

Reduction of Hepatotoxicity Induced by Acetaminophen Overdoses in a Mouse Model of Inflammation Induced by Freund's Adjuvants

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Abstract

Acetaminophen (APAP) is often used as an analgesic and antipyretic during the inflammatory process. Its toxicity in overdoses depends on the integrity of the hepatic cytochrome P450 (CYP). The oxidative drug metabolism mediated by CYP can be inhibited during inflammatory diseases or after use of immuno-stimulants drugs and vaccines. The objective of this work was to evaluate if inflammation is able to modulate the toxicity of APAP. Five female Balb/c mice were injected subcutaneously with Freund Complete Adjuvant (FCA), and boosted with Freund's Incomplete Adjuvant (FIA) at 14th day. Then, they were treated with 360 mg/kg of acetaminophen orally during the 14th, 15th and 16th days. Convenient control groups were included with APAP administration without immuno-stimulation. Serum levels of IL-1 β , TNF α , IFN γ , α -1-acid glycoprotein (α -1-AGP), alanine transaminase (ALT), aspartic acid aminotransferase (AST), lactate dehydrogenase (LDH) and hepatic CYP2E1 expression were measured. Inoculation site of adjuvants and liver histopathological responses were also evaluated. FCA/FIA injection produced acute inflammatory response in the inoculation site and increased serum levels of the pro-inflammatory cytokines, α -1-AGP and LDH with reduction of hepatic CYP2E1 expression. A reduction of liver damage induced by APAP overdoses was also observed, suggesting that inflammatory processes can be protective against APAP hepatotoxicity.

Keywords: Inflammation; Immunotoxicity; Freund's adjuvants; Acetaminophen; Hepatotoxicity

Introduction

It has been documented that when host defense mechanisms are stimulated, there is a concomitant decrease in different hepatic cytochrome P450 enzymes (CYP), with impact in the drug biotransformation and elimination. This has resulted in a number of clinically important unwanted drug responses in patients with infections or inflammatory responses [1]. Moreover, several reports has shown that the administration of immuno-stimulating drugs, such as influenza and Bacillus Calmette Guerin (BCG) vaccinations, endotoxins or interferons, can affect the metabolism of several drugs [2-4]. Analgesics and antipyretics are often co-administered with immuno-stimulatory drugs, after vaccine application or during disease states with an inflammatory component; consequently, the study of these interactions is very important for the prediction of toxicity manifestation in treated patients.

Acetaminophen (*N*-acetyl-*p*-aminophenol) (APAP), also referred to as paracetamol, is one of the common analgesics and antipyretic drugs. It is generally considered harmless at therapeutic doses, but in overdose causes severe and sometimes fatal hepatic damage in humans and experimental animals [5,6]. The main metabolic pathways of acetaminophen in humans are hepatic glucuronidation and sulphation. Furthermore, several CYP isoenzymes metabolize a small proportion of APAP at therapeutic doses being oxidized to *N*-acetyl-*p*-benzoquinimine (NAPQI), a highly reactive intermediate metabolite that yields hepatotoxicity. In overdoses the production of NAPQI increases producing severe hepatic damage [6] (Figure 1).

Pharmacokinetics studies in human volunteers and animal models have evidenced that CYP2E1 is the main CYP isoform involved in the hepatotoxicity of APAP, although other isoforms such as: CYP1A2,

CYP3A4 and CYP2A6 may also participate [7-9]. The importance of CYP2E1 in acetaminophen toxicity was demonstrated by the finding that CYP2E1 null mice were much less sensitive to acetaminophen hepatotoxicity than the wild-type mice or CYP1A2 null mice. The double null mice (CYP2E1 $-/-$; CYP1A2 $-/-$) were only mildly sensitive to the toxic effects of acetaminophen. Moreover, in CYP2E1 null mice in which the human CYP2E1 gene was introduced as an artificial chromosome genomic clone of bacterial origin, the hepatotoxic effects of acetaminophen were restored to a significant extent [10,11].

Oxidative stress is also considered to be involved in the induction of hepatotoxicity by APAP overdose, causing decreasing in antioxidant enzyme activities such as catalase and glutathione peroxidase, increasing the effect of reactive oxygen species (ROS) like hydrogen peroxide, superoxide and peroxy nitrite [12-15].

