

## Histological changes and impairment of liver mitochondrial bioenergetics after long-term treatment with $\alpha$ -naphthyl-isothiocyanate (ANIT)

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### Abstract

This study was designed to evaluate the effects of long-term treatment with  $\alpha$ -naphthyl-isothiocyanate (ANIT) on liver histology and at the mitochondrial bioenergetic level. Since, ANIT has been used as a cholestatic agent and it has been pointed out that an impairment of mitochondrial function is a cause of hepatocyte dysfunction leading to cholestatic liver injury, serum markers of liver injury were measured and liver sections were analyzed in ANIT-treated rats (i.p. 80 mg/kg/week  $\times$  16 weeks). Mitochondrial parameters such as transmembrane potential, respiration, calcium capacity, alterations in permeability transition susceptibility and ATPase activity were monitored. Histologically, the most important features were the marked ductular proliferation, proliferation of mast cells and the presence of iron deposits in ANIT-treated liver. Mitochondria isolated from ANIT-treated rats showed no alterations in state 4 respiration, respiratory control ratio and ADP/O ratio, while state 3 respiration was significantly decreased. No changes were observed on transmembrane potential, but the repolarization rate was decreased in treated rats. Consistently with these data, there was a significant decrease in the ATPase activity of treated mitochondria. Associated with these parameters, mitochondria from treated animals exhibited increased susceptibility to mitochondrial permeability transition pore opening (lower calcium capacity). Since, human cholestatic liver disease progress slowly overtime, these data provide further insight into the role of mitochondrial dysfunction in the process.

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**Keywords:** Liver mitochondria; Bile acids; Cholestasis; Membrane potential; Respiratory activity; Mitochondrial permeability transition

**Abbreviations:** ANIT,  $\alpha$ -naphthyl-isothiocyanate; BSA, bovine serum albumin; MPT, mitochondrial permeability transition.

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## 1. Introduction

Bile formation is a vital function of the liver and its impairment is central in the pathogenesis of cholestasis. In clinical settings, retention of hydrophobic bile acids within the liver is the major pathophysiologic mechanism during cholestasis and liver failure (Greim et al., 1972; Fischer et al., 1996). The mechanisms resulting in cell dysfunction in animal models and human liver disease remain obscure. Understanding the mechanisms underlying bile acids toxicity will provide new therapeutic strategies to intervene in the clinical management of cholestatic disease. Cholestasis was mimicked by  $\alpha$ -naphthyl-isothiocyanate (ANIT) administration to rats, a well characterized cholestatic agent (Plaa and Priestley, 1976). Liver injury caused by ANIT is the ultimate consequence of increased permeability of the tight junctions between hepatocytes and bile duct epithelial cells, and the subsequent diffusion of osmotically active solutes from the bile into plasma.

Mitochondria are essential for cellular energy metabolism and calcium homeostasis. It is therefore not surprising that mitochondrial dysfunction can cause cell death through ATP depletion and calcium dysregulation, playing a central role in the initiation of both apoptotic and necrotic cell death (Petit et al., 1995; Wallace and Starkov, 2000). A major player in this process is the mitochondrial permeability transition (MPT), widely implicated in the pathophysiology of cell death caused by a number of agents (Lemasters et al., 1998). It is characterized by an increase in non-specific permeability of the inner membrane to low-molecular-weight solutes leading to mitochondrial membrane depolarization, mitochondrial calcium release, mitochondrial swelling and inhibition of oxidative phosphorylation (Zoratti and Szabo, 1995), and specifically inhibited by CyA (Broekemeier et al., 1989).

In recent studies, we were able to show that rats with chronic cholestasis have impaired mitochondrial function and this finding can be explained entirely by the disruption of mitochondrial calcium homeostasis (Rolo et al., 2002a). This mechanism may therefore contribute to bile acids

hepatotoxicity with alteration of some important cell functions. Additionally, it may explain the characterization of cholestatic liver diseases by accumulation of hepatotoxic substances, mitochondrial dysfunction (Schaffner et al., 1971; Krahenbuhl et al., 1992; Gores et al., 1998; Rolo et al., 2000) and impairment of liver antioxidant defense (Sokol et al., 1993).

Our next questions were: (i) which mechanisms contribute to impaired mitochondrial function in rats with long-term cholestasis, (ii) are these changes similar to the ones observed with the short-term model of cholestasis, and (iii) are these changes histologically evident. Concerning the relevance and actuality of these questions, this study was undertaken to clarify the persistence of liver injury associated with mitochondrial dysfunction, in an ANIT-induced model of long-term cholestasis.

## 2. Materials and methods

### 2.1. Materials

ANIT, which was dissolved in olive oil, and cyclosporin A (CyA), used as ethanolic solution, were purchased from Sigma Chemical Co. (St. Louis, USA); Calcium Green 5-N was purchased from Molecular Probes (Leiden, The Netherlands). All other reagents and chemicals used were of the highest grade of purity commercially available.

### 2.2. Induction and characterization of cholestasis

Female Wistar rats (12 weeks) from our colony were used (Animal Research Center Laboratory, University Hospitals, Coimbra, Portugal). The use and care of the animals for these studies were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Coimbra. Animals were kept under controlled light (12 h day/night cycle), temperature (22–24 °C) and humidity (50–60%) conditions and with free access to powdered rodent chow (diet URF1, Charles Rivers, France) and water (pH 5.5). Animals were divided randomly into two

groups of eight animals each. To induce cholestasis, ANIT (80 mg/kg) was administered intraperitoneally, and this treatment was repeated for 16 weeks. Control animals received an equivalent volume of the vehicle. One week after the 16th ANIT treatment, rats were anesthetized (i.m.) with ketamin chloride (88.5 mg/kg, Park Davies, USA) and chlorpromazine chloride (2.65 mg/kg, Lab. Victoria, Portugal) and their weight was recorded. A blood sample (2 ml) was collected from cardiac puncture for measurement of serum markers of liver injury. Analyses were performed using commercial kits from Olympus, Tokyo, Japan and from Beckman, Fullerton, CA.

### 2.3. Liver morphology

Livers from four untreated female Wistar rats and from four treated female Wistar rats were removed quickly and immediately after the sacrifice and fixed in 10% buffered formaline, processed routinely, and embedded in paraffin. The 4- $\mu$ m thick sections were deparaffinized with xylene and rehydrated through decreasing concentrations of ethanol for staining with haematoxylin and eosin (H&E). To identify mast cells in supporting tissues and iron deposits, liver sections were also stained, respectively, with toluidine blue (Caballero et al., 1993) and Perls' Prussian blue (Churukian, 2002).

