

# Cumulative Mitoxantrone-Induced Haematological and Hepatic Adverse Effects in a Subchronic *In vivo* Study

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**Abstract:** Mitoxantrone (MTX) is an antineoplastic agent that can induce hepato- and haematotoxicity. This work aimed to investigate the occurrence of cumulative early and late MTX-induced hepatic and haematological disturbances in an *in vivo* model. A control group and two groups treated with three cycles of 2.5 mg/kg MTX at days 0, 10 and 20 were formed. One of the treated groups suffered euthanasia on day 22 (MTX22) to evaluate early MTX toxic effects, while the other suffered euthanasia on day 48 (MTX48), to allow the evaluation of MTX late effects. An early immunosuppression with a drop in the IgG levels was observed, causing a slight decrease in the plasma total protein content. The early bone marrow depression was followed by signs of recovery in MTX48. The genotoxic potential of MTX was demonstrated by the presence of several micronuclei in MTX22 leucocytes. Increases in plasma iron and cholesterol levels in the MTX22 rats were observed, while in both groups increases in the unconjugated bilirubin, C4 complement, and decreases in the triglycerides, alanine aminotransferase, alkaline phosphatase and transferrin were found in plasma samples. On MTX 48, the liver histology showed more hepatotoxic signs, the hepatic levels of reduced and oxidized glutathione were increased, and ATP hepatic levels were decreased. However, the hepatic total protein levels were decreased only in the livers of MTX22 group. Results demonstrated the MTX genotoxic effects, haemato- and direct hepatotoxicity. While the haematological toxicity is ameliorated with time, the same was not observed in the hepatic injury.

Mitoxantrone (MTX) is an anticancer and immunosuppressive drug that has been used in the treatment of tumours such as acute leukaemia, lymphoma, prostatic and breast cancer [1] and in the active forms of relapsing-remitting or secondary progressive multiple sclerosis [2]. MTX acts as an intercalating agent that causes double breaks in DNA by stabilization of a complex formed between DNA and topoisomerase II [1,3]. The regimen of MTX administration varies depending on disease progression and the clinical condition of the patient. Additionally, MTX is a substrate of membrane efflux pumps, namely the transporter breast cancer resistance protein (BCRP), which could interfere in MTX biodisposition [4]. However, the doses commonly employed range between 12–14 mg/m<sup>2</sup> and the maximum recommended cumulative dose is 140 mg/m<sup>2</sup> [1].

One of the most serious adverse events related to MTX therapy is the late and irreversible cardiotoxicity [1], whose underlying mechanisms have been studied by us [5–7]. It is known that cardiotoxic drugs such as MTX can cause acute liver failure as a result of a primary congestive heart failure

with low cardiac output and consequent reduced hepatic blood flow [8]. However, the precise origin of liver failure, due to a direct effect or secondary to the cardiomyopathy, is not easy to define, because clinical signs of cardiac decompensating can be undetected in a first moment delaying the diagnosis [8]. Additionally, MTX has been considered more hepatotoxic than other antineoplastic agents such as doxorubicin [9].

In human beings, the MTX-induced hepatotoxicity manifests as transient increases in the serum bilirubin concentration and in the activity of hepatic enzymes, occurring in about 15% of treated patients (12 mg/m<sup>2</sup>) [2,10]. Indeed, the inhibition of CYP450 metabolism in HepG2 cells and rat isolated hepatocytes prevented the MTX cytotoxicity [11], demonstrating the relevance of MTX oxidative metabolism to its noxious effects. MTX has at least one pharmacologically and toxicologically relevant metabolite, naphthoquinoline (the 8, 11-dihydroxy-4-[2-hydroxyethyl]-6-[[2-[[2-hydroxyethyl]amino]ethyl]amino]-1,2,3,4,7,12-hexahydronaphto-[2,3]-quinoline-7-12-dione), which has proven antitumour effects and has been related to MTX toxicity [5,10]. Recently, we demonstrated that, as it happens with MTX, the naphthoquinoline metabolite also distributes to highly perfused tissues and accumulates in organs such as liver and heart in rats [5]. The liver is subjected to a prolonged exposure to MTX and, possibly, to

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this toxic metabolite that may contribute to its direct hepatotoxicity.

Haematological parameters should be monitored during therapy and the occurrence of neutropenia and thrombocytopenia can limit the dose administered or oblige the cessation of MTX therapy [1,12], demonstrating the haematological effects induced by MTX. The late haematological effects of MTX involves the development of MTX-associated leukaemia, which is also related to other topoisomerase II inhibitors [1].

Considering that the occurrence of liver dysfunction, as well as MTX-induced haematological toxicity in the treatment of complex diseases such as cancer and multiple sclerosis, can compromise the therapy [13], this work aimed to characterize the potential hepatotoxicity and haematological toxicity after therapeutic doses of MTX administration in male Wistar rats in 2 experimental settings. Animals were subjected to 3 cycles of 2.5 mg/kg MTX (cumulative dose of 7.5 mg/kg, which corresponds to 48 mg/m<sup>2</sup> by the allometric relationship). The toxic effects were evaluated 48 hr (day 22) after the last administration to assess immediate toxicity and also 28 days after the last administration (day 48) to determine whether the effects were cumulative or whether adaptation responses occurred in the hepatic tissue and blood.

## Materials and Methods

**Chemicals.** All chemicals and reagents were of analytical grade. MTX hydrochloride, reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR, EC 1.6.4.2), 2-vinylpyridine, reduced  $\beta$ -nicotinamide phosphate adenine dinucleotide ( $\beta$ -NADPH), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), luciferin and luciferase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Perchloric acid (HClO<sub>4</sub>) was obtained from Merck (Darmstadt, Germany). The reagents for biochemical analyses were purchased from PZ Cormay S.A.

