FERNANDA CAROLINA DE CAMPOS

ANÁLISE DO ESTRESSE OXIDATIVO SISTÊMICO EM RATOS SUBMETIDOS AO TRATAMENTO COM PACLITAXEL E SEU AGENTE SOLUBILIZADOR, CREMOPHOR EL

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Patologia Experimental.

Orientador: Prof. Dr. Rubens Cecchini

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“A dúvida é o princípio da sabedoria”.

Aristóteles

RESUMO

INTRODUÇÃO E OBJETIVO: A quimioterapia com paclitaxel (PTX) é uma das principais formas de tratamento de diversas neoplasias. Devido à sua baixa solubilidade, é necessário o uso de Cremophor-EL (CREL), como veículo. Embora os efeitos tóxicos do PTX e CREL sejam bem estabelecidas *in vitro*, os mecanismos *in vivo* pelos quais isso ocorre ainda não está claro. Através de um modelo experimental mimetizando o tratamento quimioterápico realizado em humanos, o objetivo deste estudo foi investigar a participação do estresse oxidativo como um provável mecanismo de toxicidade do tratamento e elucidar se os efeitos tóxicos são devido à associação PTX-CREL ou somente ao veículo CREL.

METODOLOGIA: Este trabalho foi aprovado pelo comitê de ética em experimentação animal da Universidade Estadual de Londrina (CEEA n°82/10). Foram utilizados ratos Wistars (250g) canulados na veia femoral para infusão intravenosa (i.v.) com duração de uma hora. Foram avaliados em dois tempos, imediatamente (após 1 hora de infusão) e 24 horas após a infusão e divididos em 2 *sets* de 4 grupos. Grupo controle (CTR) que recebeu infusão i.v. de 3 mL de NaCl 0,9%, grupo etanol (ET) que recebeu infusão i.v. de 3 mL de NaCl 0,9% + etanol 50% v / v. , grupo Cremophor (CREL) que recebeu infusão i.v. de 3 mL de NaCl 0,9% + Cremophor 50% v / v. em etanol e o grupo paclitaxel (PTX) que recebeu infusão i.v. de 3 mL de NaCl 0,9% + paclitaxel 175mg/m^2 (PTX = paclitaxel + cremophor EL + etanol + NaCl 0,9%). No primeiro *set,* os grupos apenas receberam a infusão com duração de 1 hora. No segundo *set,* receberam 72 horas antes dos mesmos protocolos descritos acima, 100mg/kg i.p de vitamina E (vitE), para validar a participação do estresse oxidativo. Os animais foram sedados com éter etílico e o sangue foi coletado via punção cardíaca. Uma alíquota de sangue heparinizado total foi utilizado para determinar a cotagem de hemácias e leucócitos. O plasma foi obtido a partir da centrifugação do sangue a 1500 rpm, 10 minutos 4 °C, e armazenadas a -20°C. Os eritrócitos foram lavados três vezes com solução salina 0,9% a 4°C e utilizado imediatamente para as análises. Foram realizadas as seguintes avaliações: lipoperoxidação eritrocitária e plasmática avaliada por quimiluminescência (QL), determinação da atividade das enzimas superóxido dismutase (SOD) e catalase (CAT), glutationa reduzida (GSH), contagem de hemácias, perfil de hemoglobina para determinar metahemoglobina (metHB) e oxyhemoglobina (oxyHB), capacidade antioxidante total plasmática (TRAP), determinação de óxido nítrico (NO), hidroperóxidos e níveis de malondialdeído (MDA). Os dados foram analisados utilizando o programa GraphPad Prism 5.0, two-way ANOVA para cálculos de QL, one way ANOVA e teste *t* de Student, adotando $p < 0.05$ como significativo.

RESULTADOS: Os resultados mostraram aumento da QL eritrocitária no grupo CREL nos dois tempos analisados ($P < 0.0001$) e reversão após 24 horas com o uso da vitE ($P < 0.0001$). No grupo tratado com PTX a QL eritrocitária não obteve nenhuma diferença significante, diminuindo ainda mais a lipoperoxidação quando tratados com vitE em ambos os tempos analisados. A atividade da enzima catalase teve um aumento significativo após 24 horas da infusão de CREL ($P< 0.05$), revertido pela vitE, também 24 horas após ($P < 00001$). O grupo infundido com PTX obteve diminuição da CAT em 24 horas após infusão, e

esta diminuição foi alterada pelo prévio tratamento com vitE no mesmo tempo. A contagem de hemácias aumentou significatimente no grupo tratado com etanol 24 horas ($P < 0.05$). Os níveis de GSH não foram diferentes entre os grupos. Os níveis de oxyHB, diminuiu no grupo infundido com PTX imediatamente e previamente tratados com vitE ($P < 0.0001$), e aumentou no mesmo tempo ($P < 0.0001$), no grupo tratado com CREL e vitE. PTX obteve em 1 hora de infusão aumento de metHB e diminuição após 24 horas, ambos revertido por vitE ($P < 0.05$). O TRAP foi aumentado imediatamente no grupo CREL, diminuindo após 24 horas, mas apenas o efeito visto em 24h foi revertido pelo tratamento prévio de vitE ($P< 0.0001$). No grupo PTX foi observado diminuição imediata do TRAP e vitE reverteu este parâmetro($P < 0.05$). Houve aumento na QL plasmática no grupo PTX em ambos os tempos analisados, revertido pelo tratamento com vitE apenas imediatamente após 1 hora de infusão ($P < 0.0001$). A lipoperoxidação plasmática do grupo CREL esteve diminuida tanto em 1 hora, quanto após 24 horas, contudo o tratamento com vitE aumentou o perfil da QL no grupo CREL apenas na análise imediata ($P < 0.0001$). Nenhuma alteração significante em relação a SOD, NO, hidroperóxidos e MDA foram revelados.

CONCLUSÃO: Nossos dados mostraram que o tratamento com CREL é o principal responsável pelos danos oxidativos eritrocitários, enquanto PTX responde principalmente pelos efeitos plasmáticos, ambos através da geração de estresse oxidativo. Estes resultados fornecem novas informações sobre a presença de estresse oxidativo, como um mecanismo de toxicidade sistêmica *in vivo* do tratamento com CREL e PTX e identificam a necessidade de desenvolver novas formulações menos tóxicas de PTX.

ABSTRACT

INTRODUCTION AND AIMS: Paclitaxel (PTX) chemotherapy is one of the main forms of several cancer treatments. Due to its low solubility, it is necessary the use of Cremophor-EL (CREL) as vehicle. Although the toxic effects of PTX and CREL are well established *in vitro*, the *in vivo* mechanisms by which this occurs remain unclear. Through an experimental model mimicking chemotherapy performed in humans, the aim of this study was to investigate the role of oxidative stress as a mechanism of treatment toxicity and determining whether the toxic effects are due to the association of PTX-CREL or only the vehicle.

METHODS: This study was approved by the ethics committee in animal experimentation of the State University of Londrina (CEEA No. 82/10). Wistars rats (250g) were used. There were available in two times, immediately (after 1 hour of infusion) and 24 hours after the infusion and divided in 2 sets of four groups: Control group (CTR) received i.v. infusions of 3 ml of NaCl 0.9%, Ethanol group (ET) received i.v. infusions of 3 ml NaCl 0.9% + 50% ethanol v / v. , Cremophor group (CREL) received i.v. infusions of 3 ml NaCl 0.9% + 50% Cremophor v / v. (in ethanol) Paclitaxel group (PTX) that was infused i.v. at 3 mL NaCl 0.9% + paclitaxel 175mg/m^2 (PTX = Paclitaxel + Cremophor EL + ethanol + NaCl 0.9%). In the first set, the groups only received an infusion lasting 1 hour. The second set of animals received i.p. 100mg/kg of vitamin E (vitE) 72 hours before the same protocols described above to validate the role of oxidative stress. The animals were sedated with ethyl ether and blood was collected by cardiac puncture. An aliquot of whole heparinized blood was used to determine the red blood cells (RBCs) and leukocytes counts. Plasma was obtained by blood centrifugation at 1500 rpm, 10 minutes 4 ° C and stored at -20 ° C. The erythrocytes were washed three times with saline 0.9% solution at 4 ° C and used immediately for analysis. The following evaluations were performed: erythrocyte and plasma lipid peroxidation evaluated by chemiluminescence (CL), the activity of superoxide dismutase (SOD) and catalase (CAT), levels of reduced glutathione (GSH), RBCs counts, hemoglobin profile to determine methemoglobin (metHb) and oxyhemoglobina (oxyHB), total plasma antioxidant capacity (TRAP), levels of nitric oxide (NO), hydroperoxides and malondialdehyde (MDA). Data were analyzed using GraphPad Prism 5.0, two-way ANOVA for CL assay, one way ANOVA and Student's t test for others parameters, adopting $p < 0.05$ as significant.

RESULTS: CREL group showed an increased erythrocytic CL ($P < 0.0001$) and reversal after 24 hours with vitE treatment ($P < 0.0001$). PTX group did not differ in the erythrocyte CL and showed decreasing lipid peroxidation when treated with vitE in both periods analyzed. CAT activity increased significantly after 24 hours of infusion of CREL ($P < 0.05$), reversed by vitE ($P < 00001$), also after 24 hours. PTX group decreased CAT after 24 hours changed by prior treatment with vitE at the same time. RBCs counts increased only in the ET group at 24 hours ($P < 0.05$). GSH levels did not differ among groups. The oxyHB levels, decrease in the PTX group immediately and pretreated with vitE ($P < 0.0001$) and increase in the same time ($P < 0.0001$), in the group treated with CREL and vitE. PTX immediately increased metHB and decreased after 24 hours, both reversed by vitE ($P < 0.05$). TRAP was increased immediately in the CREL group and decreased after 24 hours, however, only the effect of the 24 hours was reversed by vitE ($P < 0.0001$). PTX group, immediately after

infusion, showed TRAP decrease and vitE reversed this parameter ($P < 0.05$). There was an increase of plasmatic CL in the PTX group in two times analyzed, reversed by vitE only immediately after infusion ($P < 0.0001$). The lipid peroxidation of CREL group was decreased in both immediately as 24 hours after infusion, however, treatment with the vitE increased QL profile of CREL group ($P < 0.0001$). No significant change in SOD, NO, MDA and hydroperoxides were revealed.

CONCLUSION: Our data showed that CREL treatment is primarily responsible for oxidative damage to erythrocytes, while PTX responds mainly by plasma effects, both through the generation of oxidative stress. These results provide new information on the presence of oxidative stress as a mechanism of systemic toxicity *in vivo* treatment with PTX and CREL and identify the necessity to develop new formulations less toxic PTX.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

4-HNE - 4-hidroxinonenal

CAT - catalase

ERNs - espécies reativas do nitrogênio

EROs - espécies reativas do oxigênio

GPx – glutationa peroxidase

GSH – glutationa reduzida

GSSG – glutationa oxidada

MDA - malondialdeído

QL – quimiluminescência

SOD - superóxido dismutase

TBARS - substâncias reativas ao ácido tiobarbitúrico

TRAP – capacidade antioxidante total

VitE – vitamina E

SUMÁRIO

1. INTRODUÇÃO

1.1. Câncer e tratamento: Quimioterapia

O Câncer um termo comum que se refere a tumores malignos, tem como característica a proliferação e crescimento descontrolado de células com capacidade de invasão e metástase (ROBBINS & COTRAN, 2010; ALMEIDA et al. 2005).

De um modo geral, o câncer é um termo empregado para designar mais de uma centena de diferentes doenças crônicas e infelizmente está se tornando cada vez mais comun no mundo todo, causando danos devastadores para muitas famílias (JEMAL et al. 2010).

No Brasil, as estimativas de 2012 válidas para 2013, definem a ocorrência de aproximadamente 518.510 novos casos, sendo as neoplasias de pele não melanoma, próstata, pulmão, estômago, cólon e reto mais incidentes no sexo masculino e as neoplasias de pele não melanoma, mama, colo do útero e glândula tireóide mais incidente no sexo feminino (INCA, 2012).

Existem vários fatores de riscos para o desenvolvimento do câncer, na maioria dos casos estão relacionados ao meio ambiente, aos hábitos de vida e também aos consumos diários de alimentos e medicamentos em geral (ALMEIDA et al. 2005).