### 2.4. Preparation of mitochondria

Normal and cholestatic rats were maintained ad libitum for at least 12 h, before being sacrificed, according to a previously established method (Gazotti et al., 1979), with slight modifications.

Homogenization medium contained 250 mM sucrose, 5 mM HEPES (pH 7.4), 0.2 mM EGTA, 0.1 mM EDTA and 0.1% defatted BSA (bovine serum albumin). EDTA, EGTA and defatted BSA were omitted from the final washing medium and adjusted to pH 7.2. The mitochondrial pellet was washed twice, suspended in the washing medium and immediately used. Protein was determined by the Biuret method, using BSA as a standard (Gornall et al., 1949).

### 2.5. Membrane potential ( $\Delta\Psi$ ) measurements

The mitochondrial transmembrane potential was estimated by calculating transmembrane distribution of tetraphenylphosphonium ion (TPP<sup>+</sup>) with a TPP<sup>+</sup>-selective electrode prepared in our laboratory, as previously reported (Kamo et al., 1979; Palmeira et al., 1994) using a calomel electrode as a reference, since accumulation and release of TPP<sup>+</sup> by coupled mitochondria is directly correlated to membrane potential (Kamo et al., 1979). Therefore, TPP<sup>+</sup> uptake was measured from the decreased TPP<sup>+</sup> concentration in the medium of the electrode. The potential difference between the selective and the reference electrodes was measured with an electrometer and recorded continuously. A matrix volume of 1.1  $\mu$ l/mg was assumed and valinomycin was used to calibrate the baseline. Reactions were carried out at 25 °C in 1 ml of the reaction media (130 mM sucrose, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES, pH 7.2), supplemented with 3  $\mu$ M TPP<sup>+</sup>, 2  $\mu$ M rotenone, 1 mg mitochondria and 5 mM succinate. To induce state 3, 200 nmol ADP were used. Oxygen consumption was measured simultaneously, as described below. In order to evaluate the induction of MPT in the presence of calcium and phosphate, membrane potential was also evaluated. Reactions were carried out at 25 °C, in 1 ml of reaction medium (200 mM sucrose, 10 mM Tris–Mops (3-[*N*-morpholino]propanesulfonic acid) (pH 7.4), 1 mM KH<sub>2</sub>PO<sub>4</sub> and 10  $\mu$ M EGTA), supplemented with 2  $\mu$ M rotenone, 1 mg mitochondria and 5 mM succinate. To induce MPT, pulses of calcium were added, until the loss of  $\Delta\Psi$  compatible with the opening of MPT was recorded.

### 2.6. Mitochondrial respiration

Oxygen consumption of isolated mitochondria was determined polarographically at 25 °C with a Clark oxygen electrode, connected to a suitable recorder in a closed chamber with magnetic stirring. Mitochondria (1 mg) and respiratory substrate (succinate, 5 mM) and rotenone (2  $\mu$ M) were added to the standard reaction medium (1 ml). To induce state 3, 200 nmol ADP were used.

The respiratory control ratio (RCR) and ADP/O ratios were calculated according to Chance and Williams (1956).

### 2.7. Measurement of mitochondrial permeability transition

Mitochondrial swelling was estimated by changes in light scattering as monitored spectrophotometrically at 540 nm (Palmeira and Wallace, 1997). The reaction medium (200 mM sucrose, 10 mM Tris–Mops (3-[*N*-morpholino]propanesulfonic acid) (pH 7.4), 1 mM  $\text{KH}_2\text{PO}_4$  and 10  $\mu\text{M}$  EGTA), supplemented with 2  $\mu\text{M}$  rotenone, 0.5  $\mu\text{g/ml}$  of oligomycin and 0.6 mg mitochondria was stirred continuously and the temperature maintained at 25 °C. The experiments were started by the addition of 5 mM succinate. Calcium (50  $\mu\text{M}$   $\text{CaCl}_2$ ) was added prior to all the other compounds.

### 2.8. Measurement of mitochondrial calcium fluxes

The uptake and following release of calcium by isolated liver mitochondria was determined using a calcium-sensitive fluorescent dye, Calcium Green 5-N (Rajdev and Reynolds, 1993). The reactions were carried out at 25 °C, in 2 ml of reaction medium (200 mM sucrose, 10 mM Tris–Mops (3-[*N*-morpholino]propanesulfonic acid) (pH 7.4), 1 mM  $\text{KH}_2\text{PO}_4$  and 10  $\mu\text{M}$  EGTA), supplemented with 2  $\mu\text{M}$  rotenone, 0.6 mg mitochondria, 0.4  $\mu\text{M}$  oligomycin and 100 nM Calcium Green 5-N, and stirred continuously in a water-jacketed cuvette holder. Fluorescence (excitation 505 nm; emission 531 nm) was monitored continuously for 50 s, prior to the addition of calcium ( $\text{CaCl}_2$ ) to a final concentration of 15  $\mu\text{M}$ . Fluorescence was monitored continuously for an additional 4 min, and stopped with excess EGTA to obtain the basal line. Calcium fluxes are expressed as relative fluorescence units (RFU).

### 2.9. Measurement of mitochondrial ATPase activity

ATPase activity was determined by quantification of inorganic phosphate released in the assay

medium by the procedure of Taussky and Shorr (1953). The reaction was carried out in 2 ml of the respiratory medium and supplemented with 2- $\mu\text{M}$  rotenone and mitochondria (1 mg). The reaction was initiated by the addition of 3 mM Mg–ATP. Mitochondrial ATPase activity was determined by the difference obtained in the presence and in the absence of oligomycin (2  $\mu\text{g}$ ), a specific inhibitor of  $\text{F}_0\text{F}_1$  ATPase.

### 2.10. Statistical data analysis

The results are presented as mean  $\pm$  S.E.M. of the number of experiments indicated. Statistical significance was determined by the one-way ANOVA Student–Newman–Keuls post-*t*-test for multiple comparisons. A *P* value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Characterization of animals

Previous reports have been shown that ANIT is a cholestatic agent that produces cholestasis in a reproducible and dose-dependent manner (Goldfarb et al., 1962; Rolo et al., 2002a). On the day of experiment (1 week after 16 weeks of ANIT administration), several markers of liver injury were measured. As described in Table 1, biochemical analyses showed the cholestatic potential of ANIT, since there was an elevation in serum markers such as conjugated bilirubin, alkaline phosphatase,  $\gamma$ -glutamyltransferase and alanine aminotransferase. Citrate synthase activity and the yield of mitochondrial preparations were also similar among the two groups of animals (data not shown).