**Animals.** Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) weighing 240–300 g were used. The animals were housed in individual cages, in a temperature- and humidity-controlled environment and acclimated for 1 week prior to the study. Food and water were provided *ad libitum*, and animals were subjected to a 12-hr light/dark cycle. Animal experiments were approved by the Ethical Committee of Faculty of Pharmacy of the University of Porto (protocol number 09/04/2013). Housing and experimental treatment of the animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Research. The experiments complied with current Portuguese laws.

**Dose regimen.** Animals were divided into three groups (five animals per group): control, MTX22 and MTX48. Animals were treated with three administrations, 5 mL/kg, intraperitoneal, of saline solution (0.9% NaCl) (control) or MTX 2.5 mg/kg (MTX22 and MTX48) on days 0, 10 and 20. The MTX-treated groups reached a total cumulative dose of 7.5 mg/kg on day 20. Animals belonging to the MTX22 group suffered euthanasia on day 22, to evaluate the early MTX-induced cumulative damage (i.e. 2 days after the last cycle of treatment). Animals from the MTX48 group suffered euthanasia on day 48, corresponding to 28 days after the last administration, to evaluate late cumulative responses [6]. Two animals of control group suffered euthanasia on day 22 and the others on day 48.

The used dose of MTX is clinically realistic (2.5 mg/kg corresponds to 16 mg/m<sup>2</sup> of a rat weighing 240 g, being similar to the dose administered in human beings and about one-tenth of the maximum dose recommended in human beings). The regimen of administration (one administration every 10 days) was scheduled aiming to simulate the human chemotherapeutic cycles. The interval of 10 days was defined considering the life cycle of the rat and the clinical conditions noted in pilot studies.

Euthanasia was performed under anaesthesia with xylazine/ketamine (10 mg/kg and 100 mg/kg), and blood was collected through cardiac puncture. A necropsy was performed to all animals.

**Plasma biochemical analysis.** On the day of euthanasia, blood was collected into heparinized tubes to perform biochemical analysis and into EDTA tubes to the haematological evaluations. Plasma levels of albumin, total proteins, IgG, IgM, IgE, C3 and C4 complement, total and conjugated bilirubin, alanine aminotransferase (ALT), alkaline phosphatase, transferrin, ferritin, iron, cholesterol, triglycerides, glucose, amylase, creatinine, urea, uric acid, potassium sodium, calcium, C-reactive protein,  $\alpha_1$ -antitrypsin,  $\delta$ -glutamyltranspeptidase (GGT) and lactate dehydrogenase (LDH) were evaluated in duplicate on an AutoAnalyzer (PRESTIGE® 24i, PZ Cormay S.A.) using the respective kits and following the manufacturer instructions.

**Haematological analysis.** Whole blood samples (collected using EDTA as anticoagulant) were used to evaluate erythrocyte count, haemoglobin (Hb) concentration, haematocrit, haematological indexes – mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count (PLT), plateletcrit (PCT), platelet distribution width (PDW), mean platelet volume (MPV) and total white blood cell (WBC) count, using an automated blood cell counter (Sysmex K1000, Hamburg, Germany). Differential leucocyte count was performed on blood smears stained according to Wright [14]. Reticulocyte count was performed by microscopic counting on blood smears after vital staining with new methylene blue (Reticulocyte stain; Sigma-Aldrich).

**Tissue preparation for light and transmission electron microscopy.** For light microscopy analysis, pieces of 2–4 mm<sup>3</sup> from all liver lobes were fixed in 4% formaldehyde during 24 hr. The pieces were further dehydrated with graded ethanol (Panreac, Barcelona, Spain) and included in paraffin blocks (MERCK) after standard procedures. Semithin sections (5  $\mu$ m) were cut (Leica Microsystems, Model RM2125) and mounted on silane-coated slides (Sigma-Aldrich). After dewaxed in xylene and hydrated through graded alcohol concentrations, tissue sections were stained with haematoxylin-eosin (Atom Scientific Ltd, Manchester, UK) and analysed under a light microscope coupled to a digital camera (Axio Imager A1; Carl Zeiss, Oberkochen, Germany).

For transmission electron microscopy, 1 mm<sup>3</sup> tissue pieces from all liver lobes were fixed in 2% glutaraldehyde, post-fixed with 2% osmium tetroxide, dehydrated in graded ethanol, and later embedded in Epon (TAAB 812 Resin, Kit Cat. No. T024) according to standard procedures. Ultrathin (100 nm) sections obtained in an ultra microtome (Reichert Ultracut) were mounted in copper grids (300 Mesh; from TAAB Laboratories Equipment Ltd, West Berkshire, UK) and further contrasted with uranyl acetate and lead citrate for analysis at an accelerating voltage of 60 Kv (Zeiss EM 10A; Carl Zeiss).

**Biochemical determinations in the hepatic tissue.** After the excision from each animal, the liver was washed in a phosphate-buffered saline solution, pH 7.4, dried and weighed to assess the relative mass of the organ (calculated as a percentage of the total body-weight on the day of euthanasia). Liver samples were homogenized [1:4 (m/v)] in ice-cold phosphate-buffered solution, pH 7.4, with an Ultra-Turrax®

homogenizer and centrifuged ( $3000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ). Aliquots of the supernatant were taken to determine the glutathione status, ATP levels and total protein levels.

