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Acute and subacute toxicological studies of metronidazole and two analogues in animals

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ABSTRACT

Metronidazole (MTZ) is an antibacterial/ antiprotozoal drug used to treat infections. This study aimed to assess the safety of two pharmacologically active MTZ analogs, MTZ-Ms and MTZ-I, in an attempt to provide scientific evidences that justify further investments in these drugs. The acute toxicity studies were performed using 300 or 2000 mg/kg doses. No treatment-related mortality occurred in MTZ and MTZ-Ms groups. By contrast, one death was detected when using 2000 mg/kg of MTZ-I. In the subacute toxicity studies, 200, 400, or 600 mg/kg doses of MTZ and MTZ-I, and a 1000 mg/kg dose of MTZ-Ms, were applied. No abnormal behavior was observed in the MTZ and MTZ-Ms group. Two deaths in the MTZ-I (600 mg/kg) group were identified. Changes in clinical biochemistry parameters were of minor nature and occurred in the absence of a clear dose-related distribution. Lymphocytes and leukocytes increased in the MTZ and MTZ-Ms groups, suggesting immunostimulation. Histopathological examination revealed hyperplasia of intestinal epithelium along with alterations in spleen and testis in rats treated with MTZ (600 mg/kg) and MTZ-Ms (1000 mg/kg). In MTZ-I groups, mortality was considered to be related to treatment with test substance in both acute and subacute toxicity assays. The results suggest that MTZ and MTZ-Ms have similar toxicological profile. However, further investigations are warranted.

KEYWORDS: acute toxicity, immunostimulation, metronidazole analogs, subacute toxicity

1. INTRODUCTION

Metronidazole (MTZ) can be found on the essential drug list of the World Health Organization [1]. MTZ is the most commonly prescribed drug for the treatment of anaerobic infection, amebiasis, trichomoniasis, and giardiasis, and exhibits activity against certain microaerophilic organisms, such as *Helicobacter pylori*. Currently, one of the most effective treatment regimens for *H. pylori* consists of a combination of proton pump inhibitors and any two of the following three antimicrobial agents: amoxicillin, MTZ, and clarithromycin [2].

MTZ synthesis studies have observed that the nitro group in the 5th position of metronidazole ring is essential for biological activity. The nitro group is activated when it is reduced through an electron donation from ferredoxin, which is reduced by pyruvate, possibly forming a hydroxylamine [3]. Activated MTZ is believed to interact directly with DNA. As such, the resultant complex can no longer function as an effective primer for DNA and RNA polymerase [4].

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Therefore, the requirement for MTZ activity is the presence of nitroreductases capable of reducing the nitro group in animal's organs and tissues. However, increased use of nitroheterocyclic drugs can induce metronidazole-resistant strains. Among the protozoa, *Trichomonas* have developed a resistance to metronidazole through a number of mechanisms, especially a decrease in the MTZ reduction, resulting from alterations in the electron transport pathway [5].

Cavalcanti *et al.* [6], when investigating metronidazole analogs with an antibacterial activity higher than that of MTZ and against metronidazole-resistant strains, synthesized two analogs of MTZ, 1-[2-methanesulfonatethyl]-2-methyl-5-nitroimidazole (MTZ-Ms) and 1-[2-iodoethyl]-2-methyl-5-nitroimidazole (MTZ-I). These compounds contain more hydrophobic aliphatic side chains when compared with MTZ. This may well contribute to the higher giardicidal activity observed in the study [7]. Those compounds, upon undergoing an *in vitro* assay against different strains of *H. pylori*, showed significant antibacterial activity even toward metronidazole-resistant strains. Busatti *et al.* studied the two analogs of MTZ-I and MTZ-Ms and observed a higher activity than that from MTZ giardicidal activity with metronidazole, thus indicating their potential as alternative drugs for giardia infections. However, it is of utmost importance to submit those drugs to safety tests before continuing further efficacy studies.

The aim of this study is to evaluate the safety of these two analogs through acute and subacute preclinical toxicity tests in an attempt to provide scientific evidences that justify further investments in these drugs.

2. MATERIALS AND METHODS

2.1. Drug

Metronidazole and the two analogues: 1-[2-methanesulfonatethyl]-2-methyl-5-nitroimidazole (MTZ-Ms) and 1-[2-iodoethyl]-2-methyl-5-nitroimidazole (MTZ-I) (Fig. 1) were provided by Pharmaceutical Chemistry Laboratory of Faculty of Pharmacy of Federal University of Minas Gerais (Brazil).

