days before the transplantation (days minus 6 to minus 4) or BEAM: carmustine (BCNU - 300mg/m2) given only 1 day before the transplantation (day minus 7) + etoposide (VP-16 – 200 mg/m2) given 4 consecutive days two times a day, starting 6 days before the transplantation (days minus 6 to minus 3) + cytarabine (100 mg/m2) given 4 consecutive days two times a day, starting 6 days before the transplantation (days minus 6 to minus 3) + melphalan (140 mg/m2) given only 1 day before the transplantation (day minus 6) for Hodgkin and non-Hodgkin lymphomas in autologous PBSCT. FluCy: fludarabine (30 mg/m2) + cyclophosphamide (300 mg/m2), both given 3 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 2) for Hodgkin and non-Hodgkin lymphomas in allogeneic BMT. BuCy120: busulphan (1 mg/kg), given 4 consecutive days four times a day, starting 8 days before the transplantation (days minus 8 to minus 5) + cyclophosphamide (60 mg/kg), given 2 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 3) for acute and chronic myeloid leukemia and syndrome mielodysplastic in autologous PBSCT or allogeneic BMT. BuCy200: busulphan (1 mg/kg), given 3 consecutive days four times a day, starting 9 days before the transplantation (days minus 9 to minus 7) + cyclophosphamide (50 mg/m2), given 4 consecutive days, starting 6 days before the transplantation (days minus 6 to minus 3) for aplastic anemia in allogeneic BMT. CyTBI: cyclophosphamide (60 mg/m2), given 2 consecutive days, starting 7 days before the transplantation (days minus 7 to minus 6) + total body irradiation, carried out on 4 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 2, three times a day and day minus 1, two times a day) for acute and chronic lymphoid leukemia in autologous PBSCT or allogeneic BMT. All patients had 2 rest days (without chemotherapy) before the transplantation (days minus 2 and minus 1), except FluCy and CyTBI CR patients, that had only one rest day (day minus 1 and minus 5, respectively).

[b] Significantly different between groups II (autol.) and III (allog.).

Table 2. Plasma lipid peroxidation (TBARS) levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I (control)	15.01(12.76/19.25)	14.40(11.66/16.06)	14.52(11.56/15.30)	15.02(12.55/16.58)
Group II (autol.)	21.12(17.76/29.70)[a]	21.89(17.60/26.40)[a]	20.46(15.45/32.21)[a]	19.36(15.62/27.61)[a]
Group III (allog.)	18.26(15.40/22.00)	19.14(16.60/23.10)[a]	29.23(21.34/40.72)[acd]	27.50(18.92/38.50)[acd]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – TBARS: nmol TBARS/mL plasma. Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).
[a] Significantly different from group I (control).
[c] Significantly different from before CR.
[d] Significantly different from CR.

Table 3. Plasma vitamin C (VIT C) levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I (control)	22.75(16.92/26.40)	17.80(12.90/21.84)	17.80(15.40/22.13)	19.20(16.38/24.26)
Group II (autol.)	12.70(8.80/19.80)[a]	9.90(8.02/11.76)[ac]	7.21(5.33/8.00)[a]	9.72(6.53/10.82)[a]
Group III (allog.)	13.08(9.96/16.66)[a]	8.09(4.63/10.50)[ac]	7.50(6.48/12.50)[ac]	9.10(4.37/12.40)[ac]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – vitamin C: µg/mL plasma.
Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).
[a] Significantly different from group I (control).
[c] Significantly different from before CR.

Table 4. Plasma protein thiol (P-SH) group levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I (control)	463.50 (445.20/487.80)	432.00 (406.20/469.35)	459.00 (433.76/498.00)	429.66 (403.75/451.75)
Group II (autol.)	364.51 (335.82/407.42)[a]	352.64 (306.15/371.64)[a]	304.44 (272.34/340.41)[a]	305.60 (272.21/352.03)[ad]
Group III (allog.)	407.07 (362.10/452.54)[a]	355.88 (335.25/371.10)[a]	358.37 (303.10/386.70)[a]	345.00 (308.48/383.40)[ac]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – P-SH: nmol P-SH/mL plasma.
Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).
[a] Significantly different from group I (control).
[c] Significantly different from before CR.
[d] Significantly different from CR

Table 5. Erythrocyte non protein thiol (NP-SH) group levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I (control)	1933.63 (1749.00/2012.72)	1758.24 (1563.10/1909.60)	1787.09 (1535.72/1920.80)	1821.60 (1612.80/1937.60)
Group II (autol.)	2052.34 (1929.70/2084.16)	1982.40 (1716.00/2279.06)	1936.18 (1766.80/2170.30)	1741.60 (1504.00/2147.58)
Group III (allog.)	1805.06 (1664.60/2308.24)	1604.40 (1263.06/1898.24)[bc]	1748.30 (1542.29/1998.24)	1803.36 (1271.20/1932.00)

CR: conditioning regimen; BMT: bone marrow transplantation; Units – NP-SH: nmol NP-SH/mL erythrocytes. Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).
[b] Significantly different between groups II (autol.) and III (allog.).
[c] Significantly different from before CR.

Table 6. Blood δ-Aminolevulinate dehydratase (δ-ALA-D) activity over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I (control)	4.67(3.95/5.01)	4.00(3.42/4.90)	4.10(3.80/4.60)	4.23(3.70/4.67)
Group II (autol.)	3.94(2.98/4.89)	1.78(1.20/2.52)[ac]	1.29(1.09/1.98)[ac]	2.16(1.36/2.62)[acd]
Group III (allog.)	3.67(2.14/5.52)	2.31(1.56/3.10)[ac]	1.91(1.49/2.42)[ac]	2.20(1.36/2.70)[ac]

CR: conditioning regimen; BMT: bone marrow transplantation; Units –ALA-D: nmol PBG/ml blood/h. Data are expressed as median (lower/ upper quartile). Group I (n=37); Group II (n=13); Group III (n=24).
[a] Significantly different from group I (control).
[c] Significantly different from before CR.
[d] Significantly different from CR

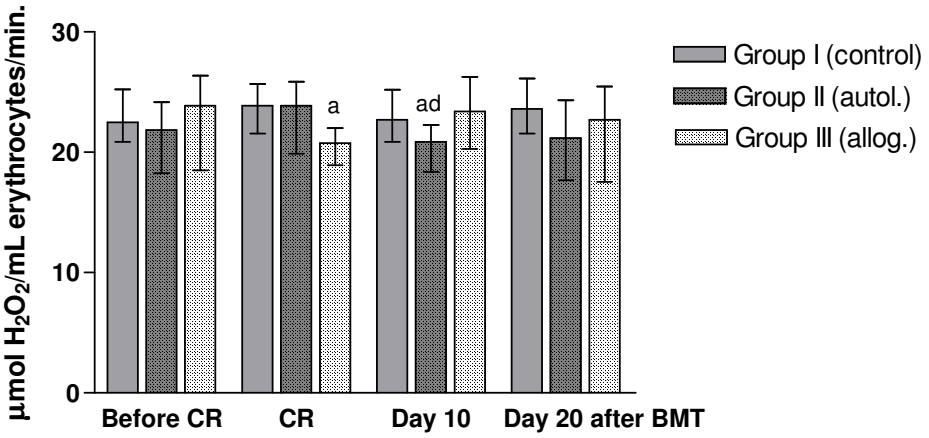

Figure 1- Erythrocyte catalase (CAT) activity over time

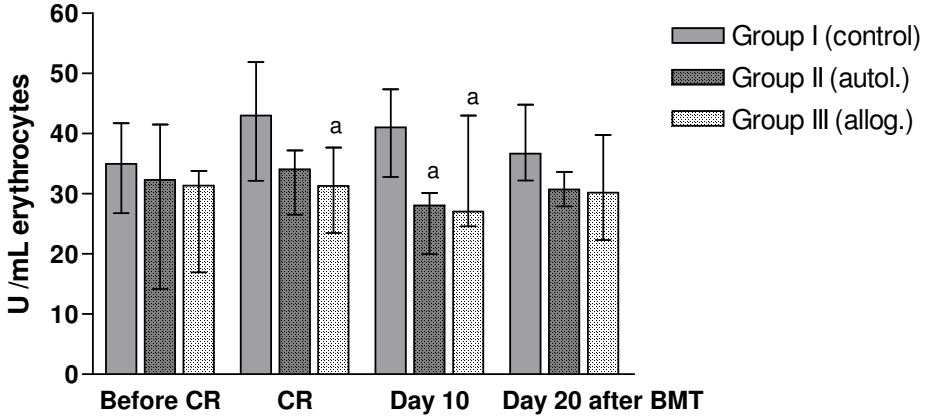

Figure 2- Erythrocyte superoxide dismutase (SOD) activity over time

A

Estimations before CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.027 (n.s.)	0.074 (n.s.)	–0.015 (n.s.)	–0.447* (p.0.005)	–0.057 (n.s.)	–0.052 (n.s.)
VIT C	_	0.143 (n.s.)	–0.152 (n.s.)	0.265 (n.s.)	–0.344 (n.s.)	0.204 (n.s.)
CAT	_	_	0.158 (n.s.)	–0.256 (n.s.)	–0.122 (n.s.)	0.324 (n.s.)
SOD	_	_	_	–0.031 (n.s.)	0.151 (n.s.)	0.357* (p.0.027)
P-SH	_	_	_	_	0.069 (n.s.)	–0.102 (n.s.)
NP-SH	_	_	_	_	_	–0.022 (n.s.)

B

Estimations CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.097 (n.s.)	–0.272* (p.0.018)	–0.202 (n.s.)	–0.327* (p.0.004)	–0.121 (n.s.)	–0.390* (p.0.000)
VIT C	_	–0.062 (n.s.)	–0.028 (n.s.)	0.332* (p.0.003)	–0.110 (n.s.)	0.360* (p.0.001)
CAT	_	_	0.254* (p.0.033)	0.015 (n.s.)	0.126 (n.s.)	0.278* (p.0.016)
SOD	_	_	_	0.075 (n.s.)	0.076 (n.s.)	0.305* (p.0.010)
P-SH	_	_	_	_	–0.012 (n.s.)	0.616* (p.0.000)
NP-SH	_	_	_	_	_	0.000 (n.s.)

