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Response of n-nitrosodiethylamine-induced hepatocellular carcinoma to treatment with curcumin vs doxorubicin

Resposta do carcinoma hepatocelular induzido por n-nitrosodietilamina ao tratamento com curcumina vs doxorrubicina

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ABSTRACT

Purpose: Hepatocellular carcinoma (HCC) is a frequent and fatal human cancer with poor diagnosis that accounts for over half a million deaths each year worldwide. Curcumin has a wide range of pharmacological activities. This study aimed to investigate the chemopreventive and therapeutical effect of Curcumin against diethylnitrosamine (DEN)-induced HCC in rats. **Methods**: HCC was induced in rats by a single injection of DEN (50 mg/kg) once a week, i.p., for four weeks. Group 1 rats were orally treated with curcumin two weeks prior to DEN injection that continued until the end of the experiment. Group 2 was treated with doxorubicin (0.72 mg/rat) and group 3 with

saline. **Results**: In the current study, a significant decrease in serum biomarkers of liver damage and cancer, including alfa-fetoprotein (AFP), gamma glutamyl transpeptidase (GGT), alanine transaminase (ALT), and aspartate transaminase (AST) was observed in curcumin-treated rats when compared to group 2 rats. The counting of red blood cells and leukocytes were significanty lower in doxorubicin group then in curcumin group ($p<0.05$). The relative weight of liver, a prognostic marker of HCC, was also reduced in curcumin group comparing with controls. No diference was observed comparing with doxo group rats. **Conclusion**: To conclude, our results clearly demonstrated that curcumin have a significant chemopreventive and therapeutical effect against primary liver cancer induced by DEN in rats. It can be suggested that the preventive activity of curcumin against hepatocarcinogenesis may have clinical relevance in further studies.

Keywords: Hepatocellular carcinoma. Pharmacological treatment. Curcumin. Doxorubicin. Diethylnitrosamine. Rats.

RESUMO

Objetivo: O carcinoma hepatocelular (CHC) é um câncer humano freqüente e fatal, com diagnóstico precário, responsável por mais de meio milhão de mortes a cada ano no mundo. A curcumina tem uma ampla gama de atividades farmacológicas. Este estudo teve como objetivo investigar o efeito quimiopreventivo e terapêutico da curcumina contra o CHC induzido por dietilnitrosamina (DEN) em ratos. **Métodos**: O CHC foi induzido em ratos por uma única injeção de DEN (50 mg / kg) uma vez por semana, i.p., durante quatro semanas. Os ratos do grupo 1 foram tratados oralmente com curcumina duas semanas antes da injeção de DEN, que continuou até ao final da experiência. O grupo 2 foi tratado com doxorrubicina (0,72 mg / rato) e o grupo 3 com solução salina. **Resultados**: Uma redução significativa nos biomarcadores séricos de dano hepático e câncer, incluindo alfa-fetoproteína (AFP), glutamil transpeptidase gama (GGT), alanina transaminase (ALT) e aspartato transaminase (AST) foi observada nos ratos tratados com curcumina quando comparados com ratos do grupo 2. A contagem de hemácias e leucócitos foi significativamente menor no grupo doxorrubicina do que no grupo da curcumina ($p<0,05$). O peso relativo do fígado, um marcador prognóstico do CHC, também foi reduzido no grupo da curcumina em comparação com os controles. Nenhuma diferença foi observada em comparação com ratos do grupo doxo. **Conclusão**: Nossos resultados demonstraram que a curcumina tem um efeito quimiopreventivo e terapêutico significativo contra o câncer primário de fígado induzido por DEN em ratos. Pode-se sugerir que a atividade preventiva da curcumina contra a hepatocarcinogênese pode ter relevância clínica em estudos futuros.

Descritores: Carcinoma hepatocelular. Tratamento farmacológico. Curcumina. Doxorrubicina. Dietilnitrosamina. Ratos.

INTRODUCTION

The incidence of cancer and cancer-related mortality is increasing worldwide. Among solid tumors, primary liver cancer, the hepatocellular carcinoma (HCC), is the fifth most common malignant disease and the third leading cause of cancer-related deaths1. One of the major causative factors is the hepatitis B virus. However, a number of other factors like hepatitis C virus, alcoholism, cirrhosis, hepatic steatosis and some environmental factors can also trigger HCC. Population aged over 50 years is generally more prone to this type of neoplasm[1].

