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Antitumor activity of *Cratylia mollis* lectin encapsulated into liposomes

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Abstract

The hemagglutinating (HA) activity of *Cratylia mollis* lectin (Cra) was evaluated and the influence of ultrasound and mechanical agitation on its activity examined. The antitumor activity of Cra-loaded liposomes was also investigated. Liposomes were obtained by the lipid thin film method. Physicochemical characterization was carried out and long-term stability of Cra-loaded liposomes assessed. Antitumor activity of Cra-loaded liposomes was investigated against Sarcoma 180 in Swiss mice. The treatment was performed intraperitoneally (7 mg/kg body weight per day) for 7 days. Histopathological analyses of tumor, liver, spleen and kidneys were carried out after treatment of the animals. The results showed that Cra–HA activity is affected under ultrasound exposure. However, Cra was successfully encapsulated into liposomes and the activity of the lectin was preserved despite the use of ultrasound in the liposome preparation. Cra-loaded liposomes were produced with an 84% encapsulation ratio (700 μg/ml) and a tumor inhibition of 71% was achieved. The encapsulation of Cra produced a decrease in its tissue toxicity and improved its antitumor activity. In particular, histopathological analysis revealed that treatment with Cra-loaded liposomes prevented Cra cytotoxicity in the liver and kidney of animals.

Keywords: Lectin; *Cratylia mollis*; Liposomes; Cytotoxicity; Antitumor activity

1. Introduction

Lectins are carbohydrate-binding proteins of non-immune origin from microorganisms, animals or plants. They are multivalent molecules, possessing two or more sugar-binding sites for agglutinating cells, and for precipitating polysaccharides, glycoproteins, peptidoglycans, teichoic acids and glycolipids (Liener et al., 1986). In general two approaches have been applied for introducing proteins into therapy. On the one hand, the protein itself presents biological activity and can be encapsulated into liposomes (Meyer et al., 1994). On the whole lectins present antitumor activity, possibly mediated by an immunomodulatory

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action (Wang et al., 1996; Haas et al., 1999; Karasaki et al., 2001; Timoshenko et al., 2001). On the other, proteins, specifically lectins, could also be binding to the surface of nanodevices for site-specific drug targeting (Bendas et al., 1997; Forssen and Willis, 1998; Wirth et al., 1998; Yamazaki et al., 2000; Kompella and Lee, 2001).

Cratylia mollis lectin (Cra) belongs to the mannose/glucose-binding class of lectins and has been purified and characterized from the seeds of a plant (*Camaratu bean*) native to the northeast of Brazil (Correia and Coelho, 1995). Cra has shown strong binding to human malignant cancerous tissues, particularly to those of the mammary glands, uterus and brain (Beltrão et al., 1998). Nevertheless, it has presented cytotoxicity against Hep-2 cells and hepatotoxicity (unpublished data).

Bearing in mind a potential application of Cra in the field of pharmaceutical nanotechnology, either as an active agent or as a surface binding-molecule for drug targeting, previous studies of Cra behavior at the air–water interface were carried out using surface tension measurements (Baszkin et al., 2000). The ability of Cra to penetrate phospholipid monolayers (Lipoid E80^{TM}, 80% phosphatidylcholine and Epikuron 200^{TM}, 96.5% phosphatidylcholine) was ascertained. The kinetics of Cra interfacial adsorption and lectin–lipid interactions data revealed that Cra is a fairly stable protein and exhibits typical protein adsorption patterns.

The use of new bioactive proteins in medical treatment depends on the development of drug delivery systems to overcome stability drawbacks related to these macromolecules. Proteins and peptides are rapidly cleared from the circulation when administered intravenously or subcutaneously, requiring frequent injection in order to maintain therapeutic levels. The pharmaceutical nanotechnology of colloidal drug carriers, especially liposomes, is particularly useful for formulating new drug derived from biotechnology (peptides, proteins, genes and oligonucleotides) because it enables drug to be kept safe from degradation in biological fluids and can lead to their penetration into cells (Barrat, 2000).

In recent years immune-stimulating effects of certain lectins have been the focus of considerable interest for application in cancer biotherapy. In vitro, a galactose-specific lectin from *Viscum album* has been reported to upregulate the gene expression and secretion of proinflammatory cytokines including interleukin (IL)-1-α, IL-1-β, IL-6, IL-12, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and granulocyte macrophage colony-stimulating factor (GM-CSF) by human leucocytes (Timoshenko et al., 2001).