C

Estimations day 10 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.543* (p.0.000)	0.094 (n.s.)	–0.389* (p.0.001)	–0.536* (p.0.000)	0.220 (n.s.)	–0.590* (p.0.000)
VIT C	_	–0.067 (n.s.)	0.375* (p.0.003)	0.601* (p.0.000)	–0.021 (n.s.)	0.610* (p.0.000)
CAT	_	_	0.376* (p.0.001)	0.001 (n.s.)	0.019 (n.s.)	0.121 (n.s.)
SOD	_	_	_	0.252* (p.0.039)	–0.130 (n.s.)	0.375* (p.0.001)
P-SH	_	_	_	_	–0.118 (n.s.)	0.624* (p.0.000)
NP-SH	_	_	_	_	_	–0.005 (n.s.)

D

Estimations day 20 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.567* (p.0.000)	–0.156 (n.s.)	–0.252 (n.s.)	–0.296* (p.0.014)	0.048 (n.s.)	–0.482* (p.0.000)
VIT C	_	0.104 (n.s.)	0.057 (n.s.)	0.386* (p.0.002)	–0.010 (n.s.)	0.565* (p.0.000)
CAT	_	_	0.295* (p.0.020)	–0.000 (n.s.)	–0.113 (n.s.)	0.170 (n.s.)
SOD	_	_	_	0.077 (n.s.)	–0.026 (n.s.)	0.174 (n.s.)
P-SH	_	_	_	_	–0.109 (n.s.)	0.644* (p.0.000)
NP-SH	_	_	_	_	_	0.034 (n.s.)

Panel 1. Correlation between biochemical estimations for all groups. A: estimations before CR; B: estimations during CR; C: estimations on day 10 after BMT; D: estimations on day 20 after BMT. CR: conditioning regimen; BMT: bone marrow transplantation; TBARS: thiobarbituric acid-reactive substances; VIT C: vitamin C; CAT: catalase; SOD: superoxide dismutase; P-SH: protein thiol groups; NP-SH: non protein thiol groups; δ-ALA-D: δ-aminolevulinate dehydratase; n.s.: no significant

4.2 Oxidative stress and δ-ALA-D activity in different conditioning regimens in allogeneic bone marrow transplantation patients

Clinical Investigation

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Abstract

Objectives: To compare different conditioning regimens (CR), in order to determine whether either of them could be less toxic to allogeneic bone marrow transplantation (BMT) patients in terms of oxidative stress and also analyze δ-ALA-D activity as a possible marker of oxidative stress.

Design and methods: Lipid peroxidation, vitamin C, thiol groups levels and catalase, superoxide dismutase and δ-ALA-D activity were determined in 21 healthy controls, 05 patients with fludarabine + cyclophosphamide (FluCy) CR, 12 with busulfan + cyclophosphamide (BuCy) and 04 with cyclophosphamide + total body irradiation (CyTBI).

Results: There were a decrease in enzymatic and non enzymatic antioxidants and in δ-ALA-D activity, in all CRs and a increase in lipid peroxidation more pronounced in CyTBI CR.

Conclusions: All CRs promoted oxidative stress in allogeneic BMT patients, but this was more pronounced with CyTBI and δ-ALA-D activity seemed to be an additional biomarker of oxidative stress in these patients.

Keywords: oxidative stress; bone marrow transplantation; conditioning regimens; ALA-D; antioxidants.

Introduction

Bone marrow transplantation (BMT) is a therapeutic method used in various malignant, hematologic, immunologic, and genetic diseases [1]. Allogeneic transplantation involves the transfer of marrow from a donor to another person, to reestablish hematopoietic cell function after the administration of high-dose chemotherapy, in this case the stem cells can come from the donor's bone marrow or peripheral blood. One of the main problems in this procedure is the graft-versus-host disease [2]. Allogeneic transplantations are preferred for patients with leukemias or myeloproliferative diseases [3].

In all procedures of preparation, conditioning regimen (CR), and during hospitalization, BMT patients show intense and variable organic toxicity, in addition to severe and prolonged myelosuppression, a period of high vulnerability for patients to develop complications. Administration of high doses of chemotherapy (often including busulfan and cyclophosphamide) with or without total body irradiation (TBI) is a feature of BMT protocols known as CR. These regimens are designed to cause immunosuppression in order to avoid destruction of the allograft, and to destroy any residual cancer cells in allogeneic BMT [2]. Chemotherapy and radiation therapy are associated with the generation of large amounts of reactive oxygen/nitrogen species and depletion of antioxidants [4-7]. The production of free radicals and the deficiency of antioxidants can to lead to a condition known as oxidative stress, which can be associated with serious complications such as hepatic failure [8].

Chemotherapy has been shown to decrease plasma antioxidant concentrations in BMT patients, including vitamin C, α-tocopherol and β-carotene [9,10]. GSH concentrations also are reduced by chemotherapeutic agents [11,12]. The –SH groups are important antioxidants and play important roles in a variety of activities of cell. The –SH groups are easily modified by oxidants or alkylating agents [13]. The decrease of –SH group content can be attributed to oxidation by free radicals or formation of adducts with reactive chemicals [14].

δ-Aminolevulinate dehydratase (δ-ALA-D) is a sulfhydryl-containing enzyme [15,16] essential for aerobic organisms that catalyses the synthesis of tetrapyrrolic compounds such as billins and hemes. δ-ALA-D contains sulfhydryl (-SH) groups and zinc, which are essential for its activity [17,18]. Therefore, δ-ALA-D is inhibited by substances that compete with zinc and/or that oxidize the –SH groups [19] and is sensitive to situations associated with oxidative

stress [20-23]. Furthermore, enzyme inhibition can lead to accumulation of substrate 5-aminolevulinate (ALA) in the blood, which in turn can intensify oxidative stress by generating carbon-centered reactive species or by releasing iron from proteins such as ferritin [24].

BMT procedure is toxic and excessive free radical production has been implicated in the action of many cytostatic drugs [25], but little is known about how cytostatic drugs affect the antioxidative system in human beings and only a few studies have been performed in the clinical setting [5]. In this context, the objective of this study was to look for further evidence of oxidative stress and also analyze δ-ALA-D activity as a possible marker of oxidative stress in allogeneic BMT patients and to compare three common CR utilized preceding allogeneic BMT, in order to determine whether either of them could be less toxic to patients, using markers of oxidative stress as the end point of toxicity.

Materials and methods

Reagents

δ-Aminolevulinate dehydratase (δ-ALA-D) and zinc chloride ($ZnCl_2$) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Subjects

Altogether, 21 patients undergoing allogeneic BMT, 10 female and 11 male, mean age 39.60±14.87 and 21 controls (matched by age and sex with the patients) were included in the investigation (samples were obtained from March 2007 to March 2008). The patients were under treatment in the 'Hospital Universitário' from the 'Universidade Federal de Santa Maria' (HUSM), RS, Brazil. They were treated with allogeneic BMT which was preceded by CR.

The CR patients were divided according to diseases. FluCy: fludarabine (30 mg/m^2) + cyclophosphamide (300 mg/m^2), both given 3 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 2) for Hodgkin and non-Hodgkin lymphomas. BuCy:

busulfan (1 mg/kg), given 4 consecutive days four times a day, starting 8 days before the transplantation (days minus 8 to minus 5) + cyclophosphamide (60 mg/ m^2), given 2 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 3) for acute and chronic myeloid leukemia and syndrome myelodysplastic. CyTBI: cyclophosphamide (60 mg/m^2), given 2 consecutive days, starting 7 days before the transplantation (days minus 7 to minus 6) + total body irradiation, carried out on 4 consecutive days, starting 4 days before the transplantation (days minus 4 to minus 2, three times a day and day minus 1, two times a day) for acute and chronic lymphoid leukemia. The BuCy CR patients had 2 rest days (without chemotherapy) before the transplantation (days minus 2 and minus 1), and FluCy and CyTBI CR patients had only one rest day (day minus 1 and minus 5, respectively).

The subjects included in the study were classified in four groups: Group I – Control: healthy volunteers of HUSM Blood Bank served as control group and they were receiving no treatment for any diseases (n=21 volunteers); Group II: CyTBI CR patients (n=04); Group III: FluCy CR patients (n=05); Group IV: BuCy CR patients (n=12).

Table 1 shows the characteristics of the patients included in this study.

The present study was approved by the Human Ethical Committee of the Universidade Federal de Santa Maria, protocol number 0152.0243.000-06.

Sample collection

The blood (4 mL) was collected during routine examinations by venous arm puncture in EDTA vaccutainer tubes in the Hematology and Oncology Laboratory. The blood used in this study was the leftover of this 4 mL sample. Four samples of each patient were taken, the first before CR, the second during CR (the last day that the patient received chemotherapy), the third 10 days after BMT and the fourth 20 days after BMT. Control group samples were the leftover blood of HUSM Blood Bank donors, which was collected on the same day of the matched patient. Only one sample was used from these volunteers (before CR, CR, 10 and 20 days after BMT, consequently, control values for each time point are from different subjects). The plasma and cells were separated by centrifugation at 1000 rpm for 12 min. Then a portion of the collected plasma and erythrocytes were stored for analysis at –20^{o}C for less than 3 weeks. The other analyses were performed on the same day.

Biochemical estimations

All biochemical assays were made in duplicates or triplicates, depending on the availability of samples.

Thiobarbituric acid–reactive substances

Thiobarbituric acid-reactive substances (TBARS) assay measures the peroxidative damage to lipids that occurs by excessive ROS generation. Lipid peroxidation was estimated in plasma according to the method of Lapenna et al. [26], using 1% phosphoric acid and 0.6% thiobarbituric acid (TBA). The pink chromogen produced by reaction of TBA with MDA, was measured spectrophotometrically at 532 nm. The results were expressed as nmol TBARS/mL plasma, using MDA as standard.

Catalase enzyme activity

Catalase (CAT) enzyme activity was measured by the method of Aebi [27]. Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water, then, 20 μL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mM H_2O_2 in phosphate buffer (50 mM, pH 7.0) to give a final volume of 1 mL. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. The catalase activity was expressed as μmol H_2O_2/mL erythrocytes/min.

Superoxide dismutase enzyme activity

Superoxide dismutase (SOD) activity was performed according to the method of Misra and Fridovich [28]. Briefly, epinephrine rapidly auto oxidizes at pH 10.5 producing adrenochrome, a pink colored product that can be detected at 480 nm. The addition of samples containing SOD inhibits epinephrine auto-oxidation. The inhibition rate was monitored during 150 s at intervals of 10 s. The amount of enzyme required to produce 50% inhibition at 25 °C was defined as one unit of enzyme activity. The SOD activity was expressed as U/mL erythrocytes.

