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Cytotoxicity of monocrotaline in isolated rat hepatocytes: Effects of dithiothreitol and fructose

Marcos A. Maioli [a], Larissa C. Alves [a], Diego Perandin [a], Andréa F. Garcia [a], Flávia T.V. Pereira [b], Fábio E. Mingatto [a,*]

[a] Laboratório de Bioquímica Metabólica e Toxicológica, UNESP-Univ Estadual Paulista, Campus de Dracena, 17900-000 Dracena, SP, Brazil
[b] Laboratório de Morfofisiologia da Placenta e Embrião, UNESP–Univ Estadual Paulista, Campus de Dracena, 17900-000 Dracena, SP, Brazil

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ABSTRACT

Monocrotaline (MCT) is a pyrrolizidine alkaloid present in plants of the *Crotalaria* species that causes cytotoxicity and genotoxicity, including hepatotoxicity in animals and humans. It is metabolized by cytochrome P-450 in the liver to the alkylating agent dehydromonocrotaline (DHM). In previous studies using isolated rat liver mitochondria, we observed that DHM, but not MCT, inhibited the activity of respiratory chain complex I and stimulated the mitochondrial permeability transition with the consequent release of cytochrome c. In this study, we evaluated the effects of MCT and DHM on isolated rat hepatocytes. DHM, but not MCT, caused inhibition of the NADH-linked mitochondrial respiration. When hepatocytes of rats pre-treated with dexamethasone were incubated with MCT (5 mM), they showed ALT leakage, impaired ATP production and decreased levels of intracellular reduced glutathione and protein thiols. In addition, MCT caused cellular death by apoptosis. The addition of fructose or dithiotreitol to the isolated rat hepatocyte suspension containing MCT prevented the ATP depletion and/or glutathione or thiol oxidation and decreased the ALT leakage and apoptosis. These results suggest that the toxic effect of MCT on hepatocytes may be caused by metabolite-induced mitochondrial energetic impairment, together with a decrease of cellular glutathione and protein thiols.

1. Introduction

Monocrotaline (MCT), a pyrrolizidine alkaloid phytotoxin, has well-documented hepatic and cardiopulmonary toxicity for animals, including ruminants and humans (Mclean, 1970; Mattocks, 1986; Huxtable, 1989; Souza et al., 1997; Schultze and Roth, 1998; Stegelmeier et al., 1999; Nobre et al., 2004, 2005). This compound is frequently ingested accidentally because of food grain contamination or intentionally in the form of herbal medicine preparations (Huxtable, 1989). It has been reported that its toxicity depends on cytochrome P-450 mediated bioactivation to the reactive pyrrolic metabolite dehydromonocrotaline (DHM) (Butler et al., 1970; Lafranconi and Huxtable, 1984; Roth and Reindel, 1990; Wilson et al., 1992; Pan et al., 1993; Schultze and Roth, 1998). This metabolite, despite having a half-life of only a few seconds in aqueous media, is a powerful alkylating agent that binds to DNA and proteins (Petry et al., 1984; Hincks et al., 1991; Niwa et al., 1991; Wagner et al., 1993; Yan and Huxtable, 1995; Lamé et al., 2005).

We previously demonstrated that DHM, but not MCT, inhibits the activity of NADH-dehydrogenase when added at micromolar concentrations to isolated rat liver mitochondria, an effect associated with significantly reduced ATP synthesis (Mingatto et al., 2007). Because the activity of complex I is regulated by thiol groups, it was suggested

* Corresponding author. Tel.: +55 18 3821 8200; fax: +55 18 3821 8208.
E-mail address: fmingatto@dracena.unesp.br (F.E. Mingatto).

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that the inhibition of complex I NADH oxidase activity resulted from oxidation of cysteine thiol groups by DHM. In a recent study, we also demonstrated that DHM induces membrane permeability transition (MPT) and the release of cytochrome c associated with oxidation of protein thiol groups in isolated rat liver mitochondria (Santos et al., 2009).