**Total glutathione (GSHt), GSH and GSSG levels in the hepatic tissue.** An aliquot of previous supernatant was added to an equal volume of  $\text{HClO}_4$  10% (5% final concentration). Samples were again homogenized, centrifuged ( $16\,000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ), and the supernatant was used to determine the glutathione status [15]. The GSHt and GSSG levels were evaluated by the DTNB-GSSG reductase recycling assay, as previously described [16,17]. Briefly, for GSHt quantification, 200  $\mu\text{L}$  of the acidic supernatant was neutralized with 200  $\mu\text{L}$  of 0.76 M  $\text{KHCO}_3$  and centrifuged ( $16\,000 \times g$ , 5 min.,  $4^{\circ}\text{C}$ ). For GSSG quantification, 10  $\mu\text{L}$  of 2-vinylpyridine was added to 200  $\mu\text{L}$  of acidic supernatant, and the samples were shaken during 1 hr on ice prior to the neutralization step. In 96-well plates, 100  $\mu\text{L}$  of sample, standard or blank was added in triplicate and mixed with 65  $\mu\text{L}$  of fresh reagent solution containing DTNB and  $\beta$ -NADPH. Plates were incubated at  $30^{\circ}\text{C}$  for 15 min. in a plate reader (PowerWaveX; Bio-Tek Instruments, Winooski, VT, USA) prior to the addition of 40  $\mu\text{L}$  glutathione reductase solution (10 U/mL). The final product of this reaction is a coloured substance, and its formation was monitored for 3 min., at 415 nm, and compared with a standard curve. GSH and GSSG standard solutions were prepared in  $\text{HClO}_4$  5% [15]. Results were expressed as nmol/mg protein.

**Hepatic ATP levels.** An aliquot of the supernatant obtained as described above was added to an equal volume of  $\text{HClO}_4$  10% (5% final concentration). Samples were again homogenized, centrifuged ( $16\,000 \times g$ , 10 min.,  $4^{\circ}\text{C}$ ), and the supernatant was used to determine the ATP levels as described before [6]. One hundred microliter of tissue homogenate was neutralized with 100  $\mu\text{L}$  of 0.76 M  $\text{KHCO}_3$  and centrifuged ( $16\,000 \times g$ , 10 sec.,  $4^{\circ}\text{C}$ ). The ATP levels were quantified by the bioluminescence test after the

reaction of 100  $\mu\text{L}$  neutralized supernatant with luciferin/luciferase solution. ATP standards were prepared in 5%  $\text{HClO}_4$ . ATP intracellular levels were normalized to total protein content (nmol/mg of protein).

**Hepatic total protein quantification.** An aliquot of the supernatant obtained after centrifugation described in the section 'Biochemical determinations in the hepatic tissue' was diluted in 0.3 M NaOH and used to assess the total hepatic protein levels. The protein levels were determined by the Lowry method using a microplate reader (750 nm), as previously described [16].

**Statistical analysis.** Results are presented as means  $\pm$  standard deviation. Statistical comparisons between groups were performed with one-way ANOVA (in case of normal distribution: haematological and plasma biomarkers) or the Kruskal-Wallis test (one-way ANOVA on ranks – in case of not normal distribution: hepatic protein levels, hepatic glutathione levels and ATP levels). Significance was accepted at  $p$  values  $< 0.05$ . Details of statistical analysis are found in the legend of the figures in the results section.

## Results

### Plasma and haematological changes induced by MTX cumulative doses.

The results from plasma and haematological biomarkers at the end of the experiment which presented significant changes (in at least one of the treated groups) compared with control rats, are shown in Table 1. A transient decrease in total protein and IgG plasma levels was observed in the MTX22 group. Transient decreases in RBC count, HCT, Hb, WBC and reticulocytes were evident in the MTX22 group. The leucocyte group

Table 1.

Plasma and haematological parameters of the animals treated with MTX (three cycles of 2.5 mg/kg) and control group.

Parameter	Groups		
	Control	MTX22	MTX48
Total proteins (g/dL)	56.8 $\pm$ 3.3	48.3 $\pm$ 4.9*	52.4 $\pm$ 2.1
IgG	48.6 $\pm$ 19.9	10.2 $\pm$ 6.5**	32.0 $\pm$ 13.9
Complement C4 (mg/dL)	2.4 $\pm$ 0.9	4.8 $\pm$ 0.5**	3.7 $\pm$ 0.6*
Conjugated bilirubin (mg/dL)	0.10 $\pm$ 0.02	0.07 $\pm$ 0.02*	0.06 $\pm$ 0.02*
ALT (U/L)	34.8 $\pm$ 7.4	13.0 $\pm$ 5.3**	19.5 $\pm$ 5.0**
Alkaline phosphatase (U/L)	278.6 $\pm$ 61.0	133.2 $\pm$ 54.9**	149.0 $\pm$ 35.8**
Cholesterol (mg/dL)	44.6 $\pm$ 2.8	65.28 $\pm$ 11.3**	52.47 $\pm$ 7.6
Triglycerides (mg/dL)	93.8 $\pm$ 9.9	40.6 $\pm$ 7.28***	64.3 $\pm$ 19.5*
Iron ( $\mu\text{g}/\text{dL}$ )	176.9 $\pm$ 27.8	250 $\pm$ 27.0**	169.7 $\pm$ 14.3
Transferrin (mg/dL)	105.2 $\pm$ 5.2	73.6 $\pm$ 17.0**	82.7 $\pm$ 15.5*
RBC ( $\times 10^6/\text{mm}^3$ )	7.8 $\pm$ 0.9	6.0 $\pm$ 1.0*	6.6 $\pm$ 0.6
Haematocrit (%)	43.78 $\pm$ 4.9	32.7 $\pm$ 5.5*	43.7 $\pm$ 3.2
Hb(g/dL)	14.6 $\pm$ 1.1	11.4 $\pm$ 1.8**	14.4 $\pm$ 0.7
MCH (pg)	18.8 $\pm$ 0.9	19.1 $\pm$ 0.3	22.1 $\pm$ 1.2***
RDW (%)	12.5 $\pm$ 0.4	12.9 $\pm$ 0.9	17.9 $\pm$ 1.1***
MCV (fL)	56.2 $\pm$ 1.1	54.5 $\pm$ 1.3	66.3 $\pm$ 2.1***
Platelet ( $\times 10^3/\text{mm}^3$ )	599.4 $\pm$ 79.24	592.0 $\pm$ 246.2	785.0 $\pm$ 74.0
MPV (fL)	7.4 $\pm$ 0.4	8.4 $\pm$ 1.0*	6.9 $\pm$ 0.4
Reticulocytes (%)	1.6 $\pm$ 0.5	0.0 $\pm$ 0.0**	2.9 $\pm$ 0.6**
WBC ( $\times 10^3/\text{mm}^3$ )	2.8 $\pm$ 1.3	1.6 $\pm$ 0.8*	2.3 $\pm$ 0.7

