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The role of mitochondria and biotransformation in abamectin-induced cytotoxicity in isolated rat hepatocytes

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ABSTRACT

Abamectin (ABA), which belongs to the family of avermectins, is used as a parasiticide; however, ABA poisoning can impair liver function. In a previous study using isolated rat liver mitochondria, we observed that ABA inhibited the activity of adenine nucleotide translocator and F_oF_1-ATPase. The aim of this study was to characterize the mechanism of ABA toxicity in isolated rat hepatocytes and to evaluate whether this effect is dependent on its metabolism. The toxicity of ABA was assessed by monitoring oxygen consumption and mitochondrial membrane potential, intracellular ATP concentration, cell viability, intracellular Ca^{2+} homeostasis, release of cytochrome *c*, caspase 3 activity and necrotic cell death. ABA reduces cellular respiration in cells energized with glutamate and malate or succinate. The hepatocytes that were previously incubated with proadifen, a cytochrome P450 inhibitor, are more sensitive to the compound as observed by a rapid decrease in the mitochondrial membrane potential accompanied by reductions in ATP concentration and cell viability and a disruption of intracellular Ca^{2+} homeostasis followed by necrosis. Our results indicate that ABA biotransformation reduces its toxicity, and its toxic action is related to the inhibition of mitochondrial activity, which leads to decreased synthesis of ATP followed by cell death.

1. Introduction

Avermectins are metabolites derived from the fermentation of the fungi *Streptomyces avermitilis*; these metabolites belong to the family of macrocyclic lactones and exhibit extraordinarily potent anthelmintic activity (Burg et al., 1979; Fisher and Mrozik, 1989). Abamectin (ABA) is a mixture of avermectins containing $\geqslant$80% B_{1a} and $\leqslant$20% B_{1b} (Meister, 1992; Zeng et al., 1996; Agarwal, 1998). Avermectin B_{1a} and B_{1b} differ chemically by the presence of a methylene or ethylene group at C-26 (Zeng et al., 1996). According to Hayes and Laws (1990), these molecules have similar biological activities and toxicological properties. ABA is widely used because of its potent anthelmintic and insecticidal action and wide spectrum of action. ABA is also used as an insecticide to control citrus, nut culture and household pests, such as fire ants (Elbetieha and Daas, 2003). In veterinary medicine, ABA is administered to animals in a systematic way to control endoparasites and ectoparasites (Shoop et al., 1995).

The mechanism of ABA action is related to its effect on the γ-aminobutyric acid (GABA) system and Cl^- channels. GABA receptors are responsible for regulating the neural basal tone of the brain (Turner and Schaeffer, 1989) and are in virtually all neurons of the central nervous system (CNS). The symptoms of ABA poisoning exhibited in laboratory animals include pupil dilation, vomiting, convulsions and/or tremors and coma (Lankas and Gordon, 1989). In addition, some studies have reported genotoxic effects of ABA (Molinari et al., 2010).

As demonstrated by the *in vivo* studies (Lowenstein et al., 1996; Hsu et al., 2001) and the *in vitro* study conducted with isolated hepatocytes (El-Shenawy, 2010), the liver can also be affected by ABA. ABA caused an increase in the concentration of the enzyme aspartate aminotransferase (AST) in serum *in vivo* and an increase in the concentration of AST and alanine aminotransferase (ALT) *in vitro*, which are used as indicators of damage to the hepatic parenchymal cells (Klaassen and Eaton, 1991). We previously demonstrated that ABA inhibits the activity of F_oF_1-ATPase and adenine nucleotide translocator (ANT) when added at micromolar concentrations to isolated rat liver mitochondria, an effect associated with significantly reduced ATP synthesis (Castanha Zanoli et al., 2012).

F_oF_1-ATPase is an enzyme present in the inner mitochondrial membrane that is responsible by ATP synthesis driven by the proton electrochemical gradient generated in the respiratory chain. The main components of the enzyme are F_o, an integral membrane protein that works as a proton channel, and F_1, a hydrophilic moiety which contains the catalytic and regulatory sites (Hatefi,

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1993; Pedersen, 1996). ANT is other important component of the mitochondrial machinery of ATP synthesis because of its intrinsic adenine nucleotide translocase activity. ANT has been involved in both pathological (mitochondrial permeability transition formation/regulation and cell death) and physiological (adenine nucleotide exchange) mitochondrial events, making it a prime target for drug-induced toxicity (Oliveira and Wallace, 2006).

The xenobiotic metabolism in the liver is accomplished by cytochrome P450 and its main function is to increase the polarity of these substances, so excretion occurs more easily (Oga, 2008). However, this process is responsible for the toxic effects of numerous chemical compounds. The metabolites may cause adverse effects in the animal (Ioannides and Lewis, 2004; Mingatto et al., 2007; Maioli et al., 2011) by changing a fundamental cellular component (mitochondria, for example) at the cellular and molecular level, thus modulating its function (Meyer and Kulkarni, 2001).

Due to the important functions of the liver in animals and previous studies that indicated the occurrence of liver damage after the use of ABA, this study aims to characterize the mechanisms of ABA toxicity on parameters related to bioenergetics and cell death and determine whether the toxicity induced by the compound is due to a possible activation following its metabolism in the liver.

2. Materials and methods

2.1. Chemicals

Abamectin, containing 92% avermectin B_{1a} and 8% avermectin B_{1b}, was kindly supplied by the company Ourofino Agribusiness (Cravinhos, SP, Brazil), proadifen was purchased from Sigma–Aldrich (St. Louis, MO, USA), and sodium pentobarbital was a gift from Cristália (Itapira, SP, Brazil). All other reagents were of the highest commercially available grade. Abamectin and proadifen were dissolved in anhydrous dimethyl sulfoxide (DMSO). All stock solutions were prepared using glass-distilled deionized water.

2.2. Animals

Male Wistar rats aged 7–8 weeks and weighing approximately 200 g, were used in this study. The animals, which were obtained from the Central Bioterium of UNESP – Univ Estadual Paulista, Campus de Botucatu, SP, Brazil, were maintained with a maximum of 4 rats per cage under standard laboratory conditions with water and food provided *ad libitum*. The experimental protocols were approved by the Ethical Committee for the Use of Laboratory Animals of the UNESP – Univ Estadual Paulista, Campus de Dracena, SP, Brazil.