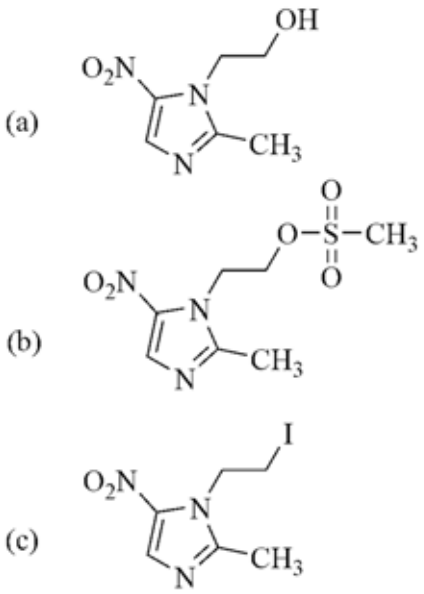

Fig. 1. (a) Metronidazole (MTZ): 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol; (b) metronidaloze anologue MTZ-Ms: 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl methanesulfonate; (c) metronidaloze anologue MTZ -I: 1-(2-iodoethyl)-2-methyl-5-nitro-1H-imidazole.

2.1.1. Suspension for use

The drugs were suspended in Polvix® (vehicle) immediately prior to the administration. The Polvix® was supplied by Galena (Galena Ltda., São Paulo, Brazil) and is constituted by microcrystalline cellulose, carboxymethylcellulose, laurylsulfate sodium and methyl-4-hidroxybenzoate. The suspensions were prepared in suitable concentrations to permit administration in rats of no more than 1mL/100g body weight [8, 9].

2.2. Animals

Adult Wistar rats (200 g ± 20%), both sex, aged 8 to 10 weeks were used. All rats were provided by the Animal Shelter from the School of Pharmacy at UFMG. They were kept in cabinets for five days before the experiment to allow acclimatization under controlled conditions of temperature (25 ± 2 ºC), humidity (50-60%) and 12 h light/darkness (07:00-19:00). The animals were fed with food in *pellets* (*Nuvilab*®) and water *ad libitum* [8, 9]. The females were nulliparous and non-pregnant.

The experimental protocols were approved by the Ethics Committee on Animal Experimentation (CETEA) of the UFMG (protocol n° 180/2006).

The present study was conducted in compliance with OECD good laboratory practices [8, 9] which essentially conforms with The United States food and drug administration good laboratory practice regulations.

2.3. Toxicity studies

2.3.1. Acute toxicity

Groups of 10 female rats were used, which received either a vehicle (Polvix®) or a single oral dose of the test drugs (MTZ-I or MTZ-Ms). Rats were fasted for one night, with free access to water, prior to administration of drugs through gastric gavage. Suspensions were prepared as described above in the item 2.1.1, in 300 or 2000 mg/kg doses. After the administration of the suspensions, food was withheld for another 3-4 h. Animals were observed individually at 15, 30, 60, 120 and 240 minutes after dosing and daily thereafter for a period of 14 days. Daily cage-side observations included changes in skin and fur, eyes and mucous membrane (nasal) as well as respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urination, and defecation), and central nervous system (ptosis, drowsiness, gait, tremors, and convulsion) changes. Individual animal weights were determined shortly before the administration of doses and weekly thereafter. Food and water consumption were also evaluated. Mortality, if any, was determined over a 2-week period. At the end of the test, all animals were subjected to gross necropsy and all pathological changes were recorded [8].

2.3.2. Subacute toxicity

Eighty Wistar rats, both sex, were randomly and equally divided into 8 groups (5 males and 5 females each group). One group received vehicle (control group) and the others received MTZ or MTZ-I at doses of 200, 400 or 600 mg/kg.

Since data on acute toxicity of MTZ-Ms (2000 mg/kg) show no toxic effect, it was used a single MTZ-Ms dose (1000 mg/kg - limit test dose). One level dose in MTZ-Ms group was also performed to reduce the number of animals in the study. This procedure is in accordance with protocol OECD 407.