Results are presented as means  $\pm$  standard deviation.

Statistical analysis was performed using one-way ANOVA followed by the Student Newman Keuls *post hoc* test (\* $p < 0.05$  versus control, \*\* $p < 0.01$  versus control, \*\*\* $p < 0.001$  versus control).

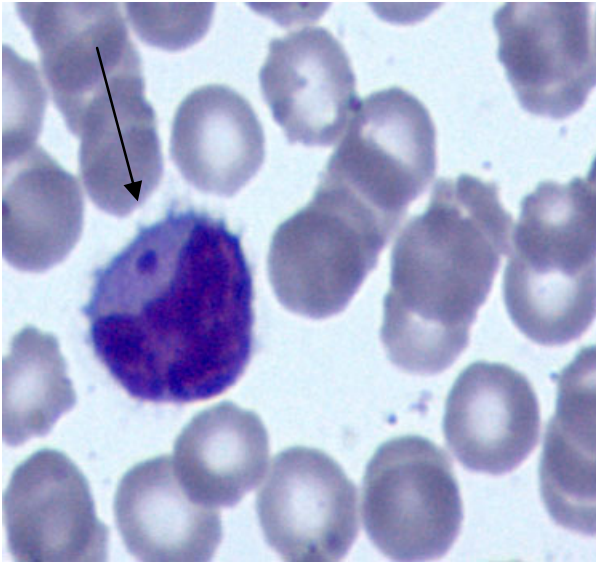


Fig. 1. Representative blood smears from the MTX22 group, demonstrating a monocyte with the presence of a micronucleus (arrow).

that mostly contributed to this decrease were lymphocytes (diminished in both groups). Additionally, increased levels of MPV were observed in the MTX22 group and microscopic analysis of the blood smears from the MTX22 group revealed the presence of several nucleated cells with micronucleus for all the animals (Fig. 1). Transient bone marrow suppression was observed in the MTX22 group, as shown by the absence of circulating reticulocytes. However, in MTX48, a recovery was observed and circulating reticulocytes were significantly higher than controls, representing an adaptation process to MTX exposure (Table 1).

A significant increase in the C4 complement and reduced levels of transferrin, conjugated bilirubin, ALT and alkaline phosphatase were evident in both treated groups. The MTX48 group had significant increases in the MCH, RDW and MCV levels, while WBC returned to control levels at MTX48.

Glucose, amylase, albumin, creatinine, urea, uric acid, calcium, potassium, sodium, C-reactive protein,  $\alpha_1$ -antitrypsin, GGT, C3 complement, LDH, total bilirubin and IgM, and IgE plasma levels in the treated animals did not show any changes in comparison with control levels (data not shown). PLT, PCT, MCHC, platelet distribution width, neutrophil, eosinophil, basophil and monocytes levels remained constant in all groups at the time-points evaluated.

#### *MTX administration induced macro- and microscopic changes in hepatic tissue.*

Hepatic macroscopic alterations were evident in the necropsy of MTX-treated rats (Fig. 2). The livers, presenting a dark red colour with brilliant surface, were swollen with all lobes showing rounded edges. Their consistency was firm but friable during the cut. These alterations were more notorious in the MTX48 group. No macroscopic changes were observed in control livers.

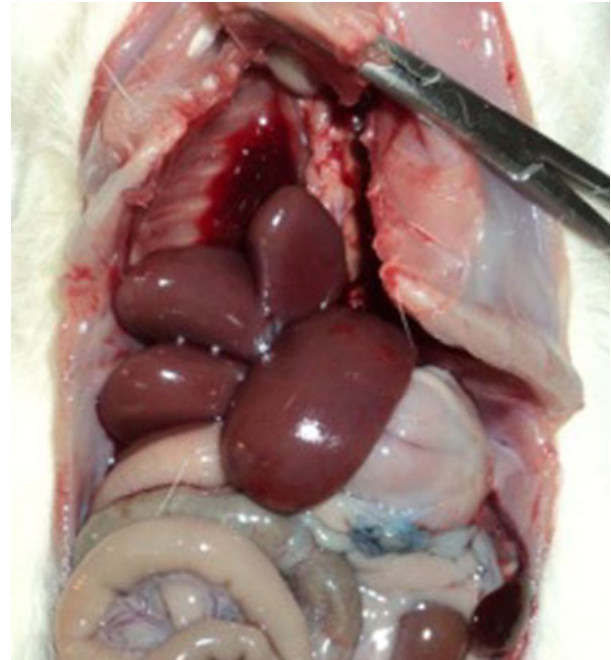


Fig. 2. Necropsy of an animal from the MTX48 group, demonstrating the liver macroscopic alterations observed in all MTX-treated rats, being more evident in the MTX48 group. The liver presents a swollen aspect with rounded edges in all lobes, having a dark red colour and brilliant surface. Male Wistar rats were subjected to MTX treatment (three cycles of 2.5 mg/kg) and suffered euthanasia 28 days after the last MTX administration.