2.3. Isolation and incubation of hepatocytes

For the surgical procedure, the rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The hepatocytes were isolated by a collagenase perfusion of the liver as described previously (Guguen-Guillouzo, 1992). The hepatocyte viability after isolation was determined by Trypan blue (0.16%) uptake, and the initial cell viability in all experiments was more than 85%. The hepatocytes were suspended in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM Hepes and 0.1% bovine serum albumin (BSA), and maintained at 4 °C. The cells (1×10^6/mL) were incubated in 25-mL Erlenmeyer flasks, which were maintained under constant agitation (30 rpm) at 37 °C under a 95% O_2 and 5% CO_2 atmosphere. The reactions in the experiments of cell viability, cellular ATP content, mitochondrial membrane potential, release of cytochrome *c*, caspase 3 activity and necrotic cell death were initiated by the addition of abamectin (ABA) at concentrations of 25, 50, 75 and 100 μM. Aliquots (1 mL) of the suspension were removed from the mixture at appropriate times for the determination of cell death and biochemical parameters. In some experiments, the cells were incubated with 100 μM proadifen 15 min before the addition of ABA.

2.4. Oxygen uptake

Oxygen uptake by the isolated hepatocytes was monitored using a Clark-type oxygen electrode (Strathkelvin Instruments Limited, Glasgow, Scotland, UK). The respiration buffer contained 250 mM sucrose, 2 mM KH_2PO_4, 10 mM HEPES, pH 7.2, 0.5 mM EGTA, 0.5% BSA, and 5 mM $MgCl_2$, at 37 °C. The 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). ABA at concentrations of 5, 10, 15 and 25 μM was added to the medium immediately after the initiation of state 3 or state 4 respirations.

2.5. Mitochondrial membrane potential

The mitochondrial membrane potential was determined using the fluorescent probe TMRM (tetramethyrodamine, methyl ester). The cell suspensions incubated with different concentrations of abamectin were collected and centrifuged at 50*g* for 5 min. The pellet was suspended and incubated for 10 min at 37 °C with TMRM solution at a final concentration of 6.6 μM. After the incubation, the samples were centrifuged twice at 50*g* for 5 min, and the pellet was suspended with 1 ml of Triton X-100, 0.1% (v/v). Subsequently, the samples were centrifuged at 2000*g* for 5 min, and the fluorescence of the TMRM captured and retained by the mitochondria was determined in the supernatant using a fluorescence spectrophotometer RF-5301 PC (Shimadzu, Tokyo, Japan) at excitation and emission wavelengths of 485 and 590 nm, respectively. The results are expressed as a percentage of the fluorescence intensity over the control group.

2.6. Cellular ATP content

Cellular ATP content was determined by the firefly luciferin–luciferase assay. 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 65 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. Evaluation of cell viability

Cell viability was assessed by the leakage of alanine transaminase (ALT) and aspartate transaminase (AST) from hepatocytes. After incubation with ABA at concentrations of 25, 50, 75 and 100 μM the cell suspensions were collected at time 0, 30, 60, 90 and 120 min and centrifuged (50*g* for 5 min). The presence of ALT and AST in the supernatant was determined using Enzyme Activity Assay Kits (Bioclin, Quibasa, Brazil) according to the manufacturer's instructions. The absorbance was measured at 340 nm with a spectrophotometer DU-800 (Beckman Coulter, 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.

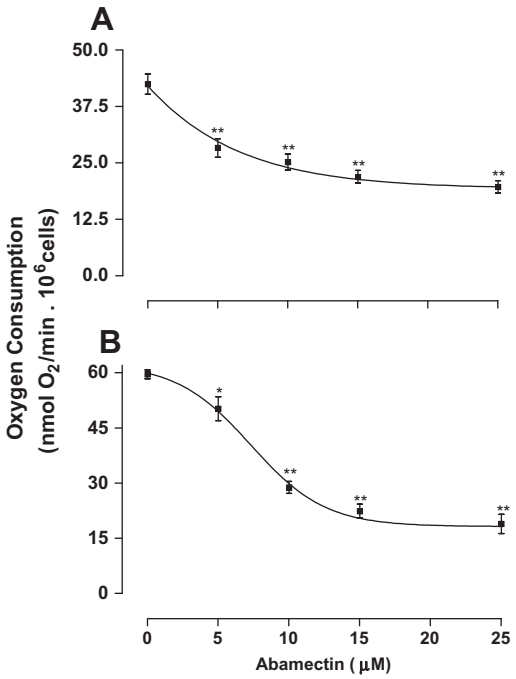

Fig. 1. Effects of abamectin (ABA) on glutamate-plus-malate-supported (A) or succinate-supported (B) state 3 (ADP stimulated) respiration of mitochondria in digitonin-permeabilized isolated rat hepatocytes. The figure is representative of five experiments with different cell preparations. *,**Significantly different from the control (without ABA) ($P < 0.05$ and $P < 0.01$, respectively).

2.8. Intracellular Ca^{2+} homeostasis

Hepatocytes (2×10^6/ml) were incubated in Krebs-Henseleit medium supplemented with 2% BSA, 12.5 mM HEPES and 10 mM glucose, pH 7.4. In this medium, 0.005% pluronic acid and 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM) were added. The hepatocytes were maintained under constant agitation at 32 °C for 60 min to capture the probe.

The cell suspension loaded with Fura-2 AM was collected and subjected to two centrifugations at 50*g* for 3 min to remove residual Fura-2 AM and maintained at 4 °C for later use. The fluorescence of Ca^{2+} was determined by the ratio of the excitation wavelengths at 340 and 380 nm and emission wavelength at 505 nm using the fluorescence spectrophotometer RF-5301 PC (Shimadzu, Tokyo, Japan). The calibration and calculations in $[Ca^{2+}]_c$ were performed as previously described (Grynkiewicz et al., 1985). Maximum fluorescence (F_{max}) was obtained by the addition of 1% Triton X-100, and minimum fluorescence (F_{min}) was obtained by the addition of 10 mM EGTA. The equilibrium constant for the calculations was 225 nM. Changes in free $[Ca^{2+}]_c$ in the cytoplasm of hepatocytes were evaluated with increasing additions of ABA (25, 50, 75 and 100 μM) every 300 s.

2.9. Release of cytochrome *c*

The release of cytochrome *c* was determined as previously described (Appaix et al., 2000). The hepatocytes (2.7 mg protein/ml) were incubated in Krebs-Henseleit medium supplemented with BSA (2 mg/mL), 0.002% digitonin and different concentrations of abamectin at 25 °C for 30 min. After the incubation, the cells were centrifuged at 10,000*g* for 30 min at 4 °C, and the supernatant was collected and filtered through a 0.2 μm Millipore membrane. The absorbance was determined in a spectrophotometer DU-800 (Beckman Coulter, Fullerton, CA, USA) by the difference in absorbance at wavelengths 414 and 600 nm. The results are expressed in nmol cytochrome *c* released/10^6 cells using a molar extinction coefficient (ε) of 100 mM^{-1} cm^{-1}.