Disseminated neoplasic disease and the development of drug resistance remain the major complications of cancer, leading ultimately to the failure of treatment. The use of liposomes in cancer therapy has proved promising due to their ability to extravasate at sites of active tumor growth (Gabius et al., 1994). More recently, the intraperitoneal administration of liposomes has been shown to prolong the drug's circulation in the blood, owing to their slow absorption from the abdominal cavity. Furthermore, the low clearance of liposomes from this cavity prolongs the contact between tumor cells and the encapsulated drug, thereby producing an increase in antitumor activity (Sadzuka et al., 2000).

On the basis of previous available figures about Cra's surface properties and biological activity, the present study was undertaken to further investigate the efficacy of Cra encapsulation into liposomes with regard to its antitumor activity. The influence of liposome preparation methods on the stability of Cra was considered through the hemagglutinating (HA) activity assay. Furthermore, the antitumor activity of Cra-loaded liposomes against Sarcoma 180 was evaluated.

2. Materials and methods

2.1. Materials

Soya phosphatidylcholine (PC) (Epikuron 200, 98% PC) was obtained from Lucas Meyer (Hamburg, Germany). Cholesterol, stearylamine and bovine serum albumin (BSA) with 98% purity levels were purchased from Sigma (St. Louis, USA). Chloroform, methanol, potassium phosphate, sodium hydroxide and all analytical grade reagents were purchased from Merck (Darmstadt, Germany). *C. mollis* lectin was purified from seeds of *C. mollis* Mart. (*Camaratu bean*) seeds and characterized at the Glycoprotein Laboratory of the Federal University of Pernambuco, Brazil (Correia and Coelho, 1995).

2.2. Methods

2.2.1. Stability evaluation of Cra in solution after exposure to ultrasound and mechanical agitation

A preliminary assay was performed to ascertain the effect of ultrasound and mechanical agitation on Cra–HA activity using glutaraldehyde-treated rabbit erythrocytes. Cra in solution (5 ml) was submitted to continuous mode ultrasonication using an ultrasound probe (Vibra Cell, BRASON, USA) operating at 200 W, 40 Hz and 4 °C. Samples of Cra solution (5 ml) were submitted to mechanical agitation using a shaker (Polytest® 20 Bioblock Scientific, France) operating at 150 strokes per min at 37 °C for 24 or 48 h. After these procedures the hemagglutinating activity was measured using Cra samples (50 μl) serially diluted twofold in 0.15 M NaCl, in microtiter dishes. Rabbit erythrocyte suspension (2.5%, v/v, in 0.15 M NaCl, 50 μl) previously treated with glutaraldehyde (1%) was added and the titer read after 45 min (Correia and Coelho, 1995). A positive control assay was performed using carbohydrate binding specifically to Cra (glucose or mannose) in microtiter dishes. Initially, 100 μl of a solution containing either glucose or mannose (0.2 M) in 0.15 M sodium chloride was mixed with 100 μl of lectin preparation (7 μg/ml). The inhibition of the Cra–HA activity was then verified in identical conditions for the determination of Cra–HA activity.

2.2.2. Formulation of Cra-loaded liposomes

Liposomes were obtained using a modified version of the method of Amselen et al. (1990), based on a lipid film formed by the evaporation of solvents from a phospholipid organic solution. Positively surface-charged liposomes were prepared in the following manner: initially the lipid constituents, soybean–phosphatidylcholine, cholesterol and stearylamine (7:2:1, 36 μmol lipids per 10 μl 0.2 M phosphate buffer solution, pH 7.4) were dissolved in chloroform/methanol 3/1 (v/v) under magnetic agitation (150 rpm/min, 15 min). Next, the organic solution was subjected to evaporation under reduced pressure (25 min at 40 ± 1 °C) and agitation at 120 rpm to remove solvents. The dried lipid film was hydrated through the addition of an aqueous phase (10 ml) consisting of 0.2 M phosphate buffer solution (pH 7.4) containing Cra at 700 μg/ml, leading to multilamellar vesicle (MLV) formation. The suspension was then kept under magnetic stirring (40 min). Large unilamellar vesicles (LUV) were obtained by MLV ultrasonication using a probe (Vibra Cell, BRASON, USA) at 200 W with 40 Hz for 250 s. Finally the suspension of liposomes was stored at 4 °C.

