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Toxicological Assessment of Beta-lapachone on Organs from Pregnant and Non-pregnant Rats

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Naphthoquinones have been studied extensively due to their activity as topoisomerase inhibitors. These enzymes are critical to DNA replication in cells. β-Lapachone (beta-lap) is an *o*-naphthoquinone chemically obtained from lapachol. This work results in a toxicological evaluation of beta-lap in Wistar rats observing the following parameters: teratology, histology, hematology and serum biochemistry. The data demonstrate teratogenic action at the doses used, as well as hematological alterations in the total leukocytes, monocytes and segmented. The biochemical data demonstrated an increase in gamma glutamyl transferase, alkaline phosphatase and glutamate pyruvate transaminase levels. Histological study showed significant alterations in the spleen, however, the liver and kidney did not present significant alterations.

Keywords: β-lapachone; biochemical and hematological study; histological study; Wistar rats.

INTRODUCTION

Naphthoquinones are widely distributed in the plant kingdom. Their molecular structures endow them with redox properties, which can intervene in biological oxidative processes (Menna-Barreto *et al.*, 2005; Silva *et al.*, 2003; Portela *et al.*, 1996). B-Lapachone (beta-lap) is a lipophilic *o*-naphthoquinone isolated from the bark of the lapacho tree, through lapachol, that is native to Central and South America and has antibiotic/antineoplasic potential (Santana *et al.*, 1968; Silva *et al.*, 2003). Initial observations proved its capability for inhibiting the growth of Yoshida sarcoma and Walker 256 rat carcinosarcoma (Santana *et al.*, 1968; Dubin *et al.*, 2001; Pu *et al.*, 2004), and against various cancer cell lines such as human ovarian and prostate tumors (Li *et al.*, 1999; Ravelo *et al.*, 2004; Duvoix *et al.*, 2004; Kumi-Diaka *et al.*, 2004; Lee and Lee, 2004). Its mechanism of action includes the inhibition or activation of topoisomerase I and II in a manner that is distinct from those of other topoisomerase inhibitors (Chen *et al.*, 2004; Park *et al.*, 2005). In recent years interest in these substances has intensified, not only due to their importance in vital biochemical processes, but also due to their more and more frequent presence in varied pharmacological studies, mainly in the levels of the cellular respiratory chain. Beta-lap inhibited DNA synthesis in *Trypanosoma cruzi* as well as topoisomerases I and II in different cells (Menna-Barreto *et al.*, 2005; Woo and Choi, 2005; Perez-Sacau *et al.*, 2005). These enzymes are essential for maintaining DNA structure. Advances in knowledge on apoptosis ('programmed cell death') and necrosis provided useful information for understanding the mechanism of cytotoxicity of beta-lap (Tagliarino *et al.*, 2001; de Witte *et al.*, 2004). The cytotoxicity of this naphthoquinone is related to inhibition of topoisomerases and the induction of apoptosis (Dubin *et al.*, 2001; Olivera-Brett *et al.*, 2002). It has been shown that beta-lap is active against *T. cruzi* and its mode of action is associated with the generation of free radicals (Portela *et al.*, 1996; Abreu *et al.*, 2002; de Witte *et al.*, 2004; Zielinska-Park *et al.*, 2004; Villamil *et al.*, 2004). The objective of this paper was to demonstrate a possible toxic effect of beta-lap on Wistar rats, through its action on pregnant rats, and in treatment for 21 days, for histological, hematological and biochemical analyses. Since there may be a risk to pregnant patients with this anticancer drug.

MATERIAL AND METHODS

Animals. Adult Wistar rats of both sexes (160–240 g), 2 months of age at the beginning of the experiment, were used. The animals were obtained from the vivarium of the Antibiotic Department of the Federal University of Pernambuco and were housed in groups of ten per

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cage, with a light/dark period of 12 h (6:00 am to 6:00 pm) and food and water *ad libitum*. All experiments were conducted between 10:00 am and 4:00 pm. Female rats were tested without monitoring the estrus cycle. All the animals were carefully monitored and maintained in accordance with the ethical recommendation of the Brazilian College of Animal Experimentation (COBEA) and the National Institute of Health Guide for Care and Use of Laboratory Animals.