2.10. Caspase 3 activity

The assessment of caspase 3 activity was performed using a Caspase 3 assay kit (Sigma–Aldrich). The hepatocytes were

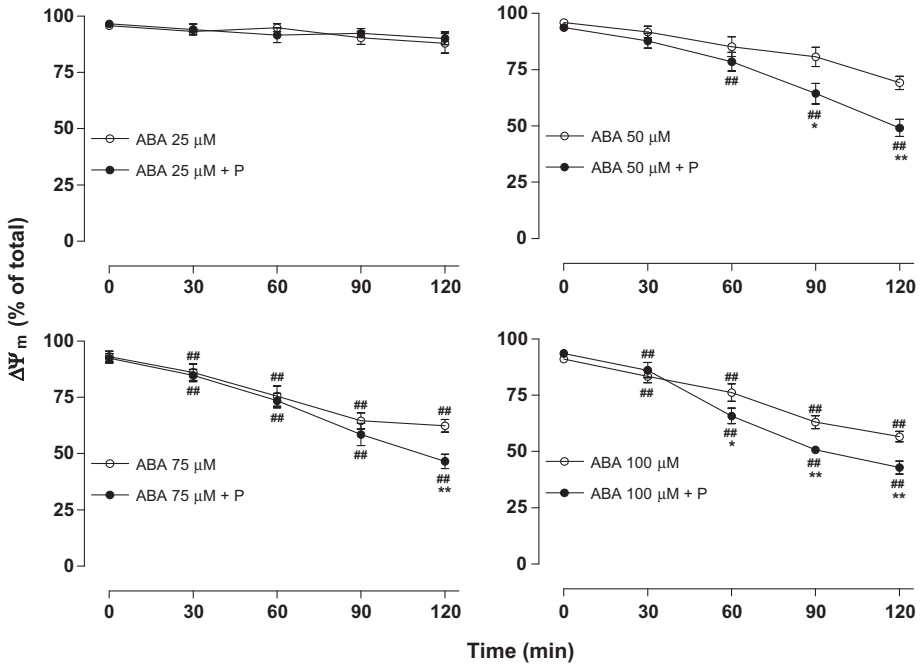

Fig. 2. Effects of abamectin (ABA) on the mitochondrial membrane potential assessed by the uptake of the fluorescent probe tetramethylrhodamine methyl ester (TMRM) in isolated rat hepatocytes (10^6 cells/ml) in the absence or presence of 100 μM proadifen (P). The results represent the mean ± SEM of six experiments with different cell preparations. ##Significantly different from control (without ABA) at the corresponding time ($P < 0.01$). *,**Significantly different from "without proadifen" at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively).

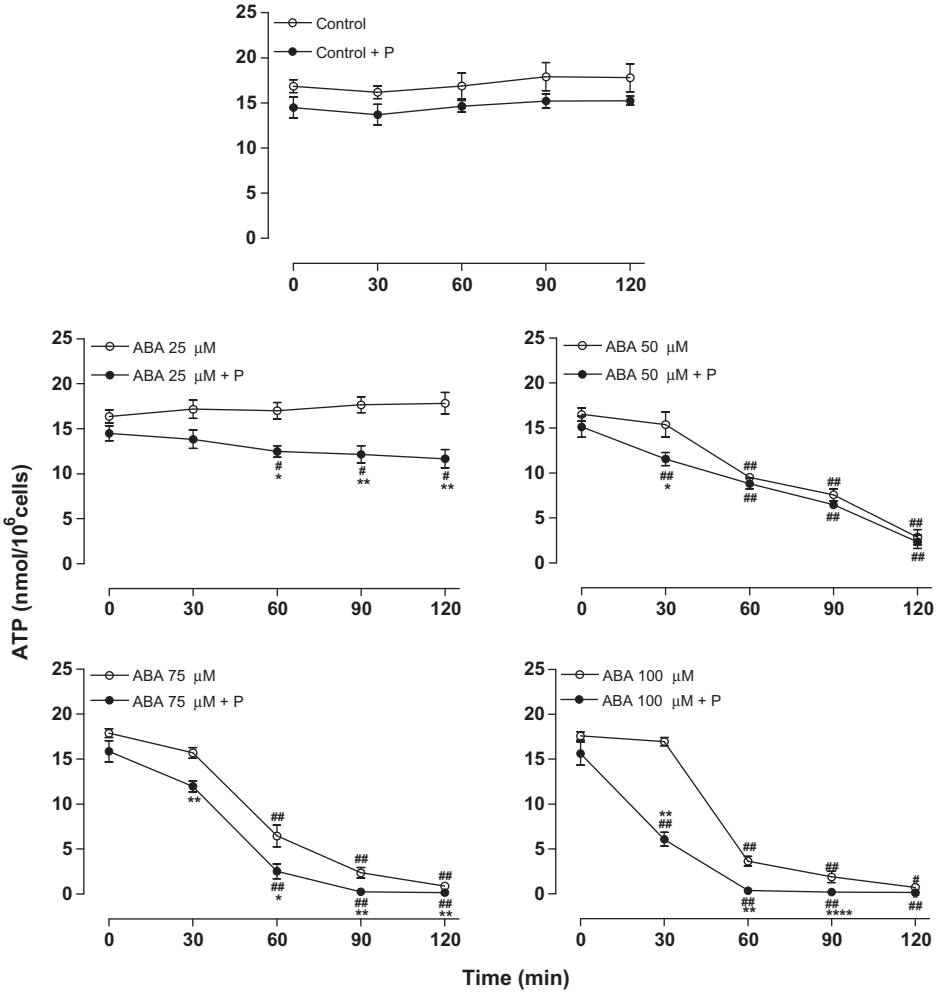

Fig. 3. Effects of abamectin (ABA) on the intracellular concentration of ATP in isolated rat hepatocytes (10^6 cells/ml) in the absence or presence of 100 μM proadifen (P). The results represent the mean ± SEM of five different cell preparations. #,##Significantly different from control (without ABA) at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively). *,**Significantly different from "without proadifen" at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively).

collected and centrifuged at 600*g* for 5 min and suspended in 1 mL of phosphate buffered saline (PBS). Further centrifugation was performed, and the precipitate was incubated for 15 min at 4 °C with 200 μL of lysis buffer for the release of caspase 3, and 300 μL of PBS was then added. The lysed cell suspension was centrifuged at 14,000*g* for 15 min at 4 °C, and the supernatant was collected. Aliquots of 50 μL of supernatant were used to assess the activity of caspase 3 according to the manufacturer's instructions. Fluorescence was determined using the fluorescence spectrophotometer RF-5301 PC (Shimadzu, Tokyo, Japan) at wavelengths of 360 and 460 nm for excitation and emission, respectively. The results are expressed as pmol of AMC/min/mL.