2.2.3. Physicochemical characterization and stability assessment of Cra-loaded liposomes

The physicochemical analysis of liposomes was carried out immediately after preparation and subsequently at regular intervals. Several features such as the macroscopic and microscopic appearance, morphological examination, pH changes and encapsulation ratio were analyzed. Morphological properties of Cra-loaded liposomes were analyzed by scanning electron microscopy (SEM). A sample of 10 μl of Cra-loaded liposomes was placed on a surface of a glass plate, which was then stuck onto a SEM stub with a carbon membrane (STR tape, Shinto Paint, Japan). Afterward, it was subjected to colloidal gold metallization using a fine coat ion sputter (BalTec SCD 050) and examined using a scanning electron microscope (JSM-5900, JEOL, Japan) at 5 kV. The mean size of the vesicle was estimated through vesicle counting using build-in software, available in the JSM-5900-JEOL apparatus.

Formulated liposomes were submitted to both accelerated and long-term stability tests with the aim of ascertaining the durability of the formulation. The purpose of accelerated stability testing is to subject preparations to stressed conditions in order to simulate processes such as sterilization, transport and storage. Liposomes were subjected to centrifugation at 1300 × *g* for 1 h (KN-70 Centrifuge, Kubota, Japan), mechanical stress at 150 strokes per min at 37 °C during 48 h (Polytest® 20 Bioblock Scientific) and freeze-thawing cycles (16 h at −18 ± 1 °C and 8 h at 25 ± 1 °C). Long-term stability of liposome suspensions was examined for samples stored at 4 ± 1 °C. The physicochemical properties of the preparations were evaluated at 7, 15, 30, 45 and 60 days or until signs of instability appeared.

The lectin was quantified by the Peterson–Lowry colorimetric method (Peterson, 1977) using UV spectroscopy at 280 nm. This method was chosen to avoid lipid interference in the protein assay. The BSA was used as the standard protein for deriving

calibration curves at concentrations ranging from 10 to 100 μg/ml.

The encapsulation ratio of lectin into liposomes was determined after ultrafiltration/ultracentrifugation at 133 570 × *g* using Ultrafree® units (Millipore, USA). The Cra concentration in the supernatant was determined as previously described and the Cra encapsulation ratio was then calculated on the basis of the total Cra content in the liposome formulation.

2.2.4. Antitumor activity of Cra-loaded liposomes

Studies were carried out in Swiss albino male mice for the purpose of investigating the in vivo antitumor activity of Cra-loaded liposomes against Sarcoma-180. Tumor ascytic cells were diluted to 5.5×10^6 cells/ml with phosphate buffer saline and subcutaneously injected into the inguinal area of mice. Four groups of 10 animals (body weight 20–25 g, 35–40-days-old) were randomly treated with i.p. injections of free Cra solution and Cra-loaded liposomes (7 mg/kg per day for 7 days) 24 h after inoculation with tumor cells. The control groups were treated with phosphate buffer saline (pH 7.4) and empty liposomes daily for seven days. After treatment, the animals were sacrificed and solid tumors excised and weighed. Tumor inhibition was expressed as the mean of body weights of tumor for the treated animal group (T) in comparison to the untreated control group (C). The tumor inhibition activity was then calculated according to: percentage tumor inhibition $= ((C - T)/C) \times 100$ (Wang et al., 1996). Animal experiments were performed according to the National Cancer Institute (NCI) protocol (Geran et al., 1972) with the approval of the Ethics Committee for Animal Experimental Assays of the Federal University of Pernambuco, Brazil.

2.2.5. Histopathological analysis

Sarcoma 180 and animal organs (liver, spleen and kidneys) were submitted to histological analysis after treatment. Tissue samples were preserved in a 10% (v/v) buffer–formalin solution and subsequently immersed in paraffin. Slices of sample tissues (4 μm) were prepared and a fixation with hematoxylin and eosin staining was developed before light microscopy examination (Olympus BH-2, Japan).

3. Results and discussion

3.1. Stability of Cra in solution after exposure to ultrasound and mechanical agitation

The influence of the exposure time of ultrasonic agitation and mechanical stress on Cra–HA activity, expressed as logarithmic values, was evaluated as shown in Table 1. As depicted, a time–effect response of HA activity after submission of Cra to stressful conditions such as long ultrasound exposure and mechanical agitation was observed. A reduction in Cra–HA activity after sonication for 250 s was observed. However, no further alterations were detected for longer periods of ultrasound exposure. Despite the small numerical difference between HA activity values, it should be borne in mind that each 0.3 log unit corresponds to double the concentration required to produce the same lectin HA activity.

In the inhibition of HA activity trial, the agglutination of rabbit erythrocytes by Cra was entirely inhibited by glucose or mannose at the minimum concentration of 0.2 M, showing that the lectin HA activity was blocked due to the binding of Cra to its specific sugars. The results thus showed that the HA activity of Cra in buffer solution is affected under ultrasound exposure.