The microscopic analysis did not reveal any abnormality in animals from the control group (data not shown). However, at light microscopy, the MTX22 livers revealed disperse regions of focal necrosis without changes in the organ architecture (Fig. 3). The hepatic tissue presented an apparent proliferation of kupffer cells, hepatocyte oedema in the periportal regions and enlargement of periportal spaces with cellular infiltration and collagen deposition (Fig. 3). In the same group, transmission electron microscopy confirmed the cellular oedema mainly located in the periportal region. Moreover, an increased density of lysosomes (Fig. 3) and mild steatosis affecting the majority of hepatocytes were observed. In general, the hepatocytes presented several nucleoli (more than 2–3 per cell) and abundant rough endoplasmic reticulum, without signs of mitochondria swelling. Frequent perisinusoidal cells containing lipid droplets, suggestive of stellate cells, as well as collagen fibres proliferation into the Disse spaces, and abundant hepatic and endothelial microvilli were also observed (Fig. 3).

In the MTX48 group, the periportal cellular oedema apparently decreased when compared with the MTX22 group, although with an enlargement of periportal spaces and a more evident fibrotic area. A higher number of areas containing cellular infiltration with apparent mild disorganization of the lobular structure were seen (Fig. 3). In general, the transmission electron microscopy revealed a high content of lysosomes in hepatocytes, abundant Kupffer and stellate cells, as well as a huge proliferation of collagen fibres into the Disse spaces.

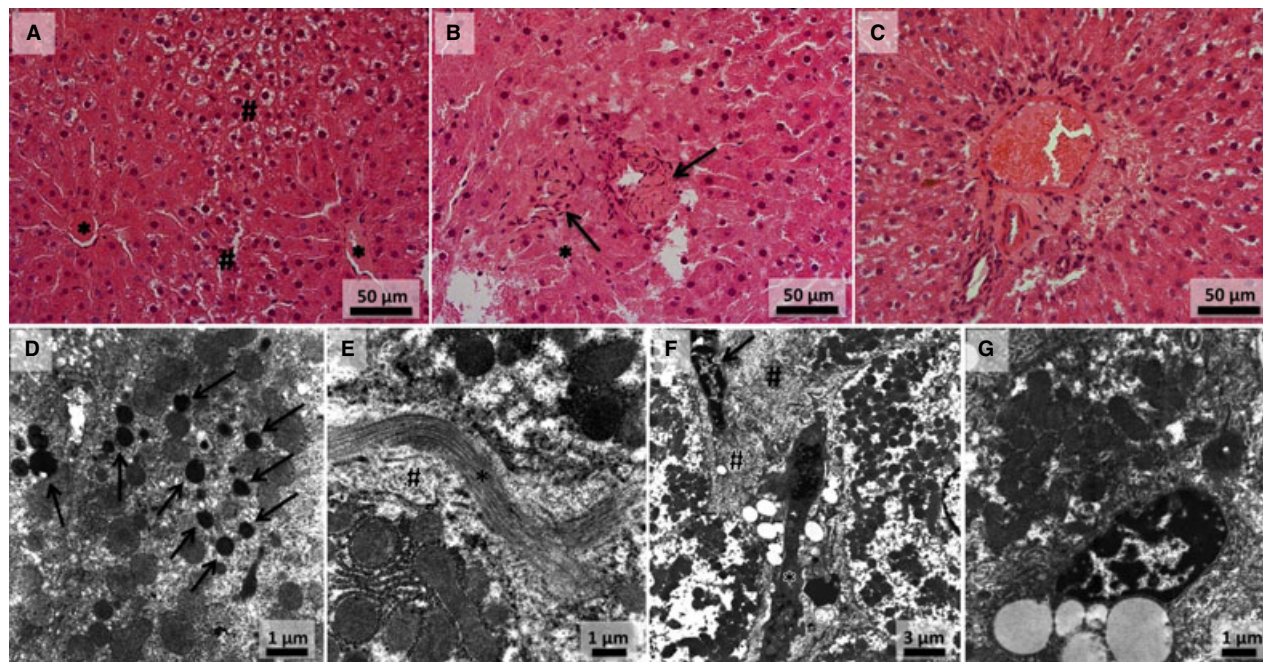


Fig. 3. Light (A, B and C) and electron micrographs (D, E, F and G) of liver sections illustrating the structural and ultrastructure alterations observed in MTX-treated animals. In (A) (from MTX22 group), two central veins (\*) surrounded by hepatocytes displaying a normal morphology are seen; however, at the periphery of the lobule, the presence of swollen hepatocytes is notorious (#); (B) (from MTX48) depicts a necrotic area (\*) infiltrated by fibroblasts like cells with deposition of connective tissue (arrows); (C) (from MTX48 group) displays a portal area with enlargement of periportal space, which is invaded by connective tissue and infiltrated by fibroblasts like cells and leucocytes; (D) (from MTX22 group) depicts a great amount of lysosomes within a hepatocyte (arrows), with mitochondria presenting a normal morphology without signals of swelling; In (E) (from MTX22 group), the presence of collagen fibres (\*) in the Disse space as well as an area of high density of hepatocyte microvilli are shown (#); (F) (from MTX22 group) shows two adjacent swollen hepatocytes, containing mitochondria with normal morphology (at left and right sides), separated by a space occupied by abundant microvilli (#) with a Kupffer cell near by (arrow); at the centre, a stellate-like cell (\*) with several lipid droplets; In (G) (from the MTX48 group), a stellate cell is depicted.