2.11. Necrotic cell death

Samples of cells (200 μL) were collected and centrifuged at 50*g* for 5 min, and the precipitate was suspended in Krebs/Henseleit medium, pH 7.4, and incubated with Hoechst 33342 (8 μg/mL) and Propidium Iodide (5 μM) dyes for 15 min at room temperature in the dark. After incubation, the samples were centrifuged twice at 50*g* for 5 min to remove excess dye. After the washes, the hepatocytes were suspended in 50 μL of Krebs/Henseleit medium, pH 7.4. The cells were analyzed with a fluorescence microscope (DM 2500 type, Leica, Rueil-Malmaison, France), and the percentage of necrotic cells was quantitated using the Qwin 3.0 software.

2.12. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). The statistical significance of the differences between control and the experimental groups was evaluated using one-way analysis of variance (ANOVA) followed by Dunnett's test, and differences between the experimental groups at the same time points was evaluated using unpaired *t* test with Welch's correction. Values of $P < 0.05$ were considered to be significant. All statistical analyses were performed using GraphPad Prism software, version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Effects of ABA on the respiration of mitochondria in isolated rat hepatocytes

Fig. 1 shows the inhibitory effect of ABA on the glutamate-plus-malate-supported and succinate-supported state 3 (ADP-stimulated) respiration of mitochondria in digitonin-permeabilized hepatocytes. ABA has an inhibitory action on cellular respiration under conditions with the substrates of the respiratory chain complex I (glutamate + malate) and with the substrate of complex II (succinate) at the same concentrations and in a concentration-dependent manner beginning at 5 μM. ABA did not stimulate state 4 (basal)

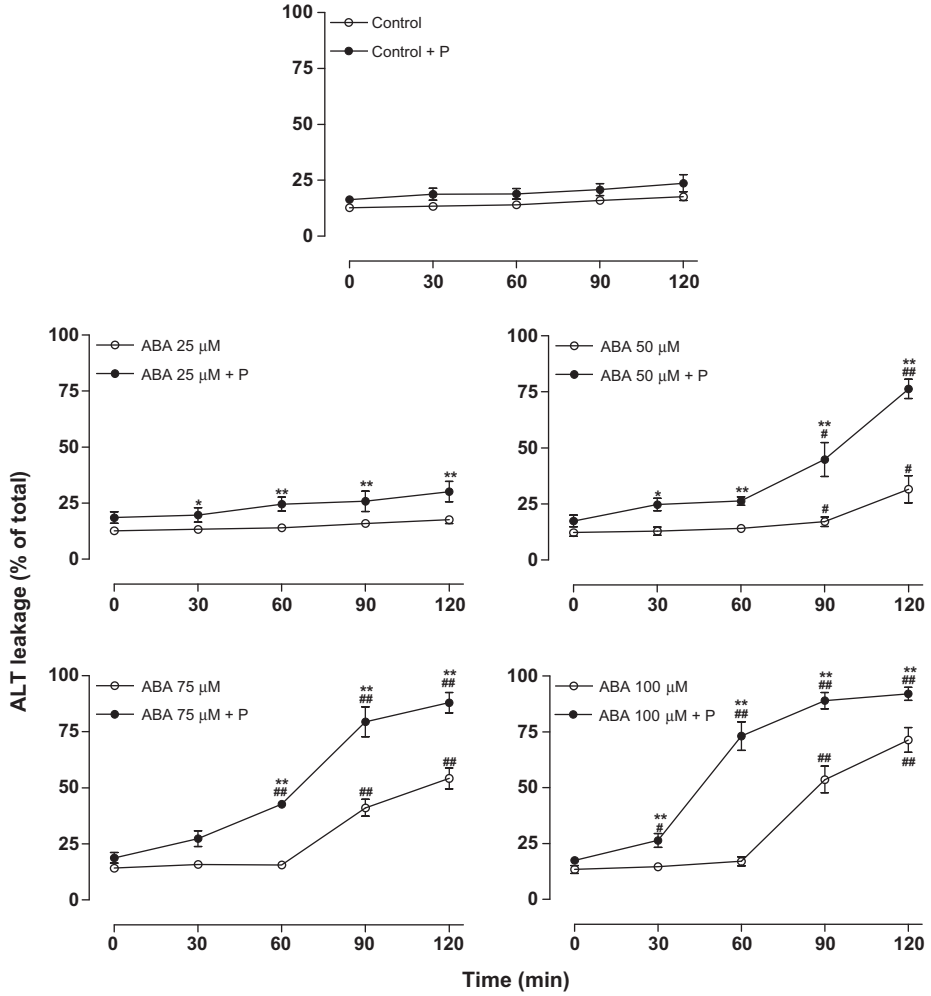

Fig. 4. Effects of abamectin (ABA) on cell viability assessed by the release of the enzyme alanine transaminase (ALT) in isolated rat hepatocytes (10^6 cells/ml) in the absence or presence of 100 μM proadifen (P). The results represent the mean ± SEM of six experiments with different cell preparations. $^{\#,\#\#}$Significantly different from control (without ABA) at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively). *,**Significantly different from "without proadifen" at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively).

respiration (results not shown). These results indicate that ABA inhibits the oxidative phosphorylation of mitochondria as assessed in isolated hepatocytes, and the results are in agreement with those previously described that show ABA as an inhibitor of the adenine nucleotide translocator (ANT) and F_oF_1-ATPase in isolated mitochondria (Castanha Zanoli et al., 2012). Proadifen (100 μM) did not present any effect on the mitochondrial respiration of hepatocytes (results not shown).

3.2. Effects of ABA on the mitochondrial membrane potential and ATP levels in isolated rat hepatocytes

The effects of ABA on the mitochondrial membrane potential and ATP levels were evaluated in the presence or absence of proadifen, a cytochrome P450 inhibitor (Figs. 2 and 3, respectively). The addition of increasing concentrations of ABA to the hepatocytes (25–100 μM) resulted in a decrease in the mitochondrial membrane potential and ATP levels in a concentration- and time-dependent manner. Proadifen stimulated an ABA-induced decrease in the mitochondrial membrane potential and ATP levels (Figs. 2 and 3, respectively), suggesting that the parent drug by itself is the main factor responsible for the toxic effect on isolated hepatocytes.