Drugs were administered by gastric gavage once a day for 28 days [8]. During the study, the intake of water and food, and body weight of each group rats were recorded weekly. Animal general behavior, clinical signs of toxicity and mortality were evaluated daily.

By the end of 28 days, the animals were fasted for one night, but had free access to water, and then were euthanized by decapitation. Their blood was collected in heparinized funnels and tubes with EDTA (used for hematological tests) and in funnels and tubes without anticoagulants (used for biochemical tests).

After collecting blood, selected organs and tissues (liver, kidney, spleen, lung, heart, thymus, testis, ovary, intestine, stomach) were carefully dissected out, inspected for macroscopic alterations and intended to histopathological analyses.

2.3.3. Blood analyses

Hematological analyses were performed in total blood by using hematology detection system (ABXMicros ABC Vet - Horiba ABX Diagnostics, Montpellier, France). The following parameters were evaluated: hemoglobin (HB), hematocrit (HT), red blood cells count (RBC), White blood cells count (WBC) and platelets count (PLT).

Biochemical analyses were performed in serum obtained after centrifugation of total blood without anticoagulants, at 2500 rpm for 15 min. Standardized diagnostic kits (Gold Analisa Diagnostica, Belo Horizonte, Brazil) and a spectrophotometer (Biotron BRT-811 - Biosystems SA, Barcelona, Spain) were used in spectrophotometrical determination of the following biochemical parameters: uric acid (AUR), albumin (ALB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol (CT), creatinine (CRE), alkaline phosphatase (FAL), amylase (AMY), glucose (GLU), total proteins (PROT) and urea (URE).

2.3.4. Histopathological analyses

The tissues and organs (liver, kidney, spleen, lung, heart, thymus, testis, ovary, intestine, stomach) were fixed in 10% buffered formalin (pH 7.2), processed, embedded in paraffin, cut into slices of 2-4 µm and stained with haematoxylin and eosin (HE).

A complete histopathological examination was performed in organs of all animals that received the vehicle (control), MTZ-Ms (1000 mg/kg) and in the ones that received MTZ and MTZ-I at higher dose (600 mg/kg). The slices were examined for abnormalities by an observer that was not aware of treatments [8, 10, 11, 12, 13, 14].

2.3.5. Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed utilizing ANOVA, followed by test of Duncan to evaluate significant differences between the groups. Differences were considered significant for $p<0.05$. Results that did not present a normal distribution were analyzed using non-parametrical Mann & Whitney test [15].

3. RESULTS

3.1. Acute toxicity

No clinical signs of toxicity were noted and no treatment-related mortality occurred among rats treated with 300 and 2000 mg/kg of MTZ or MTZ-Ms during the observation period. However, one death occurred in the group treated with MTZ-I at the dose of 2000 mg/kg, which, after

Table 1. Evaluation of body weight and reproduction system after *per os* treatment for 28 days with MTZ, MTZ-I and MTZ-Ms.

Treatment	Sex	Body weight initial	Body weight end	Testis	Uterus with ovaries
Control	Male	174.2 ± 3.9	277.2 ± 11.6	10.94±1.18	-
	Female	155.8 ± 7.1	208.2 ± 17.5	-	3.44±1.14
MTZ 200mg/kg	Male	193.2 ± 3.2	263.0 ± 34.0	11.17 ± 0.19	-
	Female	189.6 ± 7.9	211.8 ± 25.4	-	3.66 ± 0.33
MTZ 400mg/kg	Male	185.4 ± 5.2	269.0 ± 50.2	11.63 ± 0.99	-
	Female	183.2 ± 6.8	219.4 ± 9.9	-	3.83 ± 0.87
MTZ 600mg/kg	Male	193.6 ± 3.8	264.0 ± 22.8	7.68 ± 1.98*	-
	Female	190.2 ± 5.8	209.8 ± 9.2	-	4.14 ± 0.41
MTZ-I 200mg/kg	Male	173.8 ± 1.09	258.4 ± 7.1*	12.02 ± 0.16	-
	Female	160.4 ± 3.2	194.4 ± 17.7*	-	3.90 ± 0.87
MTZ-I 400mg/kg	Male	176.4 ± 5.2	243.3 ± 10.5*	12.99 ± 0.64	-
	Female	151.6 ± 5.7	179.8 ± 18.8*	-	2.61 ± 0.44
MTZ-I 600mg/kg	Male	181.4 ± 3.4	236.8 ± 13.6*	12.81 ± 0.71	-
	Female	152.6 ± 5.1	179.5 ± 6.6*	-	3.48 ± 1.06
MTZ-MS 1000mg/kg	Male	177.4 ± 7.7	242.7±16.0	8.60 ± 1.57*	-
	Female	154.2 ± 17.6	206.8 ± 29.8	-	4.25 ± 0.84

Data are expressed as mean ± SD, n = 5. *Duncan test ($p<0.05$) vs control group. The weight of the testis and the uterus with ovaries are expressed in relative organ weights (g/kg of body weight).