### 3.2. Liver morphology

In all treated animals absence of hepatocellular damage (Fig. 1A) and moderated to marked ductular proliferation were the most important features (Fig. 1B). In control animals, no proliferation of mast cells was observed (Fig. 2A), while in ANIT-treated rats portal tracts and some

Table 1  
Characterization of the animals

Parameter	Control	One week after 16th injection
Body weight (g)	309.7±10.9	263.1±6.8*
Albumin (g/dl)	3.72±0.1	3.2±0.1
Conjugated bilirubin (mg/dl)	0.15±0.03	0.48±0.05*
Alkaline phosphatase (IU/l)	73.2±4.2	179.4±6.8*
γ-Glutamyltransferase (IU/l)	0	4.6±0.21*
Alanine aminotransferase (IU/l)	49.8±4.2	60.6±3.8*
Aspartate aminotransferase (IU/l)	149.5±17.8	140.5±18.1

Note: Data represent the mean ± S.E.M. ( $n = 7$ ) from control animals (1 week after 16th injection with vehicle) and animals 1 week after 16th ANIT treatment. IU, International units.

\*  $P < 0.05$ , when compared to controls.

sinusoids (Fig. 2B) were infiltrated by large numbers of mast cells (stain purple). Iron-deposits (stain blue) at Kupffer cells and portal macrophages were also observed in all treated rats (Fig. 3B). Only one rat had some portal edema (Fig. 1C).

### 3.3. Liver mitochondrial respiratory indexes and transmembrane potential

Respiratory parameters from liver mitochondria isolated from ANIT-treated and control rats, were evaluated using succinate as a substrate. Mitochondria isolated from ANIT-treated rats showed no significant alteration in state 4 respiration, RCR and ADP/O, compared with the rates in mitochondria from controls (Table 2). State 3 respiration was significantly decreased in ANIT-treated rats. Additionally, when both preparations were energized with succinate, they developed similar transmembrane potential ( $\Delta\Psi$ ) while after ADP addition,  $\Delta\Psi$  and the repolarization rate were significantly decreased (Table 3 and Fig. 4).

Taken together the results in ANIT-treated rats, namely the decrease in state 3 respiration, the decrease in repolarization rate, as well as the observed decrease in  $\Delta\Psi$  after ADP addition, suggest an effect at the ATP-synthase level. To

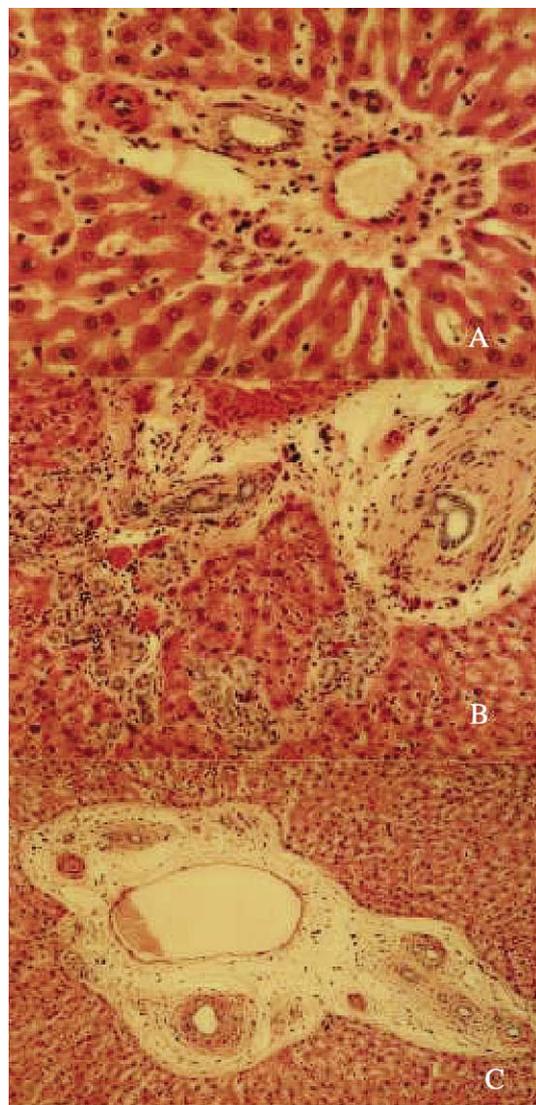


Fig. 1. Liver tissue of untreated and treated Wistar rats: (A) portal tract and liver cells of untreated Wistar rat (H & E 400 ×); (B) portal tract and liver cells of treated Wistar rat: proliferation of bile ductules and normal hepatocellular structure (H&E 200 ×); (C) portal edema in one treated Wistar rat (H&E 125 ×).

verify this hypothesis, we determined ATPase activity in both preparations (see insert in Fig. 4). There was a significant decrease in the ATPase activity of mitochondria from ANIT-treated when compared to control rats ( $85.5 \pm 19.1$  in ANIT vs.  $120.6 \pm 12.4$  nmol Pi/mg/min in control mitochondria).

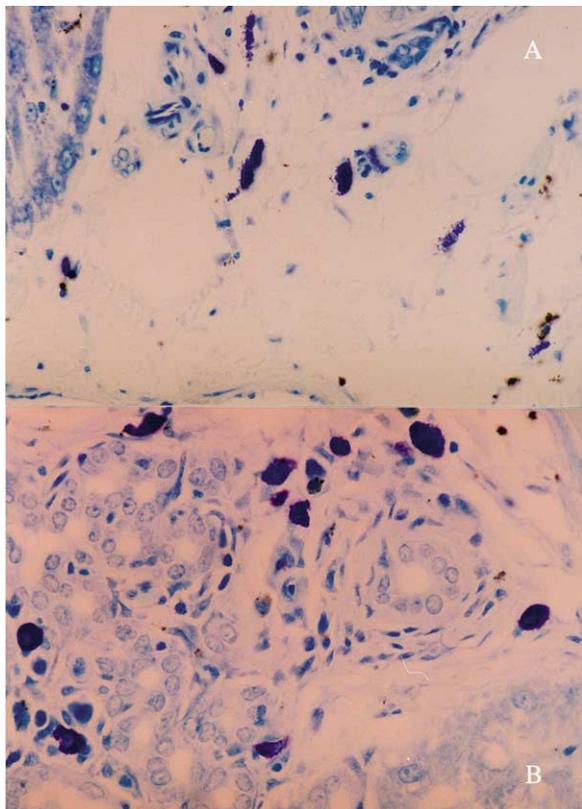


Fig. 2. Liver tissue of untreated and treated Wistar rats: (A) some mast cells confined to portal tract of untreated Wistar rats (Toluidine blue 500  $\times$ ); (B) portal tract of treated Wistar rats infiltrated by large numbers of mast cells (Toluidine blue 640  $\times$ ).

dria), confirming that after 16 weeks of treatment this key enzymatic complex is severely affected.