It is well known that the thiol group in proteins and non-proteins is involved in the maintenance of various cellular functions. Some investigators have indicated that protein thiols, more than non-protein thiols, are essential for the maintenance of cell viability during exposure to toxic chemicals (Nicotera et al., 1985; Nakagawa and Moldéus, 1992). The liver removes the xenobiotics from the body by the triad of actions oxidation (phase I), conjugation (phase II) and elimination (phase III), but in a few cases either phase I or phase II reactions can result in more toxic species (Boelsterli, 2007). The metabolism of MCT seems to be one of these cases. Within this context, in the present study, we evaluated the mechanisms responsible for MCT toxicity in isolated rat hepatocytes and the roles of its metabolism, thiol groups and mitochondria.

2. Material and methods

2.1. Chemicals

The MCT was purchased from Sigma-Aldrich (St. Louis, MO), and DHM was prepared from MCT according to published procedures (Mattocks et al., 1989). The purity of the resulting pyrrole was confirmed using NMR. All other reagents were of the highest commercially available grade. Dexamethasone was purchased from DEG, Brazil. Sodium pentobarbital was a gift from Cristália, Brazil. MCT was solubilized in 2 M HCl and was neutralized with 0.5 M phosphate buffer. All stock solutions were prepared with glass-distilled deionized water. MCT and DHM were dissolved in anhydrous dimethyl sulfoxide (DMSO).

2.2. Animals

Male Wistar rats weighing approximately 200 g were used in this study. Animals were maintained at a maximum of 4 rats per cage under standard laboratory conditions. Water and food were given *ad libitum*. In experiments with dexamethasone induction, rats were dosed intraperitoneally (50 mg/kg body weight) daily for 3 consecutive days and used 24 h after the last dose. The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Dracena.

2.3. Isolation and incubation of hepatocytes

For the surgical procedure, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The hepatocytes were isolated by collagenase perfusion of the liver as described previously (Guguen-Guillouzo, 1992). Hepatocyte viability after isolation was determined by Trypan blue (0.16%) uptake, and initial cell viability in all experiments was more than 85%. Hepatocytes were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM Hepes and 0.1% albumin (BSA) and maintained at 4 °C. Cells (1×10^6/mL) were incubated in 25-mL Erlenmeyer flasks kept under constant agitation (30 rpm) at 37 °C under a 95% O_2 and 5% CO_2 atmosphere. Reactions were initiated by the addition of MCT. Aliquots (0.5 mL) of the suspension were removed from the mixture at appropriate times for the determination of cell death and biochemical parameters. In some experiments, cells were incubated with 20 mM fructose or 10 mM dithiotreitol (DTT) 15 min before the addition of MCT.

2.4. Oxygen uptake

Oxygen uptake by the isolated hepatocytes was monitored polarographically with an oxygraph equipped with a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Glasgow, Scotland, UK) at 37 °C. Respiration buffer contained 250 mM sucrose, 2 mM KH_2PO_4, 10 mM HEPES, pH 7.2, 0.5 mM EGTA, 0.5% bovine serum albumin, and 5 mM $MgCl_2$. Cells were treated with 0.002% digitonin, and state 4 and state 3 mitochondrial respiration rates were measured in the presence of 1 μg/mL oligomycin and 2 mM ADP, respectively (Moreadith and Fisckum, 1984). MCT and DHM were added in the medium, immediately after the initiation of state 3 respirations by ADP.

2.5. Evaluation of cell viability

Cell viability was assessed by the leakage of alanine transaminase (ALT) from hepatocytes. Cell suspensions were centrifuged (50 × g for 5 min). ALT in the supernatant was determined using an Alanine Transaminase Activity Assay Kit (Bioclin, Quibasa, Brazil) according to the manufacturer's instructions. Absorbance was measured at 340 nm with a DU-800 spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA). Enzyme activity in the supernatant is expressed as a percentage of the total activity, which was determined by lysing the cells with 0.5% Triton X-100.

2.6. Cell ATP content

Cell ATP was determined by means of the firefly luciferin-luciferase assay system. The cell suspension was centrifuged at 50 × g for 5 min at 4 °C, and the pellet containing the hepatocytes was treated with 1 mL of ice-cold 1 M $HClO_4$. After centrifugation at 2000 × g for 10 min at 4 °C, aliquots (100 μL) of the supernatant were neutralized with 70 μL of 2 M KOH, suspended in 100 mM Tris-HCl, pH 7.8 (1 mL final volume), and centrifuged again. Bioluminescence was measured in the supernatant with a Sigma-Aldrich assay kit according to the manufacturer's instructions using a SIRIUS Luminometer (Berthold, Pforzheim, Germany).