Drugs. β-Lapachone was obtained from the Antibiotic Department of the Federal University of Pernambuco. The beta-lap was dissolved in 0.025% Tween 80 and diluted with saline 0.85%. All solutions were administered by intraperitoneal (i.p.) route at a volume of 0.1 mL/100 g body weight.

Chemicals. Kits for the glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), total bilirubin, urea, creatinine, total cholesterol, triglycerides and gamma glutamyl transferase (GGT) used for the biochemical studies were supplied by diagnostic Doles®, Brazil.

The study of pregnant rats. Female Wistar rats were mated with males of previously confirmed fertility (one male for three females). Vaginal smears were examined each morning for the presence of sperm, and were considered as day 1 of pregnancy (Almeida *et al.*, 2000). Inseminated animals were isolated in cages and divided in two groups, each group with ten animals, which were treated with beta-lap. Each group treated had its corresponding control group (saline + Tween 80). The beta-lap was administered by intraperitoneal route at doses of 40, 80 and 160 mg/kg. The control group received an equivalent amount of saline 0.9% 0.1 mL/100 g (i.p.). Each animal was weighed and killed by cervical displacement on day 19 of gestation. A laparatomy was performed and the uterus and ovaries were removed. Resorptions (embryotoxicity/fetotoxicity) were counted and viable implants were examined. The number of live/dead fetuses, viability, growth and deformity of newborn and maternal weight gain were recorded (Almeida *et al.*, 2000; Barrow, 2003; Jelinek, 2005).

Long-term administration effects (21 days). Male and female Wistar rats were studied. All were submitted to administration of 40, 80 and 160 mg/kg (i.p.), ten animals for each dose, for 21 days. The blood levels of total bilirubin, total cholesterol, creatinine, urea, glucose, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), gamma glutamyl transferase (gamma GT) and alkaline phosphatase (AP) were measured at 0 and 21 days. A colorimetric method was used for each one with the Doles® diagnostic system. At the end, each animal was killed by cervical displacement 24 h after the last treatment and then the liver, kidneys and spleen were removed for histological study (Casarett and Doull, 1975; Oga, 2003).

Statistical evaluation. The data were submitted to variance analysis (ANOVA). Posthoc comparisons between individual treatments and controls were made using Student's *t*-test. The results were considered significant when $p < 0.05$ and $p < 0.01$ were obtained (Siegel, 1975).

RESULTS

Teratogenic and abortive activity in pregnant rats

As observed in Tables 1 and 2, the beta-lap at the three doses (40, 80, and 160 mg/kg) administered on days 7–12

Table 1. Effect of beta-lap in the period of days 1–6 and days 7–12 of gestation

Analysed parameter	Control group (saline + Tween 80)	Dose (40 mg/kg)	Dose (80 mg/kg)	Dose (160 mg/kg)
Variation of the maternal average weight	73 ± 2.42	44.8 ± 8.7[a]	43 ± 5.52[a]	39 ± 5.03[a]
Fetal weight	1.59 ± 0.01	1.18 ± 0.12[b]	1.13 ± 0.03[b]	1.08 ± 0.14[b]
Weight of the placenta	0.41 ± 0.01	0.34 ± 0.02[a]	0.32 ± 0.04[a]	0.26 ± 0.01[a]
Resorption index (RI)	0.0	7.27[a]	16.66[b]	18.36[b]
Malformations	0.0	4.0	2.0	3.0
Total number of corpora lutea	9 ± 0.81	10 ± 0.51	10.3 ± 0.71	10 ± 0.82
Number of viable embryos	60	5	48	49

Values are mean ± SD for 10 animals; [a] $p < 0.05$; [b] $p < 0$, 01 vs control group. Student's *t*-test followed by one-way variance analysis (ANOVA). Variation of the average maternal weight is the difference between the fetus controls for fetuses of the treated groups.

Table 2. Type of malformations observed in the embryos on days 1–6 and days 7–12 of gestation

Fetal observation and type of anomaly	Dose (40 mg/kg)	Dose (80 mg/kg)	Dose (160 mg/kg)
Enterocelia	x	x	X
Absence of tail	x	x	X
Absence of members	x	–	X
Bifid spine	–	x	X
Sindactly	x	x	X

X, presence of malformation; –, absence of malformation.