O tratamentodo câncer é definido a partir da gravidade constatada da doença, sendo possível utilizar as técnicas de cirurgia, radioterapia, quimioterapia, hormonioterapia ou mesmo as combinações destas (SOUZA, 2004). Sem metástase, a técnica cirúrgica pode ser eficaz para a remoção do tumor e geralmente é associada à radioterapia (ALMEIDA et al. 2005). Entretanto, na maioria dos casos, os pacientes são submetidos ao tratamento com quimioterapia, visando à redução do tamanho do tumor previamente a cirurgia (quimioterapia neoadjuvante), prevenção de metastatização (quimioterapia adjuvante) ou o tratamento de metástases já disseminadas (quimioterapia paliativa) (INCA, 2010).

A quimioterapia representa um avanço na busca da cura do câncer (MELO et al. 2002). Os fármacos utilizados no tratamento quimioterápico têm por finalidade impedir a reprodução celular, levando as células malignas à morte (SOUZA, 2004). Segundo Ferreira e colaboradores (2008) os agentes antineopláscios inicialmente foram desenvolvidos com a função de destruir de forma rápida as células neoplásicas, atuando sobre os processos metabólicos celulares, como o DNA, RNA e a síntese de proteínas, porém esta ação não é exclusiva a estas células durante o tratamento, devido ao fato de que há lesões agudas e crônicas em células normais. Rocha e colaboradores (2004) confirmam que ocorre o acúmulo desses agentes nos tecidos saudáveis ocasionando grave toxicidade clínica.

O estudo para o tratamento das neoplasias com a quimioterapia iniciou no final do século XIX, com a descoberta da solução de Fowler (arsenito de potássio) por Lissauer (em 1865), e da toxina de Coley (em 1890). Contudo foi na Segunda Guerra Mundial em Bari, Itália (1943) durante uma explosão de um depósito de gás mostarda, foi observado em soldados expostos ao gás, alguns efeitos como a mielodepressão intensa e morte por hipoplasia de medula óssea. E à partir destas observações, foram então administrado quimioterápicos em pacientes com linfoma de Hodgkin e leucemia crônica, desenvolvido por farmacologistas do Pentágono.(BONASSA et al. 2000).

A classificação dos quimioterápicos ocorre conforme a sua atuação sobre o ciclo celular. São divididos em: fármacos de ciclo inespecífico – que atuam nas células que estão ou não no ciclo proliferativo (Ex. mostarda nitrogenada); fármacos de ciclo específico – que atuam somente nas células que estão em proliferação (ciclosfosfamida); fármacos de fase específica – atuam em determinadas fases do ciclo celular (Ex. metrotexato na fase S e taxol na fase M) (INCA, 2012).

Estudos demonstram que tanto o câncer quanto o tratamento quimioterápico, podem levar o paciente à anemia através de diversos mecanismos, como suprimir a hematopoiese diretamente por infiltração de medula óssea, ou ainda levar à perda crônica de sangue em locais do tumor (BENNET et al. 2008). Adicionalmente, dependendo da concentração plasmática, do tempo de exposição e da droga utilizada durante o tratamento, podem ocorrer aos pacientes efeitos colaterais, como hipoplasia e aplasia medular, alopécia e alterações gastroinestinais (SANTOS & CRUZ, 2001). Apesar destes efeitos colaterais, os antineoplásicos, são drogas que têm sido amplamente utilizados no tratamento do câncer (ROCHA et al. 2004). No Brasil, dentre os quimioterápicos de escolha no Sistema Único de Saúde para tratamento dos tumores sólidos mais incidentes estão a doxorrubicina (DOX) e o paclitaxel (PTX) sendo freqüente a combinação destas duas drogas ao longo do tratamento (INCA, 2010).

O paclitaxel (PTX) é utilizado para o tratamento de diversas neoplasias (HADIZIC et al. 2010). Este antineoplásico tem atividade antitumoral significativa contra vários tipos de tumores humanos, incluindo o câncer de mama, ovário e pulmão (SCRIPTURE et al. 2005).

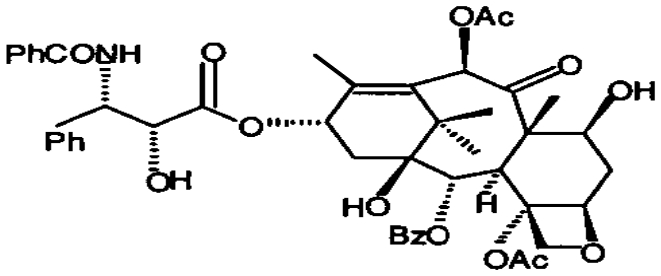

Figura 1 – Estrutura química do paclitaxel (CORRÊA, 1995).

Wall e colaboradores isolaram pela primeira vez, o PTX em 1962, a partir da casca de uma árvore do Pacífico, a *Taxus brefivolia (*BRANDÃO et al. 2010*).* Contudo esta espécie de árvore encontra-se em extinção e é necessário aproximadamente 10.000 kg da casca para

obter apenas 1 kg do PTX, além do mais demora cerca de 100 a 200 anos para esta atingir a maturidade (SOUZA, 2004). Todavia este problema foi resolvido, quando nas folhas da árvore *Taxus baccata,* fora identificado à molécula 10-desacetilbacatina-III, que apresenta o esqueleto básico e as funcionalidades do PTX (KINGSTON, 2000).

O PTX foi o primeiro composto descoberto capaz de induzir morte celular, impedido a mitose, através da estabilização dos microtúbulos, inibindo a sua despolimerização a partir estabilização dos núcleos de α e β tubulina (BRANDÃO et al. 2010; SCHIFF & HORWITZ 1980). O alvo dos fármacos que leva a célula a morte impedindo a divisão celular, como é o caso do PTX, é a fase da metáfase (M), interrompendo o ciclo celular da fase G2 (fase de síntese de proteína) para M (SOUZA 2004).

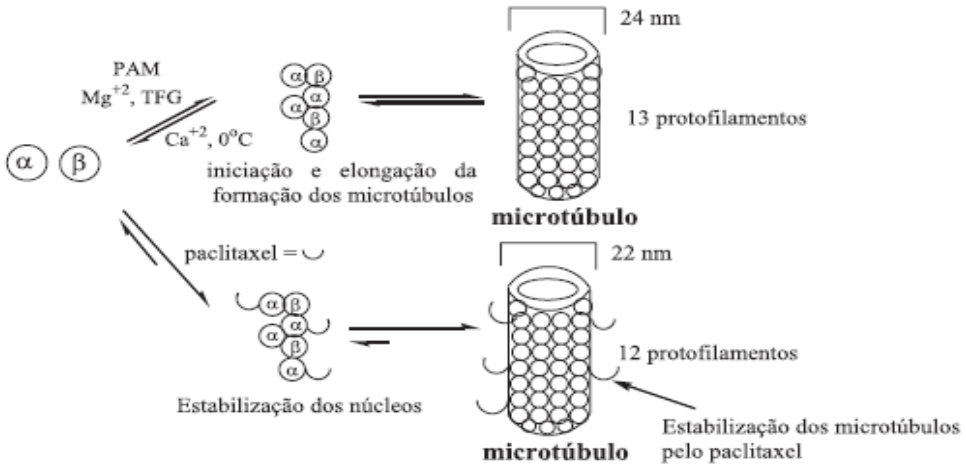

Figura 2 - Estrutura e estabilização dos microtúbulos pelo paclitaxel (SOUZA, 2004).

Devido à eficácia do PTX, a companhia americana Bristol-Meyer Squibb, o comercializou na década de 90 com o nome de Taxol®, e este fármaco encontra-se disponível para tratamento quimioterápico em mais de 60 países (SOUZA 2004).

Pesquisa com pacientes com carcinoma de ovário e administração de 135 e 175mg/m^2 de PTX em infusões de 3 e 24 horas, demonstraram que após a administração, o fármaco

apresenta um declínio bifásico em suas concentrações plasmáticas (Bristol–Myer Squibb, 2009). Todavia PTX apresenta uma elevada taxa de ligação às proteínas do plasma, aproximadamente 95%, e é extensivamente metabolizada no fígado, pelo sistema do citocromo P450 através da izoenzima CYP3A (SCHRIJVERS, 2003; BIGANZOLI et al. 2009).

1.2 Estresse oxidativo e toxicidade do paclitaxel

Uma perturbação generalizada das funções das células podem ser causada pelo estresse oxidativo, que caracteriza-se pelo desequilíbrio entre o sistema de geração e de eliminação de radicais livres ou seja pró e antioxidante, resultando em dano tecidual ou na produção de compostos tóxicos ou danosos aos tecidos (BURTON & YUNG, 2011; ROBBINS & COTRAN, 2010; SCHNEIDER & OLIVEIRA, 2004).

Radicais livres são todas as espécies que possuem um ou mais elétrons desemparelhados, sendo que este elétron livre pode estar centrado em um átomo de oxigênio, hidrogênio, nitrogênio, carbono, enxofre ou átomos de metais de transição (JUNIOR et al. 2001). Estes radicais podem iniciar reações em cadeia originando novos radicais e amplificando a capacidade de produzir lesões em proteínas, DNA e lipídios (FILHO, 2011).

O organismo sofre ação constante de espécies reativas de oxigênio (EROs) e nitrogênio (ERNs), gerados tanto em processos inflamatórios quanto por disfunções biológicas ou mesmo provenientes de alimentos (HALLIWELL & GUTTERIDGE, 2007).

Outra via de formação das EROs, os chamados de oxidantes, é através da redução unieletrônica do oxigênio à água, onde a entrada de 4 elétrons na molécula do oxigênio promove aparecimento do radical peróxido de hidrogênio (H_2O_2), ânion superóxido (O_2^-), e do radical hidroxila (OH^-) (HALLIWELL & GUTTERIDGE, 2007).

O O_2^- é produzido durante a auto-oxidação nas mitocôndrias, ou enzimaticamente pelas enzimas citoplasmáticas, como a xantina oxidase e Citocromo P-450 (ROBINS & COTRAN, 2010). O radical superóxido é formado a partir da adição de um elétron (e^-) a uma molécula de oxigênio:

$O_2 + e^- \longrightarrow O_2^-$ (SCHNEIDER & OLIVEIRA, 2004).

O O_2^- é pouco reativo em solução aquosa sendo convertido em H_2O_2 e oxigênio molecular (O_2), que pode ser espontânea ou acelerada pela enzima superóxido dismutase (SOD):

$O_2^- + \quad O_2^- + 2H \xrightarrow{SOD} H_2O_2 + O_2$ (FILHO, 2011).

Quando o H_2O_2 recebe mais um elétron e um íon hidrogênio, é formado o radical hidroxil (OH^-), que pode reagir e alterar qualquer estrutura celular que esteja próxima e assim influenciar enzimas, membranas ou ácidos nucléicos (JENKINS, 1988).

Os radicais livres gerados durante o estresse oxidativo têm muitos alvos celulares, mas um dos principais são os lipídios de membrana, causando a lipoperoxidação (SCHNEIDER & OLIVEIRA, 2004). Esta oxidação de lipídios, resulta em produtos primários como o alcoxil ($RO^\bullet$) e peroxil, e secundários, como o malondialdeído (MDA) e o 4-hidroxinonenal (4-HNE), estes ultimos são compostos eletrofílicos mais estáveis que podem se propagar por toda a célula danificando componentes celulares e interferindo em suas funções (CONKLIN, 2004) .

Quando a produção de EROs e ERNs é exacerbada, o organismo dispõem de um eficiente sistema antioxidante endógenos ou exógenos que bloqueiam o início da formação destes radicais ou os inativa, restabelecendo o equilíbrio entre pró e antioxidantes (ROBBINS & COTRAN, 2010; VASCONCELOS et al, 2007; HALLIWEL & GUTTERIDGE, 2007).

Existem defesas antioxidantes enzimáticas e não enzimátcas. O sistema enzimático inclui as enzimas SOD, catalase (CAT) e glutationa peroxidase (GPx). A SOD catalisa o radical O_2^- (esquematizado acima) e a CAT degrada H_2O_2

$2H_2O_2 \xrightarrow{CAT} O_2 + 2H_2O$ (BARREIROS & DAVID, 2006).

O sistema composto pela glutationa (GSH) funciona como mecanismo de proteção contra estresse oxidativo, convertendo a GSH em glutationa oxidada (GSSG), removendo H_2O_2 e formando água:

$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2\ H_2O$ (SCHNEIDER & OLIVEIRA, 2004).

Os antioxidantes SOD, CAT e sistema glutationa, formam as primeiras linhas de defesa contra espécies reativas e protegendo as células do estresse oxidativo, desempenhando um papel crucial nos efeitos dos produtos de peroxidação lipídica (PARK et al. 2007; GAGO-DOMINGUEZ et al. 2007).