Various authors reported a rapid decrease in total CYP contents in FCA-induced inflammation in rat liver and the selective down-regulation of specific CYP isoforms, as illustrated by decreased mRNA

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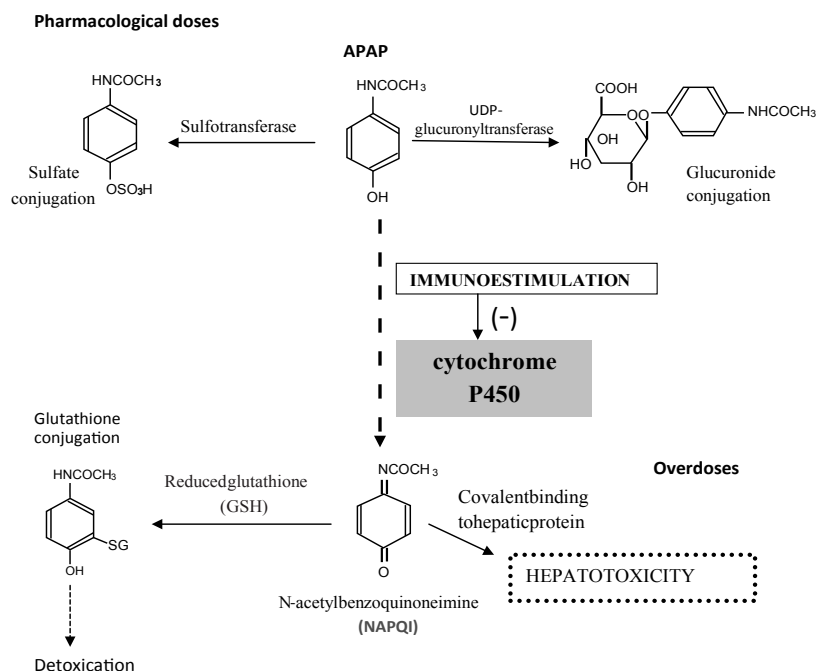


Figure 1: Simplified scheme of the metabolic pathways of APAP

Acetaminophen (APAP) is biotransformed and eliminated as nontoxic sulfate and glucuronic acid conjugates. Cytochrome P450 (CYP) participates in metabolizing a small proportion of APAP at therapeutic doses. The metabolism of APAP by CYP leads to the formation of N-acetylbenzoquinoneimine (NAPQI), a highly reactive intermediate metabolite, which is normally detoxified by conjugation with reduced glutathione (GSH). After high doses of APAP, the capacity for its removal by hepatic conjugation with glucuronide and sulfate is exceeded, and more of the reactive metabolite NAPQI is formed. Consequently, more NAPQI is conjugated with GSH, and when hepatic GSH is depleted, more NAPQI will bind covalently to cellular macromolecules; this leads to a loss of protein thiol-groups and ultimately to cell necrosis (hepatotoxicity)

levels (CYP2B, CYP2C11, CYP3A1, and CYP2E1), protein contents (CYP2B, CYP2C11, and CYP2E1) or catalytic activities (CYP2C6, CYP2C11, and CYP2E1) [16]. In animal models, using endotoxins as an immuno-stimulation model, also diminished the catalytic activity of CYP2E1 [17,18], including decreased expression of CYP1A, 2B1/2, and 3A subfamily associated with reversible changes in the pharmacokinetic parameters of theophylline [18]. In 1994, Raiford and Thigpen reported that Kupffer cells stimulated with *Corynebacterium parvum* reduced some CYP dependent activities and it was protective against the hepatotoxicity induced by an APAP overdose in rat [19]. After that, Projean reported a rapid decrease in total CYP contents, within 24 h, in the liver of FCA-treated rat and the selective down-regulation of specific CYP isoforms including CYP2E1, accompanied by significant increment of the levels of IL-6 as biomarkers of inflammation [16]. In another study it was showed that both FCA and FIA, elicited a hepatic acute phase response and induction in this organ of TLR2 expression in mice [20]. More recently Kusunoki demonstrated that early hepatic inflammation induces down-regulation of hepatic CYP expression, including CYP2E1, in the dextran sulfate sodium-induced murine colitis [21]. Also it was demonstrated higher plasma concentrations and greater area under the curve associated with manifestations of toxicity of theophylline, in rats treated with FCA that developed inflammatory reactions in the site of injection [22].

