J Bras Patol Med Lab. 2018 Oct; 54(5): 279-287.

10.5935/1676-2444.20180048

Evaluation of acute toxicity of β-lapachone associated with chitosan as a cytoprotective agent

Avaliação da toxicidade aguda da β-lapachona associada à quitosana como agente citoprotetor

Maria Eduarda F. A. G. Oliveira[1]; Érika Cristina G. M. Silva[2]; Celso A. Câmara[3]; Ivone Antônia de Souza[1]; Rosa Valéria S. Amorim[1]

1. Universidade Federal de Pernambuco (UFPE), Pernambuco, Brazil. 2. Faculdade Maurício de Nassau, Pernambuco, Brazil.
3. Universidade Federal Rural de Pernambuco, Pernambuco, Brazil.

ABSTRACT

Introduction: β-lapachone (β-LAP), a potent antitumor agent, has limited therapeutic use due to its low solubility and high toxicity. A possible strategy to overcome these drawbacks may be the use of adjuvants such as chitosan (CS), a cationic polysaccharide with biological properties of biocompatibility and biodegradability. **Objective**: Evaluate the adjuvant action of CS as a cytoprotectant associated with β-LAP, through acute toxicity studies, evaluating histopathological changes in organs such as liver and kidneys. **Methods:** The β-LAP-CS conjugate was prepared in a 1:1 ratio, administered orally, with a single dose of β-LAP of 80 mg/kg, in Swiss mice. Histomorphological and histomorphometric analyses of the kidneys and liver were performed. **Results:** In the histomorphological studies of the tested groups, we observed that the hepatocytes of animals treated with the free drug presented morphological alterations, such as cytoplasmic vacuolization, cellular extravasation, atypical and pyknotic nuclei. In this same group, the kidneys presented granular aspects suggestive of glomerulonephritis. These changes were not found in the control group and in animals treated with CS-conjugated β-LAP. There was no statistical difference in the histomorphometric analyses of the distal tubules and the renal glomeruli between the three groups analyzed, even with evident histomorphological alterations. After histomorphometric studies, it was observed that the area of hepatocytes and their cell nuclei presented a statistically significant difference between the animals treated with free β-LAP and the β-LAP-CS. **Conclusion:** The decrease in β-LAP toxicity after conjugation may be related to the hepatoprotective property of CS.

Key words: chitosan; acute toxicity; histology; β-lapachone.

INTRODUCTION

β-lapachone (β-LAP) is an ortho-naphthoquinone sinthesized from lapachol, obtained from purple lapacho tree, belonging to the *Bignoniaceae* family, *Tabebuia* sp. (**Figure 1**), a tree native to the Brazilian Atlantic Forest. It displays a wide range of pharmacological properties, such as antibacterial[1, 2], antiviral[3], antifungal[4], and antiprotozoal activity against *Plasmodium falciparum*[5] and *Trypanosoma cruzi*[6, 7]. Additionally, other effects were reported, such as anti-inflammatory[8] and, especially, anticancer properties, with activity against several types of cancer[9, 10].

A limitation in clinical use is its poor aqueous solubility and high toxicity, what prevents it from being commercialized using conventional formulations[12]. New strategies have been developed to ensure higher bioavailability and biological action of β-LAP, such as the use of lower doses and convenient administration via, aimed at minimizing the adverse effects of its high toxicity[13].

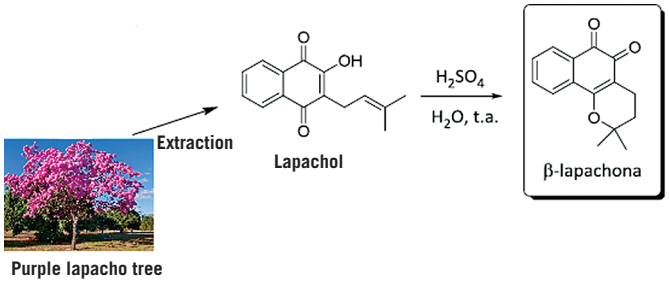

FIGURE 1 – *Synthesis of β-lapachone from lapachol under acid catalysis Adapted from Aires* et al. *(2014)*[11].

First submission on 02/13/17; last submission on 10/08/18; accepted for publication on 10/09/18; published on 10/20/18

An alternative is the use of bioadhesive polymers, which can deliver the drug and be employed in sustained releases with applicability in antitumor oral administration[14].

Chitosan (CS), an N-deacetylated derivative of chitin (β-(1-4)-2-amino-2-deoxy-D-glucopyranose), is a promising biopolymer that can be used to reduce toxicity and protect hepatocytes during administration of cytotoxic drugs. The hepatoprotective effect of CS is probably owed to an action against free radicals, by its antioxidant nature and/or capacity to inhibit lipid accumulation by its antilipidemic properties[15]. Some of the advantages associated with CS are low toxicity, low immunogenicity and biocompatibility[16, 17].

For those reasons, in order to reduce the undesired toxicity of β-LAP, as well as to improve its solubility, the objective of this study was to evaluate the use of CS and its adjuvant effect in the reduction of hepatic and renal acute toxicity, by means of a comparative assessment of the toxicological potential of β-LAP, free and conjugated to CS, after oral administration, through histopathological and histomorphometric analyses.

METHODS

Materials

β-LAP and low molecular weight CS was obtained from Sigma-Aldrich (St. Louis, USA). Sulfuric acid, sodium nitrite, acetic acid, dichloromethane and other chemical products of analytical quality that were used directly with no additional purification belonged to the collection of chemical substances of our research laboratory (Labipol).

β-lapachone synthesis

β-LAP was synthesized from lapachol, extracted from the heart of *Tabebuia avellanedae* (*Bignoneaceae*), with a yield of 76%, by the use of sulfuric acid, following the method adapted from Cavalcante[18]. β-LAP was purified by silica gel column using an eluent system of *n*-hexane-dichloromethane (8:2, v/v). The product was characterized by the usual spectroscopic methods, including hydrogen nuclear magnetic resonance (^{1}H-RMN) and infrared (IR) spectroscopy. The physicochemical properties and melting point were assessed chromatographically by comparing the product profile with a pure β-LAP.