Já o sistema não enzimático inclui compostos ingeridos através da dieta regular como ácido ascórbico (vitamina C), α-tocoferol (vitamina E) e β-caroteno e ou sintetizados pelo organismo humano, como ácido úrico, bilirrubina, hormônios sexuais, ceruloplasmina e melatonina (SCHNEIDER; OLIVEIRA, 2004).

Dados na literatura relatam diversos efeitos colaterais relacionados ao tratamento com PTX, muitos deles relacionados a geração de estresse oxidativo. Mark e colaboradores (2001) destacam a importância de investigar os efeitos cardiovasculares do tratamento. Biganzoli e colaboradores (2009) apontam como principal efeito adverso do PTX, a toxicidade hematológica relacionada a mieloaplasia e anemia de grau moderado a intenso. Recentemente estudo realizado por Panis e colaboradores (2011) em pacientes com câncer de mama submetidos à quimioterapia com PTX, demonstrou estresse oxidativo sistêmico e danos

eritrocitários com desenvolvimento da anemia, imediatamente após o tratamento quimioterápico.

Ramanathan e colaboradores (2005) demonstraram em seu trabalho, *in vitro,* que EROs e ERNs estão envolvidos na citotoxidade do tratamento com PTX. Hadizic e colaboradores (2010) mostraram ainda, que o efeito oxidativo do PTX seja devido principalmente à produção excessiva de peróxido de hidrogênio (H_2O_2). Alexandre e colaboradores (2007) confirmam em outro estudo que o PTX induz efeitos tóxicos sobre as células tumorais através da ativação de NADPH oxidase, promovendo a geração de H_2O_2 extracelular.

Considerando que PTX é insoluvel, este é formulado no veículo Cremophor EL (CREL), para aumentar a hidrossolubilidade da droga e permitir o tratamento endovenoso (SCRIPTURE et al. 2005). Por esta razão a fórmula PTX é composta de PTX mais 50% de CREL, mais etanol na mesma proporção (50%) (ADAMS et al, 1993; SPARREBOOM et al, 1998).

Scripture e colaboradores (2005) hipotetizaram que o transporte do PTX na circulação sistêmica pode ser através do seu aprisionamento dentro das micelas do CREL. Sendo provável que a interação farmacocinética do PTX seja dentro do compartimento sanguíneo central (GELDERBLOM et al. 2001; SPARREBOOM et al. 1999).

Estudos *in vitro* e *in vivo* sugerem que o tratamento com PTX induz aumento da viscosidade do sangue e a estomatocitose, sugerindo que o solvente CREL seja o responsável por essas alterações (MARK et al. 2001; NYGREN et al. 1995). Entretanto, o mecanismo pelo qual isso ocorre ainda é desconhecido.

O CREL, um óleo de alta viscosidade e cor amarelado pálido, obtido das sementes de *Ricinus communis*, tem sido utilizado para solubilização de uma ampla variedade de fármacos

hidrofóbicos, como sedativos, anestésicos, imunossupressores e drogas antineoplásicas (GUTIERREZ et al. 2006).

Em média a quantidade de CREL utilizada como solvente, é de 5mL por dose, já na formulção do PTX o montante por administração é muito maior (GUTIERREZ et al. 2006; SPARREBOM, 1998). Sendo assim um fator importante, é a compreensão do comportamento biológico e farmacológico do CREL, em especial na formulação do PTX (GELDERBLOM et al. 2001).

Estudos têm demonstrado alterações no comportamento farmacocinético em medicamentos administrados por via intravenosa que tenham este solvente, como é o caso de antraciclinas e PTX (SCRIPTURE et al. 2005). No tratamento quimioterápico com PTX, existem evidências de que o CREL seja o responsável por causar uma série de efeitos adversos graves, como hiperlipidemia, hipersensibiliade, padrões anormais de lipoproteína, neuropatia periférica e agregação eritrocitária (HENNENFENT & GOVINDAN, 2005; GELDERBLOM et al. 2001).

Há confirmações de que o CREL provoca alterações do acúmulo de PTX eritrocitário reduzindo a fração celular do fármaco livre (SPARREBOOM et al. 1999). Por outro lado um estudo avaliando a distribuição de PTX em coelho, rato e sangue humano, *in vitro* e *in vivo,* concluiu que a adição de CREL na formulação aumenta a distribuição de PTX no plasma para todas as espécies estudadas (LIU et al. 2008).

Gutierrez e colaboradores (2006) em estudo com fígados de animais tratados com CREL avaliaram os níveis de estresse oxidativo através da medida de substâncias reativas ao ácido tiobarbitúrico (TBARS) e GSH, evidenciando a geração de dano oxidativo durante o tratamento com este solvente. Em outro estudo, Iwase e colaboradores (2004) demonstraram *in vitro* que o uso do CREL depleta a GSH e induz a morte celular via produção de H_2O_2,

defendendo a idéia de que CREL potencializa a ação das drogas antineoplásicas via estresse oxidativo.

Devido aos efeitos deletérios dos radicais livres no organismo, atualmente existe um grande interesse no emprego de terapias antioxidantes capazes de neutralizar os danos oxidativos causados pelas espécies reativas, sem, entretanto, interferir no efeito antineoplásico da quimioterapia. Estudos demonstram que o tratamento experimental com vitamina E (vitE) pode ser um eficiente inibidor de peroxidação de lipídios causada pelo estresse oxidativo (BARREIROS & DAVID, 2006). Adicionalmente, embora os efeitos tóxicos do PTX, e seu agente solubilizador, o CREL, tenham sido estabelecidos *in vitro*, os mecanismos *in vivo* pelos quais isso ocorre ainda não está claro. Portanto, o objetivo deste trabalho foi avaliar a toxicidade hematológica induzida pela quimioterapia experimental com PTX, com base na infusão empregada no tratamento do câncer de mama humano através de parâmetros oxidativos e hematológico. Adicionalmente, foram conduzidas análises visando investigar o papel do solvente Cremophor EL neste modelo.

2. OBJETIVO

Avaliar a toxicidade sistêmica imediata e 24 horas após o tratamento com o antineoplásico Paclitaxel e seu agente solubilizador, o Cremophor EL, e sua relação com estresse oxidativo em modelo experimental.

2.1 Estratégias

Neste trabalho pretendeu-se avaliar o perfil pró-oxidativo e antioxidante de hemácias e plasma de ratos Wistar imediatamente após a infusão endovenosa de paclitaxel (175mg/m^2) ou Cremophor através dos seguintes parâmetros:

1-Análise da lesão oxidativa em eritrócitos através da mensuração da lipoperoxidação de membrana, contagem de hemácias e perfil de hemoglobina;

2- Determinações dos níveis plasmáticos de lipoperoxidação, óxido nítrico (NO), de hidroperóxidos e malondialdeído (MDA);

3- Avaliação do *status* antioxidante através da atividade das enzimas eritrocíticas superóxido dismutase e catalase, níveis de glutationa reduzida e capacidade antioxidante total plasmática.

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4. ANEXOS

4.1 ANEXO 1 – Normas técnicas para publicação na revista –Plos One"

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4. Conclusions are presented in an appropriate fashion and are supported by the data.
5. The article is presented in an intelligible fashion and is written in standard English.
6. The research meets all applicable standards for the ethics of experimentation and research integrity.
7. The article adheres to appropriate reporting guidelines and community standards for data availability.

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The results must be interpreted appropriately, such that all conclusions are justified. However, authors may discuss possible explanations for their results as long as these are clearly identified as speculations or hypotheses, rather than as firm conclusions. Inappropriate interpretation of results is a justifiable reason for rejection.

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Solar Drinking Water Disinfection (SODIS) to Reduce Childhood Diarrhoea in Rural Bolivia: A Cluster-Randomized, Controlled Trial

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Provide the first names or initials (if used), middle names or initials (if used), surnames, and affiliations—department, university or organization, city, state/province (if applicable), and country—for all authors. One of the authors should be designated as the corresponding author. It is the corresponding author's responsibility to ensure that the author list, and the summary of the author contributions to the study are accurate and complete. If the article has been submitted on behalf of a consortium, all author names and affiliations should be listed at the end of the article.

Abstract

The abstract succinctly introduces the paper and should not exceed 300 words. It should mention the techniques used without going into methodological detail and should summarize the most important results. Please do not include any citations in the abstract. Avoid specialist abbreviations if possible.

Registration

Registration details should be included when reporting results of a clinical trial (see "Reporting Clinical Trials" for details). For each location that your trial is registered, please list: name of registry, registry number, and URL of your trial in the registry database.

Introduction

The introduction should put the focus of the manuscript into a broader context. As you compose the introduction, think of readers who are not experts in this field. Include a brief review of the key literature. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Results

The results section should provide details of all of the experiments that are required to support the conclusions of the paper. There is no specific word limit for this section. The section may be divided into subsections, each with a concise subheading. Large datasets, including raw

data, should be submitted as supporting information files; these are published online alongside the accepted article. We advise that the results section be written in past tense.

Discussion

The discussion should spell out the major conclusions of the work along with some explanation or speculation on the significance of these conclusions. How do the conclusions affect the existing assumptions and models in the field? How can future research build on these observations? What are the key experiments that must be done? The discussion should be concise and tightly argued. Conclusions firmly established by the presented data, hypotheses supported by the presented data, and speculations suggested by the presented data should be clearly identified as such. The results and discussion may be combined into one section, if desired.

Materials and Methods

This section should provide enough detail to allow full replication of the study by suitably skilled investigators. Protocols for new methods should be included, but well-established protocols may simply be referenced. We encourage authors to submit, as separate supporting information files, detailed protocols for newer or less well-established methods. These are published online only, but are linked to the article and are fully searchable.

Acknowledgments

People who contributed to the work but do not fit the criteria for authors should be listed in the Acknowledgments, along with their contributions. You must also ensure that anyone named in the Acknowledgments agrees to being so named.

Details of the funding sources that have supported the work should be confined to the funding statement provided in the online submission system. Do not include them in the acknowledgments.

References

Only published or accepted manuscripts should be included in the reference list. Meetings abstracts, conference talks, or papers that have been submitted but not yet accepted should not be cited. Limited citation of unpublished work should be included in the body of the text only. All personal communications should be supported by a letter from the relevant authors.

PLoS uses the numbered citation (citation-sequence) method. References are listed and numbered in the order that they appear in the text. In the text, citations should be indicated by the reference number in brackets. Multiple citations within a single set of brackets should be separated by commas. Where there are three or more sequential citations, they should be given as a range. Example: "... has been shown previously [1,4-6,22]." Make sure the parts of the manuscript are in the correct order before ordering the citations.

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of the references is crucial. Please use the following style for the reference list:

Figure Legends

The aim of the figure legend should be to describe the key messages of the figure, but the figure should also be discussed in the text. An enlarged version of the figure and its full legend will often be viewed in a separate window online, and it should be possible for a reader to understand the figure without switching back and forth between this window and the relevant parts of the text. Each legend should have a concise title of no more than 15 words. The legend itself should be succinct, while still explaining all symbols and abbreviations. Avoid lengthy descriptions of methods.

Tables

Tables should be included in the text file, at the very end of the manuscript. All tables should have a concise title. Footnotes can be used to explain abbreviations. Citations should be indicated using the same style as outlined above. Tables occupying more than one printed page should be avoided, if possible. Larger tables can be published as online supporting information. Please ensure that table formatting conforms to our Guidelines for Figure and Table Preparation.

Ethical Treatment of Animals

For studies involving animals, all work must have been conducted according to relevant national and international guidelines. Approval must have been obtained for all protocols from the author's institutional or other relevant ethics committee and the institution name and permit numbers provided at submission (see example). For research involving non-human primates, all studies must be performed in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research". Where unregulated animals are used or ethics approval is not required by a specific committee, the article should include a clear statement of this and the reasons why ethical approval is not required. A statement should also be provided confirming that all efforts were made to ameliorate suffering of animals (and details should be provided in the methods).

Example: *This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Permit Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.*

4.2 ANEXO 2 – Aprovação do comitê de ética em experimentação animal

CEEA n° 82/10

Universidade
Estadual de Londrina

COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL

OF. CIRC. CEEA Nº 05/2011 Londrina, 14 de fevereiro de 2011

Prezado Pesquisador

O CEEA/UEL, reunido aos 14 de dezembro de 2010, avaliou o projeto de pesquisa intitulado **"Estudo da resposta medular em mecanismo oxidativo e da anemia hemolítica em animais portadores do tumor de Walker-256 submetidos à quimioterapia com doxorrubicina"**, registrado no CEEA sob o nº 82/10, pesquisa do Centro de Ciências Biológicas, desenvolvido sob sua responsabilidade. Esclarecidos os aspectos metodológicos solicitados, o projeto está *aprovado* para execução entendendo-se que os princípios éticos postulados pelo Colégio Brasileiro de Experimentação Animal estão respeitados.