Considering that APAP hepatotoxicity is mediated mainly by CYP2E1 biotransformation, that the inflammation decreases the expression or activity of these enzyme families and taking into account the wide use of APAP as an analgesic/antipyretic during inflammatory process, the aim of the present study is to assess the effect of the inflammation in the hepatotoxicity patterns in APAP mild overdoses.

Materials and Methods

Adjuvants

Freund Complete Adjuvant (263810) (FCA); is a suspension of *Mycobacterium butyricum* in a mixture of paraffin oil and an emulsifying agent, mannide monooleate. Formulation per 10 mL ampule: Mannide Monooleate (1.5 mL), Paraffin oil (8.5 mL) and *Mycobacterium butyricum* (5 mg). Freund Incomplete Adjuvant (263910) (FIA): is essentially FCA without *Mycobacteria*. Formulation per 10 mL ampule: Mannide Monooleate (1.5 mL) and Paraffin oil (8.5 mL). DIFCO Laboratories, Detroit, MI). They were prepared immediately before inoculation according to a well-established method. Briefly adjuvant and a solution of NaCl 0.9% (QUIMEFA, Cuba) were mixed in proportion v/v using two glass syringes with Luer Lock connector during 10 minutes.

Acetaminophen

APAP purchased from QUIMEFA, Cuba, was prepared as oral suspension at a final concentration of 50 mg/mL in sterile distilled water.

Animals and treatment

Female Balb/c mice (20-25 g) were obtained from the National Center for the Laboratory Animals Production (CENPALAB, Havana, Cuba). The present study was conducted according to the Guiding Principles for the Care and Use of Laboratory Animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Toxicology and Biomedicine Center. All efforts were made to minimize suffering. The animals were maintained under controlled conditions

Groups of mice	Product	Dose	Via	Day
Control s.c	NaCl 0.9% solution	100 µl	sc	0, 14
Control i.m	NaCl 0.9% solution	100 µl	i.m	0, 14
APAP	APAP suspension	360 mg/kg	v.o	14,15,16
FA s.c	FCA emulsion	100 µl	s.c	0
	FIA emulsion	100 µl	s.c	14
FA i.m	FCA emulsion	100 µl	i.m	0
	FIA emulsion	100 µl	i.m	14
APAP/FA s.c	FCA emulsion	100 µl	sc	0
	FIA emulsion	100 µl	sc	14
	APAP suspension	360 mg/kg	v.o	14,15,16
APAP/FA i.m	FCA emulsion	100 µl	i.m	0
	FIA emulsion	100 µl	i.m	14
	APAP suspension	360 mg/kg	v.o	14,15,16

Table 1: Protocol of treatment in Balb/C mice.

(22°C, 55% humidity, and 12-hour day/night rhythm) and fed ratonine (purchased from CENPALAB) and acidulate water at libitum. Five mice by groups were treated according to the protocol shown in Table 1. All mice were clinically observed during the experiment.

Measurement of cytokines in serum

Cytokine concentrations were determined 8 h after the second application of Freund's Adjuvant (FA) (14th day) to all groups of mice, using commercially available ELISA kits (eBioscience, San Diego, CA, USA). Briefly, each well of a 96-well plate was coated with 100 µL of capture antibody, and incubated overnight at 4°C. After washing and blocking with assay dilution, serum or standard were added to individual wells; then the plates were maintained for 2 h at room temperature. The plates were washed while biotin-conjugated detecting mouse antibody was added to each well and incubated at room temperature for 1 h. The plates were washed again and further incubated with avidin-HRP for 30 min before detection with TMB solution. Finally, reactions were stopped by adding 1 M H₃PO₄, and the absorbance at 450 nm was measured with an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). The amount of cytokine was calculated from the linear portion of the generated standard curve.