Table 2. Biochemical parameters after *per os* treatment for 28 days with MTZ.

Treatment	Sex	Parameter							
		Glucose (mg/dL)	Albumin (g/dL)	Creatinine (mg/dL)	HDL (mg/dL)	Urea (mg/dL)	ALT (U/L)	AST (U/L)	Alkaline phosphatase (U/L)
Control	Male	109.8 ± 7.95	3.08 ± 0.10	0.76 ± 0.21	52.8 ± 3.96	39.4 ± 9.4	87.20 ± 4.55	65.20 ± 52.31	463.40 ± 103.37
	Female	111.8 ± 10.7	3.26 ± 0.16	0.62 ± 0.13	71.6 ± 6.62*	44.2 ± 8.23	108.80 ± 23.61	33.60 ± 19.88*	293.60 ± 134.89
200mg/kg	Male	98.0 ± 25.33	3.42 ± 0.25	0.26 ± 0.11*	91.4 ± 13.11*	59.2 ± 7.26*	109.2 ± 22.21	217.2 ± 49.44*	679.4 ± 136.99*
	Female	103.20 ± 6.06	3.19 ± 0.25	0.26 ± 0.3*	75.4 ± 8.76*	67.4 ± 13.92*	87.60 ± 14.19	242.6 ± 66.93*	338.0 ± 155.42*
400mg/kg	Male	98.4 ± 12.9*	3.28 ± 0.29	0.2 ± 0.1*	62.8 ± 13.85	53.4 ± 8.65*	96.0 ± 18.0	222.2 ± 51.78*	467.8 ± 50.54
	Female	80.5 ± 8.35*	3.34 ± 0.24	0.4 ± 0.08*	86.0 ± 9.09*	67.25 ± 4.57*	48.25 ± 5.74*	167.75 ± 74.41*	205.0 ± 139.13
600mg/kg	Male	63.20 ± 9.63*	3.29 ± 0.16	0.34 ± 0.05*	62.2 ± 6.61*	45.2 ± 4.71	54.8 ± 10.8*	123.6 ± 12.54*	297.4 ± 180.51*
	Female	67.2 ± 8.73*	3.53 ± 0.39	0.38 ± 0.17*	78.2 ± 24.38*	53.2 ± 13.08	39.2 ± 11.43*	149.6 ± 18.0*	209.8 ± 60.07*

AST - aspartate aminotransferase, ALT - alanine aminotransferase. Data are expressed as mean ± SD, n = 5. *Duncan test ($p<0.05$) vs control group.

necropsy, proved to be not related to gavage errors. All other animals survived up to their scheduled termination. Animals treated with MTZ-I (2000 mg/kg) showed moderate aggressiveness during the observation period.

No significant changes in the consumption of food and water could be observed in the MTZ-Ms and MTZ-I groups, as compared to the control. After 14 days, there were no significant changes in body weight and in the macroscopic aspects of the organs.

3.2 Subacute toxicity

In clinical evaluation, no abnormal behavior was observed in the groups treated with MTZ (200, 400 or 600 mg/kg) and MTZ-Ms (1000 mg/kg). In the groups treated with MTZ-I (600 mg/kg), the animals presented moderate aggressiveness and vocalization. As compared to control group, there were no statistically significant difference ($P<0.05$) in the water and food intake and body weight of rats treated with MTZ (200, 400 or 600 mg/kg) and MTZ-Ms (1000 mg/kg). In these groups, no animals died. However, in the groups treated with MTZ-I a significant reduction in the weight gain and food intake was noted in all doses (200, 400 or 600 mg/kg) as compared to control groups. A reduced body weight gain (Table 1) and reduced food intake were recorded for rats treated at 200, 400 or 600 mg/kg MTZ-I. Some rats showed slight weight loss during the treatment period. Moreover, in the group treated with high dose (600 mg/kg) of this drug, two animals died in the 27^{th} day.