Serão utilizados 64 ratos Wistar, machos, pesando entre 250-250g, provenientes do Biotério Central do CCB-UEL. Os animais serão divididos em 8 grupos com 8 animais cada. Serão constituídos 8 grupos: 1.Controle; 2.Grupo doxorrubicina; 3.Grupo tratado com vitamina E; 4. Grupo tumor de Walker; 5.Controle; 6.Grupo doxorrubicina; 7.Grupo tratado com vitamina E; 8. Doxorrubicina. Os animais serão submetidos à eutanásia e terão seu sangue coletado por punção cardíaca, fígado, coração e rins, fixados e preparados para análises histológicas, e outras avaliações serão realizadas para avaliação do sistema antioxidante e do grau de estresse oxidativo. O projeto está previsto para ser executado entre junho e setembro de 2011.

Cumpre orientar que caso se pretendam quaisquer alterações no protocolo experimental aprovado, deve-se submeter o novo protocolo à apreciação do CEEA/UEL anteriormente à execução das modificações.

Sem mais para o momento, subscrevo-me.

Cordialmente,

Profa. Dra. Mirian Siliane Batista de Souza
Coordenadora do CEEA/UEL

Ilmo. Sr.
Prof. Dr. Rubens Cecchini
Coordenador do Projeto
Departamento de Ciências Patológicas
Centro de Ciências Biológicas

Com cópia para Prof. Luiz Carlos Juliani (Diretor do Biotério Central da UEL).

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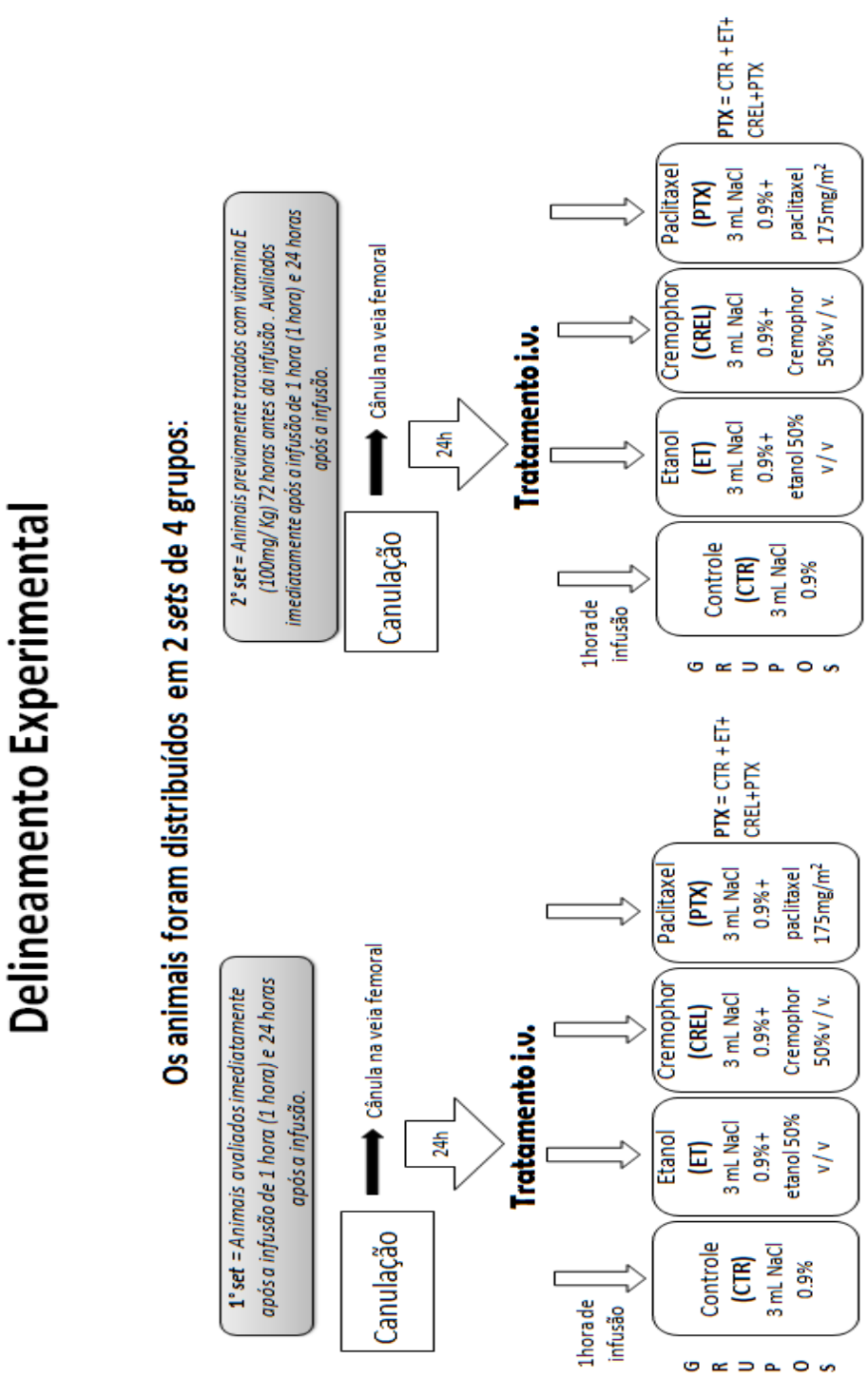
LONDRINA - PARANÁ - BRASIL

4.3 ANEXO 3 – Delineamento Experimental



4.4 ANEXO 4 – Artigo: Paclitaxel-induced oxidative stress *in vivo* is related with Cremophor EL.

Este trabalho foi realizado no Laboratório de Fisiopatologia dos Radicais Livres da Universidade Estadual de Londrina, formado pelo artigo científico: **Paclitaxel-induced oxidative stress *in vivo* is related with Cremophor EL.** Fernanda C. Campos, Carolina Panis, Vanessa J. Victorino, Ana Cristina A. Herrera, Marli C.M. Pinge, Alessandra L. Cecchini, Rubens Cecchini.

As formatações do artigo seguem as normas da revista Plos One (Anexo 1)

Paclitaxel-induced oxidative stress *in vivo* is related with Cremophor EL.

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The authors have no conflict of interest to declare.

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Abstract

Background: The toxic effects of paclitaxel (PTX) and its solubilizing agent cremophor EL (CREL) have been well established *in vitro*, nevertheless, *in vivo* mechanisms remain unclear. Due oxidative stress is associated with chemotherapy toxicity, the aim of this study was to investigate the involvement of oxidative stress as a mechanism of toxicity during PTX chemotherapy and further investigate if such effects are associated with its vehicle CREL.

Methodology/Principal Findings: It was evaluated the oxidative profile of Wistar rats treated with PTX and CREL immediately (1 hour) and 24 hours after infusion. Vitamin E (vitE) was employed to certify the oxidative stress participation. We evaluated the erythrocytic lipid peroxidation, superoxide dismutase (SOD) and catalase activity, reduced glutathione (GSH) levels, red blood cells (RBCs) count, hemoglobin profile, plasmatic total antioxidant capacity (TRAP), plasmatic lipid peroxidation, nitric oxide (NO), hydroperoxide and malondialdehyde (MDA) levels. Our findings showed enhanced erythrocytic lipid peroxidation in CREL group after 24 hours, reversed by vitE. CREL group showed a significant increase in catalase activity after 24 hours reversed by vitE, while in PTX group occurred a reduction in this parameter not neutralized by vitE. TRAP was increased by CREL infusion and a decreased after 24 hours was reversed by vitE. PTX immediately after infusion also decreased TRAP and vitE reversed it. Plasmatic lipid peroxidation increased in both PTX and CREL immediately and 24h after infusion and vitE did not reverse it. The metahemoglobin increased in PTX and decreased 24h after infusion and vitE reversed this parameters.

Conclusion: Our data show that CREL treatment is primarily responsible for the oxidative damage of RBCs, while PTX responds mainly for plasmatic effects, both through oxidative stress. These findings provide new information regarding the presence of oxidative stress as *in vivo* mechanism of systemic toxicity of CREL and PTX treatment.

Keywords: Paclitaxel, Cremophor, Toxicity, Oxidative Stress

Introduction

Cancer is a term used to designate more than one hundred different chronic diseases and has becoming increasingly common worldwide, causing devastating damage to families [1]. Chemotherapic treatment constitutes in the main cancer treatment, mainly in advanced tumors [2]. Chemotherapy interfere in cell proliferation, leading malignant cells to death [2], but its mechanisms of action can damage both healthy and neoplastic cells [3], resulting in severe toxicity to both [4]. Several side effects have been reported during chemotherapic regimens, mostly related to direct and indirect interactions between drugs and blood cells generated during the oxidative metabolism of drugs [5].

Paclitaxel (PTX), is a widely anticancer drug that acts against several human tumor types, especially breast, ovarian and lung [6]. This drug is isolated from *Taxus brefivolia*, which main mechanism of action consists in the depolymerization of cell microtubules, resulting in cell death [7]. Due to this efficiency the U.S. company Bristol-Meyer Squibb, developed and marketed this natural product as Taxol in the 90's, and is currently available as a medicine in more than 60 countries [2].

However, due to its insolubility, PTX is formulated in association with the solvent Cremophor EL (CREL) plus ethanol dehydrated [6] in the proportion of 50% PTX in 50% of CREL and dehydrated alcohol [8]. Although CREL have been employed as solvent for several drugs formulations, the PTX-CREL mixture present the higher amount of CREL described in pharmacology [9]. CREL is a polyethylated oil obtained from the seeds of *Ricinus communis*, with high viscosity and good lipophilicity, employed for solubilization of a wide variety of hydrophobic drugs [10]. Studies have shown that there are changes in the pharmacokinetic behavior of intravenously administered drugs that have been solubilized by CREL [6], suggesting that CREL is not an inert vehicle [10].

The introduction of this protocol based on the CREL-PTX mixture has been associated with the enhancement of response when patients present fail to respond to anthracyclins treatment [11], overcoming drug resistance. However, several toxic events have been associated to CREL-PTX infusion. The main clinical adverse effect of treatment with the CREL-PTX mixture is the acute hypersensitivity [12], which is characterized by dyspnea, flushing, rash, tachycardia, chest pain, generalized urticaria and hypotension [10]. Further, *in vitro* and *in vivo* studies show that PTX induce increased blood viscosity and stomacytic transformation of erythrocytes, suggesting that the solvent Cremophor EL can be able to intercalate in plasma membrane of these cells and provoke morphological changes [13].

Recently, our group showed that breast cancer patients that undergo to PTX treatment develop anemia immediately after chemotherapy infusion, suggesting that oxidative stress occurrence in such patients could be a probable causative mechanism [5]. Oxidative stress is characterized by the imbalance between the generation of reactive species and their neutralization, generating oxidative damage to proteins, lipids and DNA [14]. Antioxidant defenses, such as the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), as well as low molecular weight antioxidants, protect cells from oxidative damage, playing a crucial role in the effects of lipid peroxidation products, reducing the molecules reactivity [15].

Studies have been reported that PTX induces toxic effects on cancer cells through the generation of extracellular H_2O_2 from the membrane-associated NADPH oxidase, contributing to the potent anticancer drug [16, 17]. Evidences point that some of these toxic effects may be related mainly to CREL effects. *In vitro* treatment of thymic lymphocytes with CREL accelerates the cell death induced by H_2O_2, suggesting that CREL is able to enhance oxidative stress [18]. Further experimental treatment with CREL induces hepatic toxicity in an oxidative stress-dependent manner [19].

Although the toxic effects of PTX and CREL have been well established *in vitro*, the *in vivo* mechanisms by which it occurs remains unclear. Due oxidative stress is a common pathway associated with chemotherapy toxicity, the aim of this study was to investigate the oxidative stress as a probable mechanism of toxicity of this treatment and further elucidate if the toxic effects are due to the association PTX-CREL or only to CREL vehicle. We showed that CREL treatment is primarily responsible for the oxidative damage of red blood cells (RBCs), while PTX responds mainly for plasmatic effects, both through the generation of oxidative stress. These findings provide new information regarding the presence of oxidative stress as *in vivo* mechanism of systemic toxicity of CREL and PTX treatment.