3.3. Effects of ABA on cell viability in isolated rat hepatocytes

The activity of ALT (Fig. 4) and AST (Fig. 5) was used to monitor the viability of hepatocytes following exposure to different concentrations of ABA (25–100 μM) in the absence and presence of proadifen.

The addition of increasing concentrations of ABA to hepatocytes resulted in decreased cell viability, as assessed by ALT and AST leakage into the incubation medium, in a concentration- and time-dependent manner (Figs. 4 and 5, respectively). A significant increase in the concentration of ALT and AST was observed with 50 μM ABA at 90 min.

Proadifen stimulated the ABA-induced decrease in cell viability because the cells showed a significant release of both enzymes in the presence of ABA (Figs. 4 and 5).

3.4. Effects of ABA on intracellular Ca^{2+} homeostasis in isolated rat hepatocytes

Intracellular Ca^{2+} homeostasis was evaluated by changes in the fluorescence probe Fura-2 in hepatocytes exposed to increasing concentrations of ABA (25–100 μM) in the absence of proadifen (Fig. 6).

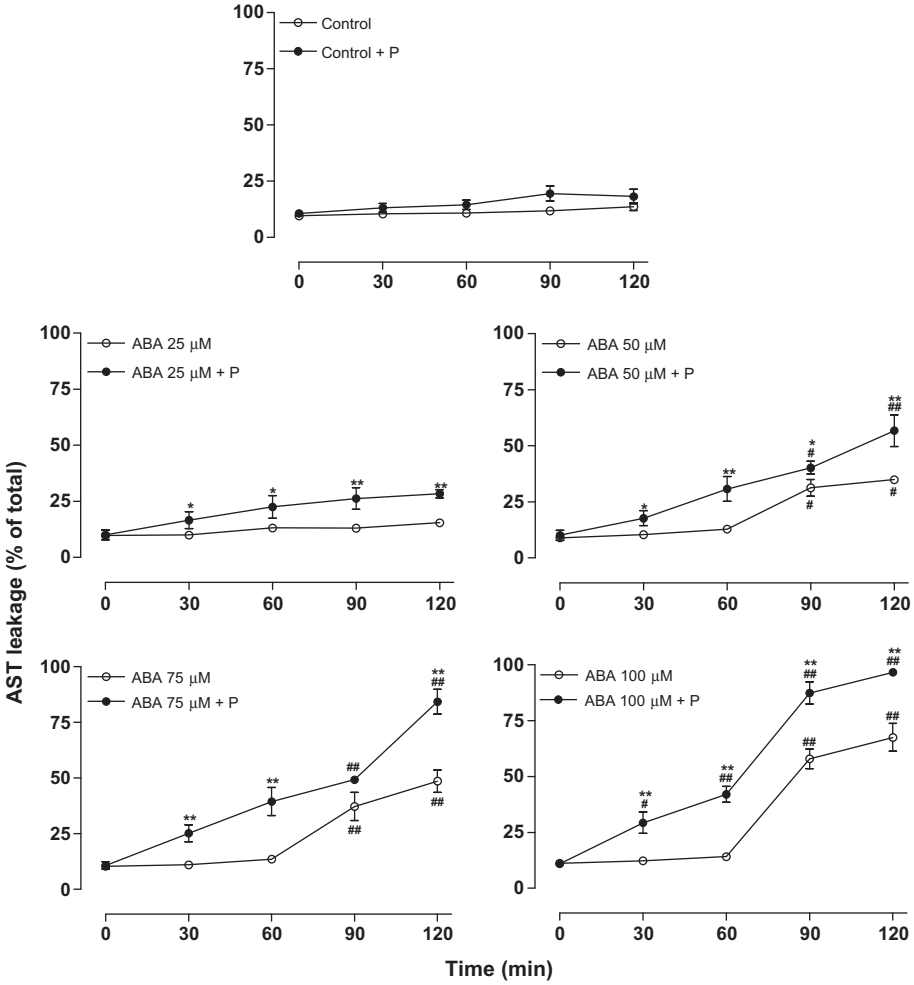

Fig. 5. Effects of abamectin (ABA) on cell viability assessed by the release of the enzyme aspartate transaminase (AST) in isolated rat hepatocytes (10^6 cells/ml) in the absence or presence of 100 μM proadifen (P). The results represent the mean ± SEM of six experiments with different cell preparations. $^{\#,\#\#}$Significantly different from control (without ABA) at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively). *,**Significantly different from "without proadifen" at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively).

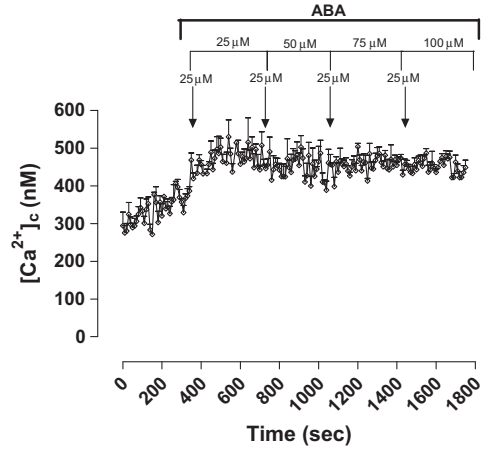

Fig. 6. Effects of abamectin (ABA) on intracellular Ca^{2+} homeostasis in isolated rat hepatocytes (2×10^6 cells/ml) permeabilized with pluronic acid (0.005%). The results represent the mean ± SEM of five experiments with different cell preparations.

The cytosolic Ca^{2+} concentration was increased after the addition of 25 μM ABA and did not change following the addition of higher concentrations (50, 75 and 100 μM) of the drug.

3.5. Effects of ABA on cytochrome c release in isolated rat hepatocytes

The release of cytochrome *c* by the mitochondria was determined in hepatocytes exposed to increasing concentrations of ABA (25–100 μM) in the absence of proadifen. The addition of ABA to the incubation medium of hepatocytes did not result in a significant release of mitochondrial cytochrome *c* (results not shown).

3.6. Effects of ABA on caspase 3 activity in isolated rat hepatocytes

Caspase 3 activity was evaluated in hepatocytes previously incubated with proadifen and exposed to increasing concentrations of ABA (25–100 μM). However, the addition of ABA to the incubation medium did not cause caspase 3 activation in hepatocytes throughout the experimental period (results not shown).