3.2. Preparation of Cra-loaded liposomes

Optimization studies were performed varying the formulation parameters with the aim of obtaining stable liposome preparations containing encapsulated

Table 1
Effects of ultrasound exposure and mechanical stress on the hemagglutinating (HA) activity of Cra in solution

	Ultrasonic exposure (continuous mode, 200 W)				Mechanical stress (150 strokes per min)		
Time of exposure	0 s	100 s	250 s	500 s	0 h	24 h	48 h
HA activity (log)	3.01	3.01	2.71	2.71	3.01	3.01	2.71

Cra. In order to avoid auto-oxidation and hydrolysis of phospholipids and to maintain the optimal pH range (from 5.4 to 7.4) for Cra stability, a 0.2 M pH 7.4 phosphate buffer solution was chosen as the aqueous phase. Several batches of positively surface-charged liposomes containing Cra at different concentrations were obtained and evaluated according to their accelerated and long-term stability.

Cra–HA activity in solution was affected by ultrasound exposure. The entrapment of Cra into liposomes, however, preserved its HA activity despite the fact that liposome preparation involved a stage of sonication. A highly efficient Cra encapsulation (84%) into liposomes was achieved for an initial concentration of 700 μg/ml. The Cra encapsulation ratio was remarkable considering that Cra-loaded liposomes are positively surface-charged, which generally produce a low protein encapsulation ratio as previously reported (Van Slooten et al., 2001). In fact, the authors found a very low association efficiency of 9% for interferon-γ with liposomes consisting of egg phosphatidylcholine and stearylamine. Moreover, our results are in agreement with those reported by Meyer et al. (1994), even though interferon-γ-loaded liposomes are negatively surface-charged. In fact, they found 90% encapsulation ratio of granulocyte colony stimulating factor (rhG-CSF, 4 mg/ml) in liposomes prepared with a mixture of 0.03 M of lipids (dimyristoylphosphatidylglycerol, distearoyl phosphatidylcholine and cholesterol) by using the lipid film hydration method followed by freeze-thaw cycles.

3.3. Physicochemical characterization and stability assessment of Cra-loaded liposomes

Without exception, Cra-loaded liposome formulations presented an initially similar macroscopic appearance of fluid colloidal yellowish suspension, ranging from opaque to clear, with a bluish appearance reflection. This latter attribute was taken for granted as preliminary evidence for the formation of stable nanometric liposomes. The SEM morphological analysis showed spherical-shaped and well-dispersed vesicles (Fig. 1). The mean size of the vesicle was estimated at 127 ± 5 nm.

Liposome suspensions were submitted to mechanical stress testing. It was observed that liposomes maintained their stability after transport simulation

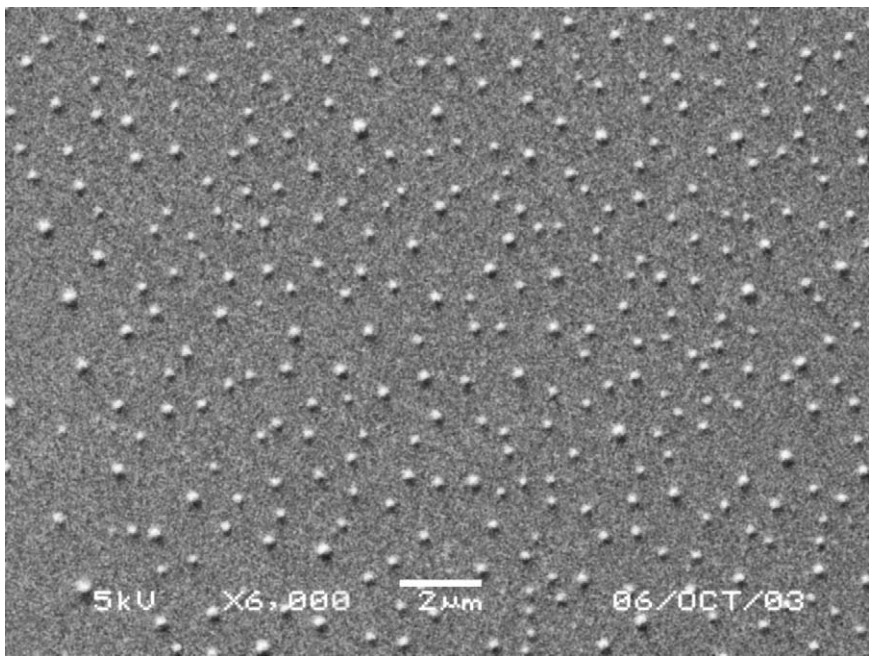

Fig. 1. Scanning electron micrograph of Cra liposomes (6000×).

testing by mechanical agitation. Furthermore, they remained stable without noticeable physicochemical changes after centrifugation or under freeze-thaw cycles. The formulations presented the same macroscopic appearance and holding stability even after the sixth freeze-thaw cycle.