Vitamin C

Plasma vitamin C (VIT C) was estimated as described by Galley et al. [29] with some modifications by Jacqes-Silva et al. [30]. Plasma was precipitated with 1 volume of a cold 5% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 μL of the supernatants were mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL) and 13.3% trichloroacetic acid and incubated for 3h at 37° C. Then, 1 mL 65% sulfuric acid was added to the medium and the orange red compound was measured at 520 nm. The content of ascorbic acid was calculated using a standard curve (1.5 – 4.5 μmol/L ascorbic acid freshly prepared in sulfuric acid) and expressed as μg vit C/mL plasma.

Protein thiol groups

Protein thiol groups (P-SH) were assayed in plasma by the method of Boyne and Ellman [31] modified by Jacques-Silva et al. [30], which consisted of the reduction of 5.5´-dithio(bis-nithrobenzoic) acid (DTNB) in 0.3 M phosphate buffer, pH 7.0, measured at 412 nm. A standard curve using glutathione was constructed in order to calculate the protein thiol groups. The results were expressed as nmol P-SH/mL plasma.

Non protein thiol groups

Erythrocyte non protein thiol groups (NP-SH) were determined as described by Boyne and Ellman [31] modified by Jacques-Silva et al. [30]. Red blood cell pellets (300 μL) obtained after centrifugation of whole blood were hemolyzed with 10% triton solution (100 μL) for 10 min. Then, the protein fraction was precipitated with 200 μL of 20% trichloroacetic acid followed by centrifugation. The colorimetric assay was carried out in 1 M phosphate buffer, pH 7.4. A standard curve using glutathione was constructed in order to calculate non protein thiol groups. The NP-SH level was measured at 412 nm and expressed as nmol NP-SH/mL erythrocytes.

δ-aminolevulinate dehydratase (δ-ALA-D) activity

δ-aminolevulinate dehydratase (δ-ALA-D) activity was assayed in whole blood by the method of Berlin and Schaller [32] by measuring the rate of porphobilinogen (PBG) formation in 1 h at 37°C. The enzyme reaction was initiated after 10 min of pre-incubation of blood with 1mM $ZnCl_2$. The reaction was started by adding δ-aminolevulinic acid (ALA) to a final concentration of 4mM in a phosphate buffered solution, and incubation was carried out for 1 h at 37° C and the reaction product was measured at 555 nm.

Statistical analysis

Biochemical assay results were expressed as median (lower/ upper quartiles) and ages were expressed as mean ± SD.

Since data had no homogeneity of variance, statistical analysis was performed using Kruskal-Wallis ANOVA followed by Mann-Whitney U test to compare the difference among the groups, Friedman ANOVA followed by Wilcoxon test to analyze changes in blood indices over time and Spearman Correlation to analyze the correlations between the biochemical estimations in all groups. Test T was used to compare the difference in the ages and sex among the groups. A value of $p<0.05$ was considered statistically significant.

Results

Patients' sex, age and diagnosis of groups II, III and IV are shown in table 1. Groups II, III and IV were not significantly different from each other (table 1) and also were not significantly different from group I (control) in sex and age (data not show).

Plasma TBARS levels were significantly higher in groups II, III and IV than group I (control), during CR, on day 10 and day 20 after BMT, ($p<0.005$). TBARS levels were higher in group II than III on day 20 after BMT and than group IV during CR and on day 20 after BMT, ($p<0.05$). TBARS levels increased from during CR to days 10 and 20 after BMT in group IV ($p<0.05$), (table 2).

Plasma vitamin C (VIT C) concentrations were significantly lower in groups II and III, on day 10 and 20 after BMT ($p<0.05$) and in group IV during CR and on day 10 and 20 after

BMT ($p<0.0005$) than group I (control). VIT C levels were lower in group IV than in group II, during CR ($p<0.05$). In group IV, VIT C levels decreased significantly from before CR to CR and to day 10 after BMT ($p<0.05$), (table 3).

Erythrocyte catalase (CAT) activity was significantly lower in groups II and IV than in group I (control), during CR ($p<0.05$) and also was lower in group III than in group I (control) during CR and on day 20 after BMT ($p<0.05$). Groups II, III and IV were not significantly different from each other in erythrocyte CAT activity. CAT activity did not change over time in groups (table 4).

Erythrocyte superoxide dismutase (SOD) activity was significantly lower in group IV than in group I (control), during CR ($p<0.005$) and on day 10 after BMT ($p<0.05$) and also was lower in group III than in group I (control) on day 10 after BMT ($p<0.005$). Groups II, III and IV were not significantly different from each other in erythrocyte SOD activity. SOD activity did not change over time in groups (table 5).

Plasma protein thiol (P-SH) group levels were significantly lower in groups III and IV, during CR, on day 10 and on day 20 after BMT ($p<0.05$) when compared with group I (control). P-SH levels in groups II, III and IV were not significantly different from each other. P-SH levels decreased from before CR to day 20 after BMT in the patients of group IV ($p<0.05$), (table 6).

Erythrocyte non protein thiol (NP-SH) group levels were significantly lower in group II, before CR, during CR and on day 20 after BMT ($p<0.05$), than in the group I (control). NP- SH levels were lower in group II, before CR ($p<0.05$), than in group III, and were lower in groups II and III, on day 20 after BMT ($p<0.05$), than in group IV. Erythrocyte NP-SH levels decreased from before CR to CR ($p<0.05$) and increased from during CR to day 20 after BMT ($p<0.01$) in group IV (table 7).

Blood δ-ALA-D activity was significantly lower in group II, on days 10 and 20 after BMT ($p<0.01$) and in groups III and IV, during CR ($p<0.05$) and on days 10 and 20 after BMT ($p<0.01$) than in group I (control). Blood δ-ALA-D activities in groups II, III and IV were not significantly different from each other. δ-ALA-D activity decreased from before CR to CR, days 10 and 20 after BMT ($p<0.05$) in group IV, (table 8).

Spearman correlation showed a negative correlation between TBARS with CAT, during CR, with SOD and P-SH during CR and on day 10 after BMT, with δ-ALA-D during

CR and on days 10 and 20 after BMT, with VIT C on days 10 and 20 after BMT. VIT C had a positive correlation with δ-ALA-D, during CR and on days 10 and 20 after BMT, with SOD and P-SH on day 10 and with CAT on day 20 after BMT. CAT had a positive correlation with SOD before CR and during CR, and with δ-ALA-D during CR. SOD had a positive correlation with δ-ALA-D before CR, during CR and on day 10 after BMT and P-SH with NP-SH before CR, with δ-ALA-D during CR and on days 10 and 20 after BMT, (panel 1).

Discussion

Treatment with radiotherapy and chemotherapy has been shown to cause peroxide accumulation [4,7,33-36], that can cause a profound alteration in the structural integrity and functions of cell membranes and free radical-induced lipid peroxidation has been implicated in the pathogenesis of several disorders, including cancer [33]. In our study, the three CRs, preceding allogeneic BMT, were associated with higher TBARS levels during CR, and after BMT when compared with group I (control). In group II (CyTBI), this increase was higher than in groups III (FluCy) and IV (BuCy). In group IV, there was increase in lipid peroxidation on days 10 and 20 after BMT with relation during CR, showing one late effect of CR. TBARS also had a negative correlation with antioxidants. This confirms, that all CRs analyzed were related with an increase in lipid peroxidation and this increase was more pronounced in the CyTBI group that also caused a pronounced decrease in antioxidant defenses, resulting in oxidative stress. This results are in agreement with data published by Durken et al.[36] that showed oxidative stress after high-dose radiochemotherapy preceding BMT.

Vitamin C is a powerful reducing agent and an important water-soluble vitamin for humans and certain other animals. It has been shown to scavenge $O_2^{\cdot-}$, H_2O_2, OH, aqueous peroxy radicals and singlet oxygen. Vitamin C also protects plasma lipids against lipid peroxidation and has an important role in the regeneration of α-tocopherol [37]. VIT C levels were lower in the plasma of patients from groups II, III and IV than in group I (control), at different time points analyzed. However, in the group IV (BuCy), VIT C levels during CR, were lower than groups II (CyTBI) and III (FluCy), indicating that this antioxidant defense was impaired in all CR groups analyzed, but more pronouncedly in BuCy group.

Thiols fulfill important antioxidant functions in cells and biological fluids. The changes in the content of -SH groups, especially of free -SH groups in membrane proteins may indicate membrane protein damage [38]. Alkylating agents, produce free radicals that can directly interact with thiol groups of proteins [13], oxidizing them to disulfides [14]. In our study, groups III (FluCy) and IV (BuCy) presented plasma P-SH levels lower than those of healthy volunteers, demonstrating that the protein thiol groups were probably oxidized by alkylating agents, cyclophosphamide and busulfan, utilized during CR. Erythrocyte NP-SH group levels in the patient of groups III and IV were not significantly different from those of the control group. However, NP-SH levels were lower in CyTBI patients (group II) than in controls (group I) and group II, also were lower than groups III (FluCy) and IV (BuCy) at different times. In group IV, there was a decrease in NP-SH levels from before CR to during CR, demonstrating the effect of BuCy CR in this antioxidant.

As demonstrated, CAT and SOD enzymatic antioxidants were similar in the patient groups and control group before the initiation of chemotherapy or radiochemotherapy. SOD is the key enzyme required for the removal of O_2 by converting it to hydrogen peroxide (H_2O_2), which is further eliminated by CAT and peroxidases. Catalase helps in neutralizing the toxic effect of H_2O_2 converting it to water and non-reactive oxygen species, thus it prevents the generation of hydroxyl radicals and protects cells from oxidative damage [39]. Our study demonstrated that after the transplant, SOD and CAT activity were lower in both groups II (CyTBI) and III (FluCy), when compared with the control group, at different times. This shows an imbalance in enzymatic antioxidants caused by these two CRs, which together with lipid peroxidation, also observed in this study, result in oxidative stress, leading to complications in patients undergoing bone marrow transplantation.

Blood δ-ALA-D activity before CR in all patient groups was similar to that of the control group and chemotherapy or radiochemotherapy CR led to a decrease in ALA-D activity over time in the patient groups and ALA-D had a positive correlation with all antioxidants analyzed and a negative with TBARS. ALA-D is a zinc metalloenzyme, essential for all aerobic organisms, that requires reduced thiol groups for its activity [40]. Reactive oxygen species, produced during CR [4-7], can oxidize thiol groups located inside the active site of mammalian δ-ALA-D [41,42], decreasing the enzyme activity as observed here. Finally, δ-ALA-D inhibition can result in ALA accumulation, which can have pro-oxidant effects [43] and contribute to oxidative stress caused by CR.