3.7. Effects of ABA on the induction of necrotic cell death in isolated rat hepatocytes

After 120 min of incubation, cell necrosis was evaluated by Hoechst-propidium-iodide double staining in hepatocytes in the absence or previously incubated with proadifen and exposed to increasing concentrations of ABA (25–100 μM) (Fig. 7).

ABA triggers cell death by necrosis in a concentration- and time-dependent manner, becoming significant at 60 min for concentrations of 75 and 100 μM (Bottom panel). Fifty micromolar of ABA triggered necrosis after only 120 min of incubation. Proadifen stimulated the ABA-induced cell necrosis.

4. Discussion

In this study, we used isolated rat hepatocytes to study the toxicity mechanism induced by ABA *in vitro* and the influence of biotransformation of the drug. The interference of ABA in the functioning of the mitochondrial respiratory chain in isolated rat hepatocytes was monitored by measuring oxygen consumption. The results showed a clear inhibition of the rate of oxygen consumption in state 3 of mitochondrial respiration with both substrates of complex I (glutamate + malate) and complex II (succinate) at all of the tested concentrations (5–25 μM). These results are consistent with those obtained by Castanha Zanoli et al. (2012), in which the effects of ABA on the isolated mitochondria of rat liver were evaluated and an inhibitory effect on the ANT and F_oF_1-ATPsintase was shown.

During the biotransformation of xenobiotics in the liver, the metabolites generated can be even more toxic than the parent compound (Ioannides and Lewis, 2004). In a study using rat liver

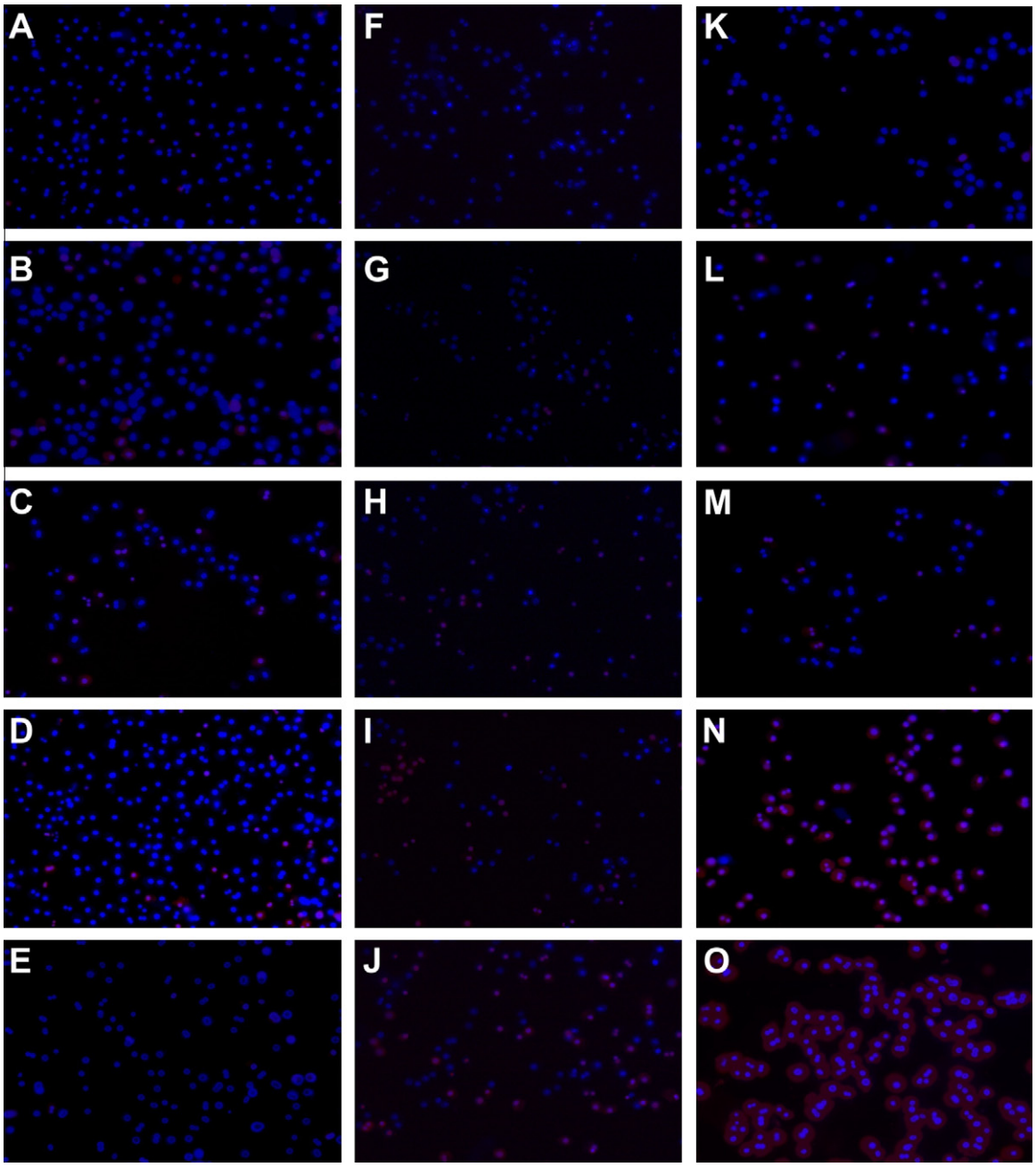

Fig. 7. (Top panel) Representative figures showing the effects of abamectin (ABA) on cell death by necrosis at 0 and 120 min as monitored by the fluorescent dyes, Hoechst and propidium iodide. (A, B, C, D and E) 0, 25, 50, 75 and 100 μM ABA, respectively, at 0 min, representatives of experiments without or with proadifen, (F, G, H, I and J) 0, 25, 50, 75 and 100 μM ABA, respectively, at 120 min, without proadifen, (K, L, M, N and O) 0, 25, 50, 75 and 100 μM ABA, respectively, at 120 min, with proadifen. (Bottom panel) Quantitation of necrotic cells expressed as the percentage of total cells counted. The results are shown as the mean ± S.E.M. of three experiments with different cell preparations in the absence or presence of 100 μM proadifen. $^{\#,\#\#}$Significantly different from control (without ABA) at the corresponding time ($P < 0.05$ and $P < 0.01$, respectively). **Significantly different from "without proadifen" at the corresponding time ($P < 0.01$).