2.7. Reduced glutathione (GSH) levels

The levels of GSH were determined by a fluorimetric reaction with o-phthalaldialdehyde (OPT) (Hissin and Hilf,

1976). The cell suspension was treated with 0.2 mL of 30% TCA and centrifuged at 2000 × g for 6 min. Aliquots (100 μL) of the supernatant were mixed with 2 mL of 100 mM NaH_2PO_4 buffer, pH 8.0, containing 5 mM EGTA. One hundred microliters OPT (1 mg/mL) was added, and fluorescence was measured 15 min later using the 350/420 nm excitation/emission wavelength pair with RF-5301 PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan). The values are expressed as nanomoles of GSH/10^6 cells using a standard curve. A blank with DTT was performed to eliminate its interference in the fluorescence intensity.

2.8. Protein thiols

Protein thiol groups were determined using Ellman's reagent according to Sedlak and Lindsay (1968) with some modifications. A sample (0.5 mL) of cell suspension was centrifuged at 50 × g for 5 min and the supernatant was discarded. The cell pellet was treated with 1 mL of 5% trichloroacetic acid, 5 mM EDTA. The protein precipitate was washed twice with the same trichloroacetic acid-EDTA solution. When DTT was used, this procedure was repeated four times. Protein was redissolved in 3 mL of 0.1 M Tris-HC1 buffer, pH 7.4, containing 5 mM EDTA and 0.5% sodium dodecyl sulfate. Aliquots of this solution were reacted with 0.1 mM (final concentration) 5,5′-dithiobis(2-nitrobenzoic)acid (DTNB) in 2 mL of Tris-EDTA buffer, pH 8.6. Absorption was measured at 412 nm and subtracted from blank value obtained by treating sample aliquots with 5 mM N-ethylmaleimide before reaction with DTNB. The values are expressed as nanomoles of –SH equivalents/10^6 cells using GSH as a standard.

2.9. Apoptosis assays

Cell death by apoptosis was determined by observing morphological changes in the nuclei of cells incubated with the fluorescent dye Hoechst 33342 (Kurose et al., 1997). Samples (200 μL) were collected and centrifuged at 50 × g for 5 min, and the supernatants were discarded; the pellet was suspended in Krebs/Henseleit medium, pH 7.4, and incubated with 8 μg/mL of Hoechst 33342 for 15 min at room temperature. After incubation, the samples were centrifuged twice at 50 × g for 5 min to remove excess dye. After the washes, the cells were suspended in 100 μL of Krebs/Henseleit medium, pH 7.4. Cells were analyzed with a fluorescence microscope (DM 2500 type, Leica, Rueil-Malmaison, France), and the percentage of apoptotic cells was quantitated using QWin software.

2.10. Statistical analysis

Comparisons of the several treated groups and the relevant controls were made by analysis of variance (ANOVA) followed by Dunnett's test. Comparisons between multiple groups were made using Newman–Keuls's test implemented in GraphPad Prism software, version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effects of MCT and DHM on the respiration of mitochondria in isolated rat hepatocytes

Fig. 1 shows the inhibitory effect of DHM on glutamate plus malate-supported state 3 (ADP-stimulated) respiration of mitochondria in digitonin-permeabilized hepatocytes. The effect was immediate and concentration-dependent, beginning at 50 μM DHM; the parent compound MCT did not inhibit state 3 respiration even at a concentration of 2 mM (Fig. 1). Neither MCT nor DHM stimulated state 4 (basal) respiration (results not shown). These results indicate that the metabolite DHM inhibits the respiratory chain, whereas neither the parent compound nor the metabolite effectively uncouples the oxidative phosphorylation of mitochondria, as assessed in isolated hepatocytes. These results are in agreement with those previously described showing that DHM is a respiratory chain complex I inhibitor in isolated mitochondria (Mingatto et al., 2007).

