Food and Chemical Toxicology 68 (2014) 78–86

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Systemic toxicity induced by paclitaxel *in vivo* is associated with the solvent cremophor EL through oxidative stress-driven mechanisms

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ARTICLE INFO

Article history:
Received 10 October 2013
Accepted 7 March 2014
Available online 19 March 2014

Keywords:
Paclitaxel
Cremophor
Oxidative stress
Hematological toxicity

ABSTRACT

The toxic effects of paclitaxel (PTX) and its solubilizing agent cremophor EL (CREL) have been well established *in vitro*; however, the *in vivo* mechanisms underlying this toxicity remain unclear. Thus, the aim of this study was to analyze the *in vivo* toxicity induced by infusion of PTX and CREL and to investigate the involvement of oxidative stress as a potential mechanism for this toxicity. We treated male Wistar rats with PTX and/or CREL for 1 h using human-equivalent doses (PTX + CREL/ethanol + NaCl 175 mg/m^2 or CREL + ethanol + NaCl) and sacrificed immediately or 24 h after these drug infusions to systemic biochemical evaluations. Hidrosoluble vitamin E (vitE, Trolox) was added as a control in some groups. The oxidative profile was determined by measuring erythrocyte and plasma lipid peroxidation, superoxide dismutase and catalase activities, reduced glutathione (GSH) levels, red blood cell (RBC) counts, hemoglobin profile, plasma total radical-trapping antioxidant parameter (TRAP), plasma lipid peroxidation, nitric oxide levels and malondialdehyde levels. Our findings showed that CREL infusion triggered immediate high plasma lipid peroxidation and augmented TRAP, while PTX caused immediate TRAP consumption and metahemoglobin formation. Pronounced oxidative effects were detected 24 h after infusion, when CREL treatment enhanced RBC counts and plasma lipid peroxidation, increased catalase activity, and decreased TRAP levels. On the other hand, after 24 h, PTX-infused rats showed reduced catalase activity and reduced metahemoglobin levels. These data indicate the existence of a continuous oxidative stress generation during CREL-PTX treatment and highlight CREL as primarily responsible for the *in vivo* oxidative damage to RBCs.

1. Introduction

Chemotherapy constitutes in the main treatment for cancer, especially in patients bearing advanced tumors (Jemal et al., 2011; Souza, 2004). At the same time that a chemotherapeutic agent acts on tumor cells, it can trigger several toxic effects in normal cell types (Ferreira et al., 2008; Rocha et al., 2004). Paclitaxel (PTX) is an anticancer drug widely employed against solid tumors (Panis et al., 2012; Scripture et al., 2005) its main mechanism of action is to depolymerize microtubules, which results in cell death (Brandão et al., 2010). However, PTX is not very water-soluble and must be formulated in association with lipophilic agents such as the solvent Cremophor EL (CREL) (Scripture et al., 2005; Adams et al., 1993). CREL, a polyethylated oil obtained from the seeds of *Ricinus communis*, is highly viscous and lipophilic, properties that make this solvent suitable for solubilizing a wide variety of hydrophobic drugs (Gelderblom et al., 2001). Although CREL has been employed as a solvent for several drug formulations, the PTX-CREL mixture represents the highest concentration of CREL described in pharmacology (Sparreboom et al., 1998).

Abbreviations: ABAP, 2,2′azo-bis, 2 amidinopropane; CAT, catalase; CL, chemiluminescence; CREL, cremophor; CTR, control; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, ethylenediaminetetraacetic acid; ET, ethanol; GSH, glutathionreductase; 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.

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http://dx.doi.org/10.1016/j.fct.2014.03.013
0278-6915/

Studies have shown changes in the pharmacokinetic behavior of intravenously administered drugs solubilized by CREL (Scripture et al., 2005), suggesting that CREL is not an inert vehicle (Gelderblom et al., 2001). Although the CREL-PTX mixture has been used as a strategy to overcome drug resistance and is associated with an enhanced response when cancer patients fail to respond to anthracyclins (Fjällskog et al., 1994), several toxic events have been associated with CREL-PTX infusion, mainly the occurrence of acute hypersensitivity (Irizarry et al., 2009). In addition, *in vitro* and *in vivo* studies have shown that CREL-PTX can increase blood viscosity and the stomacytic transformation of erythrocytes, demonstrating that the solvent CREL is able to intercalate into plasma membrane of red blood cells and induce morphological change (Mark et al., 2001).

Recently, our group showed that breast cancer patients who undergo PTX treatment develop anemia immediately after chemotherapy infusion, suggesting oxidative stress as a probable causative mechanism (Panis et al., 2012). Oxidative stress is a condition characterized by the imbalance between the generation and neutralization of reactive species, resulting in oxidative damage to cellular components such as proteins, lipids, and DNA (Halliwell and Gutteridge, 2007; Gago-Dominguez et al., 2007). PTX induces toxic effects on cancer cells *in vitro* through oxidative mechanisms, that is, the generation of oxidative stress by membrane-associated NADPH oxidase, giving rise to extracellular H_2O_2 (Hadzic et al., 2010; Alexandre et al., 2007). Additional evidence indicates that some of these toxic effects may be primarily related to the effects of CREL (Iwase et al., 2004; Gutiérrez et al., 2006).