CS purification and depolymerization

A 0.5% CS solution in 1% acetic acid was prepared under constant agitation, for approximately 12 h, filtered with a pre-filter, followed by filtration in Millipore filters 3.0 and 0.8 µm. For precipitation, 40% NaOH was dropped, until pH reached 9. The precipitate was separated by centrifugation at 4,000 rotations per minute (rpm) for 20 min; the supernatant was discarded; the precipitated was washed three times with distilled water, three times with ethanol; and once with ether. The precipitate was dried in the oven at 40°C for later depolymerization, according to Janes[19], with some modifications. The CS purified solution in 0.5% acetic acid was added to 0.1 M $NaNo_2$, kept under agitation for 3 h and precipitated with NaOH up to pH 9. Later, it was centrifuged for 30 min at 4,000 rpm; the supernatant was discarded, and the precipitate was washed three times with distilled H_2O and three times with ethanol. The depolymerized CS solution (DpCS) was dried in the oven at 40°C for later use, in the preparation of β-LAP/DpCS conjugate.

Preparation and characterization of β-LAP/DpCS conjugate

The β-LAP/DpCS conjugate was prepared by the association of β-LAP in 70% ethanol (EtOH) with a DpCS solution in 0.5% acetic acid, in the 1:1 proportion. This mixture was kept at 25°C, under agitation, for a period of 18 h; then it was frozen at -80°C and lyophilized for later use. The spectrophotometric profiles of the β-LAP/DpCS conjugate, DpCS, and free β-LAP, here analyzed, were obtained from the technique of Fourier-transform infrared spectroscopy (FTIR). Solid samples were pressed against a diamond crystal, using a pressure clamp attached to the device, followed by reading and recording in the Agilent 630 spectrometer, with wavelength between 4,000 and 750 cm^{-1}.

Acute toxicity testing

In the acute toxicity study, female Swiss albino mice were used, weighing 40 g, aged between 6 and 8 weeks. The animals were kept in cages with water and feed, and were managed as recommended for experimental procedures following the institutional policy of Animal Research Ethics Committee. The Project was approved by the Animal Research Ethics Committee of Universidade Federal de Pernambuco (UFPE), under no. 23076.016084/2014-29.

The acute toxicity of β-LAP was analyzed by administering a single dose of 80 mg/kg of the drug in the free and conjugate presentations. The animals were divided in groups of six animals each: free β-LAP group (G1), β-LAP/DpCS conjugate group (G2), and a control animal (with no administration of free or conjugated drug). For G1 group, β-LAP was dissolved into 10% dimethyl sulfoxide (DMSO); and group G2 the conjugate, in distilled water, to form the suspension to be administered. After administrations by gavage, the animals were observed for the development of toxicity, regarding

the presented clinical signs, for 30 minutes, and afterwards, each 24 h for 14 days, being kept under room temperature, with available food and water during the whole day.

Histological analyses

Fourteen days after the acute toxicity experiment, the animals were sacrificed by the method of cervical dislocation. During exploratory laparotomy, organs were taken (liver and kidneys) for histological processing. The organs of G1 and G2 animals, besides the control animal, were stored and fixed in 10% formaldehyde, for a period of 48 h. Next, fragments underwent cleavage, were processed according to routine techniques for inclusion in paraffin blocks, cut in microtomes and stained by the hematoxylin and eosin (HE) technique. After mounting histological preparations, slides of the animals' liver and kidneys were observed through optical microscopy for analysis of possible morphological alterations and degenerative lesions, at hepatic and renal level.

Histomorphometry

A total of 30 histological preparations were studied by the analysis of five random fields. The histological preparations were scanned, using a digital video camera (Samsung-Color Digital), connected to an optical microscope (Olympus BX-50). Morphometrical analysis of structures was carried out with the software Image J. Final magnification to obtain photographs was 400×. In order to measure nuclear and cytoplasmic areas of hepatocytes, renal corpuscle, glomerulus, and glomerular capsule in μm^2, preparations of the control animal and each animal of groups G1 and G2 were chosen at random, besides cells of entirely visible contour, by means of five different fields of the same preparation. In each photograph, the nuclear area and the total cellular area were measured at random. The area of the subcapsular space of Bowman's capsules was obtained by subtracting it from the total area of the renal corpuscle, by the value of the area of the glomerulus[20].

Statistical analysis

Mean, median, and standard deviation (SD) were calculated from the morphometric data of tested animals. Inferential analysis comprised Kruskal-Wallis test, and in the case of significant differences, tests of multiple comparison of the mentioned test with later tests were performed. The statistical significance level or the error margin was 5%. Data were entered in an Excel spreadsheet, and the programs used for obtainment of statistical calculations were Statistical Package for the Social Sciences (SPSS) version 23 and MedCalc version 14.

RESULTS

Formation and characterization of β-LAP/DpCS conjugate

For β-LAP/DpCS conjugate formation, commercial CS was previously purified and depolymerized for elimination of impurities and inconsistencies, in relation to variations in molecular mass. The β-LAP/DpCS conjugate presented a size of 1.3 ± 0.15 µm and polydispersity (PDI) 0.04 ± 0.002.

In **Figure 2**, FTIR of isolated β-LAP showed characteristic absorption with bands at 2951cm^{-1}, corresponding to C-H bonds of the β-LAP aromatic region. In the region 1700-1000 cm^{-1}, we found the band 1696 cm^{-1} corresponding to C=O group; we also observed bands of 1315-1122 cm^{-1} corresponding to C-O-C group, all according to the β-LAP[21, 22] corresponding spectra.

With the infrared spectrum of depolymerized CS (Figure 2), 3362 cm^{-1} large bands were observed, normally in the region of the range 3200-3600 cm^{-1}, which are attributed to groups OH and/or $-NH_2$ corresponding to CS[23]. Bands that appear in 1710 cm^{-1} were observed, attributed to the amine stretching (C=O). Besides bands in 1249 cm^{-1} that can be attributed to $-NH_2$ deformation, occurred probably due to CS depolymerization, we observed the 2892 cm^{-1} band corresponding to C-H group[24].