N-diethylnitrosamine (DEN) is a potent hepatocarcinogenic nitrosamine present in cigarette smoke, cheddar cheese, cured and fried meals, cosmetics, agricultural chemicals and pharmaceutical agents[1]. DEN produces pro-mutagenic products, 6-ethyl deoxy guanosine and 4 and 6-ethyl deoxy thymidine in the liver that lead to oxidative stress and inflammation at an early stage and HCC at a later stage[3]. However, the detailed progress and mechanism of the inflammation induced by DEN, fibrosis and HCC C in rats are not clearly defined[4]. HCC is characterized by a rising incidence and poor prognosis. Due to the highly vascular nature of the liver, HCC is prone to both intrahepatic and extrahepatic metastases, which are the main cause of treatment failure. Conventional anticancer drugs such as doxorubicin, cysplatin and 5-fluorouracil have limited efficacy[5,6]. Thus, the need to find alternative therapy is indispensable and has been the main focus of recent research on HCC treatment. Other therapeutic options, such as percutaneous ethanol injection, radiofrequency ablation, intraarterial therapy, and chemotherapy, provide only transient relief, but not complete cure of disease or prevent recurrence. Since early detection of HCC is difficult due to the lack of specific diagnostic markers and the asymptomatic nature of the disease, tumors are often discovered in the advanced stage of HCC. Recent developments in chemotherapeutic agents, such as sorafenib, have shown promising results in the transient reduction of tumor burden[7]. However, chemotherapeutic agents are generally

not specific for cancer in segmentation and often exhibit a wide spectrum of toxicities (systemic and/or neuronal). It is therefore necessary to find treatment options with agents that target and block the specific pathways of HCC.

In addition to doxorubicin, sorafenib is one of approved drug that is commercially available. However, in the case of developing countries, the high cost of such cancer drugs becomes a major obstacle in chemotherapy[8].

Curcumin is a constituent of turmeric, a bright yellow spice, derived from the roots of the Curcuma longa plant[9]. It is easily available, inexpensive and has a long history as home drug for different diseases. The main component of it is a volatile oil, containing turmerone[10]. It interacts with several molecules, including inflammatory mediators, enzymes, transporter proteins, metal ions, tumor suppressors, transcription factors, oncoproteins and nucleic acids[11]. Its chemical structure has the ability to bond with different molecules and its ability to interact with various targets is vital. Solvents such as ethanol and sodium hydroxide are used to dissolve curcumin. However, studies have shown that their solubility in water is significantly increased with the application of heat[12]. The therapeutic properties of curcumin include antioxidant, antiarthritic, antiamyloid, anti-ischemic, and anti-inflammatory[13].

It has been shown to have protective and therapeutic efficacy against cancers of the skin, oral cavity, lung, pancreas, liver and intestine and suppress angiogenesis and tumor metastasis[14-18]. Since the recognition of the potential effect of curcumin on different cancer cells, several studies have clarified their mechanisms on tumor cells. The multimodal cleavage ability of curcumin enhances its potential therapeutic effect against cancer. It has been shown to exert its antineoplastic effect by modulating different molecular stages of carcinogenesis[19-21].

Based on the data above described, the present study investigated whether curcumin can attenuate the development of HCC and evaluate its antitumor effect against DEN-induced HCC in rats.

METHODS

Animals

Wistar adult male rats (Rattus norvegicus) were obtained from the Facility of the Health Sciences Center of the Federal University of Rio Grande do Norte. The project was submitted to the Institutional Ethics Commission on Animal Use (CEUA/HUOL) for evaluation and approved (protocol 02/2018). All experimental procedures were performed based on the guidelines of the Brazilian College of Animal Experimentation, as well as Law No. 11,794 (CONCEA).

Adult rats were used both in the control group and in the test groups. The animals were kept in individual cages with water and food for rodents (Presence ®) ad libitum, in an environment with temperature 22°C, particle control and 12 hours light-dark.

Induction of hepatocellular carcinoma (HCC)

Induction of HCC was done by the injection of N-diethylnitrosamine (DEN) (Sigma, St. Louis, MO, US) dissolved in 0.9% normal saline. The rats received intraperitoneal injections of DEN at 100 mg / kg body weight once weekly for 4 weeks..

After the evaluation period (24, 48 and 72 hours after immersion), the PDO suture segments were taken from the tube with sterile tweezers, dried over a sterile field at room temperature for 15 minutes, mounted on a microscope slide, and observed ultramicroscopically under a Nikon® reflected light optical microscope at 4x and 10x. We carried out a microphotographic scan of each segment obtaining at least 12 images three for each of the groups.