Thus, although the toxic effects of PTX-CREL have been well established *in vitro* and using cancer models, the *in vivo* mechanism by which toxicity occurs remains unclear. Therefore, the aim of this study was to investigate the *in vivo* toxic effects induced by the experimental treatment with PTX and/or CREL using human equivalent doses and to determine whether oxidative stress was involved as a mechanism in this toxicity.

2. Methods

2.1. Animals

This study was approved by the Ethics Committee on Animal Experimentation at the Universidade Estadual de Londrina (CEEANo.82/10). Wistar rats (220–250 g) were bred in the mouse breeding facilities of the Departmento de Ciências Patológicas da Universidade Estadual de Londrina, Brazil, under standard conditions. Mice were housed five per cage and fed 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 the Research and Ethics Committee at the Universidade Estadual de Londrina.

2.2. Chemicals

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

2.3. Treatment and sample collection

Animals were cannulated in the femoral vein for intravenous infusion of PTX and/or CREL and categorized into the following groups ($n = 6$ animals in each group):

Group	Treatment
Control (CTR)	Intravenous infusion of 3 mL NaCl 0.9%
Ethanol (ET)	Intravenous infusion of 3 mL NaCl 0.9% + Ethanol 50% v/v
Cremophor (CREL)	Intravenous infusion of 3 mL NaCl 0.9% + Cremophor 50% v/v in ethanol
Paclitaxel (PTX)	Intravenous infusion of 3 mL of the commercial formulation of PTX, as Taxol® (Paclitaxel 175mg/m^2 + NaCl 0.9% + Cremophor 50% v/v in ethanol)

A second cohort of rats received the same experimental treatment preceded of injections of Trolox (hidrosoluble E vitamin analog, 100 mg/kg i.p.), 72 h before the start of drug treatments (vitE group). This cohort was included to determine whether oxidative stress participated as a mechanism for the toxicity of the evaluated drugs. Animals were euthanized immediately after continuous drug infusions (1 h) or 24 h after the experimental treatment by ether anesthesia and their blood collected by cardiac puncture. An aliquot of heparinized blood was used to count red blood cells (RBCs). Plasma was obtained following blood centrifugation at 1500×*g* for 10 min at 4 °C and stored at −80 °C until analysis. RBCs were washed three times with saline solution (0.9% at 4 °C) and used immediately for further analysis.

2.3. Oxidative stress analysis and hematological toxicity evaluation

2.3.1. Lipid peroxidation profiling

To evaluate lipid peroxidation in both RBC and plasma samples, a high-sensitivity chemiluminescence-based method was employed (Panis et al., 2012; Gonzales-Flecha et al., 1991). RBC was diluted 1200× in 10 mM monobasic phosphate buffer, at 37 °C. The reaction was started with 10 μL of 3 mM tert-butyl hydroperoxide added to 1 mL of this dilution. The plasma chemiluminescence reaction was started by the addiction of 10 μL of 3 mM tert-butyl hydroperoxide in 125 μL of plasma and 865 μL of 30 mM disodium phosphate-KCl 120 mM buffer, pH 7.4, 37 °C. Readings were performed in Glomax luminometer (Promega) for 40 min, one reading/s. Results were expressed in relative units of light (RLU).

2.4. Superoxide Dismutase and catalase activities

Superoxide activity was determined as a method described by Marklund and Marklund (1974). RBC was hemolysed in distillated water in a proportion of 1:20. Then, three runs of 5 μL, 10 μL and 20 μL of samples were measured. To each run, destilated water, 1 M 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.

Erythrocytic catalase activity was determined as described by Aebi (1984). RBCs were diluted in distillated water in a proportion of 1:80. Then, 10 μL of sample were incubated in a system containing 1 M TRIS buffer and 200 mM hydrogen peroxide (H_2O_2). Kinetic of absorbance disappearance was monitored in spectrophotometer at 240 nm (Shimadzu UV-1650 PC). The results were expressed in absorbance values/min/mL of sample.

2.5. Reduced glutathione (GSH) content

To determine levels of GSH (Sedlak and Lindsay, 1968), RBCs 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 min of incubation at room temperature, samples were centrifuged at 2400×*g* for 15 min. Next, 1 mL of supernatant was added to 2 mL of 0.4 M TRIS buffer, pH 8.9. Finally, 50 μL 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.

2.6. Cell counts and hemoglobin profiling

RBCs and total leukocytes counting were determined employing a Neubauer chamber in a light microscope, after appropriated dilutions (Lewis et al., 2006). The results were expressed in cells/mm^3. To determine Oxyhemoglobin and Methemoglobin, we used a method developed by Winterbourn. Aliquots of 20 μL of RBC were hemolysed in 10 mL of H_2O and the readings were taken in scan ranging from 700 to 400 nm in spectrophotometer (Shimadzu UV-1650 PC). The results were expressed in μM applying the following equations:

$$\text{OxyHb} = (119 \times \text{abs575}) - (39 \times \text{abs630}) - (89 \times \text{abs560})$$

$$\text{MetHb} = (28 \times \text{abs575}) + (307 \times \text{abs630}) - (55 \times \text{abs560})$$

2.7. Total Radical Antioxidant Parameter (TRAP)

TRAP was measured as described by Repetto et al. (1996). 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