3.2. Toxic effects of MCT on isolated rat hepatocytes

The incubation of MCT at concentrations such as 10 mM with hepatocytes isolated from normal rats did not produce toxic effects (results not shown). Thus, in order to stimulate the production of MCT metabolites by isolated hepatocytes, rats were previously treated with dexamethasone, an inducer of cytochrome P-450 3A (Gonzales, 1990). Metabolism of MCT has been attributed to this cytochrome (Reid et al., 1998). The addition of increasing concentrations of MCT to hepatocytes of rats pre-treated with dexamethasone resulted in decreased cell viability, as assessed by ALT leakage into the incubation medium (Fig. 2A). ALT leakage was concentration- and time-dependent, with a significant increase being observed at MCT concentrations of 5 and

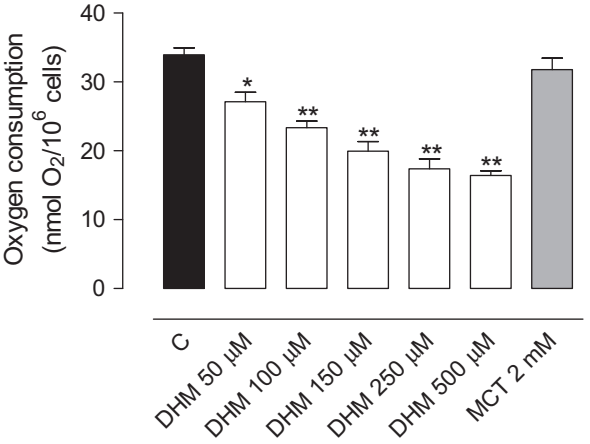

Fig. 1. Effects of monocrotaline (MCT) or its metabolite dehydromonocrotaline (DHM) on glutamate plus malate-supported state 3 (ADP-stimulated) respiration of mitochondria in digitonin-permeabilized isolated rat hepatocytes. The figure is representative of four experiments with different cell preparations. *,**Significantly different from control (absence of MCT or DHM) ($P < 0.05$ and $P < 0.01$, respectively).

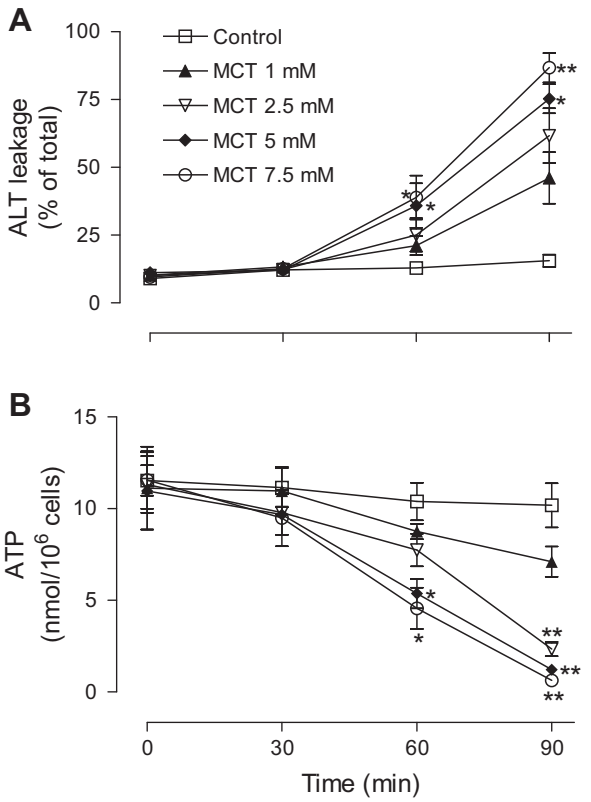

Fig. 2. Time course of the decrease in cell viability (A) and ATP levels (B) in isolated rat hepatocytes incubated without (Control) or with different concentrations of monocrotaline (MCT). Results are shown as the mean ±S.E.M. of four experiments with different cell preparations. *,**Significantly different from "without MCT" for the corresponding time points ($P < 0.05$ and $P < 0.01$, respectively).