In conclusion, the results presented here indicate that CyTBI, FluCy and BuCY, promoted similar modifications in the levels of non-enzymatic and enzymatic antioxidant defenses and in ALA-D activity. However, in relation to lipid peroxidation, this was more pronounced in CyTBI group, demonstrating thus, oxidative stress in FluCy, BuCY and principally in CyTBI CR. Moreover, blood δ-ALA-D activity seems to be an additional biomarker of oxidative stress in BMT patients.

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Table 1. Characteristics of patients

Number of patients (n=21)	CyTBI (Group II)	FluCy (Group III)	BuCy (Group IV)
Mean age (years)	35.66±17.92	41.00±16.98	40.00±13.59
Sex			
Male	02	04	05
Female	02	01	07
Diagnosis			
Lymphoma			
Hodgkin	00	03	00
No Hodgkin	02	01	00
Leukemia			
Acute myeloid	00	01	02
Chronic myeloid	00	00	06
Acute lymphoblastic	01	00	00
Chronic lymphoid	01	00	00
Myelodysplasia	00	00	04

Group II: CyTBI: cyclophosphamide + total body irradiation (n=04); Group III: FluCy: fludarabine + cyclophosphamide (n=05); Group IV: BuCy: busulfan + cyclophosphamide (n=12). Data are expressed as means ± S.D. for ages.

Table 2. Plasma lipid peroxidation (TBARS) levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	05.20 (04.80/05.60)	04.00 (03.52/04.82)	04.65 (03.20/05.26)	04.54 (03.85/05.10)
Group II	04.69 (04.25/08.65)	08.14 (07.26/08.85)[a]	12.26 (07.89/15.07)[a]	14.15 (13.87/16.64)[a]
Group III	05.72 (05.21/06.34)	07.48 (06.82/08.80)[a]	07.33 (05.64/07.55)[a]	08.43 (06.30/09.16)[ab]
Group IV	06.67 (05.53/07.33)	06.01 (05.28/06.82)[ab]	10.67 (08.43/14.56)[af]	09.82 (06.23/12.83)[abf]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – TBARS: nmol TBARS/mL plasma.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I.
[b] Significantly different from group II.
[f] Significantly different from CR.

Table 3. Plasma vitamin C (VIT C) levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	18.15 (12.93/23.40)	16.11 (09.29/20.75)	17.80 (15.40/22.29)	19.60 (16.45/24.26)
Group II	14.56 (12.13 /15.00)	11.20 (09.80/11.76)	09.56 (09.10/14.84)[a]	08.40 (06.00/12.20)[a]
Group III	14.98 (03.58/15.09)	09.30 (09.23/22.20)	08.80 (07.50/09.33)[a]	12.87 (07.52/13.95)[a]
Group IV	14.87 (07.56/25.26)	05.08 (02.80/08.73)[abce]	06.60 (04.05/13.00)[ae]	07.85 (03.74/09.60)[a]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – vitamin C: µg/mL plasma.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).
[b] Significantly different from group II.
[c] Significantly different from group III.
[e] Significantly different from before CR.

Table 4. Erythrocyte catalase (CAT) activity over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	25.79(23.16/26.60)	25.11 (23.62/27.86)	24.54 (21.55/25.45)	25.68 (22.70/26.83)
Group II	26.37 (24.54/27.98)	21.21 (19.27/22.00)[a]	20.87 (17.43/26.60)	19.95 (17.20/27.06)
Group III	23.07 (21.02/25.80)	21.10 (20.64/21.79)[a]	23.96 (22.00/25.91)	20.18 (18.58/23.85)[a]
Group IV	20.09 (10.09/26.03)	19.32 (18.34/26.37)[a]	24.08 (20.35/26.71)	23.16 (17.20/25.46)

CR: conditioning regimen; BMT: bone marrow transplantation; Units – CAT: µmols H_2O_2/mL erythrocytes/min.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCyl) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).

Table 5. Erythrocyte superoxide dismutase (SOD) activity over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	35.11 (31.01/43.88)	44.13 (33.49/53.76)	42.23 (33.49/56.25)	38.88 (32.71/46.90)
Group II	32.35 (18.34/34.49)	36.08(23.46/55.10)	25.25 (23.06/46.92)	23.45 (16.45/41.37)
Group III	28.09 (27.03/31.99)	29.73 (23.45/53.13)	25.41 (24.36/27.03)[a]	31.07 (22.92/56.62)
Group IV	24.27 (11.93/33.11)	31.26 (22.53/35.72)[a]	27.27 (21.20/55.65)[a]	31.70 (20.42/38.45)

CR: conditioning regimen; BMT: bone marrow transplantation; Units – SOD: U /mL erythrocytes.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).

Table 6. Plasma protein thiol (P-SH) group levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	462.22 (406.20/487.80)	414.30 (367.80/469.72)	453.93 (396.91/553.80)	418.20 (377.88/462.82)
Group II	372.00 (352.20/395.30)	361.20 (357.76/450.00)	355.55 (312.00/517.92)	370.56 (266.40/424.78)
Group III	402.09 (386.07/480.09)	321.16 (224.40/367.62)[ad]	367.15 (216.00/373.20)[a]	308.48 (271.20/436.59)[a]
Group IV	436.70 (380.40/466.48)	362.84 (340.82/420.60)[a]	372.60 (273.25/398.28)[a]	352.23 (341.25/383.40)[ae]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – P-SH: nmol P-SH/mL plasma.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).
[d] Significantly different from group IV.
[e] Significantly different from before CR

Table 7. Erythrocyte non protein thiol (NP-SH) group levels over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	1985.20 (1937.60/2172.80)	1769.60 (1471.05/1957.20)	1785.12 (1506.40/2100.50)	1780.92 (1435.20/2409.00)
Group II	1713.60 (1408.96/1797.60)[ac]	1187.00 (0838.40/1632.40)[a]	1716.40 (1699.60/1992.00)	1203.84 (1098.06/1595.52)[ad]
Group III	2051.28 (1859.78/2388.99)	1568.00 (1481.04/2236.00)	1697.52 (1534.00/2108.78)	1461.60 (1161.60/1876.00)[d]
Group IV	1741.60 (1615.60/2212.00)	1722.90 (1261.49/1954.00)[e]	1946.70 (1349.60/2082.20)	1932.00 (1895.60/2046.20)[f]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – NP-SH: nmol NP-SH/mL erythrocytes.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).
[c] Significantly different from group III.
[d] Significantly different from group IV.
[e] Significantly different from before CR.
[f] Significantly different from CR.

Table 8. Blood δ-Aminolevulinate dehydratase (δ-ALA-D) activity over time

Groups	Before CR	CR	Day 10 after BMT	Day 20 after BMT
Group I	4.52 (1.61/5.89)	4.12 (3.20/4.90)	4.23 (3.70/4.71)	3.89 (3.60/4.81)
Group II	3.56 (2.68/4.72)	2.57 (2.29/4.72)[a]	1.25 (1.11/1.93)[a]	2.25 (0.84/3.25)[a]
Group III	4.22 (1.61/5.89)	2.67 (1.28/2.84)[a]	2.83 (1.60/3.77)[a]	1.63 (0.77/2.59)[a]
Group IV	3.78 (1.24/6.51)	2.28 (1.56/3.56)[ae]	1.84 (1.49/2.37)[ae]	2.27 (1.36/2.77)[ae]

CR: conditioning regimen; BMT: bone marrow transplantation; Units – ALA-D: nmol PBG/ml blood/h.
Group I (control) n=21; Group II (CyTBI) n=04; Group III (FluCy) n=05; Group IV (BuCy) n=12.
Data are expressed as median (lower/ upper quartile).
[a] Significantly different from group I (control).
[e] Significantly different from before CR.

A

Estimations before CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	0.156 (n.s.)	–0.004 (n.s.)	0.207 (n.s.)	–0.103 (n.s.)	–0.207 (n.s.)	–0.028 (n.s.)
VIT C	_	0.232 (n.s.)	0.107 (n.s.)	0.012 (n.s.)	–0.073 (n.s.)	0.393 (n.s.)
CAT	_	_	0.629* (p.0.003)	–0.255 (n.s.)	–0.201 (n.s.)	0.230 (n.s.)
SOD	_	_	_	–0.173 (n.s.)	0.069 (n.s.)	0.509* (p.0.030)
P-SH	_	_	_	_	0.475* (p.0.039)	–0.269 (n.s.)
NP-SH	_	_	_	_	_	–0.041 (n.s.)

B

Estimations CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.130 (n.s)	–0.521* (p.0.000)	–0.369* (p.0.022)	–0.491* (p.0.001)	–0.231 (n.s.)	–0.345* (p.0.028)
VIT C	_	–0.021 (n.s.)	–0.086 (n.s.)	0.059 (n.s.)	–0.074 (n.s.)	0.350* (p.0.026)
CAT	_	_	0.414* (p.0.009)	0.249 (n.s.)	0.185 (n.s.)	0.385* (p.0.013)
SOD	_	_	_	0.299 (n.s.)	0.112 (n.s.)	0.398* (p.0.013)
P-SH	_	_	_	_	–0.041 (n.s.)	0.565* (p.0.000)
NP-SH	_	_	_	_	_	–0.061 (n.s.)

C

Estimations day 10 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.537* (p.0.000)	0.174 (n.s.)	–0.537* (p.0.000)	–0.504* (p.0.000)	0.161 (n.s.)	–0.686* (p.0.000)
VIT C	_	–0.082 (n.s.)	0.440* (p.0.007)	0.515* (p.0.000)	–0.043 (n.s.)	0.521* (p.0.000)
CAT	_	_	0.226 (n.s.)	0.000 (n.s.)	0.041 (n.s.)	0.063 (n.s.)
SOD	_	_	_	0.311 (n.s.)	–0.142 (n.s.)	0.398* (p.0.013)
P-SH	_	_	_	_	–0.110 (n.s.)	0.555* (p.0.000)
NP-SH	_	_	_	_	_	–0.009 (n.s.)

D

Estimations day 20 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.639* (p.0.000)	–0.330 (p.0.045)	–0.292 (n.s.)	–0.220 (n.s.)	–0.102 (n.s.)	–0.567* (p.0.000)
VIT C	_	0.329* (p.0.049)	0.123 (n.s.)	0.265 (n.s.)	–0.136 (n.s.)	0.542* (p.0.000)
CAT	_	_	0.210 (n.s.)	0.192 (n.s.)	–0.065 (n.s.)	0.271 (n.s.)
SOD	_	_	_	0.263 (n.s.)	–0.078 (n.s.)	0.320 (n.s.)
P-SH	_	_	_	_	0.198 (n.s.)	0.522* (p.0.000)
NP-SH	_	_	_	_	_	0.060 (n.s.)