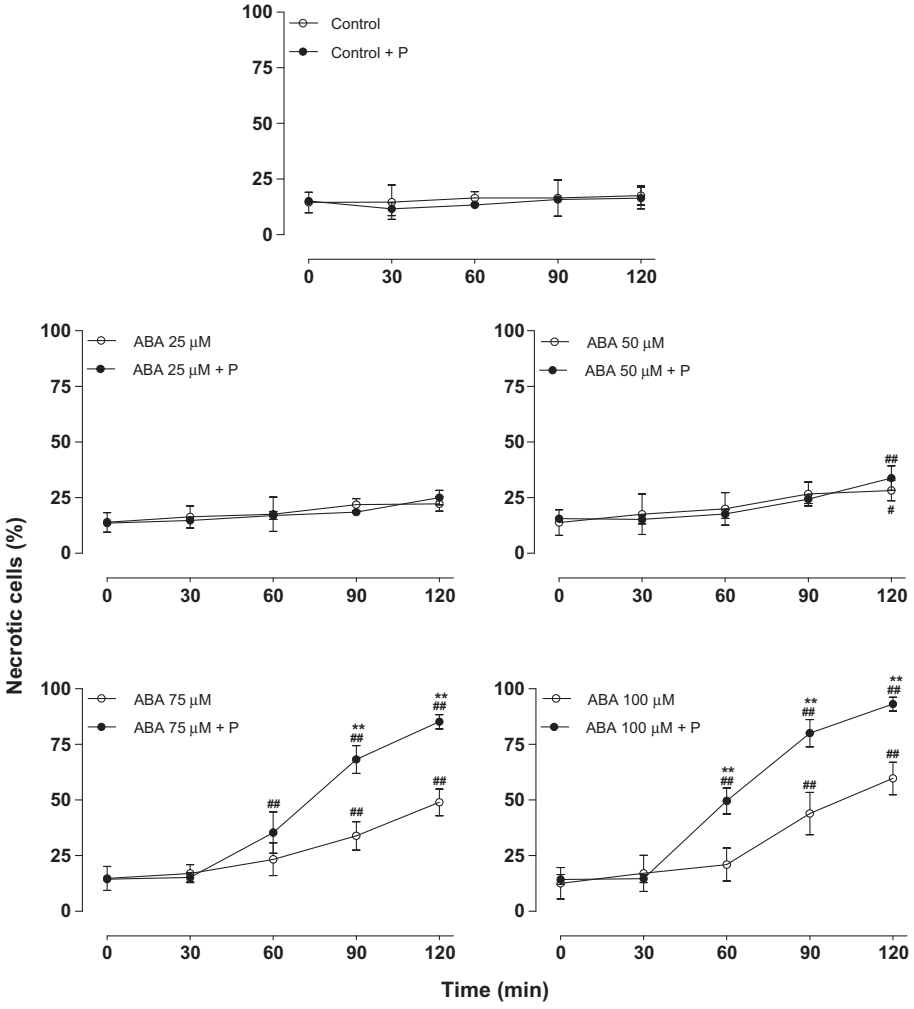

Fig. 7. (*continued*)

microsomes, Zeng et al. (1996) showed that the major metabolites produced from abamectin are 3″-O-Desmethyl $B1_a$ (3″-ODMe $B1_a$), 24-Hydroxymethyl $B1_a$ (24 OHMe-$B1_a$) and 26-Hydroxymethyl $B1_a$ (26 OHMe-$B1_a$). The authors attributed the metabolism of ABA to cytochrome P450 isoforms 1A1 and 3A as responsible for the metabolism of ABA, being the production of the metabolite 3″-ODMe $B1_a$ attributed to isoform 3A and the production of metabolites 24 OHMe-$B1_a$ and 26-OHMe $B1_a$ to isoform 1A1.

Therefore, to evaluate the effect of the biotransformation on ABA toxicity, the hepatocytes were incubated in the absence or presence of proadifen, a broad inhibitor of cytochrome P450 isoforms (Khan et al., 1993; Bort et al., 1998; Mingatto et al., 2002; Somchit et al., 2009; Shi et al., 2011), which was previously shown to inhibit about 90% of the metabolism of ABA (Zeng et al., 1996).

ABA metabolism interferes with the mitochondrial membrane potential because a more significant decrease in this parameter was observed in hepatocytes in the presence of proadifen. Due to the inhibition of oxidative phosphorylation and the formation of a mitochondrial membrane potential induced by ABA, a reduction in the intracellular ATP concentration is expected. This effect was observed in liver cells incubated with or without proadifen. The effect was more pronounced in the cells incubated with the P450 inhibitor, indicating that the parent drug is more toxic than the metabolites.

Castanha Zanoli et al. (2012) observed an inhibitory effect of ABA on the activity of ANT and F_oF_1-ATPase, thus blocking oxidative phosphorylation. However, the researchers did not observe a decrease in the mitochondrial membrane potential as was observed in this study. A possible explanation for the dissipation in the membrane potential caused by ABA in isolated hepatocytes may be related to a loss of intracellular Ca^{2+} homeostasis (Skulachev, 1999). When hepatocytes were exposed to 25 μM ABA, a loss of intracellular ion homeostasis occurred. As the Ca^{2+} concentration increased in the cell cytoplasm, the mitochondria captured the surplus using the uniporter (UP) channel. According to Brookes et al. (2004), the UP ion uptake is dependent on the membrane potential, so the movement of charges due to the uptake of calcium consumes the membrane potential that was formed.

Furthermore, ABA-induced mitochondrial dysfunction reduces cellular ATP levels and can promote in other organelles such as endoplasmic reticulum, the inactivation of the pump responsible for the maintenance of the Ca^{2+} ion gradient in the cytoplasm. Invariably, the result of inhibition of the transport system is the disruption of intracellular calcium homeostasis. The increase in intracellular Ca^{2+} can activate proteases, phospholipases and ion-dependent endonucleases (Trump and Berzesky, 1992).

The activation of proteases and phospholipases induces changes in the cytoskeleton and plasma membrane. When combined, these processes culminate in the disruption of cytoskeleton-plasma membrane interactions, which results in destabilization of the lipid bilayer, bleb formation on the cell surface and, in more severe cases, leakage and cellular necrosis (Nicotera et al., 1986; Gores et al., 1990; Sakaida et al., 1992).