Changes in the hepatic macro- and microscopic architecture were not accompanied by significant alterations in the hepatic relative mass (data not shown).

#### MTX-induced hepatic alterations in the protein levels, glutathione status and ATP levels.

The hepatic protein levels were significantly decreased in the MTX22 group ( $54.1 \pm 3.5$  mg/g) when compared to control ( $107.3 \pm 28.6$  mg/g). The values observed in the MTX48 group ( $165.5 \pm 91.8$  mg/g) suggest a recovery of hepatic proteins, being similar to control values (Fig. 4). Moreover, as shown in Fig. 5, late significant increases were observed in the hepatic GSHt levels ( $47.3 \pm 11.0$  nmol/mg in the MTX48 group versus  $26.3 \pm 7.6$  nmol/mg and  $25.4 \pm 3.6$  nmol/mg in the control and MTX22 groups, respectively). This increase in GSHt corresponds to the late increase in the GSH ( $37.4 \pm 8.5$  nmol/mg in the MTX48 group compared with  $22.9 \pm 7.7$  nmol/mg in the control and  $19.5 \pm 2.5$  nmol/mg in the MTX22 group), and a great increase in the GSSG levels in the MTX48 group ( $4.9 \pm 1.3$  nmol/mg versus  $1.7 \pm 0.3$  nmol/mg in the control and  $2.9 \pm 1.1$  nmol/mg in the MTX22 group). Regarding hepatic ATP levels, a significant decrease in the MTX48 group ( $5.2 \pm 1.5$  nmol/mg) compared with control ( $10.1 \pm 1.5$  nmol/mg) or MTX22 ( $9.0 \pm 1.5$  nmol/mg) groups was seen (Fig. 6).

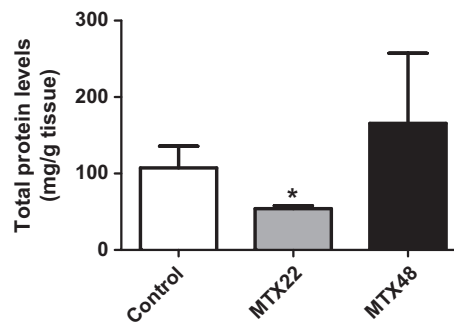


Fig. 4. Hepatic protein levels of the animals treated with MTX (three cycles of 2.5 mg/kg) and control group. Results are presented as means  $\pm$  standard deviation. Statistical analysis was performed using Kruskal–Wallis test (ANOVA on ranks) followed by the Student Newman Keuls *post hoc* test (\* $p < 0.05$  versus control).

#### Discussion

This work aimed to investigate the potential hepatotoxicity and haematotoxicity of MTX using a multiple dose *in vivo* model. To evaluate whether the toxic effects were also cumulative and long-lasting, as it happens in some cardiac events, two time-points were selected: 2 and 28 days after the last administration of MTX. The choice of two different time-points represented by the MTX22 and MTX48 groups is

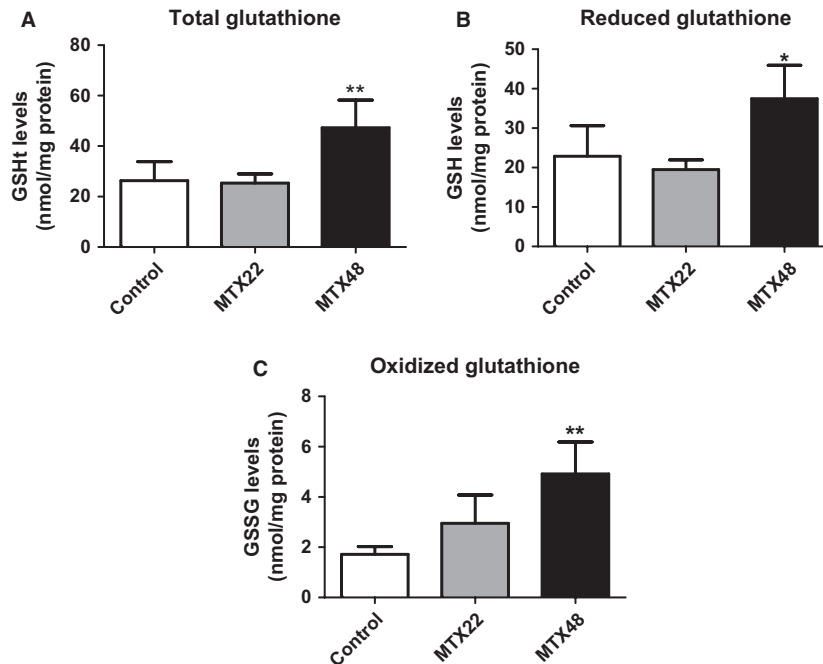


Fig. 5. Hepatic (A) GSHt, (B) GSH and (C) GSSG levels of the animals treated with MTX and control group. Results are presented as means  $\pm$  standard deviation. Statistical analysis was performed using the Kruskal–Wallis test (ANOVA on ranks) followed by the Student Newman Keuls *post hoc* test (\* $p < 0.05$  versus control).