In the spectrum of β-LAP/DpCS conjugate (Figure 2), we did not observe significant displacements of the corresponding peaks to 1759, 1712, and 1678 cm^{-1} corresponding to C=O group, what suggests there was no chemical interaction among the compounds in this group.

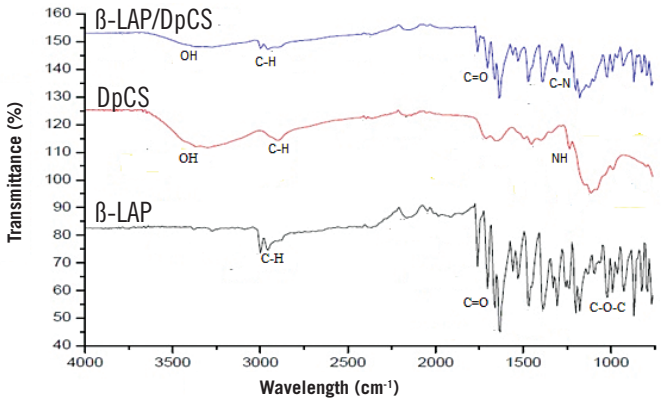

FIGURE 2 – *FTIR of β-LAP, DpCS and the β-LAP/DpCS conjugate*

FTIR: Fourier-transform infrared spectroscopy; β-LAP: free β-lapachone; DpCS: depolymerized low molecular weight chitosan.

Acute toxicity testing

The behavior of animals treated with the free and conjugated drug was observed and, 14 days after the experiment, no death occurred in the dose of 80 mg/kg.

Histopathological analyses

In the histological preparations, histopathological changes were observed in the liver of animals treated with the free drug after oral administration of a single dose of 80 mg/kg β-LAP. In hepatocytes of group G1, we observed vacuolization of cytoplasm and nucleus; some cells presented cellular extravasation, exposing atypical and pyknotic nuclei, suggestive of a degenerative necrosis process (**Figure 3A2**). In the control animal and in group G2, histopathological alterations were not observed in the analyzed hepatocytes (**Figure 3A3**). In kidney cells, we observed that in glomeruli of group G1 there were, internally, characteristics similar to the presence of hyaline (**Figure 4**). Besides, hypertrophic tubules were found, with dilated lumens (**Figure 3B2** and **B3**), presenting the granular aspect suggestive of chronic or sclerosing glomerulonephritis[25]. These alterations were neither found in histological sections of group G2 animals, nor in the control animal (**Figure 3B1**). Some glomeruli of group G1 animals also presented membranous glomerulonephritis with subcapsular space greater than that of the control animal and group G2 (**Figure 3C1**, **C2**, and **C3**).

Histomorphometry

In the histomorphometric analysis of histological liver preparations, areas in the nucleus of hepatocytes of the treated groups and the control animal were compared and their results were shown in **Table 1**. The control animal presented mean nuclear area ± 7.96 μm². Groups G1 and G2 did not present significant statistical variation of the nuclear area when compared one with the other and with the control animal ($p > 0.05$). In relation to the mean area of hepatocytes, which ranged from 24.14 μm² to 50.55 μm², the G1 group presented a cellular area twice as small as that of the control animal, and also smaller than that presented in group G2, with significant differences ($p < 0.05$).

In the histomorphometrical analysis of kidneys (**Table 2**), we observed that the mean size of Bowman capsules was larger in group G1 and smaller in group G2, when compared with the control animal, however with no significant difference between them ($p > 0.05$). The average of glomeruli presented larger area in the control animal; groups G1 and G2 presented a glomerular area smaller than the control animal, with significant difference ($p < 0.05$).

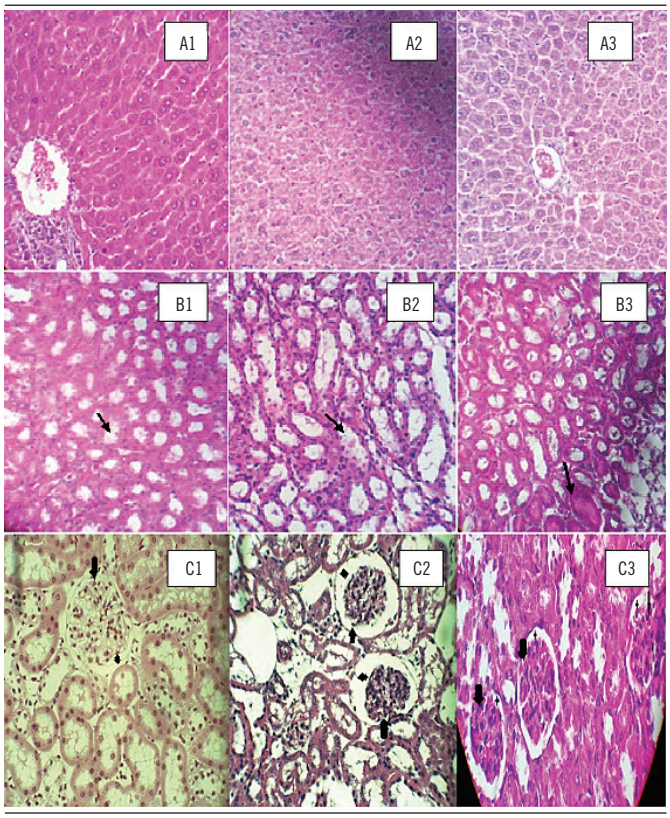

FIGURE 3 – *Analyses of histological sections representative of hepatocytes (A1, A2, and A3) distal and proximal tubules (B1, B2, and B3) and renal glomeruli (arrows) with subcapsular space (*) from mice used in the acute toxicity test, including control (A1, B1, and C1), free β-LAP (A2, B2, and C2), and β-LAP/DpCS (A3, B3, and C3). Magnification 200×, HE*

β-LAP: free β-lapachone; DpCS: depolymerized low molecular weight chitosan; HE: hematoxylin and eosin.