The stability of liposome suspensions stored at $4 \pm 1\,^{\circ}C$ was maintained for over 24 months. After this period the suspension of liposomes presented signs of instability such as creaming, lipidic aggregates and loss of protein content. A slight gradual decrease in pH values was observed from 7.58 to 7.40 over a period of 60 days for liposome suspension stored at $4 \pm 1\,^{\circ}C$. This might indicate a slow lipid degradation that induced fatty acid formation. Pontes et al. (1999) have already shown that the main degradation pathway for a lipidic medium leads to the formation of fatty acids, impairing electrical conductivity and gradually reducing the pH of preparations.

The adjustment of the initial pH minimizes the phospholipid hydrolysis rate. Although such a procedure has been routinely used to control pH variation of liposome suspensions, it was not effective in the case of Cra-loaded liposomes. The phosphate buffer solution chosen as the aqueous phase was not sufficient to prevent the pH decrease in liposome suspension under storage conditions.

3.4. Antitumor activity of Cra loaded-liposomes

In the current study the effect of the intraperitoneal administration of Cra-loaded liposomes on the

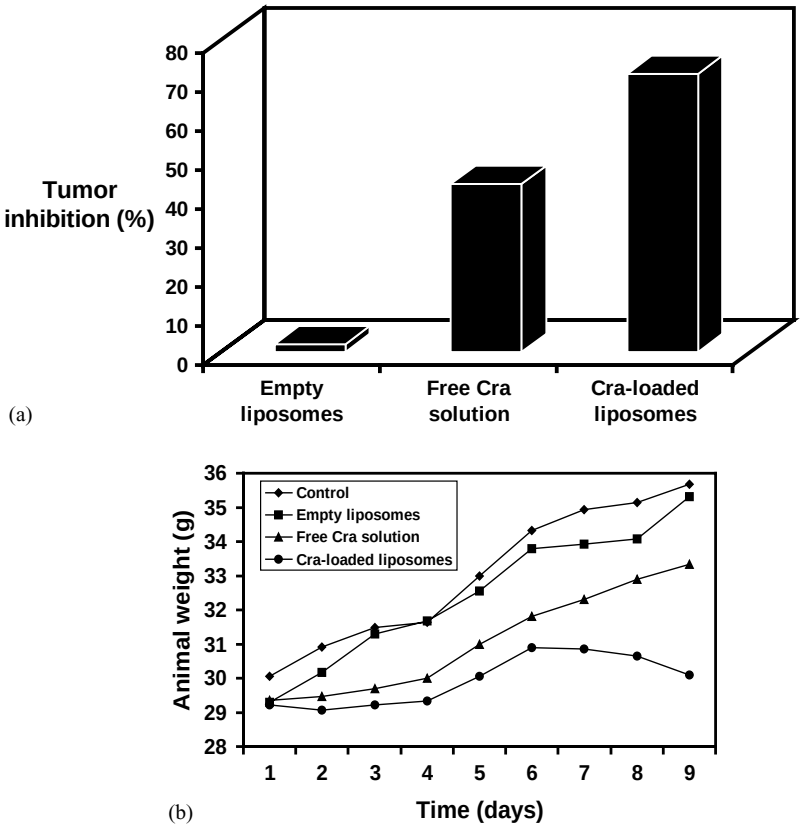

Fig. 2. Evaluation of the effect of free *Cratylia mollis* lectin (Cra) in solution and Cra-loaded liposomes on the inhibition of mice-bearing Sarcoma 180 (5×10^6 inoculated cells/animal). Animal treatment regimens are performed as follows (7 mg/kg per day for 7 days, 10 animals/group): control received saline solution (Group 1), empty liposomes (Group 2), free Cra solution (Group 3), and Cra-loaded liposomes (Group 4). Cra activity was assessed on: tumor inhibition in comparison with the control group (a); and body weight of treated animals (b).

tumor inhibition of Sarcoma 180-bearing mice was examined (Fig. 2). Tumors underwent a remarkable shrinkage under Cra treatment, which reflects an anti-tumor action on the part of the lectin. Measurements of tumor weight for the experimental animal groups were carried out. Broad data dispersion was observed, especially in the control group. This discrepancy can be explained by the individual animal response to the implanted tumor. However, the treatment with Cra-loaded liposomes produced a much more uniform response with tumor weights ranging from 0.383 to 0.498 g (S.D. 0.029). A dramatic shrinkage