#### 3.4. Calcium-induced mitochondrial permeability transition

We evaluated the calcium concentration that should be applied to assess the susceptibility of both preparations to MPT induction. Calcium capacity of liver mitochondria from ANIT-treated and control rats was determined by monitoring the effect of the addition of small pulses of calcium in mitochondrial  $\Delta\Psi$ , measured with a TPP<sup>+</sup>-selective electrode. Our results shown that mitochondria from ANIT-treated rats presented a lower capacity of accumulating calcium, since they could

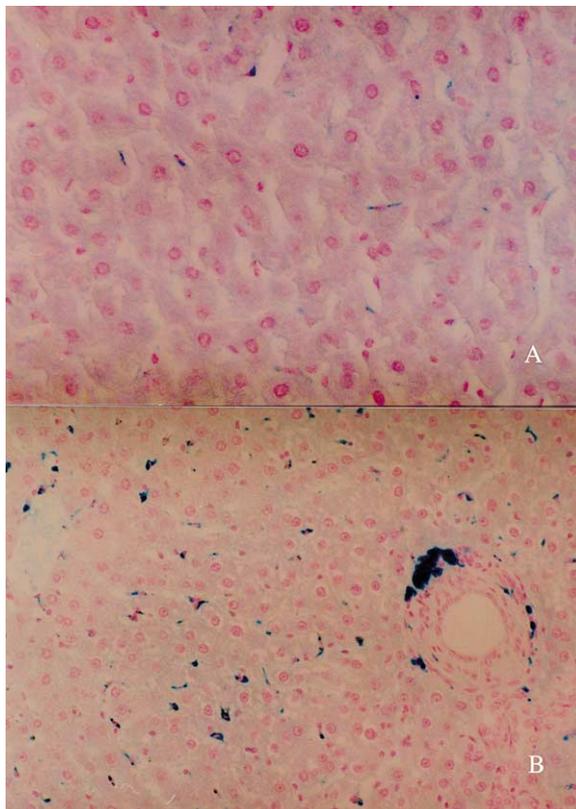


Fig. 3. Liver tissue of untreated and treated Wistar rats: (A) focal iron-deposits at Kupffer cells of untreated Wistar rats (Perls' Prussian blue 400  $\times$ ); (B) iron-deposits at Kupffer cells and portal macrophages of treated Wistar rats (Perls' Prussian blue 250  $\times$ ).

carry a small number of pulses before the loss of  $\Delta\Psi$ , compatible within the opening of MPT pore (Fig. 5). This decline in  $\Delta\Psi$  was abolished in the presence of CyA, a well known inhibitor of the MPT (data not shown).

Another evidence of the lower calcium capacity (consistent with a higher susceptibility to MPT pore opening) of mitochondria from ANIT-treated rats, comes from determinations of extramitochondrial calcium movements using the fluorescent calcium-sensitive probe Calcium Green 5-N. Data obtained with this technique confirm that mitochondria from control rats were able to retain calcium longer than those from ANIT-treated rats, indicating that mitochondria from treated animals are more susceptible to the induc-

Table 2  
Effect of ANIT-treatment on liver mitochondria respiratory indexes

Condition	State 4 (nmol O/mg/min)	State 3 (nmol O/mg/min)	RCR	ADP/O (nmol ADP/nmol O)
Control	15.0 ± 4.31 (n = 6)	71.1 ± 5.74 (n = 6)	4.45 ± 1.05 (n = 6)	1.53 ± 0.29 (n = 6)
ANIT	14.3 ± 3.07 (n = 7)	57.3 ± 3.9* (n = 7)	3.82 ± 0.44 (n = 7)	1.56 ± 0.24 (n = 7)

Mitochondria (1 mg protein) were incubated in 1 ml of the respiratory standard medium. RCR and ADP/O were determined accordingly to Chance and Williams (1956). Values are given as mean ± S.E.M. of the number of independent experiments indicated, performed with at least 3 different mitochondrial preparations.

\*  $P < 0.05$ , when compared to controls.

tion of MPT (Fig. 6). Cyclosporin A was able to inhibit calcium release, thus confirming MPT induction.

Concomitantly with these assays, we have evaluated the effect of long-term ANIT treatment on calcium-induced mitochondrial swelling by measuring changes in absorption at 540 nm, of a mitochondrial suspension. Our results shown that the same amount of calcium (50  $\mu$ M), when added to mitochondria from ANIT-treated rats, caused a larger amplitude decrease in absorption at 540 nm (Fig. 7). In the presence of CyA, this decrease was clearly prevented indicating that the observed swelling was due to MPT pore opening.

#### 4. Discussion

Our report for the first time describes the persistence of coexisting mitochondrial dysfunction and ductular proliferation following long-

term ANIT administration to rats. In numerous studies, ANIT has been employed as a mean to investigate the developmental aspects of the cholestatic type of hepatotoxic response (Goldfarb et al., 1962; Plaa and Priestley, 1976; Lock et al., 1982). Independently of the initiating event of cholestasis and the disease process, impairment of bile formation leads to an accumulation of bile acids within the hepatocyte, culminating in toxicity to the cells (Greim et al., 1972). Even in cholestasis caused by a primary insult to bile ducts, hepatocellular injury is an invariant feature.