Biochemical analysis of serum

The mice were anaesthetized with Pentobarbital 50 mg/kg IP diluted in a solution of NaCl 0.9% (Quimefa, Cuba) and blood (1,5 ml) was drawn from the vein of the tail 24 h after the last application of APAP (the 17th day). The serum were obtained by coagulation and centrifugation within 15 min were allowed to clot at room temperature and stored frozen at -20°C until analysis. The aspartic acid aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were quantified using a standard clinical automatic analyzer (Hitachi, 902) and specifics kit (Roche). The severity of the inflammatory reaction was assessed by measuring serum concentrations of α-1-acid glycoprotein (α-1-AGP) using a commercially available single radial immuno-diffusion method (Cardiotech Services, Inc., Louisville, KY, USA).

Isolation of hepatic microsomal fractions

Hepatic microsomal fractions of mice were prepared by differential centrifugation, washed in pyrophosphate buffer (pH 7.4) and stored in 100 mM Tris-acetate buffer (pH 7.4) containing 1 mM sodium ethylenediamine tetraacetate (EDTA). Microsomal preparations were stored in a -70°C freezer until use.

Immunoblot analysis

Hepatic microsomes were analyzed for CYP2E1 immunochemically. Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide) at 160 V for 1 h and electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper was incubated with anti-mouse cytochrome P-450 CYP2E1 antibodies, with goat antibody to mouse CYP2E1, then with rabbit anti-goat IgG conjugated with alkaline phosphatase (Bio-Rad). The color was developed with 5-bromo- 4-chloro-3-indole toluidine phosphate and p-nitroblue tetrazolium chloride (Bio-Rad) according to the manufacturer's instructions. The resulting blots were quantitated by densitometry, using Adobe Photoshop with a HP Precision Scanner and OneDScan software (Scanalytics) [23].

Necropsy and histopathology

After the blood collection, anaesthetized animals were sacrificed by cervical dislocation. Necropsy was made and all organs were macroscopically examined. Then, histological processing was performed according to standardized methods. Briefly, the sites of inoculation (dorsal subcutaneous tissue for subcutaneous or anterior quadriceps muscle groups of the left hindpaw for intramuscular) and the livers obtained from all treated mice, were fixed in 10% formalin, embedded in paraffin, sectioned at 4 µm thickness and stained with hematoxylin and eosin.

Statistical analysis

All data are reported as means ± S.D. The comparison of the results from the various experimental groups and their corresponding controls was carried out using a one-way analysis of variance followed by Turkey's post hoc test. The effects were considered significant when $p \leq 0.05$. The statistical analyses were performed using Statgraphics plus 5.1 software (StatSoft).