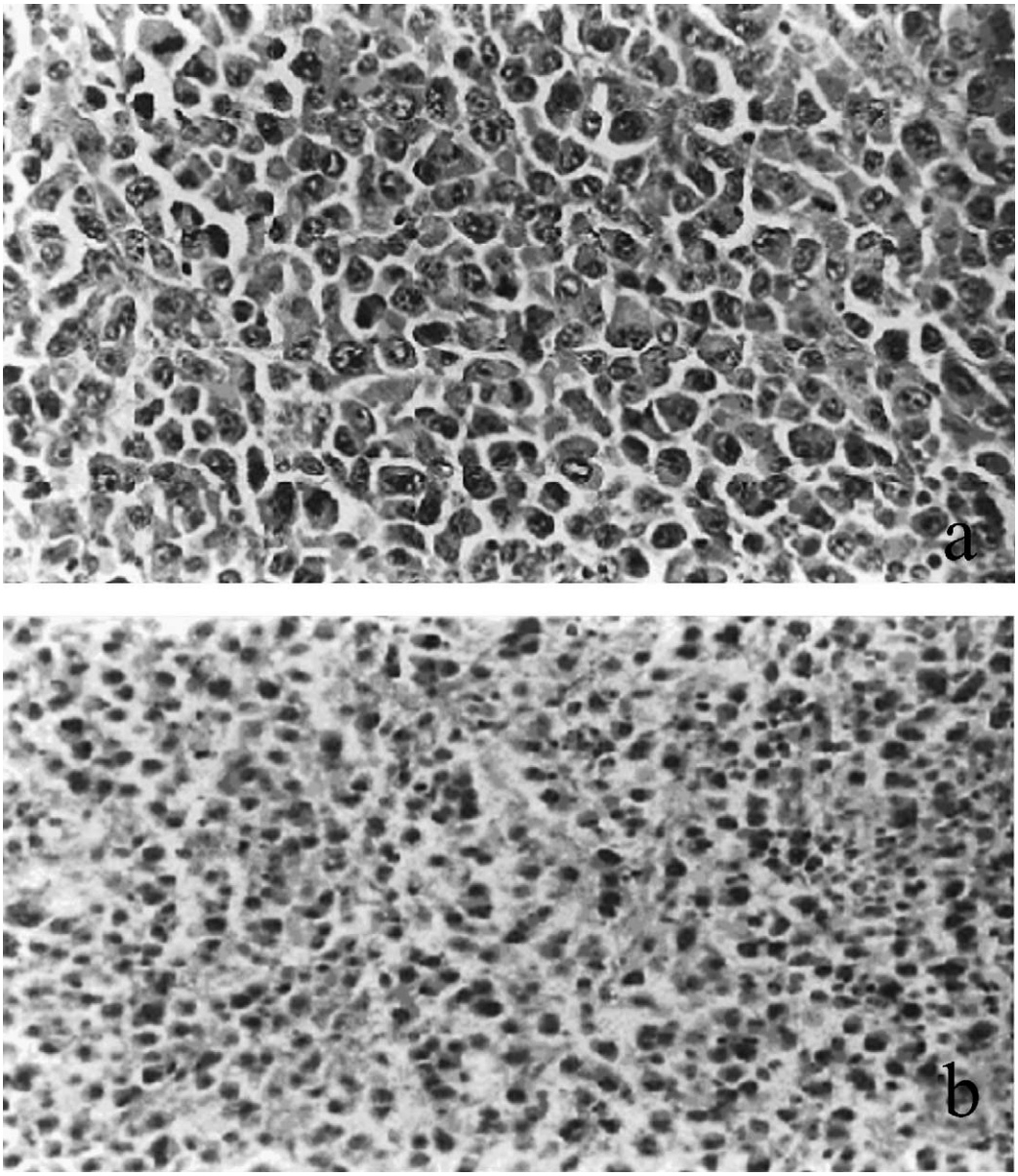

Fig. 3. Morphological appearance of Sarcoma 180 after treatments with free Cra solution and Cra-loaded liposomes, showing a normal proliferation of cells for the animal control group (a); a decrease in cellular density for the treatment with Cra-loaded liposomes (b); and the presence of necrotic areas for free Cra solution (c). Areas of necrosis are shown by the arrows (400×).