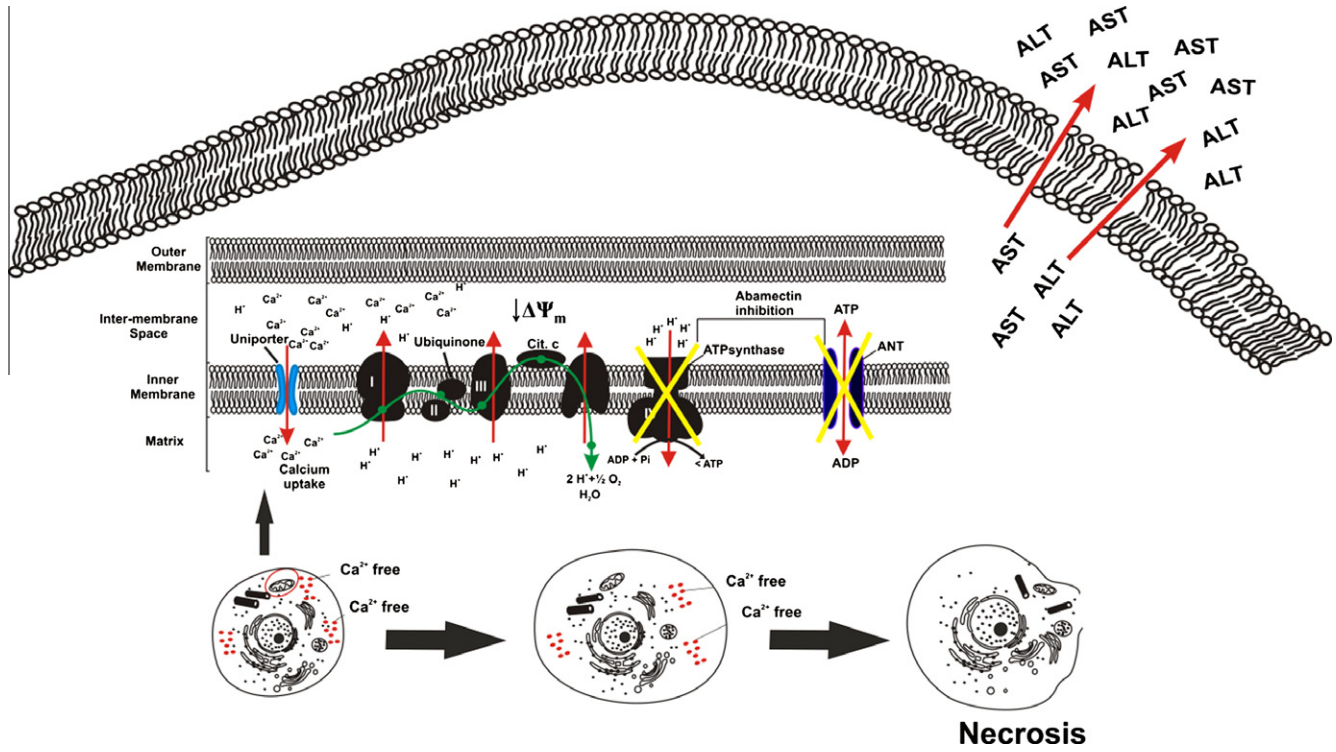

Fig. 8. Schematic representation of the mechanisms of ABA-induced impairment of mitochondrial bioenergetics, disruption of calcium homeostasis and necrosis in isolated rat hepatocytes.

The enzymes ALT and AST are used as indicators of damage to hepatic parenchymal cells (Klaassen and Eaton, 1991; Kaplowitz, 2001). According to Grisham (1979), an efflux of these enzymes in the liquid incubation of cells in culture indicates that there was a loss of membrane integrity. However, this efflux is not only associated with cell death and lysis but also with modifications that can be reversible (Grisham and Smith, 1984).

ABA increased the concentration of ALT and AST in the liquid incubation of hepatocytes, and this effect was also influenced by pre-incubation of the cells with proadifen. The changes observed in the release of these enzymes may be a reflection of the influence of ABA on mitochondrial activity. A decrease in the efficiency of energy production by the organelle affects cellular functions that are dependent on energy, and the disruption of these functions may result in cell death (Nicotera et al., 1998; Wallace and Starkov, 2000; Szewczyk and Wojtczak, 2002).

In *in vivo* studies performed by Lowenstein et al. (1996) and Hsu et al. (2001), ABA caused an elevation in the concentration of the AST in blood serum. El-Shenawy (2010) performed an *in vitro* study with isolated rat hepatocytes to compare the toxic action of several insecticides. Among the tested insecticides was ABA, which was used at concentrations of 10 and 100 μM. The results obtained by El-Shenawy (2010) showed a significant increase in ALT and AST leakage when the hepatocytes were incubated with 10 and 100 μM ABA for 30–120 min (final period of sample collection).

Necrosis and apoptosis are types of cell death. One evident physiological difference in cells undergoing apoptosis versus necrosis is in the intracellular levels of ATP. Whereas necrotic cell death occurs in the absence of ATP, apoptosis depends on intracellular ATP levels (Tsujimoto, 1997). Many key events in apoptosis focus on the mitochondria, including the release of caspase activators (such as cytochrome *c*), changes in electron transport, loss of mitochondrial transmembrane potential, altered cellular oxidation–reduction, and participation of pro- and antiapoptotic Bcl-2 family proteins (Green and Reed, 1998). Thus, in this study, the parameters related to both types of cell death were monitored, allowing the type of cell death triggered by ABA in isolated hepatocytes to be distinguished.

The release of cytochrome *c* and caspase 3 activity are steps in determining apoptosis establishment for the intrinsic pathway (Kass et al., 1996; Barros et al., 2003). For both parameters, we have not found significant variation in apoptosis induction in hepatocytes exposed to ABA.

Necrosis is characterized by changes that cause depletion of ATP, disruption of ionic equilibrium, swelling of mitochondria and the cell, and activation of degradative enzymes. These changes result in the disruption of the plasma membrane and loss of proteins, intracellular metabolites and ions (Eguchi et al., 1997; Nicotera et al., 1998; Lemasters et al., 1999). Following microscopic evaluation of Hoechst-propidium-iodide double staining, it was confirmed that ABA induces necrosis, which was initially observed at 60 min in a concentration- and time-dependent manner upon the addition of 75 and 100 μM of ABA and that proadifen stimulated this effect.

This study indicates that the mechanism of ABA hepatotoxicity involves an effect on mitochondrial bioenergetics and alteration in calcium homeostasis, which leads to a decrease in ATP synthesis with consequent cell death by necrosis (Fig. 8). Furthermore, this study shows that the metabolism of ABA, which is performed by cytochrome P450 in the liver, influences its toxicity. For all variables evaluated, there was an increase in the toxic potential of ABA in the presence of proadifen, indicating that the parent drug has greater potential than the metabolites.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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