Results

Measurement of cytokines and biochemical analysis in serum

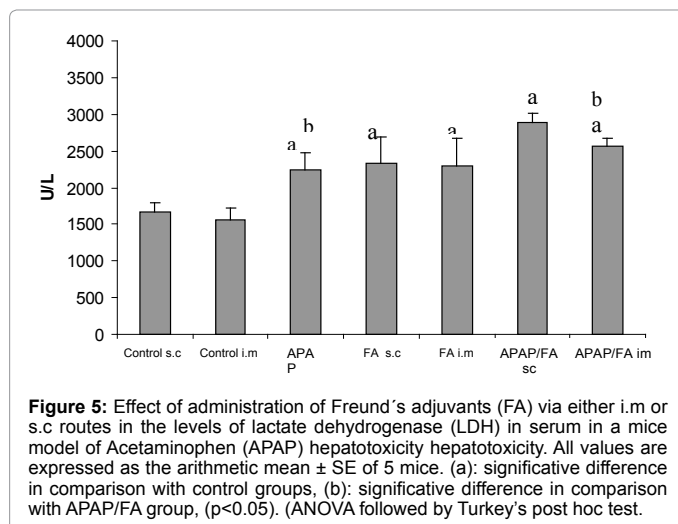
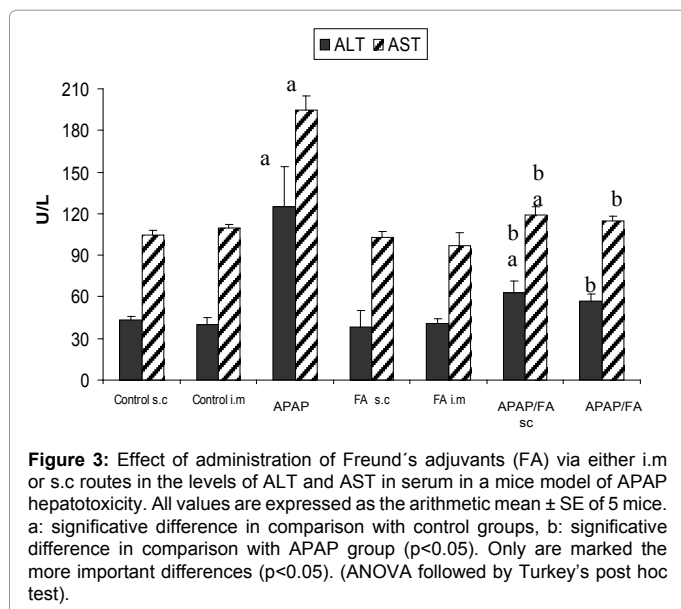
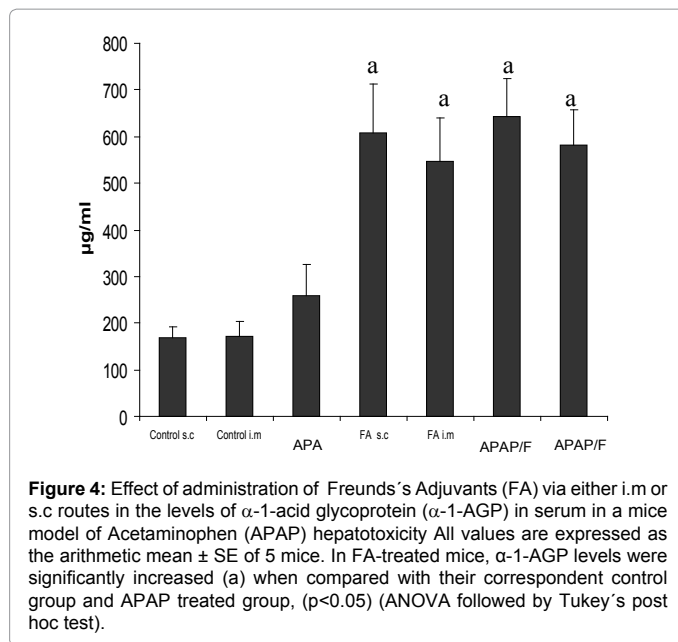
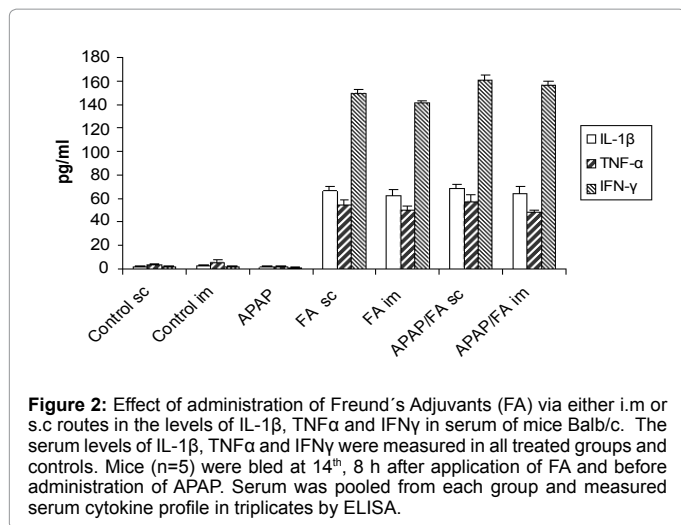
After 8 hours of applying the second dose of Freund's Adjuvants (FA), the concentration of pro-inflammatory cytokine IL-1β, TNFα and IFNγ were observed to increase in the serum of all the groups of mice that received Freund's Adjuvants (FA) (Figure 2).