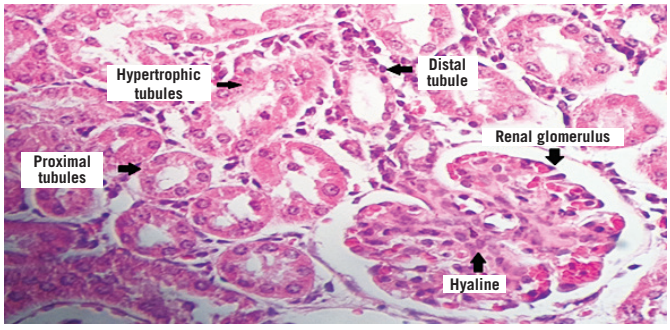

FIGURE 4 – *Histological observation of kidney treated with free β-LAP. The changes in proximal tubules and in the renal glomerulus are highlighted. Magnification 400×, HE*

β-LAP: free β-lapachone; HE: hematoxylin and eosin.

DISCUSSION

In developing countries, products based on medicinal plants are used indiscriminately, for being considered safe, as they are

TABLE 1 – Statistics of nuclear and cellular area of hepatocytes

Site	Statistics	β-LAP/ DpCS	Free β-LAP	Control group	*p* value
Nucleus	Mean (μm²)	7.09	5.59	7.96	$p^1 = 0.102$
	Standard deviation (μm²)	1.43	1.19	1.08	
	Coefficient of variation (%)	20.17	21.29	13.57	
	Median (μm²)	7.4	5.24	7.41	
Hepatocyte	Mean (μm²)	42.13[(a)]	24.14[(b)]	50.55[(c)]	$p^1 = 0.006^*$
	Standard deviation (μm²)	5.87	2.05	8.11	
	Coefficient of variation (%)	13.93	8.49	16.04	
	Median (μm²)	39.35	24.32	51.59	

β-LAP: free β-lapachone; DpCS: depolymerized low molecular weight chitosan; [1]by means of Kruskal-Wallis test with comparisons with the mentioned test[26, 27].
Observation: if letters in parentheses are distinct, significant difference between the corresponding groups is proved.

TABLE 2 – Statistics of capsular and glomerular areas

Site	Statistics	β-LAP/ DpCS	Free β-LAP	Control group	*p* value
Capsule	Number of slides	5	8	9	
	Mean (μm²)	433.05	605.47	505.99	$p^1 = 0.055$
	Standard deviation (μm²)	87.51	125.06	73.19	
	Coefficient of variation (%)	20.21	20.66	14.46	
	Median (μm²)	427.41	587.96	523.37	
Glomerulus	Number of slides	4	8	9	
	Mean (μm²)	279.57[(a)]	286.32[(a)]	374.04[(b)]	$p^1 = 0.026^*$
	Standard deviation (μm²)	62.61	61.31	72.72	
	Coefficient of variation (%)	22.4	21.41	19.44	
	Median (μm²)	281.63	271.41	396.76	

β-LAP: free β-lapachone; DpCS: depolymerized low molecular weight chitosan; [1]by means of Kruskal-Wallis test with comparisons with the mentioned test[26, 27].
Observation: if the letters in parentheses are distinct, significant difference between the corresponding groups is proved.

obtained from natural sources. Toxicity studies are necessary to supply scientific information for the safe use of herbal medicines, before they are consumed in alternative medicine[28].

The FTIR analysis is a useful technique to evaluate interaction and formation of conjugate between molecules of the drug and CS. Changes or alterations in the intensity of bands characteristic of pure substances are considered a possible existence of conjugate[29]. However, some changes are very subtle, requiring a careful interpretation of spectra. We observed that in the spectra of β-LAP/DpCS conjugate, in the region of stretching of OH, C-H, and C-O-C bonds, spectra were little modified, without significant difference from the spectra of isolated samples. The hypothesis is that carbonyl has not formed a C=N imine with amines of CS, what suggests there was no formation of conjugate in the chemical form, but they are believed to be physically attached.

β-LAP is a promising molecule of the lapachol-derivative group, being toxic for a variety of tumor cells, which are typically more susceptible to oxidative damage than normal cells[30]. Acute exposure of mice to it can produce unpredictable toxic effects, depending on the dose levels and administration via. In albino rats, a median lethal dose (DL_{50}) of 80 mg/kg β-LAP intraperitoneally is reported in the literature[31].

Medeiros (2020)[4] observed that the dose of 10 mg/kg β-LAP dissolved in 10% DMSO was not notably toxic for immunosuppressed mice, a week after the toxicity assays, while animals treated with 20 mg/kg presented lesions in the tail, which became necrotic. Analyses of possible histological alterations in organs such as liver and kidneys are important, when products of natural origin are used, besides new synthetic substances and formulations, aiming at investigating cell disorders caused by the drugs. These analyses are useful in confirming interferences with the normal activity of renal and hepatic cells[5].

The liver is an important organ that plays a significant role in the metabolism and detoxification of exogenous toxins and therapeutic agents[32, 33]. It is in the liver that drug metabolism and substance detoxification occur; therefore, verifying hepatic toxicity in pre-clinical assays is fundamental for the therapeutic use of new drugs. Lesions in this organ preclude the adoption of new therapies that could be efficient in the treatment of different diseases[34].

In histopathological analysis of the liver we can observe that in group G1 treated with free β-LAP, single dose of 80 mg/kg, there was vacuolization of cytoplasm and pyknotic nuclei, and some cells presented cellular extravasation, suggesting high toxicity of this drug in hepatic cells. In histopathology of group G2, little incidence of alteration was observed in the cytoplasmic membrane, cytoplasm, and nucleus, a picture very similar to the histology of the control animal, suggesting a hepatoprotective action of CS associated with β-LAP.

In the histomorphometric analysis of the area of hepatocyte nucleus, significant statistical differences were not observed between groups, although the findings indicate toxicological changes. However, in the histomorphometric analysis of hepatocyte area of G1 group animals, they presented significant difference when compared with the control animal and G2 group, confirming the findings of the present study. The hepatocytes of G2 group animals presented results similar to those of the control animal, that is, without cellular damage, reinforcing that CS has a hepatoprotective action. Histomorphometric studies of organs involved in the detoxification process are relevant, especially when one intends to evaluate the toxicity potential of new substances or formulations[35].