Panel 1. Correlations between biochemical estimations in all times and groups analyzed. A: before CR; B: during CR; C: on day 10 after BMT; D: on day 20 after BMT.
CR: conditioning regimen; BMT: bone marrow transplantation; TBARS: thiobarbituric acid-reactive substances; VIT C: vitamin C; CAT: catalase; SOD: superoxide dismutase; P-SH: protein thiol groups; NP-SH: non protein thiol groups; δ-ALA-D: δ-aminolevulinate dehydratase; n.s.: no significant. * Significantly different.

4.3 δ-Aminolevulinate dehydratase activity and oxidative stress during melphalan and cyclophosphamide-bcnu-etoposide (CBV) conditioning regimens in autologous bone marrow transplantation patients.

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Abstract

Severe toxicity is associated with cytotoxic drugs used during the conditioning regimen (CR) preceding bone marrow transplantation (BMT). The aim of this study was to evaluate the involvement of oxidative stress and possible use of δ-Aminolevulinate dehydratase (δ-ALA-D) activity as a marker of oxidative stress in autologous BMT patients. We have also compared common drugs that are used during CR, namely, melphalan (M-200) and cyclophosphamide-BCNU-etoposide (CBV), in order to determine whether either of them could be less toxic to patients in terms of oxidative stress. The sample consisted of 10 patients admitted for autologous BMT, 5 with M-200 CR and 5 with CBV CR and 10 healthy controls. Lipid peroxidation (estimated as thiobarbituric acid-reactive substances, TBARS), vitamin C, thiol levels, catalase, superoxide dismutase and δ-ALA-D activity were determined before CR, during CR and on days 10 and 20 after BMT. Signs of exacerbated oxidative stress were minimal before CR, except for the CVB group (patients with lymphoma) where an increase in TBARS and a decrease in P-SH were detected. Indices of oxidative stress changed in both groups (CBV and M-200) during CR and up to 20 days after BMT. There was a decrease in enzymatic and non enzymatic antioxidant defenses and in δ-ALA-D activity and an increase in lipoperoxidation in the blood of both patient groups. In conclusion, CBV and, principally, M-200 caused oxidative stress in patients undergoing autologous BMT and blood δ-ALA-D activity seems to be an additional biomarker of oxidative stress in BMT patients.

Keywords: antioxidants, autologous bone marrow transplantation, CBV, conditioning regimens, melphalan, oxidative stress, δ- aminolevulinate dehydratase

1. Introduction

Autologous bone marrow transplantation (BMT) is a therapeutic method used in various hematological neoplastic disorders, including multiple myeloma and lymphomas [1]. In BMT, normal-appearing bone marrow is removed from the patient, often treated *in vitro* with cytotoxic drugs or antibodies directed toward the cancer cells, and cryopreserved. Then patients are subjected to intensive cancer therapy, the conditioning regimen (CR), with drugs that may or may not be associated with total body irradiation (TBI), and the marrow is reinfused to support the patient's hematopoietic recovery [2].

Inadequate antioxidant protection or excessive production of reactive oxygen species (ROS) can lead to a condition known as oxidative stress, which is thought to play an important role in the etiology of various diseases and in aging. In line with this, cumulative experimental evidence supports a role for ROS in the initiation and progression of carcinogenesis [3].

Enzymes including superoxide dismutase, glutathione peroxidase and catalase, as well as β-carotene, α-tocoferol and ascorbic acid constitute major defenses against oxidative damage and they can be modified in a variety of pathological conditions [4]. Giving further support for the participation of oxidative stress in human pathologies, epidemiological studies have demonstrated a link between the consumption of diets rich in fruits and vegetables containing high levels of antioxidant vitamins and a reduced incidence of degenerative diseases, including many types of cancer [5].

Free radicals have been implicated in the action of many cytostatic drugs [6], but little is known on how cytostatic drugs affect the antioxidant system in human beings and only few studies have been performed in the clinical setting [7]. If the toxicity of chemotherapy and radiochemotherapy is partially mediated by free radicals, levels of plasma antioxidants should be affected, and markers of oxidative stress should be modified [7]. In line with this, human peripheral polymorphonuclear leukocytes from patients receiving chemotherapy produce more hydrogen peroxide and superoxide anion *in vitro* than those of healthy control subjects [8].

Limited literature data have indicated that patients undergoing BMT exhibit a decrease in the level of non-enzymatic antioxidants, including vitamin C, α-tocopherol, β-carotene and reduced glutathione (GSH) [9,10]. Similarly, chemotherapy treatment with different agents

(bussulfan, cermustine (BCNU) and cisplatin) can cause depletion of plasma GSH [11,12]. Furthermore, the CR before BMT induces nausea, emesis, oropharyngeal inflammation, abdominal pain, and diarrhea [12] and it can lead to considerable organ toxicity in the early postransplantation period, particularly in the gastrointestinal tract, liver, kidneys and lungs [13].

δ-Aminolevulinate dehydratase (δ-ALA-D) catalyzes the synthesis of tetrapyrrolic compounds such as billins and hemes. ALA-D is a sulfhydryl enzyme which depends on Zn^{2+} ion binding to display full activity and is sensitive to situations associated with oxidative stress [14,15]. Furthermore, enzyme inhibition can lead to ALA accumulation in the blood, which in turn can intensify oxidative stress by generating carbon centered reactive species or by releasing iron from proteins such as ferritin [16].

In view of the fact that the undesirable toxicity associated with CR may be related to poor antioxidant and/or excessive ROS production, one objective of this study was to look for further evidence of oxidative stress and to analyze δ-ALA-D activity as a possible marker of oxidative stress in autologous BMT patients. Moreover, we aimed to compare two common drugs that are used during CR, M-200 and CBV, among themselves and with healthy subjects (control group), in order to determine whether either of them could be less toxic to patients when oxidative stress indices are used as the endpoint of toxicity.

2. Material and methods

2.1. Subjects

Altogether, 10 male patients, with a mean age of 53.00 (SD±11.64) years (47–66) and 10 controls (matched by age and sex) were included in the investigation (samples were obtained from March 2007 to March 2008). The patients were under treatment at the 'Hospital Universitário' from the 'Universidade Federal de Santa Maria' (HUSM), RS, Brazil. They all had hematological malignancies and were treated with autologous BMT which was preceded by CR.

The subjects included in the study were classified into three groups: Group I – Control (healthy volunteers from the HUSM Blood Bank and they were not receiving treatment for any diseases; n=10); Group II – M-200 (patients treated with a single dose of melphalan-200

mg/m^2; one day (day minus 3) before the BMT; n=5); Group III (treated with 4 consecutives doses of cyclophosphamide-1,500 mg/m^2 from days minus 6 to minus 3 plus a single dose of BCNU (450 mg/m^2) one day before the BMT (day minus 6) plus 3 doses of etoposide (VP-16 – 250 mg/m^2) two times a day, before the BMT from days minus 6 to minus 4; n=5 patients). All patients had 2 resting days (without chemotherapy) before the BMT (days minus 2 and minus 1).

Patient sex, age and diagnosis in the CR of group II and III were represented in the table 1. The present study was approved by the Human Ethical Committee of the Universidade Federal de Santa Maria, protocol number 0152.0243.000-06. All persons gave their informed consent prior to their inclusion in the study.

2.2. Sample collection

Blood (4 mL) was collected by venous arm puncture in EDTA vaccutainer tubes in the Hematology and Oncology Laboratory to perform routine examinations. The left-over from this 4 mL sample was used for oxidative stress analysis. Four samples of each patient were taken: 1) before CR, 2) during CR (the last day of chemotherapy), 3) ten days after BMT and 4) twenty days after BMT. Control group samples were taken from that leftover from HUSM Blood Bank donors and they were collected on the same days of the matched patients. Only one sample was used from these volunteers (before CR, CR, 10 and 20 days after BMT, consequently, control values for each time point were obtained from different subjects). The plasma and cells were separated by centrifugation at 1000 rpm for 12 min and stored for analysis at –20^{o}C for less than 3 weeks. The others analyses were performed on the same day.

2.3. Biochemical estimations

All biochemical assays were done in duplicates or triplicates, depending on the availability of samples.

2.3.1. Thiobarbituric acid–reactive substances

Thiobarbituric acid-reactive substances (TBARS) assay measures the peroxidative damage to lipids that occurs by excessive ROS generation. Lipid peroxidation was estimated in plasma according to the method of Lapenna et al. (2001), using 1% phosphoric acid and 0.6% thiobarbituric acid (TBA). The pink chromogen produced by reaction of TBA with MDA, was measured spectrophotometrically at 532 nm. The results were expressed as nmol TBARS/mL plasma, using MDA as standard [17].

2.3.2. Catalase enzyme activity

Catalase (CAT) enzyme activity was measured by the method of Aebi (1984). Packed erythrocytes were hemolyzed by adding 100 volumes of distilled water. Then, 20 μL of this hemolyzed sample was added to a cuvette and the reaction was started by the addition of 100 μL of freshly prepared 300 mM H_2O_2 in phosphate buffer (50 mM, pH 7.0) to give a final volume of 1 mL. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm during 120 s. The CAT activity was expressed as μmol H_2O_2/mL erythrocytes/min [18].

2.3.3. Superoxide dismutase enzyme activity

Superoxide dismutase (SOD) activity was performed according to the method of Misra and Fridovich (1972). Briefly, epinephrine rapidly auto oxidizes at pH 10.5 producing adrenochrome, a pink colored product that can be detected at 480 nm. The addition of samples containing SOD inhibits the epinephrine auto-oxidation. The inhibition rate was monitored during 150 s at intervals of 10 s. The amount of enzyme required to produce 50% inhibition at 25 °C was defined as one unit of enzyme activity. The SOD activity was expressed as U/mL erythrocytes [19].

2.3.4. Plasma vitamin C

Plasma vitamin C (VIT C) was estimated as described by Galley et al. (1996) with some modifications by Jacques-Silva et al. (2001). Fresh isolated plasma was precipitated

with 1 volume of a cold 5% trichloroacetic acid solution followed by centrifugation. An aliquot of 300 μL of the supernatants was mixed with 2,4-dinitrophenylhydrazine (4.5 mg/mL) and 13.3% trichloroacetic acid and incubated for 3h at 37° C. Then, 1 mL 65% sulfuric acid was added to the medium and the orange red compound was measured at 520 nm. The content of ascorbic acid was calculated using a standard curve (1.5 – 4.5 μmol/L ascorbic acid freshly prepared in sulfuric acid) and expressed as μg VIT C/mL plasma [20,21].