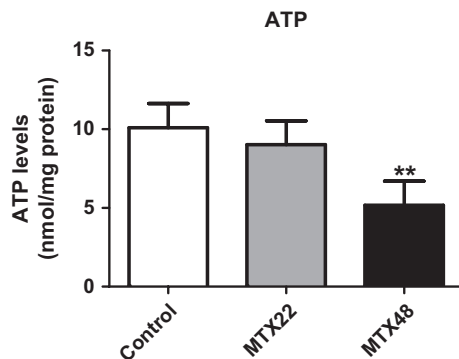


Fig. 6. Hepatic ATP levels of the animals treated with MTX and control group. Results are presented as means  $\pm$  standard deviation. Statistical analysis was performed using the Kruskal–Wallis test (ANOVA on ranks) followed by the Student Newman Keuls *post hoc* test (\* $p < 0.05$  versus control).

important to evaluate acute/adaptive and transient/ultimate effects of the liver or haematological function, because they present high turnover and plasticity [13].

We observed hepatotoxicity and haematological toxic effects after MTX administration to rats, as demonstrated by the macro- and microscopic changes in the hepatic architecture (Figs 2 and 3), and by several haematological alterations (Table 1). The reduction in hepatic protein levels in the MTX22 group, the late increases in the GSHt hepatic levels, demonstrated by an increase in GSH and GSSG contents, and late decreases in the hepatic ATP values in the MTX48 group showed mechanistic responses of hepatic tissue after MTX administration (Figs 4–6, respectively).

The MTX-induced haematological toxic effects were demonstrated by an early bone marrow depression on MTX22 group (Table 1) followed by signals of haematopoiesis recovery, observed in the MTX48 group. Indeed, MTX22 presented a reduction in WBCs, RBCs and reticulocytes, associated with an increase in non-conjugated bilirubin that was followed, at MTX48, by a recovery of WBCs and RBCs. The return to basal values of RBCs at MTX48 was probably due to a significant increase in reticulocytes, leading to macrocytosis and anisocytosis, as shown by the significant increase in the RDW and in MCV. Additionally, increased levels of plasma iron suggest an increase in iron absorption to overcome the initial reduction in RBCs. These results are in accordance with what has been observed in human beings and justify the importance of monitoring haematological parameters during MTX chemotherapy [1]. In fact, the MTX-induced myelosuppression manifests mostly as leukopaenia, thus being the main dose-limiting effect that occurs shortly after MTX treatment [18]. Also, in human beings, after administration of MTX (38 mg/m<sup>2</sup>), the WBC counting returned to normal values within 7 days [18]. However, patients treated with higher dose regimens tend to present an even faster blood count recovery [1]. The presence of micronucleus in MTX22 blood smears (Fig. 1) was constant in all animals from this treated group, suggesting a genotoxic potential for MTX. Altogether, these data elucidate the rationale for continued blood cell counts after MTX-therapy cessation due to the risk of MTX-associated secondary acute leukaemia [2]. As for other topoisomerase II inhibitors, there are only limited data on the MTX genotoxicity using microbial conventional assays [10]. However, it is well known that

MTX causes single and double breaks in the DNA [1,3] and also affects the cell cycle at various stages [3,19].

As stated before, MTX is an immunosuppressive drug that promotes apoptosis in antigen-presenting cells and reduces the levels of all types of immune cells [2,20]. Accordingly, we observed a transient decrease in the plasma IgG levels in the MTX22 rats (Table 1). Another study assessing the human safety of MTX, rituximab, ifosfamide and etoposide combined therapy demonstrated significant decrease in the IgG levels in patients treated for non-Hodgkin's lymphoma [21]. The IgG is synthesized by blood cells and corresponds to about 80% of total immunoglobulin [22]. Thus, the significant decreases in IgG levels in the MTX22 group may correlate with the observed decreases in the plasma total protein levels observed in this group. The levels of the main plasma protein albumin remained constant in the MTX-treated groups (data not shown).

The MTX-induced hepatotoxicity was clearly demonstrated by the macroscopic changes observed in the MTX-treated livers (Fig. 2). Signals of hepatic injury were found in the earlier time-point (MTX22 group), notably by changes in plasma-related liver parameters, in the hepatic macro- and microscopic changes (Fig. 3) and by alterations in biochemical hepatic values (Fig. 4). These data suggest hepatic dysfunction, demonstrated by the decrease in plasma enzyme levels, protein turn-over and higher free cholesterol. The impairment of hepatic function in both treated groups is also shown by decreases in the plasma triglycerides, which are mainly synthesized by the hepatocytes (Table 1). Furthermore, the increase in the plasma cholesterol levels in the MTX22 group is consistent with the mild steatosis observed by structural analysis, which can be related to the decreased hepatic synthesis of lipoproteins. The diminished hepatic protein synthesis was demonstrated by lower protein hepatic levels (MTX22), and lower levels of plasma biochemical parameters such as ALT, alkaline phosphatase and transferrin in both MTX-treated groups. In fact, in human beings, the acute hepatotoxicity of MTX manifests as transient increases in serum bilirubin levels and in the activity of liver enzymes [10]. However, decreases in the hepatic synthesis of these enzymes are considered a result of (sub)chronic hepatotoxicity observed with other xenobiotics [23–26]. Possibly due to the marked decrease in protein turn-over, MTX22 hepatocytes show large nucleoli and abundant rough endoplasmic reticulum, as a compensation mechanism to attempt restoring hepatic synthesis diminished by the action of the drug. The apparent recover of the hepatic total protein levels (Fig. 4) on MTX48 compared with MTX22 does not mean total functional gain, considering the changes in MTX48 hepatic ATP, glutathione and plasma protein levels.