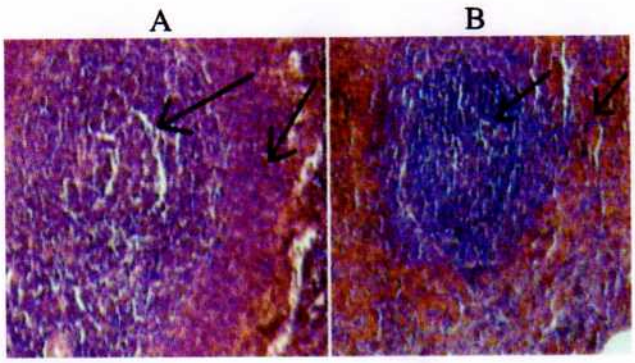

Figure 1. Microphotography of histological preparation of the spleen in the control group (**B**) increased 10× and experimental group (**A**), increased 10×. The histological analyses of the spleen structure of the experimental group (**A**), showed enlarged follicles in the white pulp when compared with control group (**B**). In these follicles, a relative hypertrophy was observed in the mantle zone as well as in the perifollicular (marginal) zone. Enlarged follicles were observed in the white pulp of the spleen of rats of the experimental group. In the follicles, hypertrophy was seen at high frequency in the mantle zone at the doses used.

of gestation led to fetal toxicity. Furthermore, malformations of the implant and viable fetus were observed in the animals killed on day 19 of pregnancy. The malformations presented were enterocelia, absence of tail, absence of members, bifid spine and sindactly, beyond resorption in the two periods of application in all the doses used (Table 2). A weight decrease of the malformed fetus in relation to the viable fetus also occurred in a significant form (Table 1). The dose of 160 mg/kg produced the most malformation and abortive activity.

Histological studies

The beta-lap at a dose of 160 mg/kg, promoted alteration in the spleen structure of the experimental group and showed enlarged follicles in the white pulp when compared with the control group. A relatively high frequency of hypertrophy in the follicles was observed in the mantle zone as well as in the perifollicular (marginal) zone (Fig. 1). No changes in the liver structure were observed in the experimental animals. Under light microscopy, the parenchyma (hepatocytes) and stroma components were well preserved. The beta-lap, at the same dose, did not produce histological alterations in the kidneys of the animals of the experimental group.

Hematological and biochemical studies

Beta-lap at doses of 40, 80 and 160 mg/kg promoted a significant increase in the number of total leukocytes, as well as in the monocytes and segmented blood cells in the animals of the experimental group (Table 3). At the biochemical level, the beta-lap promoted an increase in glutamate pyruvate transaminase (GPT), gamma glutamyl transferase (gamma GT) and alkaline phosphatase (AP) in relation to animals of the control group (Table 4).

Table 3. Blood profile after treatment with beta-lap (i.p.)

Hematological parameter	Control group (saline + Tween 80)	40 mg/kg	80 mg/kg	160 mg/kg
Total leukocytes/mm^3	11 800	17 000[a]	17 540[a]	20 600[a]
Segmented %	59	60	61	65[a]
Monocytes %	3	7[a]	10[a]	16[a]
Typical lymphocytes %	65	30	40	23
Atypical lymphocytes %	00	3[a]	3[a]	4[a]

Values are mean ± SD for 10 animals, [a] $p < 0.01$ vs control group. Student's *t*-test followed by one-way variance analysis (ANOVA).

Table 4. Blood chemistry profile after treatment with beta-lap (i.p.)