To establish a condition of long-term cholestasis, animals were treated with a single, weekly injection of ANIT, based in previous studies (Rolo et al., 2002a,b). The increase in serum markers, 1 week after the last ANIT injection, clearly shows the onset of ANIT-induced cholestasis. The increased paracellular permeability induced by ANIT causes the regurgitation of biliary contents into the blood, giving rise to the biochemical

Table 3  
Effect of ANIT-treatment on liver mitochondria transmembrane potential

Condition	$\Delta\Psi$ (-mV)	$\Delta$ ADP (-mV)	$\Delta\Psi_{\text{rep}}$ (-mV)	Rep. Rate (% control)
Control	215.5 ± 5.00 (n = 8)	30.4 ± 2.58 (n = 8)	213.0 ± 4.01 (n = 8)	100.0 ± 8.83 (n = 8)
ANIT	216.0 ± 3.06 (n = 7)	24.2 ± 3.08* (n = 7)	212.8 ± 2.38 (n = 7)	58.1 ± 3.98* (n = 7)

Values are given as mean ± S.E.M. of the number of independent experiments indicated, performed with at least 3 different mitochondrial preparations. The membrane potential was measured after the addition of 5 mM succinate, as the respiratory substrate ('energization') and after the addition of 200 nmol ADP ( $\Delta$ ADP). 'Repolarization', corresponds to the recovery of membrane potential, after the complete phosphorylation of ADP added. Repolarization rate was determined, as the time required to the complete phosphorylation of ADP added. Lag phase reflects the time required to phosphorylate the ADP added. In all experiments, the baseline was determined by addition of 10 ng/ml of valinomycin at the end of experiments, in order to completely abolish membrane potential.

\*  $P < 0.05$ , when compared to controls.

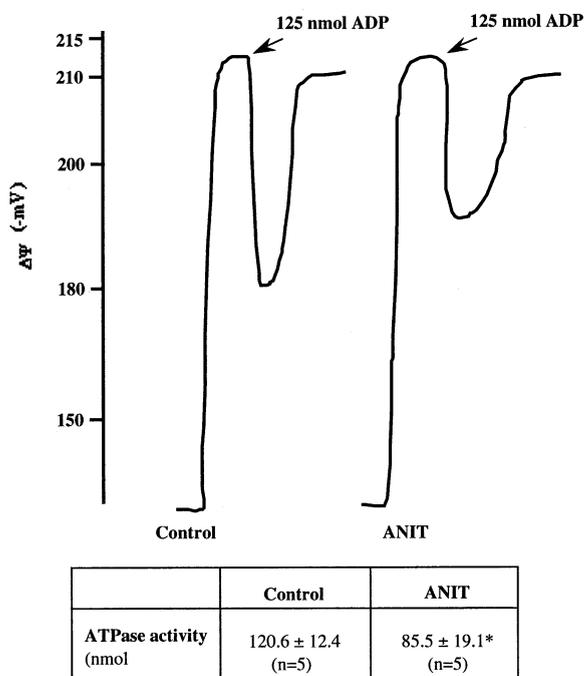


Fig. 4. Mitochondrial membrane potential measurements with a TPP<sup>+</sup>-selective electrode. Reactions were carried out in 1 ml of reaction medium, supplemented with 2  $\mu$ M rotenone and 1 mg mitochondria. Energization was achieved with 5 mM succinate. The traces are representative of experiments performed with four different mitochondrial preparations.

changes seen. Bile acids and bilirubin are described as elevated in serum, as well as the levels of enzymes normally present in bile. Although, we have not examined the levels of bile acids, in both serum or plasma, previous studies regarding short-term effects of ANIT on liver function, have clearly shown that a rise in serum bilirubin levels was associated with a reduction of bile flow (Capizzo and Roberts, 1970; Kossor et al., 1993), giving rise to bile acids accumulation. Interestingly, in a previous study regarding short-term chronic ANIT administration (Rolo et al., 2002b), we have observed that, 1-week after the sixth ANIT treatment, biochemical parameters returned to control levels. So, the evidence of serum markers of liver injury observed after this long treatment is the consequence of hepatocellular injury. Goldfarb et al. (1962) observed, in early

stages, ANIT-induced small bile ducts epithelial necrosis which results in cessation of bile flow; the surrounding stroma was edematous and periportal foci of necrosis and ductular proliferation were also observed. With chronic ANIT administration these lesions gradually regressed but proliferation of bile duct persists without interference with bile flow and liver cell injury (Goldfarb et al., 1962). Our results confirm, after 16 weeks of ANIT administration, ductular proliferation and normal hepatocellular structure as the most important features, present in all treated animals. Biliary proliferation is explained as a mechanism of bile duct repair to restore the number of intrahepatic ducts and duct secretory function. This indicator of liver disease is well correlated with the significant lower weight of treated animals at the time of the experiment. Iron deposits at portal macrophages and Kupffer cells may be related to intravascular haemolysis or previous hepatocellular necrosis.

In order to address if mitochondrial dysfunction might be one cause of the development of the pathological features associated with cholestatic diseases, we analyzed mitochondrial function. ANIT-induced changes in hepatobiliary morphology were accompanied by a significant decrease in state 3 respiration but no change in state 4 respiration of liver mitochondria isolated from treated rats, when compared with controls. Since state 4 respiration is mainly dependent on the proton leak, changes in ADP-stimulated respiration probably reflect an impairment at the level of the oxidative phosphorylative system, further substantiated by the decrease in ATPase activity. The evidence of a similar membrane potential developed upon succinate oxidation, in both preparations, further indicates that the difference in respiratory rates are the result of specific alterations on the oxidative phosphorylative system and not at the level of the  $\Delta$ pH dissipated through the subunits of the ATPase. If this was the case, an increase in state 4 respiration and a decrease in mitochondrial membrane potential should be noticed. Interestingly, RCR slightly decreased (while not statistically significant), although the great decrease in state 3 respiration. This observation may result from the fact that state 4 respira-