Results

Erytrocytic lipid peroxidation curves were evaluated employing three statistical parameters (Figure 1). Two-way ANOVA and Student's t test were employed to analyze the difference among total curves profiles, while Bonferroni's test was used to compare each point from the curves. Immediately after infusion a significant increment in lipid peroxidation was observed as shown by the higher curve profile from animals treated with CREL and PTX compared with ethanol (ET) group (*P <0.0001),* while animals that received PTX or CREL displayed no difference when compared each other. (Fig. 1A). After 24 hours of chemotherapy infusion, the enhanced lipid peroxidation profile was observed in both CREL and PTX groups compared to ethanol group (*P <0.0001*) (Fig. 1B).

Vitamin E pre-treatment did not inhibited the lipid peroxidation induced by CREL immediately after infusion. Contrarely, the animals treated with PTX the lipid peroxidation was interally inhibited both compared with control treated with vit E (Fig.1C) ($P < 0.0001$).

After 24 horus, the levels of erytrocyt lipid peroxidation of the CREL and PTX not different from control treated with ethanol (Fig.1D).

Statistical evaluation point to point of lipid peroxidation curves showed no different points among all groups. Integration of the area under the curve (AUC) revealed a small although significant difference between CREL.24.E with ET.24.E ($P < 0.05$).

No differences were detected in SOD activity in all evaluated groups (Figure 2). Regarding catalase activity, it was observed that immediately after the infusion, catalase activity did not differ among the groups (Fig.3A), but after 24 hours CREL group showed a significant increase of catalase activity when compared with CTR and ET group ($P < 0.05$) (Fig.3B). On the other hand, a significant decrease was detected in PTX.24 group compared with CREL group showed in Figure 3B ($P< 0.0001$). VitE treatment (Fig.3D) reversed the increase in catalase activity in the group CREL and decreased enzyme activity revealed in the group PTX evaluation after 24 hours ($P < 0.05$).

Table 1 shows the erythrocytic counting and intracellular content of oxyhemoglobin (oxyHB), methemoglobin (metHB) and GSH. It was detected a significant increase in RBCs counts only in the ET group compared to CTR group evaluation 24 hours after the infusion ($P < 0.05$). It was also observed an increasing of oxyHB levels in the group CREL compared with the ET group after 24 hours of the infusion and pretreatment with vitE ($P < 0.0001$). A decreasing of oxyHB levels was further observed in PTX group pretreatment with vitE and evaluation immediately after the infusion ($P < 0.0001$). PTX treatment led to a significant metHB formation immediately after its infusion compared with the CREL group ($P < 0.05$). GSH levels showed no difference among the groups.

TRAP levels increased significantly in plasma from animals treated with ET compared with CTR and CREL compared with CTR and ET group both assessed immediately after the infusion ($P < 0.0001$) (Fig.4A). PTX group decreased TRAP levels ($P <0.0001$) compared with the CREL group and evaluation immediately after the infusion. After 24 hours of treatment, ET group enhanced the TRAP when compared with CTR group ($P< 0.05$), while

CREL group showed decreased TRAP when compared with ET group ($P < 0.05$) (Fig.4B). Evaluation immediately and with the pre treatment with vitE, (Fig.4C) plasma levels of TRAP showed increased in CREL group in relation to both CTR and ET evaluation immediately ($P< 0.05$) and PTX group revealed an augment in TRAP compared to the CTR and CREL group ($P< 0.05$). All group showed no difference when pretreatment with vitE and evaluation after 24 hours.

Immediate analysis of plasmatic lipid peroxidation profile demonstrated a significant reduction in CREL group compared with ET group ($P<0.0001$) and an important increase in PTX group when compared with CREL group ($P < 0.0001$) (Fig. 5A). After 24 hours, the profile of lipid peroxidation from animals treated with CREL remained low when compared to ET group ($P < 0.0001$), while PTX group also presented enhancement of this parameter in relation to CREL group ($P < 0.0001$) (Fig.5B). The pretreatment with vitE and the immediate evaluation showed that CREL group obtained increased lipid peroxidation in relation to the ET group ($P < 0.0001$), further, the subset PTX decreased curve profile in relation to CREL ($P < 0.0001$) (Fig.5.C). However, when assessed after 24 hours and also pre treated with vitE (Fig.5D), the CREL group reduced the lipid peroxidation profile relative with the ET group ($P < 0. 0001$), while PTX significantly augmented it when compared with CREL group ($P < 0. 0001$). Statistical evaluation of lipid peroxidation curves revealed a significant difference in following points of the curve of CREL group in relation with the curve of ET group evaluation immediately oh the infusion ($P < 0.05$) point 7 of the curve; also evaluation immediately and pretreatment with vitE the curve of the CREL group compared with ET group showed difference the point 2 to 8 ($P <0.001$) and between the PTX group an CREL group the difference the point 2 to 5($P <0.001$); after the 24 hours the CREL and ET group showed difference between the curves ($P <0.01$) from point 2 to 4; and the curve of the PTX group also points had difference compared with the CREL group ($P <0.0001$) of the point 1

to 7; after 24 hours of the infusion by pretreatment with vitE the curves of the CREL group compared with PTX group showed difference the points 3 to 7*(P <0.0001*), and the PTX group compared with CREL group the points 2 to 3 to 7 (*P <0.001)*.

Levels of nitric oxide, the hydroperoxides and malondialdehyde are shown in Table 2 with no differences among groups.

Discussion

This work demonstrated for the first time *in vivo* occurrence of systemic oxidative effects during PTX treatment and its relation with CREL. An important point of this study is the fact that not previous data demonstrated the effects of experimental chemotherapy after a continuous intravenous infusion, employing the equivalent dose of chemotherapy used during human cancer treatment. Further, we pre-treated the groups with vitamin E aiming to clearly the participation of oxidative stress in this process. Relevant conclusions of this study included the differential effects of both drugs, stressing that the CREL was largely responsible for the oxidative damage caused in the RBCs during chemotherapy, due to the high level of erythrocyte lipid peroxidation and catalase activity, while treatment with PTX was responsible for oxidative damage in plasma, due to increased plasma lipid peroxidation, and of methemoglobin and reduction levels of TRAP. Noting that both the damage caused by CREL and PTX were reversed by pretreatment of vitE and highlighted the presence of oxidative stress.

Evaluation of RBCs lipid peroxidation, one of the best ways to investigate the damage caused by ROS in cellular membranes [15] showed in our study that animals infused with CREL presented enhanced lipid peroxidation immediately and 24 hours after treatment, reversed by vitE only at 24h. The antioxidant property of vitE is based on its capacity to react very fast with peroxyl radicals and intercept the lipid peroxidation chain reaction [20].

Therefore, data regarding lipid peroxidation indicate that the solvent CREL should be the main responsible for the oxidative membrane damage observed in RBCs. Mark and collaborators (2001) demonstrated that several formulations of taxanes, including Taxol®, induced a dose and time-dependent irreversible stomatocytic transformation of erythrocytes and increment in whole blood viscosity *in vitro*, confirmed in patients. The authors suggested that this event occurred due a preferential intercalation of these substances into the inner hemileaflet of the membrane lipid bilayer and is a consequence of the vehicle Cremophor EL (CrEL) employed in Taxol formulation.

It was also demonstrated that oxidative stress is an important mechanism of causing damage to RBCs membrane associated with PTX treatment, since vitE reverses the oxidation process. This finding is corroborated by previous data of our group. We demonstrated that breast cancer women undergoing PTX chemotherapy also presented higher levels of lipid peroxidation when compared to breast cancer patients before treatment [5]. Oxidative stress parameters such as SOD activity, MDA, hydroperoxide, and NO did not vary in both treatments, indicating that these markers are not involved in PTX-CREL toxicity, although *in vitro* studies suggest that H_2O_2, superoxide anion (O_2^-), and NO may all be involved in paclitaxel-induced cytotoxicity [21].

In vitro data regarding the incubation of thymocytes with two doses of CREL demonstrated that this solvent is important to enhance the chemotherapy action. Anticancer treatment can induce the augment of GSH levels during the detoxification reactions, while CREL isolated is able to consume GSH molecules, leading to cell death by apoptosis through an oxidative stress mechanism dependent on H_2O_2 [18]. In this way, our data suggest that this oxidative stress-dependent pathway is possible during CREL effects *in vivo*, because no change in intracellular levels of GSH were detected and increased activity of the H_2O_2-detoxifying enzyme catalase [22] was also detected 24 hours after treatment. However, 24

hours after infusion of PTX, it was observed a reduction in catalase activity, which could be explained perhaps by an excessive accumulation of H_2O_2 intracellularly, inactivating the catalytic moiety of this enzyme [14]. It has been demonstrated that the PTX is able to induce activation of NADPH oxidase and promote the release of extracellular H_2O_2 [17]. However, the presented data suggest that such event do not occur *in vivo*. The reestablishment of catalase activity after vitE treatment shows the importance of the tight balance between both enzymatic and non-enzymatic antioxidant system response to restore the oxidative imbalance caused by chemotherapic treatment.

It is well know that PTX treatment induce anemia in several degrees [23]. Moreover, RBCs membrane is richly composed of polyunsaturated fatty acids, highly susceptible to attack by free radicals leading to lipid peroxidation, becoming it a good experimental model to study pre hemolytic lesions [24]. Blood counting was performed in this study to verify either if this event also occurred in our model and, additionally, if vitE treatment could reverse this effect. Despite the enhanced lipid peroxidation in RBCs membrane, our findings did not evidenced the occurrence of anemia by reduction of RBCs number. Thus, either immediately or after 24 hours of chemotherapic treatment, there is the existence of pre-hemolytic lesions which became RBCs more susceptible to hemolysis in a later time not evaluated in this work. Experimental data obtained from rats treated with PTX (29 mg/kg i.p.) demonstrated this fact, since hematocrits, hemoglobin levels and total bone marrow cellularity present a decreasing around 1 -2 days after PTX treatment [25].

It was also observed that oxyHB levels were significantly reduced in PTX group analyzed 24 hours after the infusion and treated with the vitE, and increased in the CREL group analyzed in this same time and with the antioxidant treatment. In addition, in an immediate evaluation only PTX group showed higher metHB and this aspect change when this group was treated with vitE. The formation of metHB is associated with the reaction of

NO with oxyHB, rapidly destroyed inside the catalytic center iron-containing groups [26]. Thus, the reduction of oxyHB levels after PTX treatment may be due to its oxidation to metHB. Still, the augment of oxyHB suggest an increasing of oxygen transport induced by CREL treatment.

TRAP profile, composed by low molecular weight antioxidants, exhibited increased levels in CREL group immediately after infusion, while it was decreased after 24 hours. The vitE is one of the abundant components that confers the antioxidant capacity evaluated on TRAP, and the antioxidant treatment was sufficient to reverse the reduction detected 24 hours after CREL infusion. PTX group decreased TRAP levels immediately after treatment, but vitE treatment was able to completely reverse this effect. Total antioxidant capacity is a critical determinant of sensitivity to PTX treatment because the depletion of intracellular antioxidants reduces the resistance to paclitaxel [21], suggesting that TRAP impairment constitutes, in part, a mechanism of cell death induction by PTX.

Plasmatic lipid peroxidation presented enhanced in PTX group immediately and 24 hours after the treatment. These findings corroborate to recent data of our group that show high levels of plasma lipid peroxidation in breast cancer patients treated with PTX, immediately after infusion [5].Vitamin E treatment decreased the lipid peroxidation of immediately after infusion group, but after 24 hours the levels were still elevated. PTX present a high rate of plasmatic proteins binding, over than 95% [27], which could impair the antioxidant effect of vitE to neutralize its oxidative effect. Still, CREL group showed reduced lipid peroxidation in both immediately and 24 hours after, but interestingly the pre-treatment with vitE elevated its levels after 24 hours, suggesting that vitE had a pro-oxidative property when associated with CREL [28].

In conclusion, our data support that both PTX and CREL are able to induce systemic toxicity by an oxidative stress-based mechanism. CREL treatment is primarily responsible for

the oxidative damage of RBCs, while PTX responds mainly for plasmatic oxidative effects, both through the generation of oxidative stress. These findings provide new information regarding the understanding of oxidative stress as *in vivo* mechanism of toxicity of CREL and PTX treatment and corroborate to the requirement to develop new less toxic formulations of PTX.

Acknowledgements

The authors would like to thank Jesus Antônio Vargas and Pedro Raimundo Dionisio for excellent technical assistance, and CAPES, CNPq, and Fundação Araucária for providing financial support.

Material and Method

This study was approved by the ethics committee on animal experimentation of the State University of Londrina (CEEA No. 82/10).