Analysed parameter	Control group (saline + Tween 80)	40 mg/kg	80 mg/kg	160 mg/kg
Triglycerides mg/dL	153 ± 9.2	141 ± 10.1	134 ± 11.1	149 ± 10.8
Total cholesterol mg/dL	135.5 ± 10.0	141.1 ± 10.4	145.0 ± 11.0	142.9 ± 9.3
GOT UI/mL	79.85 ± 9.1	80.0 ± 9.0	80.8 ± 9.2	81.2 ± 13.2
GPT UI/mL	39.2 ± 1.0	46.2 ± 3.0[a]	49.9 ± 4.9[a]	89.9 ± 4.3[b]
Total bilirubin mg/L	1.3 ± 0.1	3.7 ± 0.2[a]	3.9 ± 0.2[b]	6.7 ± 0.4[b]
Gamma GT UI/L	3.5 ± 1.6	3.9 ± 1.2	4.9 ± 0.4[b]	6.2 ± 0.6[b]
Glucose mg/dL	99.11 ± 9.1	101.02 ± 7.2	104.9 ± 5.3	100 ± 9.3
Creatinine mg/dL	0.83 ± 0.0	0.72 ± 0.1	0.89 ± 0.0	0.9 ± 0.2
Urea mg/dL	45.9 ± 6.0	44.8 ± 5.9	46.9 ± 3.9	44.5 ± 0.3
AP UI/L	26.0 ± 2.4	54.0 ± 2.9[b]	60.6 ± 3.1[b]	95.3 ± 6.0[b]

Values are mean ± SD for 10 animals. [a] $p < 0.05$ and [b] $p < 0.01$ vs control group. Student's *t*-test followed by one-way variance analysis (ANOVA).

DISCUSSION

Beta-lap, with its molecular structure, presents redox properties that intervene in biological oxidative processes (Menna-Barreto *et al.*, 2005). It also presents inhibition of development of Yoshida sarcoma and Walker 256 rat carcinosarcoma (Santana *et al.*, 1968; Pu *et al.*, 2004). In humans, it presents an action against ovarian and prostate tumors (Ravelo *et al.*, 2004; Duvoix *et al.*, 2004; Kumi-Diaka *et al.*, 2004; Lee and Lee, 2004). Its mechanism of action includes the inhibition or activation of topoisomerase I and II in a more distinct manner than those of other topoisomerase inhibitors (Chen *et al.*, 2004; Park *et al.*, 2005). In recent years interest in these substances has intensified, not only due to their importance in vital biochemical processes, but also to their more and more frequent presence in varied pharmacological studies, mainly in the levels of the cellular respiratory chain. Beta-lap inhibited DNA synthesis in *Trypanosoma cruzi* as well as topoisomerases I and II in different cells (Menna-Barreto *et al.*, 2005; Woo and Choi, 2005; Perez-Sacau *et al.*, 2005). These enzymes are essential for maintaining DNA structure. Advances in knowledge on apoptosis ('programmed cell death') and necrosis provided useful information for understanding the mechanism of cytotoxicity of beta-lap (Tagliarino *et al.*, 2001; de Witte *et al.*, 2004). However, there is little knowledge regarding its toxicity in long-term treatment. The study of pregnant rats treated intraperitoneally (i.p.) with doses of 40, 80 and 160 mg/kg demonstrated anatomical alterations in the fetus. The beta-lap showed abortive and teratogenic action in pregnant rats (Tables 1, 2). This observation indicates that beta-lap acts at the beginning of egg division and during its implantation. The physiopathological mechanism of this effect is probably the same as the one that promotes its cytotoxic action, through the enzymes that act in the DNA chain, as well as for its action in the induction of apoptosis. On the other hand, the significant increase in the total leukocytes, segmented and monocytes could demonstrate a stimulant action of the beta-lap in the immunological system (Table 3). Histological analysis of the spleen structure showed enlarged follicles in the white pulp (Fig. 1). These data can explain the response of an increase of total leukocytes, segmented and monocytes in the blood circulation during the treatment. The data found in spleen and the immune-competent cells of the blood can be another action of beta-lap against cancer cells. However, further studies on this effect will have to be carried out in the future. The significant increase of gamma GT, GPT and AP levels can explain damage in the liver, mainly in the biliary duct, when associated with an increase in total bilirubin in blood circulation, an effect that was not observed in the histology (Table 4). In the histology and in the biochemistry of kidney (creatinine and urea) no alteration was observed in the treatment with beta-lap at the doses used. The data for the histology of spleen and the increase in the white cells of the blood could possibly indicate a new action of beta-lap as an immunostimulant and will be the subject of future studies in our laboratory.

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