There was no statistically significant difference ($P<0.05$) in organs weight among MTZ, MTZ-Ms, MTZ-I, and control group. The only exception was testis weight, which was significantly decreased in animals treated with MTZ (600 mg/kg) and MTZ-Ms (1000 mg/kg) as compared to control (Table 1).

The biochemical profiles of the MTZ treated groups and control are presented in the Table 2. A significant difference was detected in following parameters: Glucose (decreased), creatine (decreased), urea (increased in 200 and 400 mg/kg dose), ALT (decreased), AST (increased) and alkaline phosphatase (decreased). The effect on biochemical parameters of MTZ-Ms treated group are presented in the Table 3. An increase in serum cholesterol, proteins, albumin and ALT were detected. Table 4 presents the biochemical profile of MTZ-I treated group. A significant increase in serum amylase, alkaline phosphatase, glucose, cholesterol and urea was detected. Other parameters were not statistically significant (data not shown).

Most hematological parameters were in normal range, although a significant increase in certain parameters such as leukocytes, lymphocytes and red blood cells was observed (Table 5) in the groups treated with MTZ (200, 400 and 600 mg/kg) and MTZ-Ms (1000 mg/kg).

As kidney, lung, heart, thymus, and stomach of the animals that received higher doses (600 mg/kg) of MTZ and MTZ-I did not show any abnormalities in histopathological analysis, lower doses were not examined.

Histopathological examination revealed degeneration of the seminiferous epithelium (Fig. 2) along with increased incidence and/or severity of hypertrophy/ hyperplasia of intestinal epithelium (Fig. 3)

Table 3. Biochemical parameters after *per os* treatment for 28 days with MTZ-Ms.

Treatment	Sex	Parameter			
		Total cholesterol (mg/dL)	Total proteins (g/dL)	ALT (U/L)	Albumin (g/dL)
Control	Male	86.77 ± 6.78	3.68 ± 0.58	46.12 ± 8.59	2.45 ± 0.13
	Female	68.08 ± 5.56	3.58 ± 0.08	56.03 ± 9.67	2.64 ± 0.44
1000mg/kg	Male	94.96 ± 15.06*	4.46 ± 0.58*	72.98 ± 26.39*	2.64 ± 0.34*
	Female	112.85 ± 21.63*	4.91 ± 0.11*	99.37 ± 15.72*	3.0 ± 0.23*

ALT - alanine aminotransferase. MTZ-Ms = metronidazole methanosulfonate. Data are expressed as mean ± SD, n = 5. *Duncan test ($p<0.05$) vs control group.

Table 4. Biochemical parameters after *per os* treatment for 28 days with MTZ-I.

Treatment	Sex	Parameter					
		Amylase (U/dL)	Albumin (g/dL)	Alkaline phosphatase (U/L)	Glucose (mg/dL)	Total cholesterol (mg/dL)	Urea (mg/dL)
Control	Male	225.60 ± 49.83	3.84 ± 0.32	463.40 ± 103.37	109.8 ± 7.95	78.6 ± 10.41	39.4 ± 9.4
	Female	197.00 ± 35.79	4.36 ± 0.29	293.60 ± 134.89	111.8 ± 10.7	86.0 ± 10.12	44.2 ± 8.23
200mg/kg	Male	215.60 ± 27.79	3.94 ± 0.09	333.00 ± 106.56	102.2 ± 12.4*	124.4 ± 7.9*	39.6 ± 6.23*
	Female	164.00 ± 41.24	4.15 ± 0.24	227.75 ± 26.11	137.5 ± 16.05*	127.25 ± 29.06*	55.75 ± 9.64*
400mg/kg	Male	233.67 ± 28.87	4.00 ± 0.38	504.00 ± 108.84*	116.0 ± 19.8*	114.0 ± 17.09*	42.25 ± 13.33*
	Female	196.00 ± 11.35	4.33 ± 0.35	372.67 ± 136.76*	139.0 ± 8.54*	147.33 ± 36.61*	67.66 ± 4.73*
600mg/kg	Male	297.25 ± 29.35*	4.35 ± .37	494.75 ± 86.49	125.5 ± 14.84*	164.75 ± 36.36*	99.5 ± 49.7*
	Female	210.00 ± 23.73*	4.53 ± 0.29	216.50 ± 70.15	160.5 ± 19.8*	155.5 ± 26.20*	126.0 ± 30.5*

MTZ-I = iodo metronidazole. Data are expressed as mean ± SD, n = 5. *Duncan test ($p<0.05$) vs control group.