2.3.5. Protein thiol groups

Protein thiol groups (P-SH) were assayed in plasma just after it isolation by the method of Boyne & Ellman (1972) as modified by Jacques-Silva et al. (2001), which consisted of the reduction of 5.5´-dithio(bis-nithrobenzoic) acid (DTNB) in 0.3 M phosphate buffer, pH 7.0, measured at 412 nm. A standard curve using glutathione was constructed in order to calculate the P-SH groups. The results were expressed as nmol P-SH/mL plasma [22,21].

2.3.6. Non protein thiol groups

Erythrocyte non protein thiol groups (NP-SH) were determined as described by Boyne & Ellman (1972) modified by Jacques-Silva et al. (2001). Red blood cell pellets (300 μL) obtained after centrifugation of whole blood were hemolyzed with 10% triton solution (100 μL) for 10 min. Then, the protein fraction was precipitated with 200 μL of 20% trichloroacetic acid followed by centrifugation. The colorimetric assay was carried out in 1 M phosphate buffer, pH 7.4. A standard curve using glutathione was constructed in order to calculate non protein thiol groups. The NP-SH levels were measured at 412 nm and expressed as nmol NP-SH/mL erythrocytes. [22,21].

2.3.7. δ-Aminolevulinate dehydratase activity

δ-aminolevulinate dehydratase (δ-ALA-D) activity was assayed in whole blood by the method of Berlin and Schaller [23] by measuring the rate of porphobilinogen (PBG) formation in 1 h at 37°C. The enzyme reaction was initiated after 10 min of pre-incubation of blood with 1mM $ZnCl_2$. The reaction was started by adding δ-aminolevulinic acid (ALA) to a final concentration of 4 mM in a phosphate buffered solution, and incubation was carried out for 1 h at 37° C and the reaction product was measured at 555 nm and expressed as nmol PBG/mL blood/h.

2.4. Data analysis and statistics

Biochemical assay results were expressed as median (lower/ upper quartiles) and ages were expressed as mean ± SD.

Since data had no homogeneity of variance, statistical analysis was performed using Kruskal-Wallis ANOVA followed by Mann-Whitney U test to compare the difference among the groups, Friedman ANOVA followed by Wilcoxon test to analyze changes in blood indices over time and Spearman Correlation to analyze correlations between biochemical estimations.

A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Lipid peroxidation

Plasma TBARS levels were significantly higher in group II (M-200) than group I (control), on days 10 and 20 after BMT. TBARS were higher in group III (CBV) than in group I, before CR, during CR and on days 10 and 20 after BMT ($p<0.05$). Groups II and III were not significantly different from each other in TBARS levels. TBARS levels did not change over time for patient groups (Fig. 1).

3.2. Vitamin C levels

Plasma VIT C concentrations were significantly decreased in group II (M-200), during CR and on day 10 after BMT ($p<0.05$) and returned to control levels on day 20 after BMT. In group III (CBV), VIT C levels were significantly decreased during CR and on days 10 and 20 after BMT ($p<0.005$) when compared with group I (control). VIT C levels were significantly lower in group III than in group II on day 20 after BMT ($p=0.01$). In group II, VIT C levels decreased significantly from before CR to during CR and to day 10 after BMT and from during CR to day 10 after BMT and increased from day 10 to day 20 after BMT ($p<0.05$). In group III, VIT C decreased significantly from before CR to during CR and to days 10 and 20 after BMT and from during CR to day 10 after BMT ($p<0.05$), (Fig. 2).

3.3. Catalase activity

Erythrocyte CAT activity was lower in group II (M-200) than in groups I (control) and III (CBV) on day 20 after BMT ($p<0.05$). CAT activity did not change over time for patients groups (Fig. 3).

3.4. Protein thiol groups

Plasma P-SH group levels were lower in group II (M-200) during CR ($p=0.004$) and on days 10 and 20 after BMT, ($p=0.0006$) and in group III (CBV) before CR ($p=0.0006$), during CR ($p=0.007$), on day 10 ($p=0.0006$) and on day 20 after BMT ($p=0.002$) when compared with group I (control). P-SH in groups II and III were not significantly different from each other. In group II, P-SH levels decreased from CR and from day 10 to day 20 after BMT and in group III, decreased from before CR to days 10 and 20 after BMT ($p<0.05$), (Fig. 4).

3.5. Non protein thiol groups

Erythrocyte NP-SH group levels were higher in group II (M-200) than in groups I (control) and III (CBV), before CR, during CR and on day 20 after BMT ($p<0.05$). NP- SH levels in groups II and III did not change over time (Fig. 5).

3.6. Superoxide dismutase activity

SOD activity was not modified by CR treatments (data not shown).

3.7. δ-Aminolevulinate dehydratase

Blood δ-ALA-D activity was significantly lower in group II (M-200) on days 10 ($p=0.001$) and 20 ($p=0.002$) after BMT and in group III (CBV) during CR ($p=0.0006$) and on days 10 ($p=0.0009$) and 20 ($p=0.005$) after BMT than in group I (control). δ-ALA-D activity was significantly lower in group III than in group II on day 10 after BMT ($p=0.031$). In group II, δ-ALA-D activity decreased significantly from before CR to during CR and to days 10 and 20 after BMT and in group III, from before CR to days 10 and 20 after BMT and from during CR to day 20 after BMT ($p<0.05$), (Fig. 6).

3.8. Correlations of biochemical estimations

Statistical analysis (Spearman correlation) for data obtained before CR revealed a significant negative correlation between TBARS and P-SH and a positive correlation between VIT C and NP-SH as well as between SOD and δ-ALA-D for all groups (Panel 1A). For data obtained during CR, there was a significant negative correlation between TBARS and SOD and δ-ALA-D as well as a positive correlation between VIT C and P-SH and also between P-SH and δ-ALA-D for all groups (Panel 1B). For data obtained on day 10 after BMT, there was a significant negative correlation between TBARS and VIT C, SOD, P-SH and δ-ALA-D and a positive correlation between VIT C and SOD, P-SH and δ-ALA-D as well as between SOD and P-SH and δ-ALA-D and between P-SH and δ-ALA-D for all groups (Panel 1C). For data obtained on day 20 after BMT, there was a significant negative correlation between

TBARS and VIT C, P-SH and δ-ALA-D and a positive correlation between P-SH and δ-ALA-D for all groups (Panel 1D).

4. Discussion

Lipid peroxidation can cause a profound alteration in the structural integrity and function of cell membranes and free radical-induced lipid peroxidation has been implicated in the pathogenesis of several disorders, including cancer [24]. The increase in TBARS levels before the CR in patients with lymphoma (group III, CBV) indicates the presence of an oxidative state in this malignance. Treatment with alkylating agents has been shown to cause peroxide accumulation [25]. In our study, the drugs used in both CRs were associated with an increase in TBARS levels during the CR and after BMT. In fact, as described above, in the CBV group lipid damage was already evident before chemotherapy and there was no further increase in lipid peroxidation.

Thiols fulfill important antioxidant functions in cells and biological fluids [26]. The changes in the content of -SH groups, especially of the free -SH groups in membrane proteins may provide a measure of membrane protein damage [27]. Alkylating agents produce free radicals that can directly interact with thiol groups of proteins, oxidizing them to disulfides [28]. The CBV and M-200 patients presented plasma P-SH levels lower than those of the healthy volunteers, during the CR and 10 and 20 days after BMT. However, in the CBV group plasma P-SH was already lower before chemotherapy, but there was a further decrease in plasma P-SH levels, demonstrating that the protein thiol groups were oxidized by these cytostatic drugs.

VIT C is a powerful reducing agent and an important water-soluble vitamin for humans and certain other animals. It has been shown to scavenge O_2^-, H_2O_2, ·OH, aqueous peroxyl radicals and singlet oxygen. VIT C also protects plasma lipids against lipid peroxidation and has an important role in the regeneration of α-tocopherol [29]. For M-200 and CBV patients, VIT C levels declined in plasma with the CR, indicating that this antioxidant defense was impaired with both CRs. Furthermore, 20 days after BMT, VIT C levels returned to control levels in M-200 patients, but not in CBV patients.

SOD is a key enzyme required for the removal of O_2^- by converting it to hydrogen peroxide (H_2O_2), which is further eliminated by catalase and peroxidase [30]. In our study,

there was no change in erythrocyte SOD activity in either the M-200 or CBV groups at any time points analyzed (data not shown). Catalase is an enzymatic antioxidant and helps to neutralize the toxic effect of H_2O_2 [31] by converting it to water and non-reactive oxygen species, thus preventing the generation of hydroxyl radicals and protecting cells from oxidative damage [32]. We found that catalase activity was only modified 20 days after BMT in the M-200 group.

Toxicity induced by the systemic administration of cytostatic drugs entails bone marrow depression, gastrointestinal toxicity, such as nausea, vomiting, diarrhea and stomatitis, hair loss, maculopapular rashes and pruritis and these clinical manifestations have been associated with increased lipid peroxidation [33]. In this work all patients showed some of these clinical manifestations (data not shown) and this occurred simultaneously with an increase in TBARS. Here, it was shown that the M-200 is aggressive and toxic to the body, damaging both non-enzymatic and enzymatic antioxidant defenses. CBV is the most commonly used high dose preparatory regimen for Hodgkin's disease [34]. In the CBV CR, cyclophosphamide antineoplastic effects are associated with phosphoramide mustard, while its toxic side effects are linked with acrolein [35]. Acrolein interferes with the tissue antioxidant defense system [36], produces highly reactive oxygen free radicals [37] and is mutagenic to mammalian cells [38]. Irradiation or treatment with cytotoxic drugs (etoposide, camptothecin, melphalan or chlorambucil) causes a rapid increase in intracellular peroxide levels [39]. In our study, we observed that the CBV CR, which consisted of the association of three drugs, cyclophosphamide, BCNU and etoposide, caused organic toxicity. Finally, it was shown that the M-200 CR produced lipoperoxidation and affected the antioxidant defenses, while the CBV CR affected VIT C, P-SH levels and δ-ALA-D activity. In addition, TBARS levels in all groups showed a negative correlation with the antioxidants analyzed at different times. The oxidative stress caused by CBV and M-200, together with cytopenia, mucosite and other side effects, may contribute to serious complications after BMT, such as veno-occlusive disease of the liver [40] and delayed toxicity such as secondary malignancies [41].