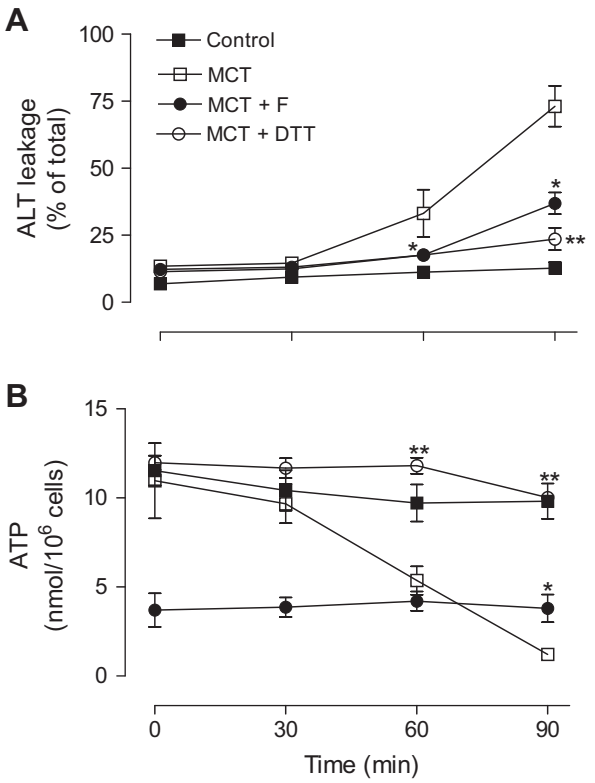

Fig. 3. Effects of 20 mM fructose (F) and 10 mM dithiotreitol (DTT) on the 5 mM monocrotaline (MCT)-induced decrease in cell viability (A) and ATP levels (B) in isolated rat hepatocytes. Results are shown as the mean ±S.E.M. of four experiments with different cell preparations. *,**Significant differences between the group of MCT alone and the group of MCT plus F or MCT plus DTT ($P < 0.05$; $P < 0.01$, respectively).

7.5 mM at 60 min incubation. In a previous report, we showed that the exposure of isolated perfused liver to MCT results in bioenergetic metabolism failure, which may reflect cell death due to decreased cellular ATP (Mingatto et al., 2008). In the current study, the incubation of isolated hepatocytes with MCT promoted a gradual decrease in ATP levels, which appeared to correlate closely with cell death (Fig. 2B). After 90 min incubation with 5 mM or more of drug, when almost all cells lost viability, ATP was almost completely depleted.

3.3. Effects of fructose and DTT on MCT-induced injury of isolated rat hepatocytes and ATP levels

In order to investigate the mechanisms involved in the cytotoxicity of MCT we evaluated the protective effects of fructose (20 mM) and DTT (10 mM) using 5 mM MCT. Fructose is an efficient substrate for glycolytic ATP formation in hepatocytes and protects against the loss of cell viability due to mitochondrial impairment. Such protection implies that cytotoxicity involves the inhibition of non-glycolytic mitochondrial ATP formation (Mingatto et al., 2002). Pre-treatment of hepatocytes with fructose prevented the decrease of cell viability caused by MCT (Fig. 3A). After 15 min pre-incubation with fructose, the intracellular levels of ATP were decreased to 30% of the control levels. This effect would be a consequence of the action of fructokinase producing fructose-1-phosphate with ATP consumption (Nakagawa et al., 1996). The addition of MCT did not further decrease the ATP levels, which remained constant for the 90-min incubation period (Fig. 3B). Because protein thiols and cellular thiol groups have long been described as important targets for reactive intermediates derived from some chemicals including MCT (Moore et al., 1985; Reed, 1990; Yan and Huxtable, 1996; Lamé et al., 2005), we also investigated the effects of DTT, a thiol reductant, on the MCT-induced cytotoxicity. Both the cytotoxicity and loss of intracellular ATP caused by 5 mM MCT were prevented by the addition of DTT (Fig. 3A and B, respectively).

3.4. Effects of MCT on intracellular levels of glutathione and protein thiols and protection by fructose and DTT

The oxidative status of hepatocytes in the presence of MCT (5 mM) was evaluated by measuring the levels of GSH and protein thiol. We observed a time-related decrease in these parameters (Figs. 4 and 5, respectively), with the GSH level being depleted more rapidly than that of protein