In the study performed by Omara[36], evaluating the cytotoxic effect of CS, 150 mg/kg and 300 mg/kg doses were tested, orally, in male and female albino Swiss mice for 35 days. The animals treated with the lowest dose did not present hepatic toxicity, compared with those treated with 300 mg/kg CS that presented degeneration, necrosis, vacuolated cytoplasm, and hemorrhagic hepatocytes. CS induces histopathological alterations in the liver just in high doses ≥ 300 mg/kg. In our study, doses of 80 mg/kg of CS conjugated with β-LAP were administered, what is below the lowest dose tested by Omara[36], therefore without toxicity. There are no reports in the literature about CS in lower doses than 150 mg/kg causing any toxic effect in the liver.

Hepatic diseases involve any physicochemical, biological, and morphological changes in the normal work of the liver. Reactive oxygen species (ROS) and free radicals have been demonstrated to play crucial roles in the pathogenesis of several hepatic diseases[37]. The β-LAP redox cycle leads to increased levels of highly reactive ROS catalyzed by NAD (P) H:quinone oxidoreductase 1 (NQO1), inducing extensive damage to deoxyribonucleic acid (DNA)[38, 39]. ROS production is known to induce cytotoxic lesions in tissues. Increased ROS and H_2O_2 deplete, subsequently, the supply of glutathione by oxidation, what could alter the activity of enzymes or vital proteins, resulting in lipid peroxidation and disorganization of proteins, and affecting cell capacity to protect themselves from free radicals, causing their death[40-42].

Several natural antioxidants have been studied aimed at reducing oxidative stress and protecting against hepatic damage. CS has attracted much attention as a hepatoprotective agent due to its antioxidative properties[43]. It can reduce oxidative stress, improve endogenous levels of antioxidants, and has an important effect on inhibition of lipid peroxidation, avoiding lesions in the liver[44]. Possibly because of these properties, it has inhibited the toxic effects of β-LAP in our study.

The administration of antituberculosis drugs caused significant increase ($p < 0.001$) in levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) in the serum of animals treated with isolated drugs. Co-administration of CS kept normal levels in the serum of treated animals, in comparison with rats of the control group, indicating the cytoprotective effect of CS[45, 46].

Great part of the armamentarium used in the treatment of cancer has a significant impact upon the gastrointestinal tract and kidneys, thus, histological observations are necessary for the detection of morphological alterations caused by antitumor compounds[35]. In the histopathological observation of kidneys, hyalinized glomeruli and hypertrophic tubules were observed in the animals of group G1.

Santana *et al*. (2016)[47], using a derivative of β-LAP, 2-hydroxy-3-anilino-1,4-naphthoquinone, in the dose of 1,000 mg/kg, observed tissue hyperemia in kidneys. Other studies also showed severe kidney damage, such as tubular necrosis in rats treated with naphthoquinone derivatives, such as 2-hydroxy-1,4-naphthoquinone[48], and 2-amino-1,4-naphthoquinone[49]. In our studies, similar results were observed, as processes characteristic of necrosis in kidneys and liver of animals treated with free β-LAP, possibly due to ROS reactions and free radicals generated by active β-LAP compounds.

Hypertrophic tubules can cause generalized tubular atrophy and decreased renal blood flow, resulting in chronic kidney disease. The hyalinized glomeruli can also cause generalized tubular atrophy, occasionally with hypertrophic tubules (with dilated lumens), corresponding to rare nephrons still functioning and the decreased renal blood flow[50].

The animals of group G2 presented renal histopathology similar to that of the control animal. However, in the histomorphometric analysis, statistical differences were not observed in the analyzed structures among the groups G1, G2, and the control animal.

Pesce (1998)[51] points out that the set of quantitative (histomorphometric) data of renal morphology is important to validate scientific findings. In the scientific literature, studies on renal histology and morphometry are abundant and significant, but there are few histomorphometric studies of kidneys treated with free β-LAP or with the use of CS associated with β-LAP. Studies related to harmful effects of β-LAP are still scarce, what impaired a wider analysis of the histopathological and histomorphometric findings of the present work.

At a study conducted to evaluate CS toxicity with doses of 150 and 300 mg/kg orally, in male and female mice for 35 days, by general histopathology kidney examination, hypercellularity and degeneration of glomeruli and tubules treated with the lowest dose were observed; the animals treated with the highest dose presented severe degeneration in relation to the control animal, besides significant difference ($p < 0.05$) from the levels of kidney markers (creatinine and urea) in a dose-dependent form[36]. Therefore, CS renal toxicity is related to administration of higher doses for a longer period, what did not happen in our study, in which 80 mg/kg were administered of the β-LAP/DpCS conjugate for 14 days orally, with no toxic findings in histology and histomorphometry.

CS has been extensively studied in the development of nanocarriers due to its unique and versatile physicochemical properties and its biodegradability[52]. The effects of its use in treatments for renal diseases, such as kidney failure, have been investigated. Patients on dialysis were treated with CS during four weeks, and their renal function improved significantly in relation to patients who did not receive CS supplementation[36]. Data revealed that the treatment with CS protects against kidney damage in animal models testing acute toxicity. The authors suggest that CS protects against severe renal lesions.

Histopathological results of kidney and liver reveal hypercellularity in glomeruli and degeneration in renal tubules, as well as decrease in hepatocytes. The morphometric study of these organs revealed toxicity statistically significant just in hepatocytes of animals treated with isolated β-LAP.

CONCLUSION

In the present study of acute toxicity in free and conjugated forms of β-LAP, when administered in a single dose of 80 mg/kg orally in Swiss mice, we found histopathological alterations in kidneys and liver just in animals of the group that received the free drug. The conjugated form did not induce morphometric and morphologic significant alterations, when compared with the control animal. However, in histomorphometric analyses, we observed that the area of hepatocytes and their cell nuclei presented statistically significant differences among animals treated with free β-LAP and β-LAP/DpCS conjugate. In renal preparations, difference was not verified among the tested groups. Therefore, decreased toxicity of β-LAP in the liver, after being conjugated, can be linked to the hepatoprotective property of CS.

ACKNOWLEDGEMENT

We thank Professors Ricardo Yara, Cláudia Sampaio, Eliete Cavalcante and Paloma Lys de Medeiros of the UFPE for assigning their facilities to the development and characterization of this study.