Animals

Wistars rats (250g) were bred in the mouse breeding facilities in the Department of Pathology Sciences, State University of Londrina, Brazil, under standard conditions. Mice were housed at a density of five animals per cage and offered special sterile food (Nuvital CR1) and water *ad libitum*. All animal procedures were performed in accordance with the principles of the Brazilian Code for the Use of Laboratory Animals and approved by Research and Ethics Committee from State University of Londrina.

Chemicals

All chemicals were provided from Sigma®. Taxol® (Paclitaxel) was purchased from Bristol-Meyer Squibb.

Treatments and sample obtention

Rats were cannulated in the femoral vein for intravenous infusion during 1 hour and were divided into 2 sets of 4 groups (n = 6). Control group (CTR) received intravenous infusion of 3 mL NaCl 0.9%, Ethanol group (ET) received intravenous infusion of 3 mL NaCl 0.9% + ethanol 50% v / v. Cremophor group (CREL) received intravenous infusion of 3 mL NaCl 0.9% + Cremophor 50% v / v (in ethanol), Paclitaxel group (PTX) received intravenous infusion of 3 mL NaCl 0.9% + paclitaxel 175mg/m^2 (PTX = PTX+CREL+ET +NaCl 0.9%). The first set of groups received only the infusions and the second set of groups received 72 hours before the treatments, plus vitamin E 100mg/kg i.p. (alpha-tocopherol dissolved in corn

oil), a gold standard antioxidant, to further validate the role of oxidative stress. Animals were euthanized immediately (after 1 hours of the duration of infusion) or 24 hours after infusion by ether anesthesia and blood collection by cardiac puncture. An aliquot of heparinized blood was used to counting leukocytes and erythrocytes. Plasma was obtained from blood centrifugation at 1500 rpm, 10 minutes, 4^0C and stored at – 20^0 C until analysis. Erythrocytes (RBCs) were washed three times with saline solution 0.9% at 4^0C and used immediately for specific further analysis.

High sensitive chemiluminescence induced by tert-butyl hydroperoxide (CL)

To evaluate oxidative stress in RBC and plasma samples, a chemiluminescence-based method was employed [5,29]. This method uses the tert-butyl hydroperoxide compound, starting a lipid chain reaction that can be detected by photon emission during the formation of lipid hydroperoxides. For RBC chemiluminescence assay, RBC was diluted 1200x in 10mM monobasic phosphate buffer, at 37°C. Then, reaction was started with 10µL of 3mM tert-butyl hydroperoxide added to 1mL of this dilution. The plasma chemiluminescence reaction was started by the addiction of 10µL of 3mM tert-butyl hydroperoxide in 125µL of plasma and 865µL of 30mM disodium phosphate-KCl 120mM buffer, pH 7.4, 37°C. Readings were performed in Glomax luminometer (TD 20/20 Turner Designers) for 40 minutes, one reading/ second. Results were expressed in relative units of light (RLU).

Superoxide Dismutase activity determination

Superoxide activity was determined as a method described by Marklund and Marklund [30]. This method is based on the inhibition of pirogalol autoxidation, which generates superoxide anion in this process, through the presence of superoxide dismutase. First, RBC was hemolysed in distillated water in a proportion of 1:20. Then, three runs of 5µL, 10µL and 20µL of samples were measured. To each run, destilated water, 1M TRIS (tris

– hydroxymethyl – aminomethane) buffer and pirogalol (1.2 mg/mL) were added. The auto-oxidation inhibition of pirogalol was measured at 420 nm in spectrophotometer (Shimadzu UV-1650 PC) during a kinetic. The results were expressed as SOD U/mL of RBC.

Catalase activity determination

Erythrocytic catalase activity was determined as described by Aebi [31,5]. In this assay, sample catalase enzyme degrades hydrogen peroxide generating oxygen and water in this process. In this method, RBC were diluted in distillated water in a proportion of 1:80. Then, 10µL of sample were incubated in a system containing 1M TRIS buffer and 200 mM hydrogen peroxide (H_2O_2). Kinetic of absorbance disappearance was monitored in spectrophometer at 240 nm (Shimadzu UV-1650 PC). The results were expressed in absorbance values/minute/mL of sample.

Erythrocytic GSH levels

To determine levels of GSH (glutathione reductase) [32], was used RBC were hemolysed at a ratio of 1:10 in distillated water and then, 1.25 mL of EDTA and 250 mL of 50% trichloroacetic acid (TCA) was added. After 15 minutes of incubation at room temperature, samples were centrifuged at 2400 x *g* for 15 minutes. Next, 1 mL of supernatant was added to 2 mL of 0.4M TRIS buffer, pH 8.9. Finally, 50µL of DTNB (5, 5' – ditiobis – 2 – nitrobenzoic acid) was added to react with GSH, forming a yellow compound. A standard curve was performed in order to determine GSH concentration in samples. The absorbance was read at 412 nm and results were expressed in nM.

RBCs and leukocytes Counts

RBCs and total leukocytes counting were determined employing a Neubauer chamber in a light microscope, after appropriated dilutions [33]. The results were expressed in cells/mm^3.

Profile of hemoglobin: Oxyhemoglobin and Methemoglobin

To determine Oxyhemoglobin and Methemoglobin, a method developed by Winterbourn, [34]. The 20µL of RBC hemolysed in 10mL of H_2O and the readings were taken in scan 700 to 400 nm in spectrophotometer (Shimadzu UV-1650 PC). The results were expressed in µM applying the following equations:

OxyHb = (119 x abs575) – (39x abs630) – (89x abs560)

MetHb = (28 x abs575) + (307x abs630) – (55x abs560)

Total Radical Antioxidant Parameter (TRAP) Thought High Sensitive Chemiluminescence Assay

TRAP was measured as described by Repetto et al. [35]. Briefly, 2, 2'azo-bis, 2 amidinopropane (ABAP), a potent free radical generator decomposes itself and emits photons in this process, which is amplified by luminol addition. The action of ABAP is neutralized as long as antioxidants are capable of inhibiting its function in the reaction. The photon emission profile of the ABAP solution was measured in a Glomax luminometer (Turner Designs TD 20/20) during 30 minutes, 5 readings/ second.

Initially ABAP emission (900µL of Glicine buffer 0.1 M pH 8.6, 50µL of luminol and 50µL of ABAP) was measured as a pro-emission standard. An antioxidant standard solution (Trolox - 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid 25µM- an

analogue of hidrosoluble vitamin E) was added in order to neutralize ABAP autoxidation (830µL of Glicine buffer 0.1 M pH 8.6, 70µL of Trolox, 50µL of luminol and 50µL ABAP) and it was used as a standard for antioxidant comparison. Subsequently, plasma samples (830µL of Glicine buffer 0.1M pH 8.6, 70µL of plasma sample, 50µL of luminol and 50µL of ABAP) were used for the measurement of TRAP. For TRAP calculation, the induction time of the sample (time which the sample antioxidants can inhibit the ABAP action) was compared to the standard antioxidant the results were expressed as µM of Trolox.

Measurement of nitrite levels

Sample nitrite, was measured as estimative of NO levels and determined as previous described Panis et al. [36]. Plasma aliquots of 60 µL were deproteinized by adding 50 µL of $ZnSO_4$ 75 mM, centrifuged at 8700 x g 2 minutes, 25^0C and then 70µL of NaOH 55mM (Merck), and centrifuged at 8700x g 5 minutes, 25^0C. The supernatant was recovered and diluted in glycine buffer solution (45g/L pH 9.7, Merck) in a proportion of 5:1.Cadmium granules (Fluka) were added to a $CuSO_4$ 5mM solution in glycine-NaOH buffer (15 g/L, pH 9.7, Merck) during 5 minutes and added to supernatant during 10 minutes. Aliquots were recovered and the same volum of Griess reagent was added (Reagent I: 50 mg of *N*-naphthylethylenediamine in 250 mL of distilled water; reagent II: 5 g of sulfanilic acid in 500 mL of 3 M HCl, Sigma). To determine sample nitrite concentration, calibration curve was prepared by dilution of $NaNO_2$ (Merck). The absorbance was determinated at 550 nm in a microplate reader.

Lipid hydroperoxides levels by ferrous oxidation – xylenol orange method (FOX)

Plasmatic lipid hydroperoxides concentrations were estimated by FOX method with modifications [37, 38]. This method consists in the oxidation of ferrous iron to ferric iron through lipid hydroperoxides present in plasma in the presence of xylenol orange. This

reaction generates a ferric-xylenol orange complex, whose color can be measured by spectrophotometer. To sample analyses, FOX reagent was prepared adding 100μM xylenol orange and 250μM ammonium ferrous sulfate in 110mM percloric acid. About 50μL of plasma sample was added to 50μL of FOX reagent. After 30 minutes in dark incubation at room temperature, samples were centrifuged 2400 x *g* for 3 minutes. Absorbance of the supernatant was evaluated at 550nm and results were expressed in μM of hydroperoxides (molar extinction coefficient = $4.3x10^4M^{-1}cm^{-1}$).

Lipid peroxidation – MDA determined by TBARS

Mainly malondialdehyde (MDA) levels in plasma were quantified spectrophotometrically by determination of thiobarbituric acid (TBA) reactive substances (TBARS), as described by Oliveira and Cecchini [39]. An aliquot of 400μL of plasma sample reacted with 1mL of TBA 1% to form MDA-$(TBA)_2$ complex, and proteins were precipitated with 1mL of trichloroacetic acid 28%. Samples were mixed in vortex and kept in boiling water bath for 15 minutes, followed by ice bath. Organic phase were extracted using 2mL of butanol. Next, samples were centrifuged 850 x *g* for 20 minutes. Readings were performed in spectrophotometer, considering the difference of 535-572nm and results were expressed in nM of TBARS.

Statistical Analysis

Statistical analysis were perfomed using GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA). Results were expressed as arithmetic means and standard error of means (SEM). Differences among groups were assessed by two-way analysis of variance (ANOVA) to lipid peroxidation curves, with Bonferroni´s test as pos-hoc , one way ANOVA to test of SOD TRAP and catalase and Student's unpaired t Test to others parameters. All data were checked in GraphPad Software to eliminate significant outliers ($P <$

0.05).). The comparisons were made with the CTR group (●); between ET group and CREL group (*) and CREL group and PTX group ($^{\#}$). Differences were considered statistically significant when $p < 0.05$.