Moreover, increases in the unconjugated bilirubin in both treated groups are suggestive of impaired conjugation with glucuronides. Subclinical unconjugated hyperbilirubinaemia is related to antituberculosis therapy, and it is attributed to the inhibition of the bile salt exporter pumps [26]. Microscopic hepatic evaluations (Fig. 3) demonstrated a huge proliferation in the Kupffer cells, leucocyte infiltration and collagen deposition, which are indicative of an inflammatory process. Indeed,

it might be related to the notorious increases observed in the plasma C4 complement levels because this component is synthesized by Kupffer cells [27]. The hepatic microscopic changes were more notorious at a later time-point (MTX48 group). As already mentioned, MTX and naphthoquinoline (an active metabolite) are retained in the hepatic tissue [5,28], and the continued hepatic exposure may result in hepatic injury. Recently, we described some significant changes related to cardiotoxicity in the MTX22 group (decreased levels of CK, CK-MB, increased cardiac relative mass and adaptive increase in the mitochondrial complexes IV and V). However, it was demonstrated that the cardiac energetic damage was established at a later time-point (increased levels of plasma lactate and cardiac relative mass, decrease in the mitochondrial complex V activity with consequent depletion of cardiac ATP levels) [6]. One hypothesis for the MTX-induced hepatotoxicity is that it was secondary to the concomitant MTX-induced cardiotoxicity. However, microscopic data do not support the theory that the classic late cardiotoxicity of MTX [6] may contribute to promote a secondary hepatic lesion [8] considering the absence of significant centrilobular damage or dilatation of centrilobular sinusoids by blood congestion. In another study, the hepatic histopathological results of mice treated with MTX (15 mg/kg) demonstrated intense hydropic vacuolization of the cytoplasm, necrosis areas, picnosis and nuclear lysis not recovered until 5 days after the single dose administration [9]. Accordingly, in the present study, the MTX-induced hepatic damage continued until the end of the evaluations.

Oxidative stress seems to assume a more important role in the MTX hepatotoxicity when compared to the oxidative damage observed in the MTX-related cardiomyopathy [6,7]. Increases in the hepatic levels of GSH were observed in the MTX48 group. As shown in Fig. 5, this increase is related to a slight (but significant) increase in the GSH hepatic levels and GSSG levels. Increases in the GSH might be interpreted as an attempt to enhance the antioxidant content to respond to injury, while increased levels of GSSG are a clear sign of oxidative stress. It is known that Kupffer cells, which were abundantly present in the MTX48 livers, are a source of reactive oxygen and nitrogen species [29]. The link between oxidative stress and the MTX-induced hepatic damage was already suggested in a study with mice, where the administration of MTX (15 mg/kg) caused increases in the lipid peroxidation, decreases in the antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and depletion of the hepatic retinol and GSH content [9]. Moreover, the incubation of MTX (100  $\mu$ M) with HepG2 cells for 6 hr was enough to promote the decrease in the GSH levels [30]. The apparent contradiction between our results (increased in the GSH hepatic levels) and the previously reported data (decreases in the GSH content) may be explained by the differences in the experimental design or models. Both referred studies assessed the GSH levels 3, 4 and 5 days after MTX administration [9] or 6 hr after high MTX concentration incubation [30]. In the present study, significant changes in the glutathione sta-

tus were seen only at the last evaluated time-point, 28 days after the last dose administration. Hence, the organism is subjected to compensatory/adaptive effects favoured by the elapsed time between the last MTX administration and rat euthanasia.

To the best of our knowledge, it is the first time that late hepatic energetic imbalance is associated with MTX-induced hepatotoxicity. The decrease in the hepatic ATP levels observed in the MTX48 group (Fig. 6) occurred 28 days after the last MTX administration. One can speculate about this result. Decreases in the ATP hepatic levels are observed in the human non-alcoholic steatohepatitis [31], and are associated with covalent binding of drugs with intracellular proteins [32]. As already stated, MTX and its toxic known metabolite accumulates in the liver tissue [5,28] and its long-lasting effects can be attributed to MTX persistence in the cells and its strong affinity for cellular macromolecules and membranes [33]. Additionally, the ATP decreases can be related to mitochondrial disruption. In other models related to myocytes, the MTX-induced decreases in the ATP levels have already been described [6,7,34]. In these models, the observed cardiac energetic depletion was already attributed to the mitochondrial toxicity induced by MTX, affecting the ATP synthesis through perturbations in the mitochondrial membrane potential and late inhibition of ATP synthase activity *in vitro* and *in vivo* [6,7]. Moreover, in a work by Shipp *et al.* [34], the MTX-metabolite naphthoquinoline also disrupted ATP homeostasis in neonatal rat heart cells. Although, in the present study, mitochondrial morphologic changes in the hepatic tissue were not observed, one cannot exclude the existence of functional mitochondrial disturbances.

In summary, this study allowed to better understand the long-term effect of MTX treatment. Moreover, the intrinsic hepatotoxic potential of chemotherapy is of great concern because an altered hepatic function might decrease the cancer therapy effectiveness and/or increase toxic adverse effects [13]. In this study, we described a severe hepatotoxicity. We did not find hepatic changes suggestive of heart failure and the location of the histological findings in the periportal region seem to reinforce the direct hepatotoxic potential of MTX. After 28 days of the last MTX cycle, hepatic tissue remained with reduced levels of glutathione and ATP, which are essential for the hepatic function.

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