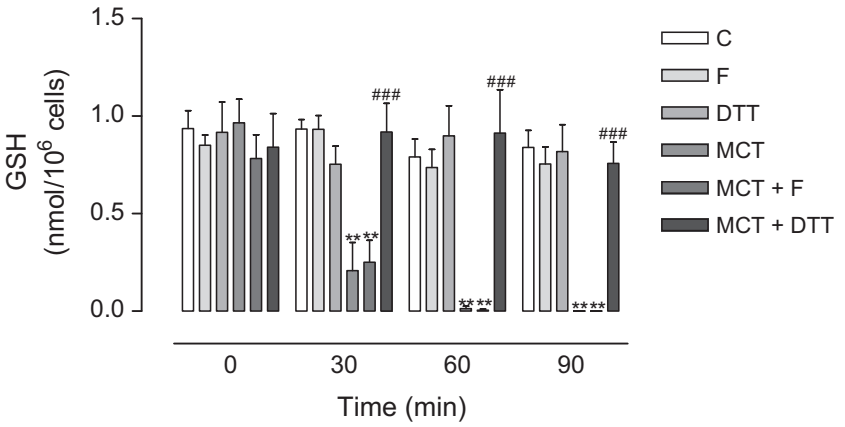

Fig. 4. Effects of 20 mM fructose (F) and 10 mM dithiotreitol (DTT) on the 5 mM monocrotaline (MCT)-induced decrease in GSH level in isolated rat hepatocytes. Results are shown as the mean ±S.E.M. of four experiments with different cell preparations. **Significantly different from Control (C) obtained without MCT ($P < 0.01$). ###Significantly different from MCT ($P < 0.001$).

thiols. As shown in Fig. 4, DTT caused a significant decrease in GSH oxidation induced by MCT, and fructose was unable to prevent this effect. Pre-incubation with DTT significantly inhibited the oxidation of protein thiol groups caused by MCT; however, in the cells that were previously incubated with fructose, we did not observe any protection (Fig. 5).

3.5. Protective effect of fructose and DTT on MCT-induced apoptosis

Fig. 6 shows that MCT induces programmed cell death. After 60 min of incubation, the cell suspension that received only MCT showed a significant increase in the number of apoptotic cells compared to the control cells (without the addition of MCT). When the hepatocytes were incubated with 20 mM fructose or 10 mM DTT prior to MCT (5 mM) treatment, however, a lower frequency of apoptotic cells was observed, and this protection was evident until the end of the incubation period (90 min).

4. Discussion

MCT, a pyrrolizidine alkaloid phytotoxin, has well-documented hepatotoxicity both for animals and humans (Mclean, 1970; Mattocks, 1986; Huxtable, 1989; Stegelmeier et al., 1999; Nobre et al., 2004, 2005). Cytochrome P-450 in the liver bio-activates MCT to an alkylating pyrrole derivative, DHM, which is considered responsible for the toxic effects of MCT (Butler et al., 1970; Lafranconi and Huxtable, 1984; Roth and Reindel, 1990; Pan et al., 1993). Previously, we have demonstrated that DHM, but not MCT, is toxic to hepatocytes by mechanisms involving mitochondrial respiration dysfunction (Mingatto et al., 2007). Furthermore, we have also shown that the exposure of isolated perfused liver of phenobarbital-treated rats to MCT results in bioenergetic metabolism failure, which may reflect cell death due to decreased cellular ATP (Mingatto et al., 2008). In addition, we demonstrated that DHM can promote cellular apoptosis by inducing MPT and cytochrome c release (Santos et al., 2009).

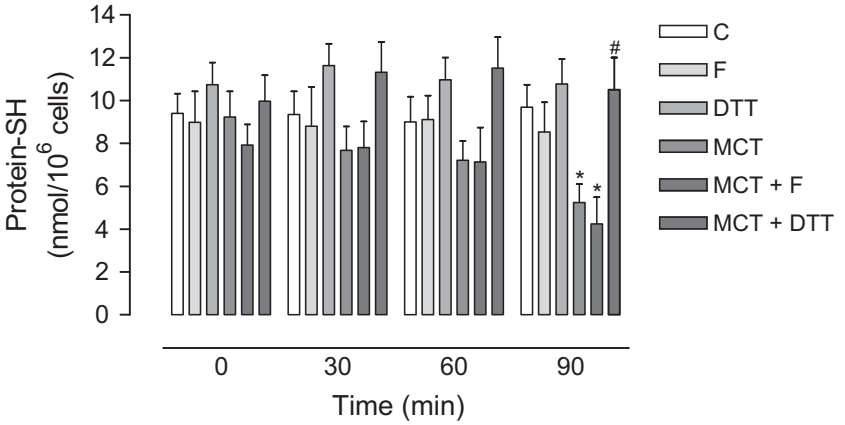

Fig. 5. Effects of 20 mM fructose (F) and dithiotreitol (DTT) on the 5 mM monocrotaline (MCT)-induced decrease in protein thiols level in isolated rat hepatocytes. Results are shown as the mean ±S.E.M. of four experiments with different cell preparations. *Significantly different from Control (C) without MCT ($P < 0.05$). #Significantly different from MCT ($P < 0.05$).