RESUMO

Introdução: *A β-lapachona (β-LAP), um potente agente antitumoral, tem uso terapêutico limitado devido a sua baixa solubilidade e elevada toxicidade. Uma possível estratégia para contornar esses inconvenientes pode ser a utilização de adjuvantes como a quitosana (QS), um polissacarídeo catiônico com propriedades biológicas, como biocompatibilidade e biodegradabilidade.* ***Objetivo:*** *Avaliar a ação adjuvante da QS como citoprotetor associada à β-LAP por meio de estudos de toxicidade aguda, verificando as alterações histopatológicas em órgãos como fígado e rins.* ***Métodos:*** *Um conjugado da β-LAP-QS foi preparado na proporção 1:1, administrado por via oral, com dose única da β-LAP de 80 mg/kg, em camundongos Swiss. Foram realizadas análises histomorfológicas e histomorfométricas dos rins e do fígado desses animais.* ***Resultados:*** *Nos estudos histomorfológicos dos grupos testados, observamos que os hepatócitos dos animais tratados com a droga livre apresentaram alterações morfológicas, como vacuolização do citoplasma, extravasamento celular, núcleos atípicos e picnóticos. Nesse mesmo grupo, os rins apresentaram aspectos granulosos sugestivos de glomerulonefrite. Essas alterações não foram encontradas no grupo-controle e nos animais tratados com a β-LAP conjugada com QS. Não houve diferença estatística nas análises histomorfométricas dos túbulos distais e dos glomérulos renais entre os três grupos analisados, mesmo com alterações histomorfológicas evidentes. Após estudos histomorfométricos, foi observado que a área dos hepatócitos e seus núcleos celulares apresentaram diferença estatística significativa entre os animais tratados com a β-LAP livre e o conjugado β-LAP-QS.* ***Conclusão:*** *A diminuição da toxicidade da β-LAP, após ser conjugada, pode estar relacionada com a propriedade hepatoprotetora da QS.*

Unitermos: *quitosana; toxicidade aguda; histologia; β-lapachona.*

REFERENCES

1. Coutre SE, Othus M, Powell B, et al. Arsenic trioxide during consolidation for patients with previously untreated low/intermediate risk acute promyelocytic leukaemia may eliminate the need for maintenance therapy. Br J Haematol [Internet]. 2014 May [cited 2014 Aug 14]; 165(4): 497-503. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24528179.

2. Jácomo RH, Melo RAM, Souto FR, et al. Clinical features and outcomes of 134 Brazilians with acute promyelocytic leukemia who received ATRA and anthracyclines. Haematologica [Internet]. 2007 Oct [cited 2014 Aug 18]; 92(10): 1431-2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18024380.

3. Schuerch AR, Wehrli W. Beta-lapachone, an inhibitor of oncornavirus reverse transcriptase and eukaryotic DNA polymerase-alpha. Inhibitory effect, thiol dependency and specificity. Eur J Biochem [Internet]. 1978 Mar [cited 2017 Feb 3]; 84(1): 197-205. Available at: http://doi.wiley.com/10.1111/j.1432-1033.1978.tb12157.x.

4. Medeiros CS, Pontes-Filho NT, Camara CA, et al. Antifungal activity of the naphthoquinone beta-lapachone against disseminated infection with Cryptococcus neoformans var. neoformans in dexamethasone-immunosuppressed Swiss mice. Brazilian J Med Biol Res [Internet]. 2010 Apr [cited 2016 May 13]; 43(4): 345-9. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-879X2010000400004&lng=en&nrm=iso&tlng=en.

5. Aucélio RQ, Peréz-Cordovés AI, Xavier Lima JL, Ferreira ABB, Esteva Guas AM, da Silva AR. Determination of lapachol in the presence of other naphthoquinones using 3MPA-CdTe quantum dots fluorescent probe. Spectrochim Acta - Part A Mol Biomol Spectrosc [Internet]. 2013; 100: 155-60. Available at: http://dx.doi.org/10.1016/j.saa.2012.04.020.

6. Menna-Barreto RFS, Corrêa JR, Pinto AV, Soares MJ, de Castro SL. Mitochondrial disruption and DNA fragmentation in Trypanosoma cruzi induced by naphthoimidazoles synthesized from β-lapachone. Parasitol Res [Internet]. 2007 Sep 2 [cited 2017 Feb 3]; 101(4): 895-905. Available at: http://link.springer.com/10.1007/s00436-007-0556-1.

7. Silva MDS, Cocenza DS, Grillo R, et al. Paraquat-loaded alginate/chitosan nanoparticles: preparation, characterization and soil sorption studies. J Hazard Mater [Internet]. 2011; 190(1-3): 366-74. Available at: http://dx.doi.org/10.1016/j.jhazmat.2011.03.057.

8. Sitônio MM, de Carvalho Jr CHR, Campos IA, et al. Anti-inflammatory and anti-arthritic activities of 3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-b]pyran-5,6-dione (β-lapachone). Inflamm Res [Internet]. 2013 Jan 28 [cited 2017 Feb 3]; 62(1): 107-13. Available at: http://link.springer.com/10.1007/s00011-012-0557-0.

9. Bey EA, Bentle MS, Reinicke KE, et al. An NQO1- and PARP-1-mediated cell death pathway induced in non-small-cell lung cancer cells by betalapachone. Proc Natl Acad Sci USA [Internet]. 2007; 104(28): 11832-7. Available at: https://www.ncbi.nlm.nih. gov/pmc/articles/PMC1913860/.

10. Moon D-O, Kang C-H, Kim M-O, et al. β-lapachone (LAPA) decreases cell viability and telomerase activity in leukemia cells: suppression of telomerase activity by LAPA. J Med Food [Internet]. 2010 Jun [cited 2017 Feb 3]; 13(3): 481-8. Available at: http://www.liebertonline.com/doi/abs/10.1089/jmf.2008.1219.