ALA-D is a zinc metalloenzyme, essential for all aerobic organisms, that requires reduced thiol groups for its activity [42]. Reactive oxygen species, produced during the CR [7,8], can oxidize thiol groups located inside the active site of mammalian δ-ALA-D [43,44], decreasing enzyme activity as observed in this study. ALA-D also had a positive correlation with some antioxidants analyzed and a negative correlation with TBARS. These results show

that this enzyme could be utilized as an additional biomarker of oxidative stress. Finally, δ-ALA-D inhibition can result in ALA accumulation, which may have pro-oxidant effects [45] and contribute to oxidative stress caused by the CR.

In summary, the results presented here indicate that patients undergoing BMT showed some signs of oxidative stress even before the CR that were possibly a consequence of their disease. In fact, the group treated with CBV (patients with lymphoma) had elevated plasma TBARS levels before the CR, and only VIT C, P-SH levels and δ-ALA-D activity were modified with the CR. On the other hand, the group treated with M-200 (patients with MM) showed only erythrocyte NP-SH levels to be increased before the CR, while the other parameters such as, TBARS, VIT C, CAT, P-SH and δ-ALA-D activity were modified with the CR. This shows that M-200 causes a greater imbalance in the oxidant/antioxidant systems than CBV.

5. Conclusion

Considering our findings that CBV and principally M-200 may cause oxidative stress in patients undergoing autologous BMT and that this was associated with a decrease in blood δ-ALA-D activity, we can suggest that δ-ALA-D may be an additional biomarker of oxidative stress in BMT patients. However, since the number of patients used in this study was small, further studies with larger numbers of patients are needed to elucidate this premise.

6. References

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Table 1. Situation of patients before bone marrow transplantation

Patient	Sex	Age	Diagnosis	CR
A	m	52	MM	M-200
B	m	47	MM	M-200
C	m	50	NHL	CBV
D	m	47	HL	CBV
E	m	66	HL	CBV
F	m	61	MM	M-200
G	m	48	NHL	CBV
H	m	49	MM	M-200
I	m	56	HL	CBV
J	m	54	MM	M-200

MM: Multiple Myeloma; NHL: No Hodgkin Lymphoma; HL: Hodgkin Lymphoma; M-200: Melphalan 200mg/m^2 (group II); CBV: Cyclophosphamide-BCNU-Etoposide (VP-16) (group III); CR: conditioning regimen; m: male.

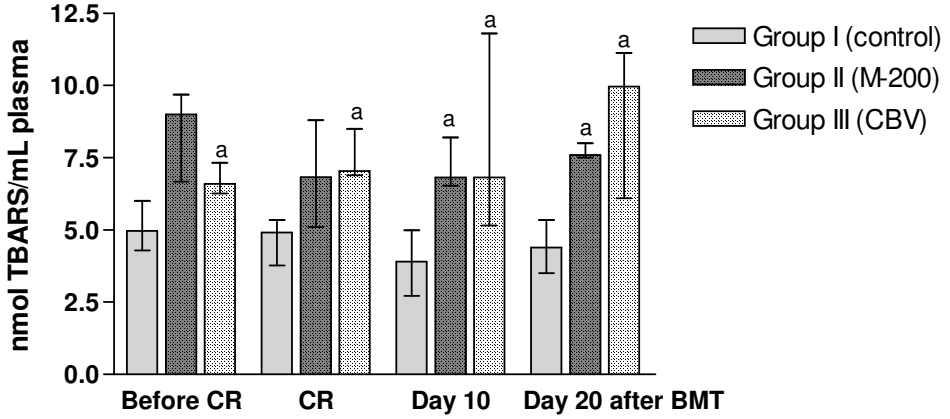

Figure. 1. Plasma lipid peroxidation (TBARS) levels over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control).

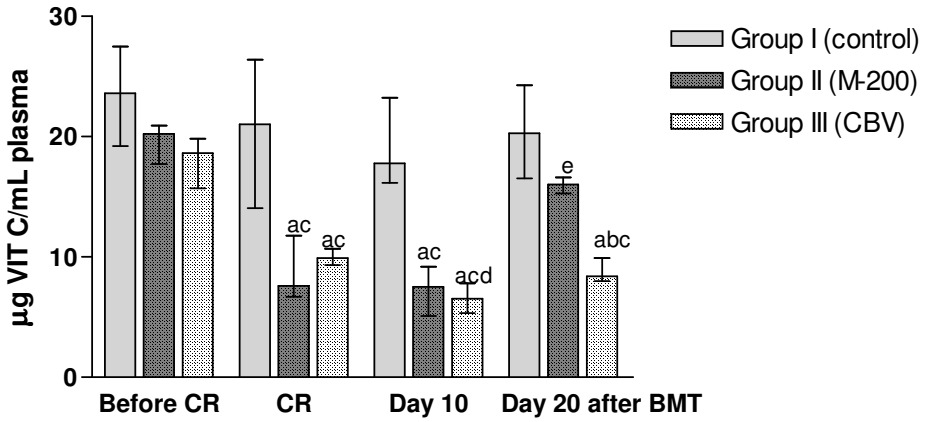

Figure.2. Plasma vitamin C (VIT C) levels over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control), [b]significantly different between groups II and III, [c]significantly different from before CR, [d]significantly different from CR, [e]significantly different from day 10 after BMT.

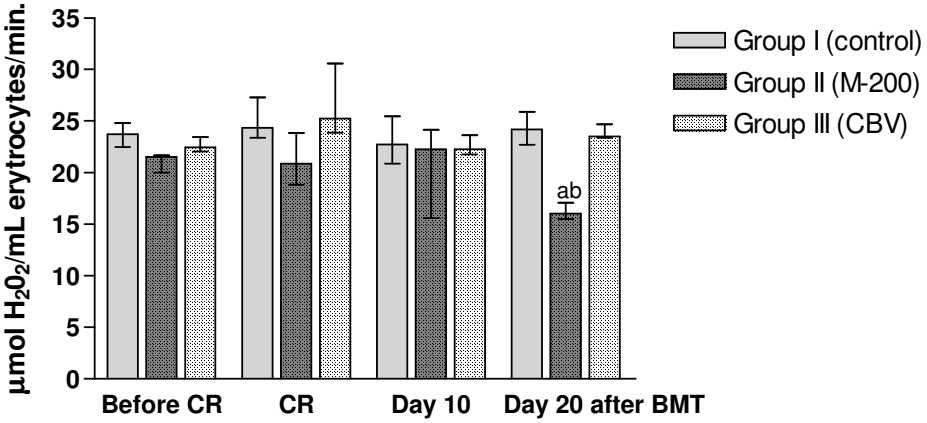

Figure 3. Catalase (CAT) erythrocyte activity over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control), [b]significantly different between groups II and III.

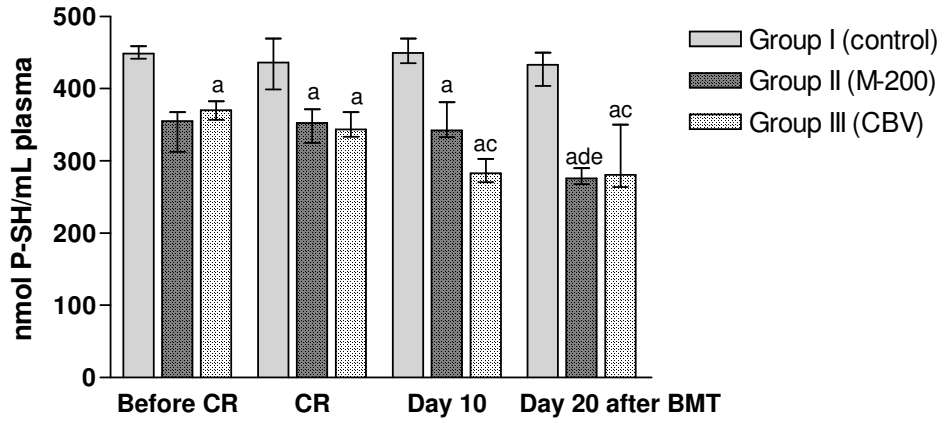

Figure. 4. Plasma protein thiol (P-SH) group levels over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control), [c]significantly different from before CR, [d]significantly different from CR, [e]significantly different from day 10 after BMT.

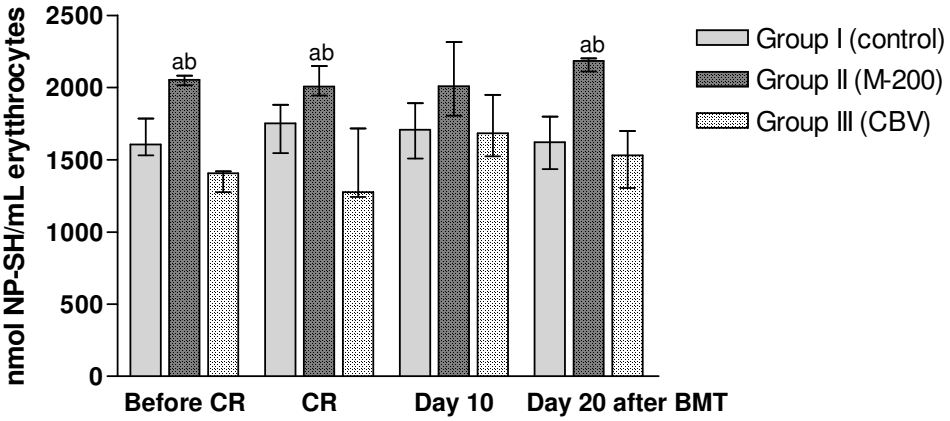

Figure. 5. Erythrocyte non protein thiol (NP-SH) group levels over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control), [b]significantly different between groups II and III.

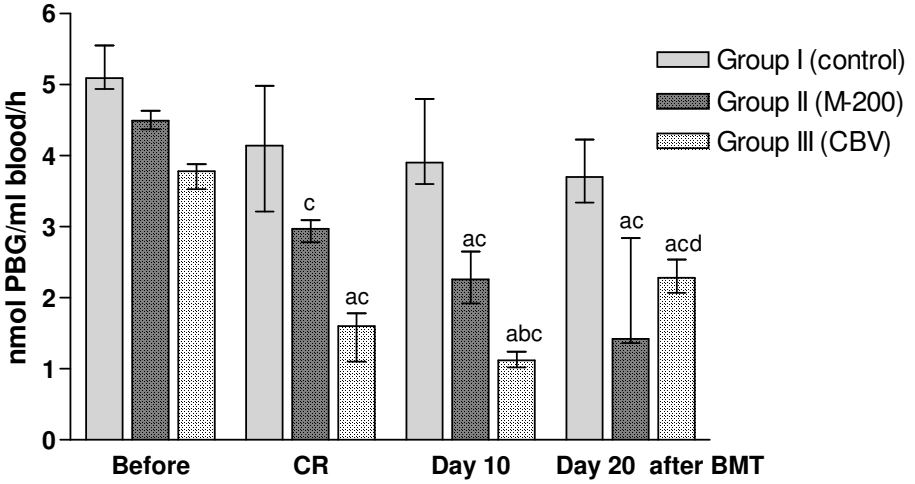

Figure. 6. Blood δ-Aminolevulinate dehydratase (δ-ALA-D) activity over time. CR: conditioning regimen; BMT: bone marrow transplantation. Data were expressed as median (lower/upper quartile). [a]Significantly different from group I (control), [b]significantly different between groups II and III, [c]significantly different from before CR, [d]significantly different from CR.