Experimental design

We used 18 animals divided into 3 groups of 6 each, according to the following model:

Treatment

After four weeks of using DEN to induce HCC, treatment of the animals was started.

Group 1: CURCUMIN - 10 mg / kg aqueous curcumin solution (Sigma, St. Louis, MO, US) were injected v.o. by gavage every 2 days for 5 weeks, starting 2 weeks before the onset of HCC induction.

Group 2: DOXORRUBICIN - HCC -bearing rats were treated intraperitoneally (ip) with doxorubicin (Janssen, SP) at a dose of 0.72 mg / rat which is equivalent to a human dose of 20 mg / m2 once weekly for 5 weeks, starting 2 weeks before the begining of HCC induction.

Group 3: CONTROL - Rats treated with saline 0.9% v.o. once a week for 5 weeks, starting 2 weeks before induction of HCC

Weighing and Survival

The animals were weighed weekly and kept in individual cages (one per cage) throughout the experiment period, the survival time of which was recorded in days.

Fluorescence imaging (ex-vivo)

The animals were anesthetized (ketamine 70 mg/kg and xylazine 7 mg/kg i.p.) and i.v. injection of 0.5 ml of indocyanine green (25 mg diluted in 2 ml saline) was done. After 10 minutes a laparotomy was performed, the liver was completely resected, washed in saline solution and dried in sterile gauze. The livers were weighted before imaging. Images of exvivo liver fluorescence were obtained using the in-vivo Image Kodak Station FX. The emission and excitation filters were 700 and 540nm respectively. The image protocol (exposure time 60 seconds, 4x binning, f-stop 2.5, field of view 160 mm and focal plane 9 mm) was maintained for all images. The images were analyzed by Kodak Molecular Imaging software (version 5.0) and quantified according to color scale. A region of interest (ROI) created by an automated tool was determined around the liver. Mean region of interest signal intensities were expressed as arbitrary fluorescent signal intensity units. Values were determined at mean fluorescence intensity. Grayscale images were colored for depiction purposes according to a color scale set to the highest and lowest levels of mean fluorescence intensity (red and purple indicate maximum and minimum light intensity, respectively).

Laboratory Dosages

Blood was collected through cardiac puncture of the animals still anesthetized, soon after imaging, for the following dosages: the level of α-fetoprotein (AFP), a tumor marker of hepatocarcinoma, was quantified based on the enzyme-linked immunosorbent assay (ELISA) using the Bayer automated system ADVIA Centaur chemiluminescent (Bayer Corp., Pittsburgh, PA, USA). Briefly, a 96-well plate precoated with AFP antibody was incubated with a peroxidase-conjugated anti-AFP antibody to measure the signal intensities generated from the enzyme substrate reactions. Serum liver marker enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) were tested with commercial Labtest kits following the manufacturer's recommendations.

Whole blood samples were used for counting red blood cells, total leukocytes and percent neutrophils using automatic cell counter (Abbott Cell-Dyn 3500R-CD 3500 5L, USA).

Statistical analysis

For the quantitative variables, the Analysis of Variance (ANOVA) was used to verify if the differences between the groups were statistically significant, followed by the analysis of comparisons paired by the Tukey test. The significance level of 5% was adopted. The analyzes were performed using BioEstat 5.0 software, Belém, PA, Brazil.

RESULTS

All animals survived after the experimental model procedures, and there was no significant difference in body weights, comparing the two study groups ($p>0.05$). At the end of the observation period, biochemical determinations showed significantly higher levels of ALT, AST, GGT and AFP in animals undergoing doxorubicin treatment compared to rats in the curcumin treated group ($p<0.05$). These data are summarized in Table 1.

Table 1 – Values of biochemical data and their statistical interpretation.

Dosages	Curcumin	Doxorubicin	Control	P-value
ALT (IU/L)	49.6 ± 3.01^{A}	71.5 ± 7.3^{A}	120.3 ± 12.3^{A}	<0.05
AST (IU/L)	58.7 ± 2.7^{A}	74.9 ±7.2^{A}	135.7 ±13.4^{A}	<0.05
GGT (IU/L)	109.6 ± 10.3^{A}	150.3 ± 12.4^{A}	178.8± 10.8^{A}	<0.05
AFP (ng/mL)	0.7 ± 0.04^{A}	1.9 ± 0.2^{A}	9.2 ± 3.1^{A}	<0.05

AST - Aspartate aminotransferase; ALT - Alanine aminotransferase; GGT-gamma-glutamyl transferase. Mean ± standard deviation. Values on the same line followed by equal letters mean statistically significant differences. Tukey test.