11. Aires ADL, Ximenes ECPA, Barbosa VX, Góes AJDS, Souza VMO, Albuquerque MCPDA. β Lapachone: a naphthoquinone with promising antischistosomal properties in mice. Phytomedicine [Internet]. 2014; 21(3): 261-7. Available at: http://dx.doi.org/10.1016/j.phymed.2013.08.012.

12. Moreno E, Schwartz J, Larrea E, et al. Assessment of β-lapachone loaded in lecithin-chitosan nanoparticles for the topical treatment of cutaneous leishmaniasis in L. major infected BALB/c mice. Nanomedicine [Internet]. 2015; 11(8): 2003-12. Available at: http://dx.doi.org/10.1016/j.nano.2015.07.011.

13. Yang R-Y, Kizer D, Wu H, et al. Synthetic methods for the preparation of ARQ 501 (beta-lapachone) human blood metabolites. Bioorg Med Chem [Internet]. 2008 May 15 [cited 2017 Feb 3]; 16(10): 5635-43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18424157.

14. Park J-S, Lee Y-Y, Kim J, Seo H, Kim H-S. β-LAPachone increases phase II antioxidant enzyme expression via NQO1-AMPK/PI3K-Nrf2/ARE signaling in rat primary astrocytes. Free Radic Biol Med [Internet]. 2016 May 27 [cited 2016 Jun 6]; Available at: http://www.sciencedirect.com/science/article/pii/S0891584916302714.

15. Laranjeira MCM, Fávere VT. Biopolímero funcional com potencial industrial biomédico. Quim Nov. 2009; 32(3): 672-8.

16. Shu XZ, Zhu KJ. The influence of multivalent phosphate structure on the properties of ionically cross-linked chitosan films for controlled drug release. Eur J Pharm Biopharm [Internet]. 2002 Sep [cited 2015 Mar 10]; 54(2): 235-43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12191697.

17. Lee M-K, Chun S-K, Choi W-J, et al. The use of chitosan as a condensing agent to enhance emulsion-mediated gene transfer. Biomaterials [Internet]. 2005 May [cited 2015 Mar 10]; 26(14): 2147-56. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15576190.

18. Cavalcante FA, Silva JLV, Carvalho VMN, et al. Spasmolytic activity of lapachol and its derivatives, α and β-lapachone, on the guinea-pig ileum involves blockade of voltage-gated calcium channels. Rev Bras Farmacogn [Internet]. 2008 Jun [cited 2017 Feb 10]; 18(2): 183-9. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-695X2008000200007&lng=en&nrm=iso&tlng=en.

19. Janes KA, Alonso MJ. Depolymerized chitosan nanoparticles for protein delivery: preparation and characterization. J Appl Polym Sci [Internet]. 2003 Jun 20 [cited 2016 Apr 27]; 88(12): 2769-76. Available at: http://doi.wiley.com/10.1002/app.12016.

20. Almeida LL, Luís D, Lucio L, Cássia V, Almeida F. Efeito do extrato aquoso de Dioclea grandiflora Mart. ex Benth. nos rins de camundongos. Mus Musculus Linnaeus. 2013; 59-63.

21. Cavalcanti IMF, Mendonça EM, Lira MCB, et al. The encapsulation of β-lapachone in 2-hydroxypropyl-β-cyclodextrin inclusion complex into liposomes: a physicochemical evaluation and molecular modeling approach. Eur J Pharm Sci [Internet]. 2011; 44(3): 332-40. Available at: http://dx.doi.org/10.1016/j.ejps.2011.08.011.

22. Cunha-Filho MSS, Dacunha-Marinho B, Torres-Labandeira JJ, Martinez-Pacheco R, Landin M. Characterization of β-lapachone and methylated β-cyclodextrin solid-state systems. AAPS Pharm Sci Tech. 2007; 8(3): E68-77.

23. Costa ES, Mansur HS. Preparação e caracterização de blendas de quitosana/poli (álcool vinílico) reticuladas quimicamente com glutaraldeído para aplicação em engenharia de tecido. Quim Nova. 2008; 31(6): 1460-6.

24. Luo Y, Teng Z, Li Y, Wang Q. Solid lipid nanoparticles for oral drug delivery: chitosan coating improves stability, controlled delivery, mucoadhesion and cellular uptake. Carbohydr Polym [Internet]. 2015; 122: 221-9. Available at: http://dx.doi.org/10.1016/j.carbpol.2014.12.084.

25. Anatpat. UNICAMP [Internet]. [cited 2016 Mar 22]. Available at: http://anatpat.unicamp.br/lamuro6.html.

26. Altman DG. Practical statistics for medical research. Chapman and Hall; 1991. 611p.

27. Conover WJ. Practical nonparametric statistics. Wiley; 1971. 462p.

28. de Witte NV, Stoppani AOM, Dubin M. 2-Phenyl-β-lapachone can affect mitochondrial function by redox cycling mediated oxidation. Arch Biochem Biophys. 2004; 432(2): 129-35.

29. Cirri M, Rangoni C, Maestrelli F, Corti G, Mura P. Development of fast-dissolving tablets of flurbiprofen-cyclodextrin complexes. Drug Dev Ind Pharm [Internet]. 2005 Jan 26 [cited 2017 Feb 8]; 31(7): 697-707. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16207617.

30. Li Y, Sun X, LaMont JT, Pardee AB, Li CJ. Selective killing of cancer cells by beta-lapachone: direct checkpoint activation as a strategy against cancer. Proc Natl Acad Sci U S A. 2003; 100(5): 2674-8.

31. de Santana CF, de Lima OG, d'Albuquerque IL, Lacerda AL, Martins DG. [Antitumoral and toxicological properties of extracts of bark and various wood components of Pau d'arco (Tabebuia avellanedae)]. Rev Inst Antibiot (Recife) [Internet]. 1968 Dec [cited 2017 Feb 13]; 8(1): 89-94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5761014.

32. Ramachandra Setty S, Quereshi AA, Viswanath Swamy AHM, et al. Hepatoprotective activity of Calotropis procera flowers against paracetamol-induced hepatic injury in rats. Fitoterapia [Internet]. 2007 Dec [cited 2017 Feb 5];78(7–8):451–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17600635.