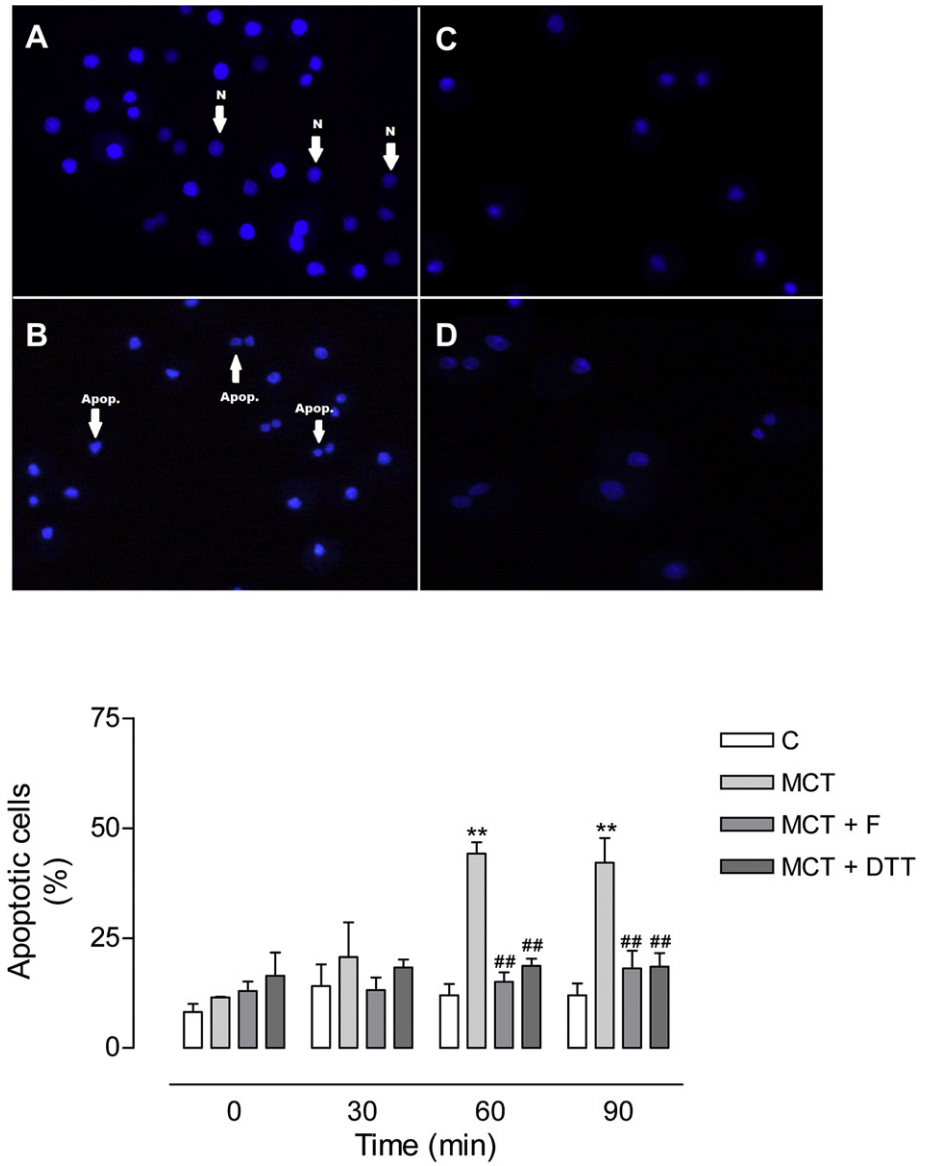

Fig. 6. (Top panel) Representative figures showing effects of 20 mM fructose (F) and 10 mM dithiotreitol (DTT) on the 5 mM monocrotaline (MCT)-induced apoptosis in isolated rat hepatocytes (Hoescht staining) after 90 min of incubation from the four experimental groups i.e. Control (A), MCT (B), MCT+DTT (C) and MCT+F (D) (N = normal cells; Apop. = apoptotic cells). (Bottom panel) Quantitation of apoptotic cells expressed as the percentage of total cells counted. Results are shown as the mean ±S.E.M. of four experiments with different cell preparations. **Significantly different from Control (C) without MCT ($P < 0.01$). ##Significantly different from MCT ($P < 0.01$).