Analyzing the enzymatic level in the treated groups, it was noted that a significant increment of ALT and AST in the APAP-treated group as evidence of hepatotoxicity induced by the overdose. However, it is interesting that in the group with co-administration of FA the levels of both enzymes were lower than positive control treated with APAP alone, indicating a hepatoprotective effect provoked by the inflammation (Figure 3).

Regarding to α-1-AGP levels, it was significantly increased in all mice treated with FA without affection for the APAP treatment (Figure 4). LDH were significantly increased in the serum of mice treated with APAP, FA and combined treatment in comparison with control groups. However, there were differences within these treated groups. Mice treated with APAP and previous s.c application of FA exhibited higher level of LDH than the other groups, being significant difference in comparison with APAP and APAP/FA i.m groups (Figure 5).

Expression of CYP2E1 in the liver

CYP2E1 was identified as a single band that appeared at approximately 56 kDa. In the groups treated with FA, the expression



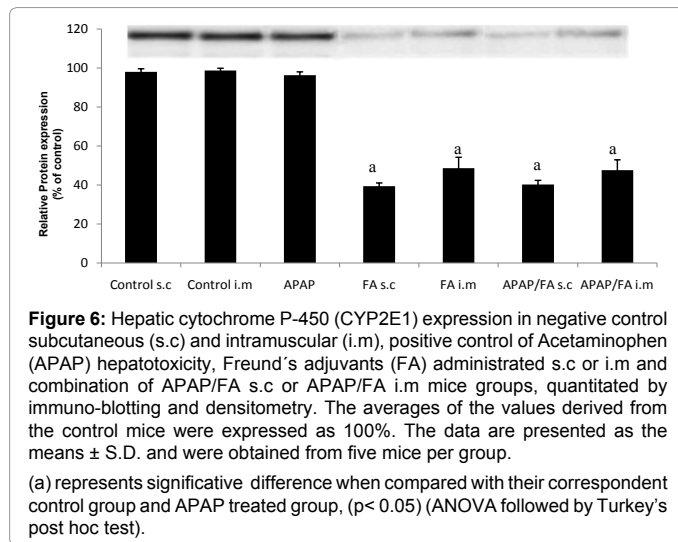
of CYP2E1 was significantly decreased to approximately 40-50% in comparison with the control groups. Apparently the groups that received FA via s.c route had lower expression in comparison with the groups treated via i.m route, but this difference was not statistically significant (Figure 6).

Pathological findings in the injection site and the liver

The main pathological finding at the injection sites and the liver in all the mice groups are detailed in Table 2. Mice previously immunostimulated with FA (i.m and s.c), showed inflammatory reactions in the injection site. The group under APAP treatment exhibited signs of mild hepatic necrosis, and other changes in the hepatic morphology according to the pattern produced by moderate overdoses of APAP, while mice with both treatment APAP and FA developed slight changes indicating a hepatoprotection, and not relevant modification on the tissue architecture in the other groups (Figure 7).

Discussion

APAP is frequently prescribed in the medical practice as analgesic



Groups	Macroscopic findings		Microscopic findings	
	Inoculation site	Liver	Inoculation site	Liver
Control s.c	Absence of visible tisular alterations	Absence of visible tisular alterations	Absence of inflammatory infiltrate or others microscopic alterations	Absence of alterations in the hepatic tissue
Control i.m	Absence of visible tisular alterations	Absence of visible tisular alterations	Absence of inflammatory infiltrate or others microscopic alterations	Absence of alterations in the hepatic tissue
Acetam	-	Moderate irregular liver's yellowish coloration	-	Focus of necrosis parenchymal, degenerated parenchymal hepatocytes multizonal, vascular congestion and swelling of hepatocytes
FA s.c	Nodule >5 mm, large amount of white tissue and haemorrhagic zones	Absence of visible tisular alterations	Severe diffuse inflammation, necrotising granuloma	Absence of alterations in the hepatic tissue
FA i.m	Localized nodular Inflammatory zone	Absence of visible tisular alterations	Inflammation with necrotising granuloma	Absence of alterations in the hepatic tissue
Acetam/ FA s.c	Nodule > 5 mm, large amount of white tissue and haemorrhagic zones	Light liver's yellowish coloration	Severe diffuse inflammation necrotising granuloma	Light degenerated parenchymal hepatocytes, with very scarce and little necrosis focus
Acetam/ FA s.c	Localized nodular Inflammatory zone	Light liver's yellowish coloration	Inflammation with necrotising granuloma	Light degenerated parenchymal hepatocytes, with scarce necrosis focus

Table 2: Main pathological finding at injection sites and liver of treated mice groups (n=5).