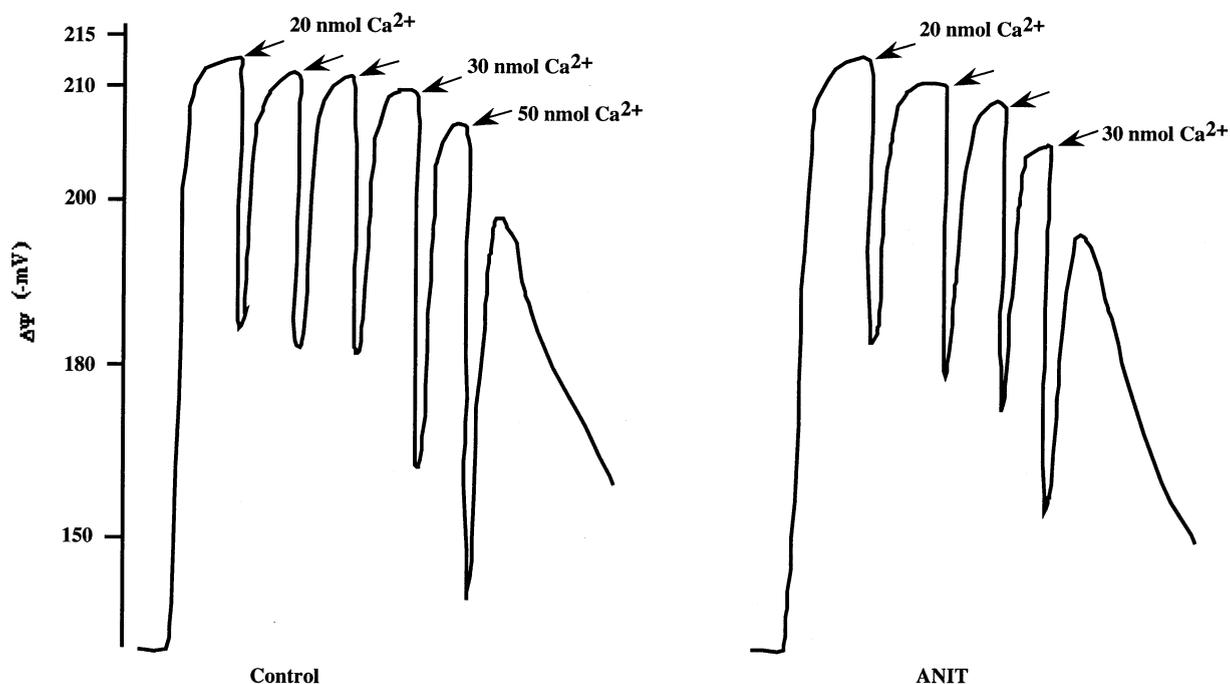


Fig. 5. Calcium accumulation capacity, measured with a  $\text{TPP}^+$ -selective electrode. Several calcium pulses were added in order to induce MPT. Experimental conditions are the same as in Fig. 4. The traces are representative of experiments performed with four different mitochondrial preparations.

tion also slightly decreased (while not statistically significant), which may lead to the observed no alteration in the ratio between state 3 and state 4 (RCR). In contrast, state 3 respiration reflects the integration of both reactions of ATP 'turnover' and those of succinate oxidation. The decrease in repolarization rate, membrane potential upon ADP addition and ATPase activity, probably are the result of an irreversible effect upon chronic ANIT administration and consequent bile acids accumulation.

Associated with alterations in oxidative phosphorylation, defective mitochondrial capacity in buffering external calcium and enhanced susceptibility to the calcium-sensitive MPT were observed, in our present study. Several reports have demonstrated toxic bile acids as potent inducers of the MPT, pointing the transition as a final common pathway responsible for ATP depletion and cytotoxicity of the accumulated compounds

during cholestasis (Botla et al., 1995; Gores et al., 1998; Rolo et al., 2000, 2003). MPT induction is a condition often related to mitochondrial and consequent cell dysfunction, and probably reflects a direct causative effect on the regulation of the CyA-sensitive MPT, since such susceptibility to calcium release is only observed upon chronic ANIT administration but not on acute cholestasis (Rolo et al., 2002b). Indeed, the fact that only MPT-dependent calcium release but not calcium uptake was affected in the treated group may suggest an effect at the often indicated as the MPT pore components. Furthermore, since state 3 respiration was significantly decreased, we may suggest that such effect may occur at the level of the adenine nucleotide translocator, explaining not only the increased susceptibility to the calcium-dependent MPT as well as the decreased state 3 respiration.

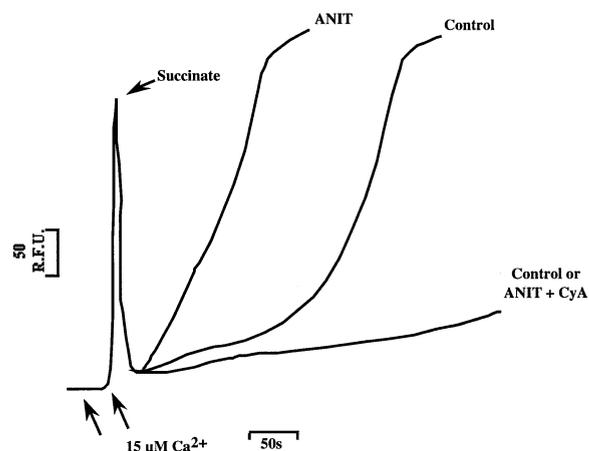


Fig. 6. Typical measurements of extramitochondrial calcium movements using the fluorescent calcium-sensitive probe Calcium Green 5-N. The reactions were carried out in 2 ml of reaction medium, supplemented with 2  $\mu$ M rotenone, 0.6 mg mitochondria, 0.4  $\mu$ M oligomycin, 5 mM succinate and 100 nM Calcium Green 5-N. Fluorescence was monitored continuously for 50 s, prior to the addition of calcium ( $\text{CaCl}_2$ ) to a final concentration of 15  $\mu$ M. Fluorescence was monitored continuously for an additional 4 min, and stopped with excess EGTA to obtain the baseline. Where indicated (first arrow), CyA (0.4  $\mu$ M) was added prior to calcium. Calcium fluxes are expressed as RFUs. The traces are representative of experiments performed with four different mitochondrial preparations.

Both mitochondrial metabolism impairment and hydrophobic bile acids accumulation are associated with increased production of oxygen free radical species and development of oxidative damage in cholestasis (Sokol et al., 1991; Krahenbuhl et al., 1995; Baron and Muriel, 1999; Sokol et al., 1993, 1995; Patel and Gores, 1997; Sokol et al., 1998; Gumprich et al., 2000; Vendemiale et al., 2002). The persistence of reactive oxygen species formation in cholangiocytes even 4 week after cessation of ANIT treatment was observed recently by LeSage et al. (2001). So, we are tempted to suggest that the observed decrease in state 3 respiration and ATPase activity may be a consequence of oxidative damage to the proteins or, since we are in the presence of a treatment of 16 weeks, the result of DNA damage by oxidative stress. This correlates well with our study of short-term chronic cholestasis, in which no reduced activity of the respiratory chain was observed

(Rolo et al., 2002a). Furthermore, other studies have shown that the activities of complexes I and III recover within days after reversal of bile-duct-ligation (Krahenbuhl et al., 1998). Additionally, conditions of oxidative stress act as negative modulators of the MPT. Being oxidative stress a phenomenon subsequent to bile acids accumulation during ANIT-induced cholestasis, oxidative damage to cardiolipin, mitochondrial DNA or proteins, may result in the observed mitochondrial dysfunction.