Regarding the hemogram, the animals treated with doxorubicin had a red cell count, total leukocytes and a percentage of neutrophils significantly lower than in the curcumin and control groups ($p<0.05$). The difference between the curcumin and control groups was statistically insignificant ($p>0.05$). Data summarized in table 2.

Table 2 – Values of hematological data and their statistical interpretation.

Variables	**Curcumin**	**Doxorubicin**	**Control**	**P-value**
Red blood cels/ µL	4.72±0.9 ^{A}	2.85±0.4^{A,C}	4.87±0.82^{B,C}	<0.05
Leukocytes/µL	8.73 ± 2.2^{A}	4.5 ± 1.3^{A,C}	8.34 ± 1.2^{B,C}	<0.05
Neutrophils (%)	66.72 ± 8.9^{A}	34.9 ±7.2^{A,C}	64.5 ±9.4^{B,C}	<0.05

Values on the same line followed by equal letters mean statistically significant differences. Tukey test.

Fluorescence Images

In figure 1 we observe representative ex-vivo images of liver fluorescence resected from experimentally induced HCC animals undergoing three different treatments. In Figures 1-Aa, the intensity of red highlighted fluorescence, which indicates carcinoma, is significantly less marked than in 1-Bb images. The red color intensity and respective area of hepatic parenchyma involvement are more pronounced in Figures 1-Cc, representative of HCC-bearing rats and treated with 0.9% saline.

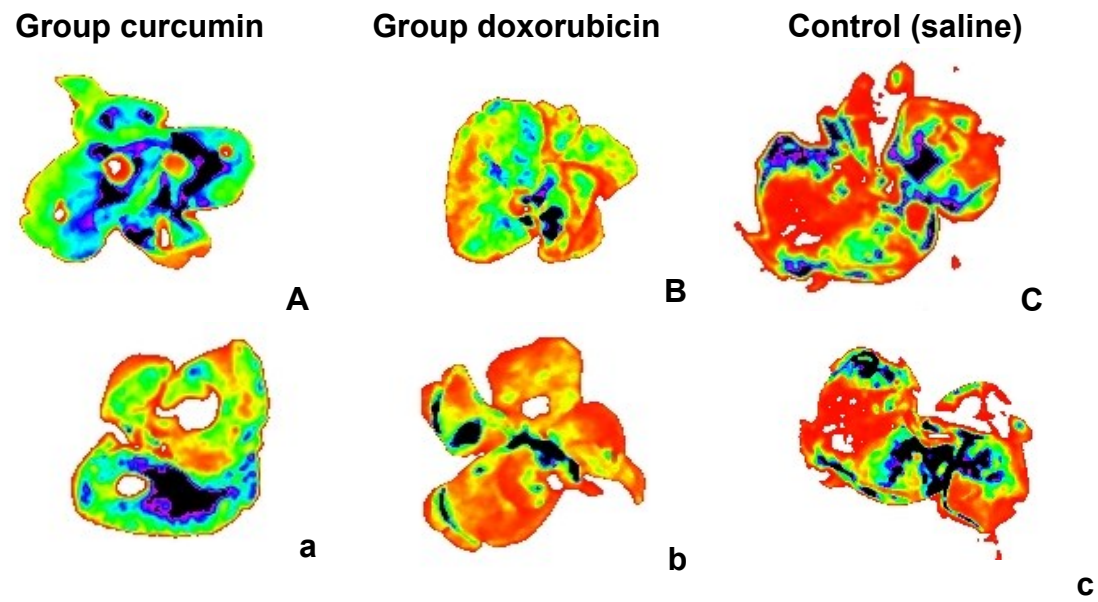

Figure 1 - Representative ex-vivo images of liver fluorescence induced by the i.v. injection of the indocyanine green marker. HCC was induced with DEN and the rats treated with curcumin, doxorubicin and 0.9% saline. In the color scale, red means neoplastic invasion of the liver. The greater the intensity of color and area, the greater the tumor invasion. Aa, animals treated with curcumin; Bb, doxorubicin-treated rats; Cc, rats treated with 0.9% saline.

Table 3 – Values of mean intensity fluorescence (MIF) and their statistical interpretation.

Variable	Curcumin	Doxorubicin	Control	P-value
MIF	240 ± 18^{B}	275 ± 25^{A}	730 ± 62^{AB}	<0.05

MIF – Mean intensity fluorescence. Mean±standard deviation. Values on the same line followed by equal letters mean statistically significant differences. Tukey test.