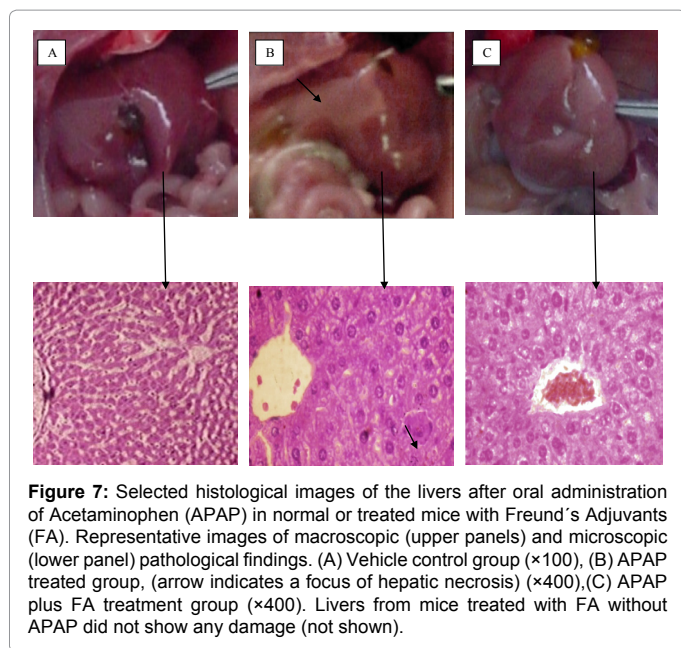


Figure 7: Selected histological images of the livers after oral administration of Acetaminophen (APAP) in normal or treated mice with Freund's Adjuvants (FA). Representative images of macroscopic (upper panels) and microscopic (lower panel) pathological findings. (A) Vehicle control group ($\times 100$), (B) APAP treated group, (arrow indicates a focus of hepatic necrosis) ($\times 400$), (C) APAP plus FA treatment group ($\times 400$). Livers from mice treated with FA without APAP did not show any damage (not shown).

and antipyretic during inflammatory process. On the other hand, overdose of APAP is the most frequent cause of drug induced hepatic failure in several countries [24]. Several studies have revealed that inflammation and immuno-stimulation can modulate the expression of different CYP enzymes, affecting the metabolism of some drugs [1-4]. In this way, Prandota has reviewed a lot of cytokines, growth factors and other substances released during inflammatory response with down-regulating effects on the metabolism of endogenous and exogenous substances [25].

This study has highlighted the role of inflammation induced by FA on the modulation of hepatotoxicity after APAP overdose, incriminating the role of proinflammatory cytokines on the inhibition of the CYP activity, reducing the APAP-induced hepatotoxicity. The dose used was 360 mg/kg via oral route, which induced an increment of biomarkers of hepatic damages and provoked a moderated hepatotoxicity that is able to detect an effect of the inflammation induced by FA in the mice model. The releasing of cytokines, such as IL-1, IL-2, IL-6, TNF α , TGF- β and

IFNs during immuno-stimulation are involved in the reduction of the expression of CYP2E1 [26] and the modulation of drug metabolism, as was evidence by Masubuchi et al., who demonstrated the role of IL-6 in hepatic heat shock protein expression and protection against acetaminophen-induced liver disease, using IL-6-knockout mice [27]. In this study was measured the concentration of the pro-inflammatory cytokines: IL-1 β , TNF α and IFN γ in sera in mice 8 h after the second application of FA, and before application of APAP, and it was observed their elevation as expression of the inflammatory reaction occurring in the inoculation site of the adjuvants.