So we propose that the initial enhancement of mitochondrial function as an adaptive response by the hepatocyte to limit ANIT-induced acute liver cholestasis (Rolo et al., 2002b), progresses to a stage characterized by disruption of mitochondrial calcium homeostasis but yet with a probable recovery on respiratory chain and biochemical indices, after ANIT removal (Rolo et al., 2002a). In a later stage, several cell functions and mechanisms are probably affected, as shown by decreased efficiency of mitochondrial oxidative phosphorylation and calcium homeostasis, resulting in general hepatocyte dysfunction as shown by the biochemical parameters, determining the outcome of the disease. These studies may be important to better understand the course of cholestasis, since human cholestatic liver disease progress slowly over time, and provide further insight into the role of mitochondrial dysfunction in the process. A progressive and differential impairment of mitochondrial function is observed, depending on the stage of chemical-induced cholestasis.

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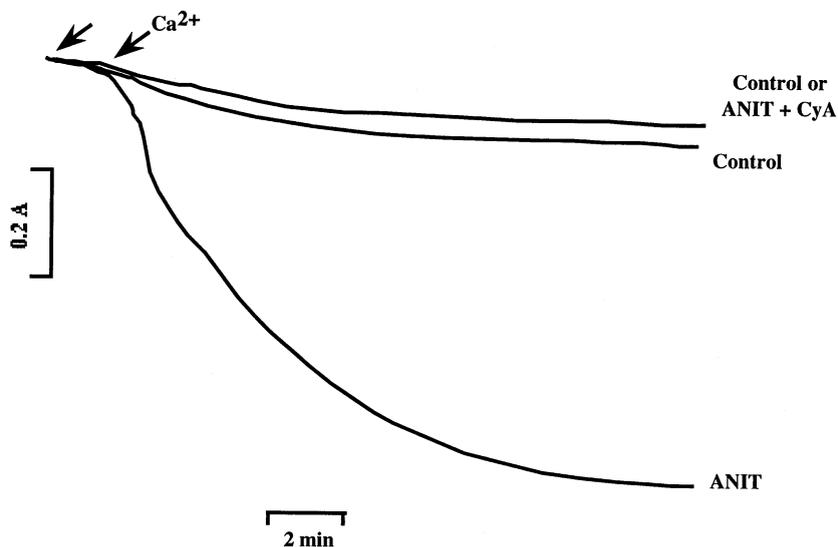


Fig. 7. Typical recording of mitochondrial swelling (decrease of absorbance at 540 nm) induced by calcium. The reaction medium, supplemented with 2  $\mu$ M rotenone, 0.5  $\mu$ g/ml of oligomycin and 0.6 mg mitochondria was stirred continuously and the temperature maintained at 25 °C. The experiments were started by the addition of 5 mM succinate. Where indicated (first arrow), CyA (1  $\mu$ M) was added prior calcium. Calcium (50  $\mu$ M  $\text{CaCl}_2$ ) was added prior to all the other compounds. The traces are representative of experiments performed with four different mitochondrial preparations.

## References

- Baron, V., Muriel, P., 1999. Role of glutathione, lipid peroxidation and antioxidants on acute bile-duct obstruction in the rat. *Biochim. Biophys. Acta* 1472, 173–180.
- Botla, R., Spivey, J.R., Aguilar, H., Bronk, S.F., Gores, G.J., 1995. Ursodeoxycholate (UDCA) inhibits the mitochondrial membrane permeability transition induced by glycochenodeoxycholate: a mechanism of UDCA cytoprotection. *J. Pharmacol. Exp. Ther.* 272, 930–938.
- Broekemeier, K.M., Dempsey, M.E., Pfeiffer, D.R., 1989. Cyclosporine A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J. Biol. Chem.* 264, 7826–7830.
- Caballero, T., Medina, M., Caballer, M.A., 1993. Histoquímica de hidratos de carbono e glúcidos. In: Garcia del Moral, R. (Ed.), *Laboratório de Anatomia Patológica*, MacGraw-Hill, Inter-americana de Espanha, Madrid, Spain, pp. 225–244.
- Capizzo, F., Roberts, R.J., 1970. Disposition of the hepatotoxin  $\alpha$ -naphthylisothiocyanate (ANIT) in the rat. *Toxicol. Appl. Pharmacol.* 17, 262–271.
- Chance, B., Williams, G.R., 1956. The respiratory chain and oxidative phosphorylation. *Adv. Enzymol.* 17, 65–134.
- Churukian, C.J., 2002. Pigments and minerals. In: Bancroft, J.D., Gamble, M. (Eds.), *Theory and Practice of Histological Techniques*. Churchill Livingstone, USA, pp. 243–267.
- Fischer, S., Beuers, U., Spengler, U., Zwiebel, F.M., Koebe, H.-G., 1996. Hepatic levels of bile acids in end-stage chronic cholestatic liver disease. *Clin. Chim. Acta* 2511, 173–186.
- Gazotti, P., Malmstron, K., Crompton, M., 1979. Membrane biochemistry. In: Carafoli, E., Semenza, G. (Eds.), *A Laboratory Manual on Transport and Bioenergetics*. Springer, New York, pp. 62–69.
- Goldfarb, S., Singer, E.J., Popper, H., 1962. Experimental cholangitis due to alpha-naphthyl-isothiocyanate (ANIT). *Am. J. Pathol.* 40, 685–698.
- Gores, G.J., Miyoshi, H., Botla, R., Aguilar, H.I., Bronk, S.F., 1998. Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases. *Biochim. Biophys. Acta* 1366, 167–175.
- Gornall, A.G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177, 751–766.
- Greim, H., Trulzsch, D., Czygan, P., Rudick, J., Hutterer, F., Schaffner, F., Popper, H., 1972. Mechanisms in cholestasis. 6. Bile acids in human livers with or without biliary obstruction. *Gastroenterology* 63, 846–850.
- Gumprich, E., Devereaux, M.W., Dahl, R.H., Sokol, R.J., 2000. Glutathione status of isolated rat hepatocytes affects bile acid-induced cellular necrosis but not apoptosis. *Toxicol. Appl. Pharmacol.* 164, 102–111.
- Kamo, N., Muratsugu, M., Hongoh, R., Kobatake, V., 1979. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J. Membr. Biol.* 49, 105–121.