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List of abbreviations

ABAP - 2, 2'azo-bis, 2 amidinopropane

CAT - Catalase

CL – Chemiluminescence

CREL – Cremophor

CTR - Control

DTNB - 5, 5' – dithiobis – 2 – nitrobenzoic acid

EDTA - Ethylenediamine tetraacetic acid

ET - Ethanol

GSH – Glutathion reductase

GPx – Glutathion peroxidase

MDA – Malondialdehyde

MetHB - Metahemoglobin

NO - Nitric oxide

OxyHB - Oxyhemoglobin

RBCs - red blood cells

RLU - relative light's units

SOD - superoxide dismutase

TBA - thiobarbituric acid

TBARS - thiobarbituric acid reactive substances

TCA - trichloroacetic acid

TRAP - Total Radical Antioxidant Parameter

TRIS - hydroxymethyl – aminomethane

TROLOX - 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

VitE – Vitamin E

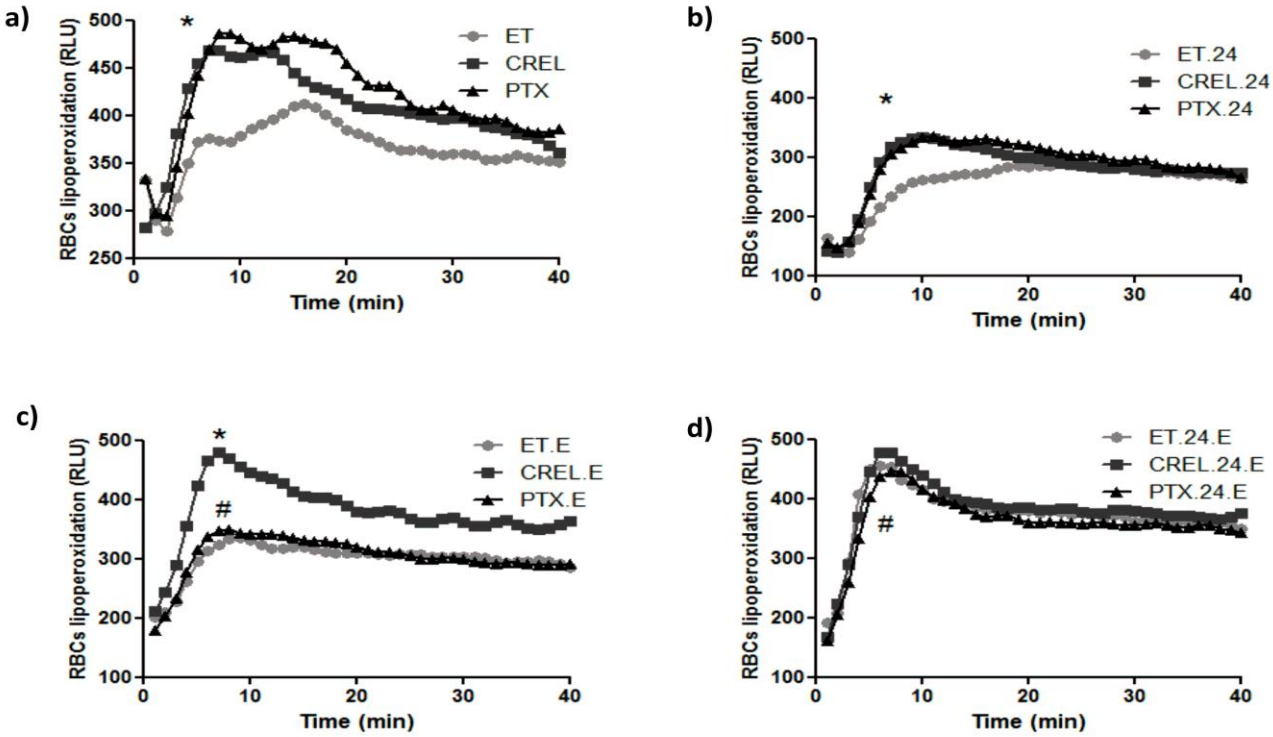


Figure 1: **Red blood cells (RBCs) profile of lipid peroxidation evaluated by high-sensitivity chemiluminescence** . Groups were evaluated immediately (1hour) and 24 hours after treatment (a and b). Groups were pre-treated with vitamin E and also evaluated (c and d). The table below indicates the parameters employed to statistical analysis of the curves. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). ET= etanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxe-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.

Statitical Analysis	**ET X CREL**	**CREL X PTX**	**ET.24 X CREL.24**	**CREL.24 X PTX.24**
Two-way ANOVA	P< 0.0001	No significance	P< 0.0001	No significance
Bonferroni Test	No points	No points	No points	No points
AUC Student Test	No signficance	No signficance	No signficance	No significance
Statitical Analysis	**ET.E X CREL.E**	**CREL.E X PTX.E**	**ET.24.E X CREL.24.E**	**CREL.24E X PTX.24.E**
Two-way ANOVA	P< 0.0001	P< 0.0001	No significance	P< 0.0001
Bonferroni Test	No points	No points	No points	No points
AUC Student Test	No signficance	No signficance	P< 0.05	No signficance

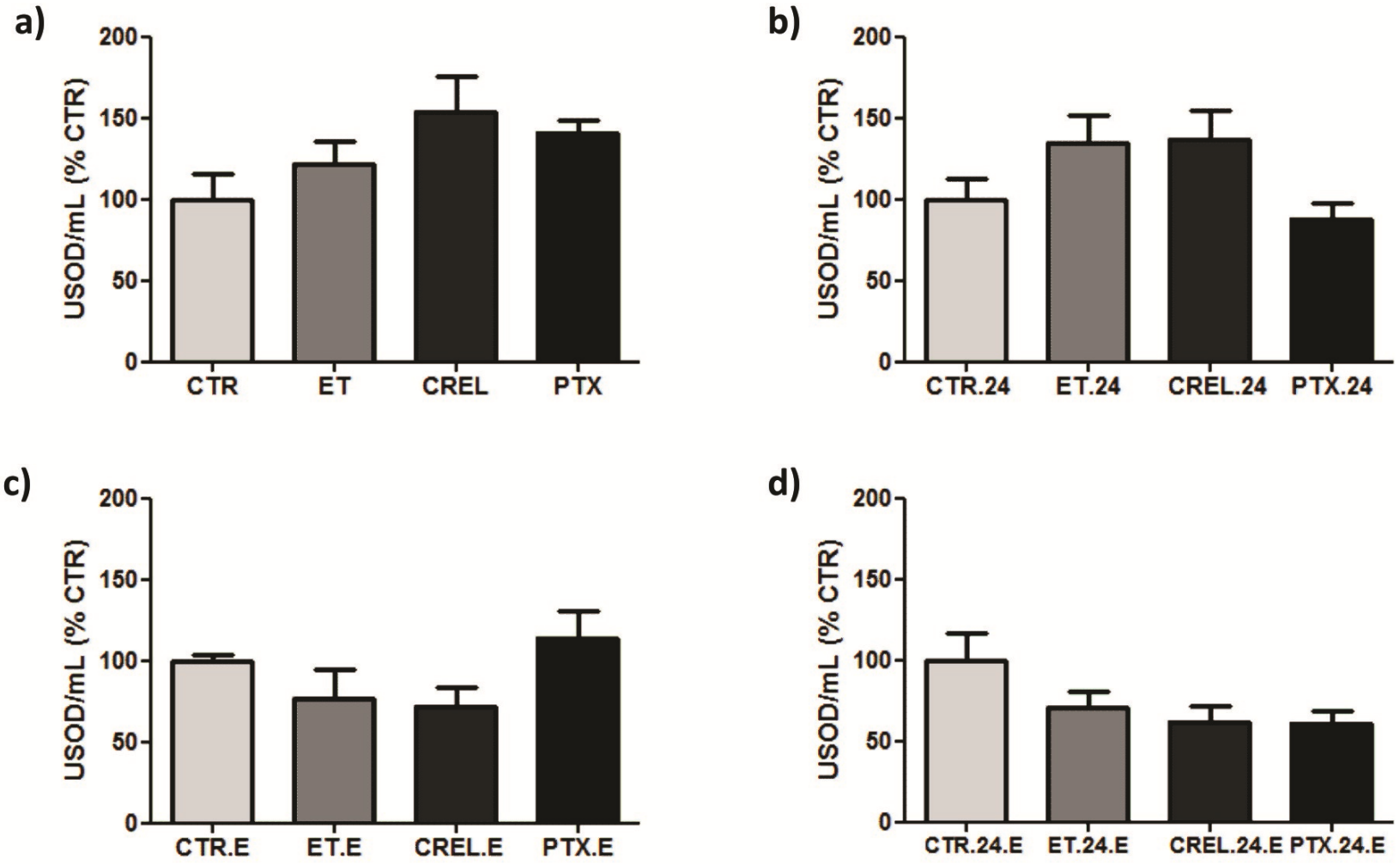


Figure 2: Erythrocytic SOD activity. (a) SOD activity was evaluated immediately (1 hour) and 24 hours after treatments (b) . Analysis of vitE pre-treatemnt (c and d). Individual distributions of values and means were evaluated by by one-way ANOVA and Bonferroni post test . • indicates a statistical difference with CTR group. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). CTR = saline-treated group, ET= etanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.

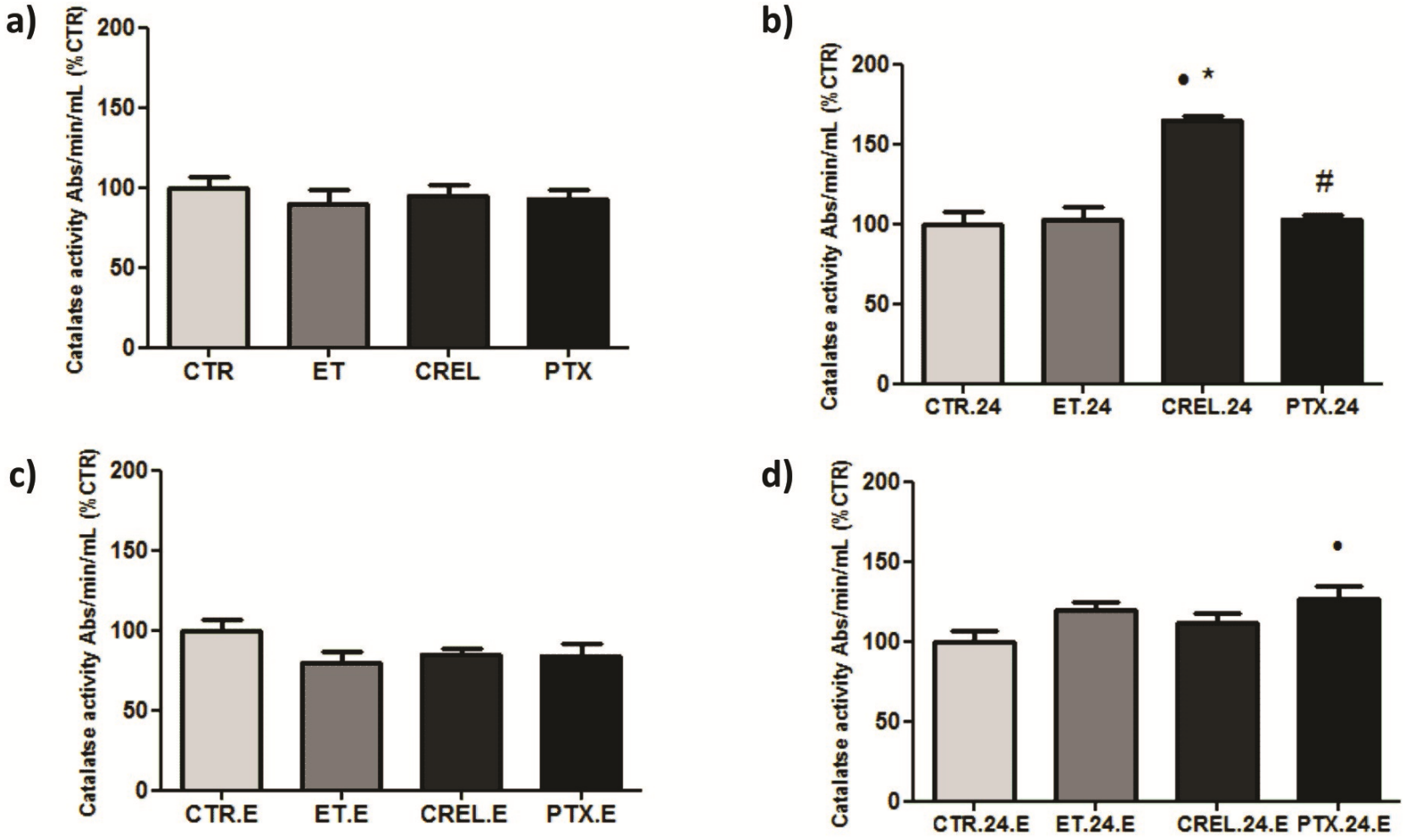


Figure 3: Erythrocytic catalase activity. (a) Catalase activity was evaluated immediately (1 hour) and 24 hours after treatments (b) . Analysis of vitE pre-treatemnt (c and d). Individual distributions of values and means were evaluated by one-way ANOVA and Bonferroni post test . • indicates a statistical difference with CTR group. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). CTR = saline-treated group, ET= etanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.

Table 1: Hematological profile and antioxidant parameter evaluated in animals subjected to infusion of 1 hour with ethanol, Cremophor EL and Paclitaxel.