AGP is other biomarker of acute inflammation elevated in the groups that received FA. AGP is an acute phase protein, which binds basic drugs, and has been proposed to have a role in immunomodulation and to be a nonspecific antimicrobial agent [28,29]. In rats treated with a single dose of FCA, the inflammatory response observed 14 days post-injection (FCA-induced arthritis) is characterized by pathophysiological changes that can alter drug disposition, namely elevated serum levels of AGP [30]. Considering that AGP is able to binds to drugs, further studies of its influence in pharmacokinetic and pharmacodynamic properties of APAP in this model needs to be studied in the future.

The hepatic enzymes ALT and AST are the best enzymatic marker of hepatic necrosis, in particular ALT is the clinical chemistry gold standard of hepatotoxicity. Damaged hepatocytes release their contents including ALT and AST into the extracellular space. The released enzymes ultimately enter into circulation and thereby increase the serum levels of ALT and AST compared to control subjects [31]. In this study, the group treated with APAP showed significant increment of these enzymes in sera due to the hepatic damaged induced by this drug, but in the groups injected with FA and APAP there was not observed such increment, as evidence of protection against APAP-induced hepatotoxicity in animal receiving FA. Regarding to LDH, despite being less specific, it has been used as a marker to evaluate the damage to hepatic tissue membranes by APAP [32]. In all the groups that received APAP and FA plus APAP, the LDH were elevated in contrast with the control groups. Taking into count that LDH is a unspecific biomarker of cellular necrosis, is possible that this result is produced by the sum of the effects of the cellular necrosis at the inoculation site induced by FA in addition to that occurred in the liver by the APAP-induced hepatotoxicity.

Many investigators reported that CYP2E1 is the main CYP isoform involved in APAP hepatotoxicity [8-11]. In this study was observed a decreased expression in mice previously treated with FA. This effect may have contributed to reduce the APAP hepatotoxicity. Similar result was reached by other authors using a model of immuno-stimulation with *C. parvum* [19]. The protective effect induced by the inflammatory response was directly evidenced in the pathological studies of the livers of the group treated with APAP plus FA with a reduction of the hepatic necrosis.

Although our study reveals one aspect of the inflammatory response and its influence in reducing hepatotoxicity by paracetamol, this phenomenon is very complex and other factors not evaluated in our study can also be involved. For example, Kupffer cells can be activated directly or indirectly by various toxicants, including paracetamol, or by the hepatocyte damage initiated by the formation of the reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine, resulting in the release of reactive oxygen and nitrogen species, all of which may contribute to liver damage with implications in the toxicity [12-15,33]. Thus was reported that the antioxidant effect of beta-D-glucan can prevent the APAP-induced toxicity [34].

Other important issue which reveals the complexity of this effect is that, hepatocyte death results in a sterile inflammatory response and activation of molecular pathways for innate immune activation, such as TLR 9 and the Nalp3 inflammasome, producing pro-inflammatory cytokines that amplifies the initial insult and increases overall tissue injury, as was demonstrated in mice deficient in Tlr9 and the inflammasome components Nalp3 (NACHT, LRR, and pyrin domain-containing protein 3) [35], and using neutralizing antibodies to TNF α or IL-1 partially prevented liver damage in mice initiated by hepatotoxic doses of APAP [36]. For this reason we measured the level of pro-inflammatory cytokines before the administration of APAP. Besides, some immuno-stimulators such as lipopolysaccharide (LPS) from Gram-negative bacteria can itself induce liver damage, and act in synergy with APAP for the production of pro-inflammatory cytokines [37]. In contrast, in the model of carbon tetrachloride (CCl₄) exposure in mice, TNF α was involved in liver repair through its ability to support hepatocyte proliferation following chemical injury [38]. In spite of the diversity of factors that participate in the APAP hepatotoxicity, our results confirm the role of the previous immuno-stimulation, reducing the hepatotoxic effect of this drug.

In conclusion, this result suggest that the use of APAP for the treatment of pain or fever as part of the flu-like syndrome induced by vaccines or immuno-modulators [39-41], and during inflammatory process, does not increase the risk of toxicity in overdose, quite the contrary, this risk probably is reduced. Further studies are required measuring glutathione (GSH), other cytokines involved in the inflammatory process and other members of the CYP in this and other models of inflammation.

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