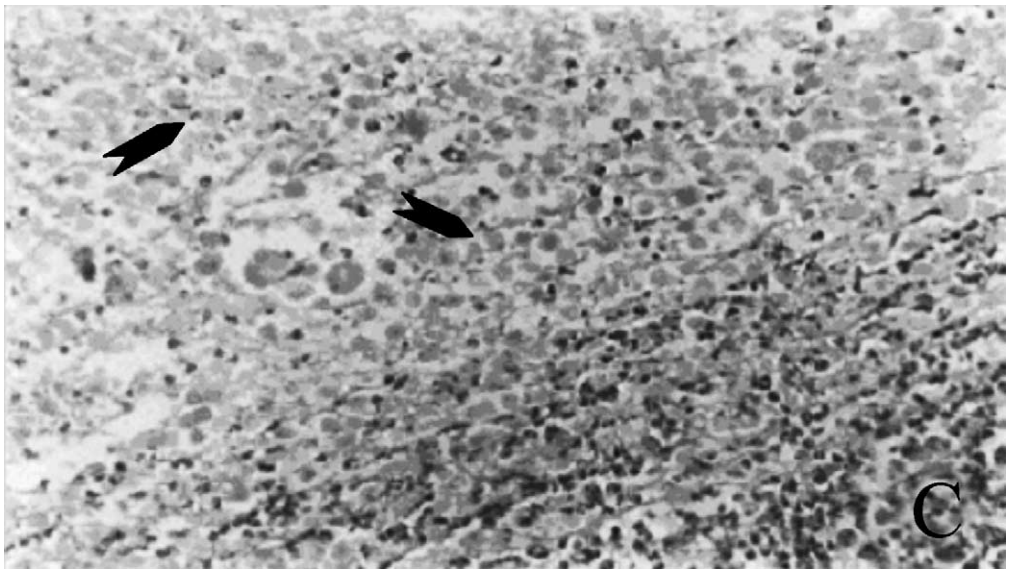

Fig. 3. (*Continued*).

of Sarcoma 180 (71%) occurred in response to the administration of Cra-loaded liposomes (Fig. 2a). A 30% improvement on tumor inhibition was achieved with the encapsulation of Cra in liposomes related to a free Cra solution treatment. The decrease in body weight in the mice was more pronounced with the treatment of Cra-loaded liposomes (Fig. 2b), which corroborates the Cra antitumor activity.

On the basis of the literature (Gabius et al., 1994; Timoshenko et al., 2001), the mechanism of Cra antitumor activity can be postulated as a stimulating effect of the lectin on the animal immune system. Indeed, it has been reported that plant lectins such as *V. album* agglutinin (VAA, Mistletoe lectin) and *N*,*N*′-diacetylchitobiose-binding lectin from *Urtica dioica* are able to affect the functional activity of cells supposedly involved in antitumor immunity (Hajto et al., 1998). The VAA has been shown to suppress growth and may reduce the extent of tumor colonization when administered in small doses, which can also affect levels of cytokines and acute phase reactants in cancer patients. More recently, it has been shown that VAA produces a significant increase in the number of macrophages and a reduction in melanoma cells in murine lungs, as well as an increase in $CD4^+$ and $CD8^+$ thymocytes (Mengs et al., 1997).

Cra encapsulated into liposomes was able to preserve its conformation and produced significant antitumor activity against Sarcoma 180. Nevertheless, the precise mechanism of the Cra action remains to be fully understood.

3.5. Histopathological analyses

The histopathological analyses of Sarcoma 180 (Fig. 3) revealed that the treatment of animals with Cra-loaded liposomes produced a reduction in the density of tumoral cells (Fig. 3b). As a result, the Cra toxicity was reduced in comparison to the control group (Fig. 3a) and the treatment with free Cra solution (Fig. 3c). The occurrence of fibrosis, extensive necrosis areas and an apparent proliferation of tumoral cells were observed (areas shown by the arrows in Fig. 3c).

The hepatotoxicity of Cra was also revealed by lymphocyte infiltration in the liver of animals treated with free Cra solution (Fig. 4a). In contrast, no liver abnormalities were observed after treatment with Cra-loaded liposomes (Fig. 4b), corroborating the ability of liposomes to diminish Cra cytotoxicity. Moreover, no morphological alterations were noticed in the spleen or kidneys after treatment with both free Cra and Cra-loaded liposomes.