A

Estimations before CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.032 (n.s.)	0.025 (n.s.)	–0.125 (n.s.)	–0.522 * (p.0.037)	–0.008 (n.s.)	–0.133 (n.s.)
VIT C	_	0.057 (n.s.)	–0.319 (n.s.)	0.312 (n.s.)	–0.512* (p.0.042)	–0.175 (n.s.)
CAT	_	_	0.339 (n.s.)	0.130 (n.s.)	–0.300 (n.s.)	0.442 (n.s.)
SOD	_	_	_	0.131 (n.s.)	0.150 (n.s.)	0.802 * (p.0.005)
P-SH	_	_	_	_	0.023 (n.s.)	–0.060 (n.s.)
NP-SH	_	_	_	_	_	–0.078 (n.s.)

B

Estimations CR	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.094 (n.s.)	–0.390 (n.s.)	–0.513* (p.0.020)	–0.150 (n.s.)	–0.120 (n.s.)	–0.470 * (p.0.036)
VIT C	_	0.053 (n.s.)	0.079 (n.s.)	0.712* (p.0.000)	–0.284 (n.s.)	0.275 (n.s.)
CAT	_	_	0.163 (n.s.)	0.069 (n.s.)	0.016 (n.s.)	0.349 (n.s.)
SOD	_	_	_	–0.307 (n.s.)	0.145 (n.s.)	0.107 (n.s.)
P-SH	_	_	_	_	–0.220 (n.s.)	0.565 * (p.0.009)
NP-SH	_	_	_	_	_	0.112 (n.s.)

C

Estimations day 10 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.723* (p.0.000)	–0.193 (n.s.)	–0.636* (p.0.002)	–0.633* (p.0.003)	0.289 (n.s.)	–0.532* (p.0.023)
VIT C	_	0.191 (n.s.)	0.611* (p.0.005)	0.793* (p.0.000)	–0.041 (n.s.)	0.734* (p.0.000)
CAT	_	_	0.310 (n.s.)	–0.071 (n.s.)	0.311 (n.s.)	0.125 (n.s.)
SOD	_	_	_	0.720* (p.0.000)	–0.209 (n.s.)	0.758* (p.0.000)
P-SH	_	_	_	_	–0.312 (n.s.)	0.808* (p.0.000)
NP-SH	_	_	_	_	_	–0.020 (n.s.)

D

Estimations day 20 after BMT	VIT C	CAT	SOD	P-SH	NP-SH	δ-ALA-D
TBARS	–0.732* (p.0.000)	–0.347 (n.s.)	–0.240 (n.s.)	–0.614* (p.0.006)	0.286 (n.s.)	–0.740* (p.0.002)
VIT C	_	0.257 (n.s.)	0.264 (n.s.)	0.402 (n.s.)	–0.044 (n.s.)	0.510 (n.s.)
CAT	_	_	0.285 (n.s.)	0.385 (n.s.)	–0.385 (n.s.)	0.774 (p.0.001)
SOD	_	_	_	0.477 (n.s.)	0.108 (n.s.)	0.500 (n.s.)
P-SH	_	_	_	_	–0.473 (n.s.)	0.714* (p.0.004)
NP-SH	_	_	_	_	_	–0.283 (n.s.)

Panel 1. Correlation between biochemical estimations for all groups. A: estimations before CR; B: estimations during CR; C: estimations on day 10 after BMT; D: estimations on day 20 after BMT. CR: conditioning regimen; BMT: bone marrow transplantation; TBARS: thiobarbituric acid-reactive substances; VIT C: vitamin C; CAT: catalase; SOD: superoxide dismutase; P-SH: protein thiol groups; NP-SH: non protein thiol groups; δ-ALA-D: δ-aminolevulinate dehydratase; n.s.: no significant.

5 DISCUSSÃO

A literatura mostra que as ações de muitas drogas citostáticas, da radioterapia e do próprio TMO, estão associadas à produção de EROs e a uma diminuição nas defesas antioxidantes (CLEMENS et al., 1989; WEIJL et al., 1997; DÜRKEN et al., 2000) . Nossos resultados estão de acordo com a literatura, já que revelaram que tanto no TMO alogênico quanto no autólogo ocorreu um aumento na peroxidação lipídica e uma diminuição nas defesas antioxidantes enzimáticas e não enzimáticas, resultando assim em estresse oxidativo (manuscrito 1).

No TMO autólogo mesmo antes do RC e do TMO, os níveis de TBARS já apresentavam um aumento e não houve posterior aumento com o RC. Este aumento no TBARS, pode estar ligado a doença dos pacientes e a procedimentos realizados anteriormente ao TMO, como a coleta das células progenitoras hematopoéticas para posterior uso no TMO. Esta coleta, por si só já é estressante e muitas vezes é acompanhada de quimioterapia (WILMORE et al., 1999). Já no caso dos pacientes alogênicos o aumento no TBARS ocorreu com o RC e o TMO, mostrando assim o efeito do TMO, das drogas citostáticas e da radioterapia no aumento da peroxidação lipídica. Conforme a literatura o TMO alogênico é um procedimento mais complexo que o autólogo, já que o paciente recebe as células progenitoras hematopoéticas de outra pessoa (CASTRO Jr. et al., 2001). E neste caso, o risco de complicações é maior e há o problema da doença do enxerto contra o hospedeiro (DECH), que é prevenida com o uso de ciclosporina e metotrexate e mesmo assim pode ocorrer, porém em graus leves (PETERS et al., 2000).

Com relação aos RCs, todos os RCs analisados, tanto para TMO alogênico quanto para autólogo, causaram aumento nos níveis de TBARS (artigos 1 e 2). Nos pacientes alogênicos este aumento foi mais evidente com o uso de CyTBI. Acreditamos que este efeito mais pronunciado, seja causado principalmente pelo uso da irradiação corporal associada à ciclofosfamida, que por si só já é bastante tóxica. A literatura mostra que tanto a ciclofosfamida quanto a TBI, são tóxicos e provocam aumento pronunciado na peroxidação lipídica (DÜRKEN et al., 2000). Já em relação às defesas antioxidantes, nos pacientes alogênicos, os três RCs analisados, CyTBI, FluCY e BuCY promoveram modificações semelhantes (artigo 1).

Nos pacientes autólogos, as modificações nos antioxidantes e também o aumento no TBARS foram mais evidentes com o uso do melfalan. No grupo do CBV, mesmo antes do RC os níveis de TBARS e P-SH já se encontravam alterados e após o RC os níveis de VIT C, P-SH e atividade da enzima δ-ALA-D foram modificados, enquanto que com o uso do melfalan, TBARS, VIT C, CAT e P-SH foram modificados. Mostrando assim que o melfalan causou um maior desequilíbrio nos sistemas oxidantes/antioxidantes. As modificações mais acentuadas em relação ao estresse oxidativo com o uso do melfalan quando comparado com o CBV, podem ser explicadas pelo fato do CBV ser uma combinação de drogas, o que permite uma diminuição na dose necessária para o efeito desejado e com isto na toxicidade. Já o melfalan por ser uma única droga necessita de dose máxima para obter o efeito desejado, e com isto a toxicidade também é maior (SILVEIRA, 2006), (artigo 2).

A δ-ALA-D é uma zinco metaloenzima, essencial para os organismos aeróbicos, que requer grupos tióis reduzidos para sua atividade (FUKUDA et al., 1988). Nossos resultados demonstraram uma diminuição pronunciada na atividade da δ-ALA-D com todos os RCs analisados e também com os TMO alogênico e autólogo. E ainda a atividade da δ-ALA-D mostrou uma correlação positiva com as defesas antioxidantes e negativa com o TBARS, indicando que a enzima δ-ALA-D pode ser utilizada como um biomarcador de estresse oxidativo nos pacientes transplantados de medula óssea. Cabe ainda salientar que a inibição da δ-ALA-D pode resultar no acumulo do ALA, que pode ter efeitos pró-oxidantes (PEREIRA et al., 1992), podendo ter contribuído com o estresse oxidativo causado pelos RCs e pelo próprio TMO. Nossos resultados estão de acordo com dados da literatura e de nosso grupo, que indicam que a enzima δ-ALA-D é sensível a situações de estresse oxidativo (GONÇALVES et al., 2005, SILVA et al., 2007, VALENTINI et al., 2007, BARBOSA et al., 2008).

6 CONCLUSÕES

De acordo com os resultados apresentados nesta tese podemos inferir o seguinte:

Os TMO do tipo autólogo e principalmente alogênico estão associados ao estresse oxidativo, já que causaram um aumento na peroxidação lipídica e uma diminuição nas defesas antioxidantes enzimáticas e não enzimáticas no sangue dos pacientes autólogos e alogênicos.

FluCY, BuCy e CyTBI causam estresse oxidativo em pacientes alogênicos. Esses 3 RCs promoveram uma diminuição nas defesas antioxidantes enzimáticas e não enzimáticas e na atividade da enzima δ-ALA-D no sangue dos pacientes alogênicos. E também um aumento na peroxidação lipídica que foi mais acentuada com o uso de CyTBI, resultando assim em estresse oxidativo.

CBV e principalmente melfalan causam estresse oxidativo em pacientes autólogos, já que, CBV e mais evidentemente melfalan, provocaram uma diminuição nas defesas antioxidantes e na atividade na enzima δ-ALA-D e um aumento na peroxidação lipídica no sangue dos pacientes autólogos, resultando assim em estresse oxidativo.

Os TMO autólogo, alogênico e seus RCs promoveram uma diminuição na atividade da enzima δ-ALA-D. E ainda, a atividade da enzima δ-ALA-D mostrou uma correlação positiva com os níveis dos antioxidantes e negativa com os de TBARS, demonstrando assim que a enzima δ-ALA-D é um biomarcador adicional de estresse oxidativo em pacientes TMO.

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