GSH is present in most cells, and it is the most abundant thiol in the intracellular medium (Meister and Anderson, 1983). Its activity in the cell may be to scavenge chemical compounds and their metabolites by enzymatic and chemical mechanisms, capturing the electrophilic substances before they can react at nucleophilic sites critical to cell viability (De Bethizy and Hayes, 2001). It may also act as a substrate for glutathione peroxidase, thereby reducing the destruction caused by free radicals and xenobiotics (Reed, 1990). After treatment of hepatocytes with MCT it was observed that the GSH levels were drastically reduced, and by adding DTT, a thiol reducing compound (Nicotera et al., 1985) at a concentration of 10 mM, no change was observed in GSH levels, protecting the cells. Accordingly, sulfur-containing amino acids such as cysteine and taurine have been shown to suppress the toxic effects of MCT (Hayashi and Lalich, 1968; Yan and Huxtable, 1996).

A link between reduced protein thiol levels and cytotoxicity has been demonstrated in a study conducted with the chemical menadione (Di Monte et al., 1984). In our laboratory, studies with isolated mitochondria showed that

DHM, but not MCT, has the ability to oxidize protein thiol groups (Santos et al., 2009). Therefore, to investigate whether this would also happen in hepatocytes, we incubated the isolated hepatocytes with MCT and observed a significant oxidation of –SH groups of proteins at 90 min of incubation. However, when DTT was added, the oxidation of these groups was prevented.

Thiol groups, in addition to participating in the antioxidant defense system previously mentioned, regulate various aspects of cellular function. Among these is the induction of cell death by apoptosis, an activity regulated by the redox state of the thiol groups (Sato et al., 1995). One of the pathways that mediate apoptosis is the mitochondrial pathway (Green and Reed, 1998; Lemasters et al., 1999), which involves the MPT, a calcium-dependent inner mitochondrial membrane permeabilization. This permeability of the inner membrane is associated with the opening of a pore called the permeability transition pore. The opening of the pore results in the potential loss of the mitochondrial membrane, swelling of the mitochondria and rupture of the mitochondrial outer membrane (Zoratti and Szabò, 1995; Halestrap et al., 2002), and it is sufficient to promote the release of cytochrome c (a component of the electron transport chain that allows the transfer of electrons between complex III and IV) into the cytoplasm of the cell (Kroemer, 1997). Cytochrome c in turn interacts with apoptotic protease activating factors (Apaf), triggering the cascade of activation of pro-caspases by proteolytic cleavage and causing death by apoptosis.

By assessing the effects of MCT on the induction of apoptosis with the dye Hoechst 33342 in parallel with monitoring the decrease in cell viability by changes in the pattern of release of the enzyme ALT, we found that MCT is able to induce programmed cell death. A possible cause for this observed effect can be found in our previous work with isolated mitochondria (Mingatto et al., 2007). We demonstrated that DHM inhibits NADH-dehydrogenase, causing a significant reduction in the synthesis of ATP, which is a critical event for the development of cell damage by necrosis or apoptosis (Nicotera et al., 1998). In addition, DHM causes the oxidation of thiol groups of proteins from mitochondria, resulting in the release of cytochrome c (Santos et al., 2009), which initiates the cascade of induction of programmed cell death. Accordingly, Copple et al. (2004) showed that MCT kills cultured hepatic parenchymal cells by apoptosis, with activation of caspase 3.

Fructose is an efficient substrate for glycolytic ATP formation and protects against cell death induced by toxic compounds (Wu et al., 1990; Nieminen et al., 1994). It protected the hepatocytes against significant programmed cell death induced by MCT, demonstrating the important role of reducing levels of ATP in this process. The protection provided by DTT indicates that the oxidation of thiol groups is also involved in the induction of apoptosis by MCT. Thus, our results suggest that the metabolite-induced mitochondrial energetic impairment, together with a decrease of cellular glutathione and protein thiol groups, can contribute to the toxic effects of MCT on hepatocytes.

Conflict of interest statement

None declared.

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