Table 5. Hematological parameters after treatment for 28 days with MTZ and MTZ-MS.

Treatment	Sex	WBC (10^3/µl)	Lynphocytes (10^3/µl)	RBC (x 10^6/µl)
Control	Male	6.74 ± 1.83	3.5 ± 1.7	7.7 ± 0.5
	Female	5.3 ± 1.35	3.1± 0.92	7.1± 0.8
MTZ 200mg/kg	Male	15.4 ± 5.7*	7.7 ± 3.2*	9.1 ± 0.4*
	Female	10.7 ± 10.7*	6.5 ± 6.9*	8.5 ±1.0*
MTZ 400mg/kg	Male	14.5 ± 6.3*	7.5 ± 3.0*	8.7 ± 1.5*
	Female	13.2 ± 5.9*	7.2 ± 2.3*	9.0 ± 0.5*
MTZ 600mg/kg	Male	15.7 ± 5.7*	10.2 ± 7.2*	8.5 ± 0.6*
	Female	12.4 ± 8.5*	7.6 ± 6.8*	8.8 ± 0.6*
MTZ-MS 1000mg/kg	Male	9.0 ± 3.6*	4.9 ± 1.4*	10.4 ± 0.4*
	Female	10.7 ± 4.1*	6.5 ± 2.9*	9.6 ± 1.0*

MTZ = metronidazole; MTZ-MS = methanosulfonate MTZ; MTZ-I = iodo MTZ; RBC = red blood cells.
MTZ-I was not represented once no significant differences were observed for all parameters. Data are expressed as mean ± SD, n = 5. *Duncan test ($p<0.05$) vs control group.

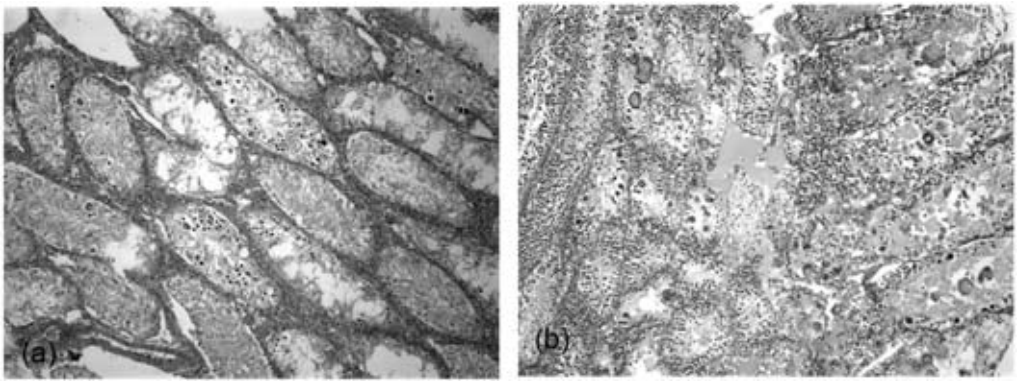

Fig. 2. Representative sections of testis of male Wistar rats treated with (a) control, (b) MTZ 600 mg/kg. Panel B shows the degeneration of germinal epithelium of affected tubules which appeared disorganized, disrupted and showed macrophages in lumina. A reduced volume of mature spermatozoa was observed. (20X, HE).