	CTR	ET	CREL	PTX	CTR.24	ET.24	CREL.24	PTX.24
RBCs counts(cells/mm^3)	4.910^6±$6.9.10^5$	4.210^6 ± 7.910^5	6.410^6±$7.3.10^5$	5.210^6 ± $7.9.10^5$	4.610^5±$3.4.10^5$	6.710^6±$7.3.10^5$ •	4.910^6 ± $4.6.10^5$	4.210^6±$1.0.10^6$
OxiHB (µM)	28.21 ± 2.046	20.94 ± 3,.95	26.49 ± 2.897	26.58 ± 1,127	21.62 ± 1.162	26,.0 ± 1.155	27.71 ± 2.906	23.85 ± 1.837
MetHB (µM)	21.98 ± 4.113	11.08 ± 2.682	9.182 ± 2.066	17.73 ± 0.7965 #	8.124 ± 1.317	8.703 ± 1.241	10.48 ± 0.9781	5.937 ±1.515 #
GSH (nM)	30.64 ± 3.397	35,44 ± 4.392	29.33 ± 3.334	23.82 ± 1.986	40.50 ± 4.532	35.00 ± 4.436	40.07 ± 5.746	38.55 ± 4.320
	CTR.E	**ET.E**	**CREL.E**	**PTX.E**	**CTR.24.E**	**ET.24.E**	**CREL.24.E**	**PTX.24.E**
RBCs counts(cells/mm^3)	5.910^6±$1.17.10^6$	5.010^6 ± $4.5.10^5$	4.910^6± $5.6.10^5$	4.710^6± $4.8.10^5$	8.610^6 ±$3.9.10^5$	9.210^6±$1.7.10^6$	1.010^6±$1.6.10^66$	7.110^6±$1.4.10^6$
OxiHB (µM)	28.64 ± 1.184	28.53 ± 1.746	70.12 ± 3.006*	22.78 ± 1.205#	22.15 ± 1.098	26.51 ± 2.881	28.24 ± 1.017	27.43 ± 1.739
MetHB (µM)	11.08 ± 2.388	6.451 ±0.7590	13.75 ± 2.630	11.87 ± 2.729	8.608 ± 1.345	9.727 ± 1.271	9.216 ± 0,5179	8.821 ± 1.260
GSH (nM)	45.25 ± 6.250	57.57 ± 3,358	55.37 ± 4.577	44.90 ± 4.973	30.23 ± 5.897	34.04 ± 7.364	23.58 ± 2.39	33.18 ± 5.741

Legend: RBCs= red blood cells, OxiHB= oxihemoglobin, MetHB= metahemoglobin, GSH= glutathione reductase. Results are represented as mean standard errors of the mean. Individual distributions of values and means were evaluated by Student's unpaired t-test. • indicates a statistical difference with CTR group. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). CTR = saline-treated group, ET= ethanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.

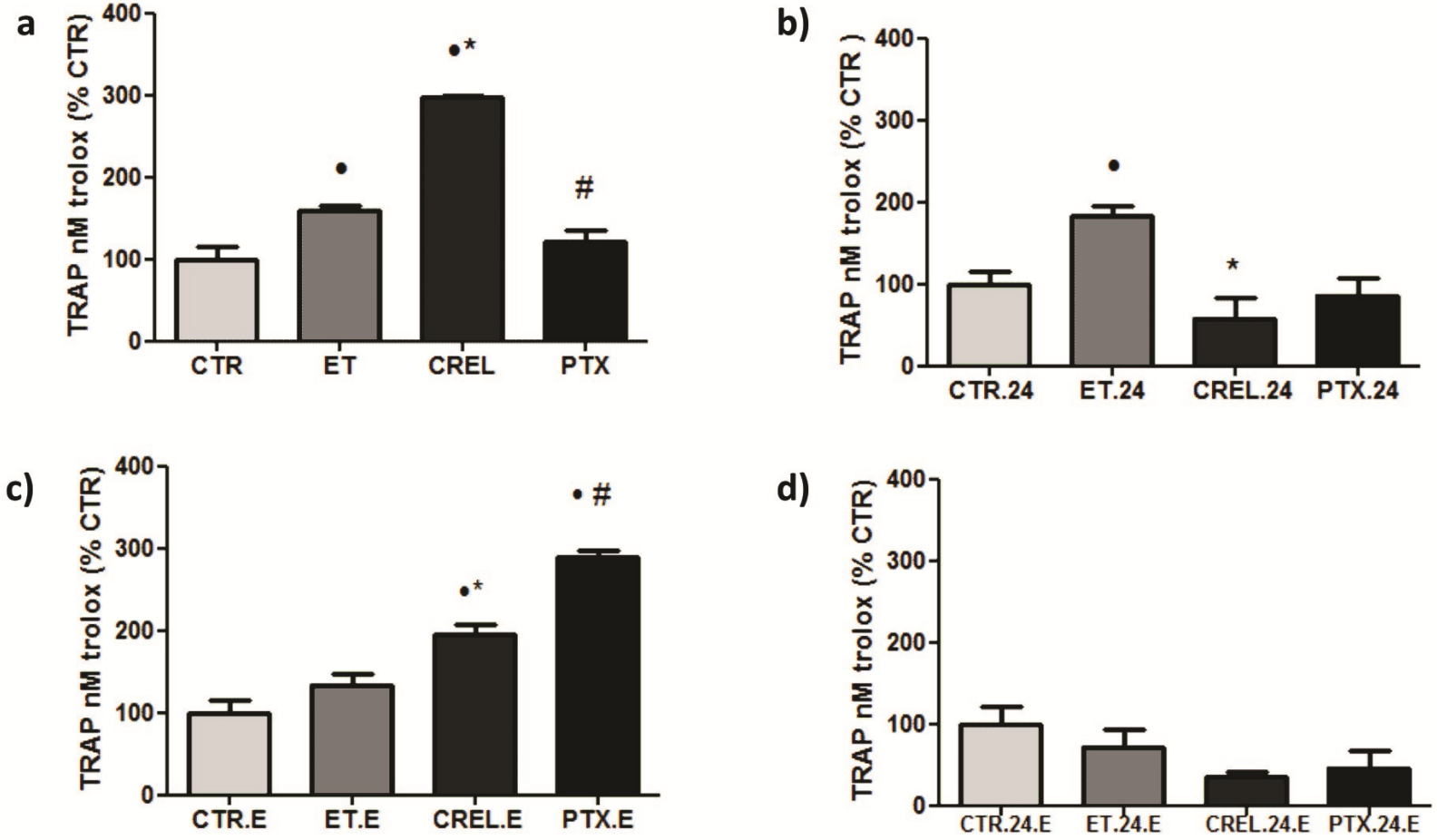
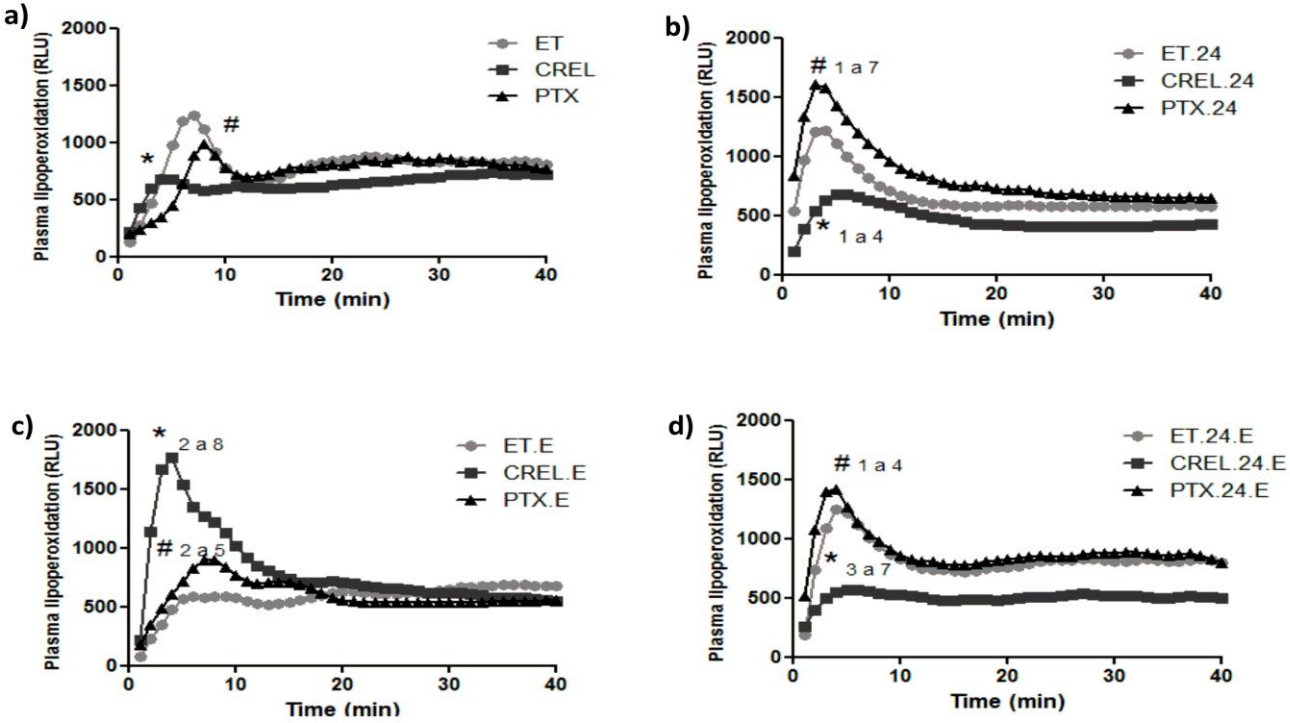


Figure 4: Total antioxidant capacity of plasma (TRAP). (a) TRAP was evaluated immediately (1 hour) and 24 hours after treatments (b) . Analysis of vitE pre-treatemnt (c and d) Individual distributions of values and means were evaluated by by one-way ANOVA and Bonferroni post test . • indicates a statistical difference with CTR group. Group. * indicates a statistical difference between ET group and CREL group. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). CTR = saline-treated group, ET= etanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.



Statitical Analysis	ET X CREL	CREL X PTX	ET.24 X CREL.24	CREL .24 X PTX.24
Two-way ANOVA	P< 0.0001	P< 0.0001	P< 0.0001	P< 0.0001
Bonferroni's Test	Point: 07	No points	Points: 2-4	Point: 1-7
AUC Student's Test	No signficance	No signficance	No signficance	P< 0.05

Statitical Analysis	ET.E X CREL.E	CREL.E X PTX.E	ET.24.E X CREL.24.E	CREL.24E X PTX.24.E
Two-way ANOVA	P< 0.0001	P< 0.0001	P< 0.0001	P< 0.0001
Bonferroni's Test	Points: 2-8	Points: 2-5	Points: 3-7	Points: 2-5
AUC Student's Test	No signficance	No signficance	P< 0.05	No signficance

Figure 5:- Plasmatic profile of lipid peroxidation evaluated by high-sensitivity chemiluminescence Groups were evaluated immediately (1hour) and 24 hours after treatment (a and b). Groups were pre-treated with vit E and also evaluated (c and d). The table below indicates the parameters employed to statistical analysis of the curves. * indicates a statistical difference between ET group and CREL group. * indicates a statistical difference between ET group and CREL group. # indicates a statistical difference between CREL group and PTX group ($P < 0.05$). ET= etanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion, while the E addition to the name of groups indicates vitE pre-treatment.

Table 2: Plasmatic parameters of lipid peroxidation evaluated in animals subjected to infusion of 1 hour with ethanol, Cremophor EL and Paclitaxel.

	CTR	**ET**	**CREL**	**PTX**
NO µM (%CTR)	100% ± 52%	68% ± 25%	59% ± 53%	69% ± 53%
Hydroperoxide µM (%CTR)	100% ±16%	75% ± 24%	91% ± 14%	93% ± 9%
MDA nM (%CTR)	100% ± 11%	83% ± 13%	85% ± 11%	93% ± 36%
	CTR.24	**ET.24**	**CREL.24**	**PTX.24**
NO µM (%CTR.24)	100% ± 60%	72% ± 34%	133% ± 58%	79% ± 42%
Hydroperoxide µM(%CTR.24)	100% ± 10%	101% ± 4%	108% ± 7%	102% ± 6%
MDA nM (%CTR.24)	100% ± 17%	78% ± 8%	91% ± 5%	84% ± 7%
	CTR.E	**ET.E**	**CREL.E**	**PTX.E**
NO µM (%CTR.E)	100% ± 50%	87% ± 58%	80% ± 48%	105% ± 26%
Hydroperoxide µM(%CTR.E)	100% ± 23%	95% ± 16%	97% ± 23%	93% ± 23%
MDA nM (%CTR.E)	100% ± 10%	101% ± 33%	1001%± 22%	103% ± 13%
	CTR.24.E	**ET.24.E**	**CREL.24.E**	**PTX.24.E**
NO µM (%CTR.24.E)	100% ± 87%	105% ± 55%	135% ± 51%	62% ± 42%
Hydroperoxide µM(%CTR.24.E)	100% ± 43%	97% ± 14%	102% ± 5%	97% ± 11%
MDA nM (%CTR.24.E)	100% ± 43%	161% ± 60%	146% ± 45%	108% ± 15%

Legend: NO= Nitric Oxide, MDA= Malondialdehyde. Results are represented in percentage of CTR group as mean standard errors of the mean. Individual distributions of values and means were evaluated by Student's unpaired t-test. Estatistical difference $P< 0.05$. CTR = saline-treated group, ET= ethanol-treated group, CREL= cremophor EL-treated group, PTX= paclitaxel-treated group. The addition of 24 to this nomenclature indicates groups analyzed 24 hours after infusion , while the E addition to the name of groups indicates vitE pre-treatment.