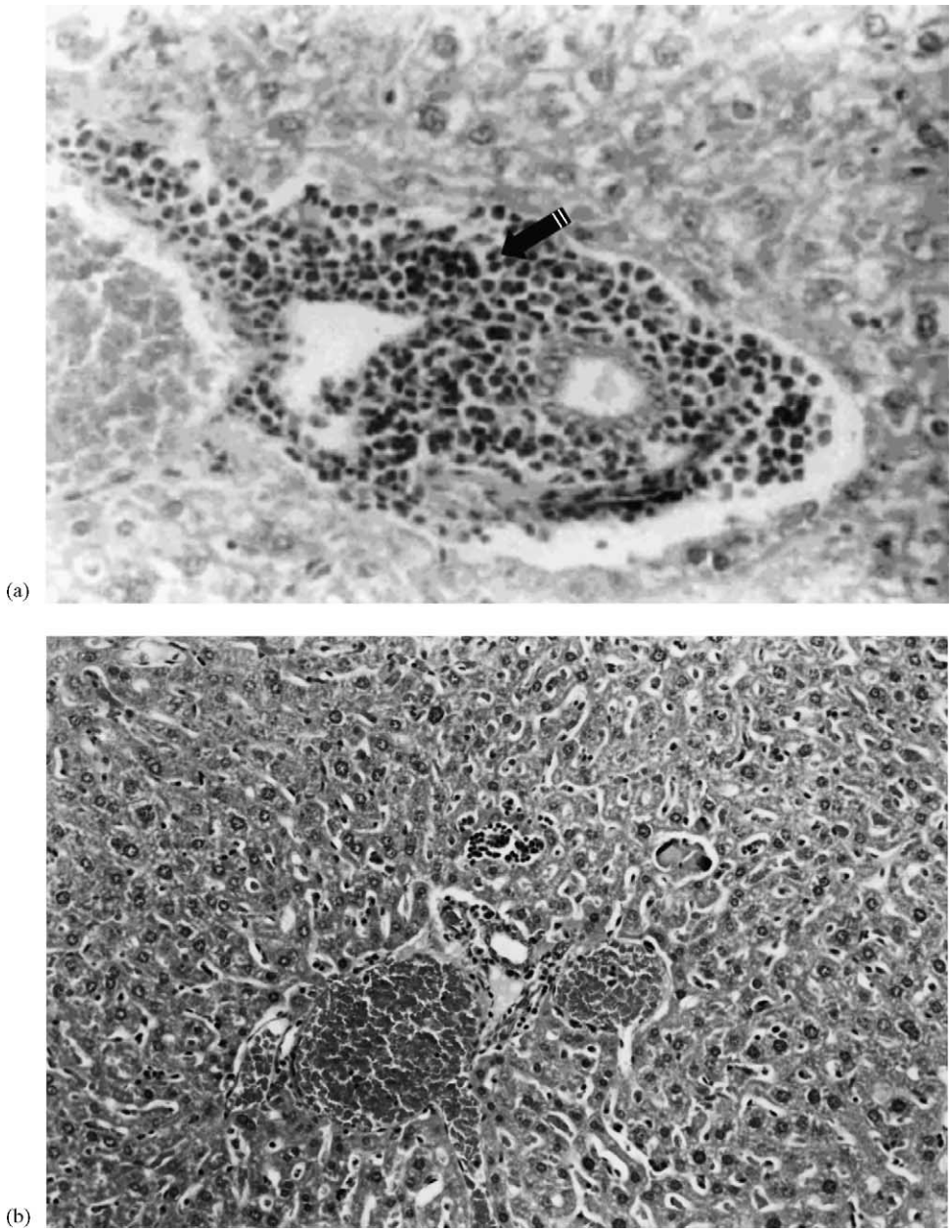

Fig. 4. Histopathological appearance of liver of animals treated with free Cra solution (a); and Cra-loaded liposomes (b). Arrows indicate focal lymphocytic infiltration (400×).

4. Conclusions

The results demonstrated that Cra hemagglutinating activity was modified under prolonged exposure to ultrasound and mechanical stress. However, the entrapment of Cra in liposomes was able to preserve HA activity despite the fact that the liposome preparation involved a stage of sonication. A highly efficient

Cra encapsulation into positively surface-charged liposomes was achieved. The encapsulation of Cra into liposomes produced an improvement in its in vivo antitumor activity against Sarcoma 180 compared with free Cra solution. Furthermore, histopathological analysis revealed that Cra encapsulation into liposomes produces a reduction in its toxic effect on the liver. The results achieved in the present study make it possible to hypothesize that Cra-loaded liposomes might exhibit an antitumor effect by stimulating the immune system of animals, thereby offering a potential new therapeutic agent to be exploited as an adjuvant in cancer therapy.

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