- Kossor, D.C., Meunier, P.C., Handler, J.A., Sozio, R.S., Goldstein, R.S., 1993. Temporal relationship of changes in hepatobiliary function and morphology in rats following  $\alpha$ -naphthylisothiocyanate (ANIT) administration. *Toxicol. Appl. Pharmacol.* 119, 108–114.
- Krahenbuhl, S., Stucki, I., Reichen, J., 1992. Reduced activity of the electron transport chain in liver mitochondria isolated from rats with secondary biliary cirrhosis. *Hepatology* 15, 1160–1166.
- Krahenbuhl, S., Talos, C., Lauterburg, B.H., Reichen, J., 1995. Reduced antioxidative capacity in liver mitochondria from bile duct ligated rats. *Hepatology* 22, 607–612.
- Krahenbuhl, L., Schafer, M., Krahenbuhl, S., 1998. Reversibility of hepatic mitochondrial damage in rats with long-term cholestasis. *J. Hepatol.* 28, 1000–1007.
- Lemasters, J.J., Nieminen, A.-L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, D.A., Brenner, D.A., Herman, B., 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta* 1366, 177–196.
- LeSage, G., Glaser, S., Ueno, Y., Alvaro, D., Baiocchi, L., Kanno, N., Phinizy, J.L., Francis, H., Alpini, G., 2001. Regression of cholangiocyte proliferation after cessation of ANIT feeding is coupled with increased apoptosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 281, G182–G190.
- Lock, S., Lavigne, J., Plaa, G.L., 1982. The effect of alpha-naphthylisothiocyanate on bile secretion prior to and during the onset of cholestasis in the rat. *Toxicol. Lett.* 10, 427–435.
- Palmeira, C.M., Moreno, A.J., Madeira, V.M.C., 1994. Interactions of herbicides 2,4-D and dinoseb with liver mitochondrial bioenergetics. *Toxicol. Appl. Pharmacol.* 127, 50–57.
- Palmeira, C.M., Wallace, K.B., 1997. Benzoquinone inhibits the voltage-dependent induction of the mitochondrial permeability transition caused by redox-cycling naphthoquinones. *Toxicol. Appl. Pharmacol.* 143, 338–347.
- Patel, T., Gores, G.J., 1997. Inhibition of bile salt-induced hepatocyte apoptosis by the antioxidant lazaroid U83836E. *Toxicol. Appl. Pharmacol.* 142, 116–122.
- Petit, P.X., Lecoer, H., Zorn, E., Dauguet, C., Mignotte, B., Gougeon, M.L., 1995. Alterations in mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. *J. Cell Biol.* 130, 157–167.
- Plaa, G.L., Priestley, B.G., 1976. Intrahepatic cholestasis induced by drugs and chemicals. *Pharmacol. Rev.* 28, 207–273.
- Rajdev, S., Reynolds, I.J., 1993. Calcium green-5N, a novel fluorescent probe for monitoring high intracellular free  $Ca^{2+}$  concentrations associated with glutamate excitotoxicity in cultured rat brain neurons. *Neurosci. Lett.* 162, 149–152.
- Rolo, A.P., Oliveira, P.J., Moreno, A.J.M., Palmeira, C.M., 2000. Bile acids affect liver mitochondrial bioenergetics: possible relevance for cholestasis therapy. *Toxicol. Sci.* 57, 177–185.
- Rolo, A.P., Oliveira, P.J., Seica, R., Santos, M.S., Moreno, A.J., Palmeira, C.M., 2002a. Disruption of mitochondrial calcium homeostasis after chronic alpha-naphthylisothiocyanate administration: relevance for cholestasis. *J. Invest. Med.* 50, 193–200.
- Rolo, A.P., Oliveira, P.J., Seica, R., Santos, M.S., Moreno, A.J., Palmeira, C.M., 2002b. Improved efficiency of hepatic mitochondrial function in rats with cholestasis induced by an acute dose of  $\alpha$ -naphthylisothiocyanate. *Toxicol. Appl. Pharmacol.* 182, 20–26.
- Rolo, A.P., Palmeira, C.M., Wallace, K.B., 2002. Mitochondrially mediated synergistic cell killing by bile acids. *Biochim. Biophys. Acta* 1637, 127–132.
- Schaffner, F., Bacchin, P.G., Hutterer, F., Scharenbeck, H.H., Sarkozi, L.L., Denk, H., Popper, H., 1971. Mechanism of cholestasis. Structural and biochemical changes in the liver and serum in rats after bile duct ligation. *Gastroenterology* 60, 888–897.
- Sokol, R.J., Devereaux, M., Khandwala, R.A., 1991. Effect of dietary lipid and vitamin E on mitochondrial lipid peroxidation and hepatic injury in the bile duct-ligated rat. *J. Lipid Res.* 32, 1349–1357.
- Sokol, R.J., Devereaux, M., Khandwala, R.A., O'Brien, K., 1993. Evidence for involvement of oxygen free radicals in bile acid toxicity to isolated rat hepatocytes. *Hepatology* 17, 869–881.
- Sokol, R.J., Winkhofer-Roob, B.M., Devereaux, M.W., McKim, J.M., 1995. Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. *Gastroenterology* 109, 1249–1256.
- Sokol, R.J., McKim, J.M., Jr., Goff, M.C., Ruyle, S.Z., Devereaux, M.W., Han, D., Packer, L., Everson, G., 1998. Vitamin E reduces oxidant injury to mitochondria and the hepatotoxicity of taurochenodeoxycholic acid in the rat. *Gastroenterology* 114, 164–174.
- Taussky, H.H., Shorr, E., 1953. A microcolorimetric method for the determination of inorganic phosphorous. *J. Biol. Chem.* 202, 675–685.
- Vendemiale, G., Grattagliano, I., Lupo, L., Memeo, V., Altomare, E., 2002. Hepatic oxidative alterations in patients with extra-hepatic cholestasis. Effect of surgical drainage. *J. Hepatol.* 37, 601–605.
- Wallace, K.B., Starkov, A.A., 2000. Mitochondrial targets of drug toxicity. *Annu. Rev. Pharmacol. Toxicol.* 40, 353–388.
- Zoratti, M., Szabo, I., 1995. The mitochondrial permeability transition. *Biochim. Biophys. Acta* 1241, 139–176.