33. Ram VJ. Herbal preparations as a source of hepatoprotective agents. Drug News Perspect [Internet]. 2001 Aug [cited 2017 Feb 5]; 14(6): 353-63. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12813598

34. Dudea M, Clichici S, Olteanu DE, Nagy A, Cucoş M, Dudea S. Usefulness of real-time elastography strain ratio in the assessment of bile duct ligation-induced liver injury and the hepatoprotective effect of chitosan: an experimental animal study. Ultrasound Med Biol. 2015; 41(1): 114-23.

35. da Silva IB, Lima IR, Santana MAN, Leite RMP, Leite SP. Indigofera suffruticosa Mill (Fabaceae): hepatic responses in mice bearing sarcoma 180. Int J Morphol [Internet]. 2014 Dec [cited 2017 Feb 5]; 32(4): 1228-33. Available at: http://www.scielo.cl/scielo.php?script=sci_arttext&pid=S0717-95022014000400017&lng=en&nrm=iso&tlng=en.

36. Omara EA, Aly HF, Nada SA. Chitosan induced hepato-nephrotoxicity in mice with special reference to gender effect in glycolytic enzymes activities. Regul Toxicol Pharmacol [Internet]. 2012; 62(1): 29-40. Available at: http://dx.doi.org/10.1016/j.yrtph.2011.11.010.

37. Albano E. Oxidative mechanisms in the pathogenesis of alcoholic liver disease. Mol Aspects Med [Internet]. 2008 Feb [cited 2017 Feb 5]; 29(1-2): 9-16. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18045675.

38. Pink JJ, Planchon SM, Tagliarino C, Varnes ME, Siegel D, Boothman DA. NAD(P)H:Quinone oxidoreductase activity is the principal determinant of beta-lapachone cytotoxicity. J Biol Chem [Internet]. 2000 Feb 25 [cited 2017 Feb 5]; 275(8): 5416-24. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10681517.

39. Huang G, Chen H, Dong Y, et al. Superparamagnetic iron oxide nanoparticles: amplifying ROS stress to improve anticancer drug efficacy. Theranostics. 2013; 3(2): 116-26.

40. Mohamed AM, Metwally NM, Mahmoud SS. Sativa seeds against Schistosoma mansoni different stages. Mem Inst Oswaldo Cruz [Internet]. 2005 Apr [cited 2017 Feb 5]; 100(2): 205-11. Available at: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762005000200016&lng=en&nrm=iso&tlng=en.

41. Kuntz AN, Davioud-Charvet E, Sayed AA, et al. Thioredoxin glutathione reductase from Schistosoma mansoni: an essential parasite enzyme and a key drug target. Loukas A, editor. PLoS Med [Internet]. 2007 Jun 19 [cited 2017 Feb 5]; 4(6): e206. Available at: http://dx.plos.org/10.1371/journal.pmed.0040206.

42. Seif el-Din SH, Al-Hroob AM, Ebeid FA. Schistosoma mansoni: N-acetylcysteine downregulates oxidative stress and enhances the antischistosomal activity of artemether in mice. Exp Parasitol [Internet]. 2011 Jul [cited 2017 Feb 5]; 128(3): 230-5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21426905.

43. Ozcelik E, Uslu S, Erkasap N, Karimi H. Protective effect of chitosan treatment against acetaminophen-induced hepatotoxicity. Kaohsiung J Med Sci [Internet]. 2014; 30(6): 286-90. Available at: http://dx.doi.org/10.1016/j.kjms.2014.02.003.

44. Jeon TI, Hwang SG, Park NG, et al. Antioxidative effect of chitosan on chronic carbon tetrachloride induced hepatic injury in rats. Toxicology. 2003; 187(1): 67-73.

45. Santhosh S, Sini TK, Anandan R, Mathew PT. Effect of chitosan supplementation on antitubercular drugs-induced hepatotoxicity in rats. Toxicology. 2006; 219: 53-9.

46. Singh C, Jodave L, Bhatt TD, Gill MS, Suresh S. Hepatoprotective agent tethered isoniazid for the treatment of drug-induced hepatotoxicity: synthesis, biochemical and histopathological evaluation. Toxicol Reports [Internet]. 2014; 1: 885-93. Available at: http://dx.doi.org/10.1016/j.toxrep.2014.10.001.

47. Santana V, Pereira DS, Bruno C, et al. Cytotoxicity, hemolysis and in vivo acute toxicity of 2-hydroxy-3-anilino-1, 4-naphthoquinone derivatives. Toxicol Reports [Internet]. 2016; 3: 756-62. Available at: http://dx.doi.org/10.1016/j.toxrep.2016.09.007.

48. Munday R, Smith BL, Fowke EA. Haemolytic activity and nephrotoxicity of 2-hydroxy-1,4-naphthoquinone in rats. J Appl Toxicol [Internet]. 1991 Apr [cited 2017 Feb 8]; 11(2): 85-90. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2061555.

49. Munday R, Smith BL, Munday CM. Effect of inducers of DT-diaphorase on the toxicity of 2-methyl- and 2-hydroxy-1,4-naphthoquinone to rats. Chem Biol Interact [Internet]. 1999 Dec 15 [cited 2017 Feb 8]; 123(3): 219-37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10654840.

50. Anatpat. UNICAMP [Internet]. [cited 2016 Mar 22]. Available at: http://anatpat.unicamp.br/lamuro9.html.

51. Pesce C. Glomerular number and size: facts and artefacts. Anat Rec [Internet]. 1998 May [cited 2017 Feb 13]; 251(1): 66-71.

52. Tao Y, Han J, Dou H. Paclitaxel-loaded tocopheryl succinate-conjugated chitosan oligosaccharide nanoparticles for synergistic chemotherapy. J Mater Chem [Internet]. 2012 Apr 10 [cited 2016 Jun 6]; 22(18): 8930. Available at: http://pubs.rsc.org/en/content/articlehtml/2012/jm/c2jm30290j.

CORRESPONDING AUTHOR

Rosa Valéria S. Amorim

Departamento de Histologia e Embriologia; Universidade Federal da Pernambuco; CEP: 50670-901; Recife-PE, Brasil; Phone: +55 (81) 2126-8516; e-mail: rosa.amorim@ufpe.br.