The mean intensity of liver fluorescence (ex-vivo) of curcumin treated rats (240±18) was lower than those treated with doxorubicin, but the difference was not statistically significant ($p>0.05$). However, in the saline treated rats the MIF was 730±72, significantly higher than in the other groups ($p<0.05$). Data summarized in table 3.

The relative weight of liver, a prognostic marker of HCC, was also reduced in curcumin group, comparing with controls. No difference was observed comparing with doxorubicin group rats. (Table 4).

Table 4 – Values of liver weight and their statistical interpretation.

Variable	Curcumin	Doxorubicin	Control	P-value
Liver weight (g)	12 ± 2.1^{B}	10 ± 2.5^{A}	6.3 ± 1.8^{AB}	<0.05

Liver weight (g). Mean±standard deviation. Values on the same line followed by equal letters mean statistically significant differences. Tukey test.

DISCUSSION

Hepatocellular carcinoma (HCC), the most common type of liver cancer, is a malignant tumor treated with difficulty. Since most patients are diagnosed at advanced stages, there is an urgent need for effective nonsurgical therapies, such as systemic chemotherapy[22]. Currently, traditional chemotherapeutic agents, such as sorafenib, doxorubicin (DOX), 5-fluorouracil and cisplatin, play a very limited role in the management of HCC. The development of multidrug resistance (MDR) to chemotherapy is a major obstacle to the effective treatment of human malignancies, including HCC[23]. Cancer cells undergo a complex transformation under hypoxic condition for cell survival, such as activation of genes involved in DNA repair and drug resistance after translocation of HIF gene from cytoplasm to the nucleus[24].

As the treatments started two weeks before induction of HCC, we considered that the effect of the treatments was therapeutic and preventive. In this report we showed the chemopreventive and therapeutical effect of curcumin in DEN-induced hepatocellular carcinoma in rats. In this study, the pretreatment of curcumin to HCC rats caused a remarkable reduction in the serum markers of liver damage and cancer. Serum alpha-feto protein (AFP) level is the golden standard among diagnostic markers for HCC[25]. AFP is a glycoprotein that is synthesized during early fetal life and after birth its serum concentration falls quickly. It has been suggested that AFP acts as a transport molecule for many ligands such as heavy metals, bilirubin, and many xenobiotics. Furthermore, AFP has a role in the regulation of cell proliferation and an immunosuppressive activity[26]. Elevated AFP serum levels are only seen in certain tumors (e.g. HCC), non-tumoral conditions (e.g. cirrhosis), and maternal serum during pregnancy. In the current investigation, the elevated level of AFP was markedly decreased by the curcumin treatment. GGT, ALT, and AST are also the most widely used HCC tumor markers. GGT is a cell surface enzyme that involves in glutathione metabolism. Its main function is to sustain cysteine levels in the body to conserve intracellular homeostasis of oxidative stress. GGT expression in tumor cells provides a selective advantage to the cells during tumor promotion[27]. Therefore, it has been suggested that GGT is an independent prognostic indicator in patients with HCC[28]. Data from our study indicated that the oral administration of curcumin to DEN treated rats

reduced significantly the GGT, ALT, and AST activities, whem compared to doxorrubicin and control groups. The treatment with doxorubicin resulted in dosages of these parameters significantly higher than with curcumin, probably due to the hepatotoxic effects of this chemotherapeutic. Doxorubicin can cause an idiosyncratic reaction and potentially contribute to liver toxicity. Impaired liver function delays excretion, increases accumulation of the drug in plasma and tissues, leading to systemic side effects like cardiomyopathy[29].

Administration of curcumin significantly prevented leukocyte loss and anemia compared to the doxorubicin group in this relevant experiment. Therefore, this drug demonstrated a good potential for the control of leukopenia and anemia in animals undergoing the experimental treatment, compared with chemotherapy. In this context, curcumin was comparable to saline treatment. Prevention of leucopenia in the treatment of cancer has been demonstrated with the use of statins[30].

Ex-vivo fluorescence imaging of this study demonstrated that the effect of curcumin as well as doxorubicin were effective in preventing and treating HCC. The ex-vivo examination was decided because of the low penetration of the fluorescence through the abdominal wall until reaching the liver. In conclusion, our results clearly demonstrated that curcumin has a significant chemopreventive and therapeutic effect against DEN-induced liver cancer in rats, without induction of toxic effects.

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