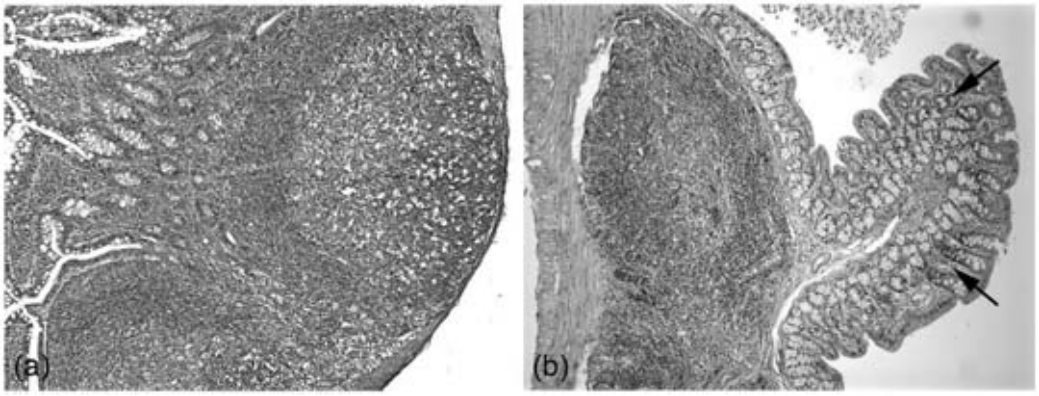

Fig. 3. Representative sections of intestinal epithelium of Wistar rats after treatment with (a) control; (b) MTZ-Ms 1000 mg/kg. The arrow points at tissue hyperplasia. (10X, HE).

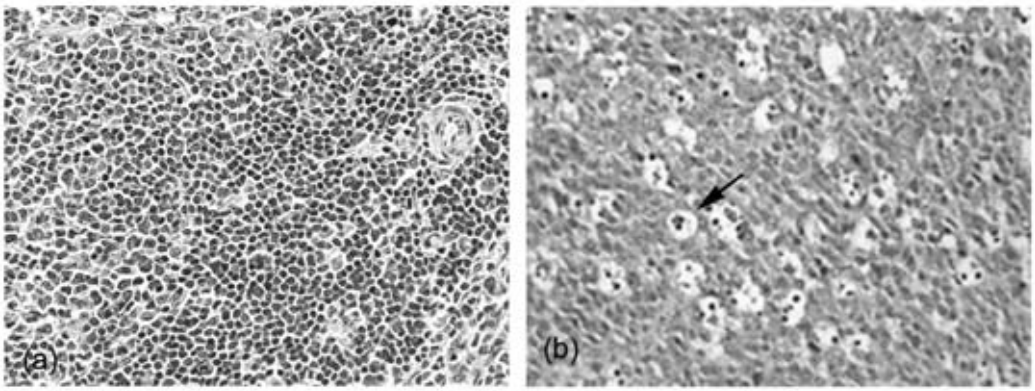

Fig. 4. Representative sections of splenic white pulp of Wistar rats after treatment with (a) control; (b) MTZ 600 mg/kg. The arrow points at a cell that has histological character an apoptotic cell, such as compaction of nuclear chromatin which is marginated against the nuclear envelope and blunt blebs are shown on the plasma membrane. (20X, HE).

in animals treated with MTZ (600 mg/kg) and MTZ-Ms (1000 mg/kg). The degeneration of seminiferous epithelium MTZ (600 mg/kg) and MTZ-Ms (1000 mg/kg) groups in most cases correlated to lower testis weight recorded at necropsy (Table 1). In these same groups, the splenic white pulp enhanced in size and showed histological characteristics of apoptotic process, such as compaction of nuclear chromatin marginated against the nuclear envelope, and blunt blebs on the plasma membrane (Fig. 4).

However, in the group treated with MTZ-I (600 mg/kg), despite the clinical signs of toxicity, no changes were noted in histopathological examination of selected organs and tissues (liver, kidney, spleen, lung, heart, thymus, testis, ovary, intestine, stomach) as compared to control.

4. DISCUSSION AND CONCLUSION

In the present study, no mortality could be observed in the evaluation of the acute toxicity of MTZ-Ms. In addition, no sign of toxicity was noticed during the experiment. These results suggest that MTZ-Ms exhibited a low acute toxicological risk, but, under certain circumstances, may be dangerous to vulnerable populations. During the evaluation of the acute toxicity of MTZ-I, one death was detected in the group treated with a 2000 mg/kg dose. Therefore, MTZ-I exhibited a toxicological risk in doses ranging from 300 mg/kg to 2000 mg/kg.

Dose level selection is a critical point in toxicity studies. Generally, it is recommended that the doses used to determine toxicity in animals should be two to four times the doses normally considered optimal [8]. Since the mean therapeutical dose of MTZ for humans is 60 mg/kg, and, according to Sohni *et al.* [16], a 200 mg/kg dose of MTZ was effective against *Entamoeba histolytica* in infected rats, in the current study, the selected doses included 200, 400, 600, or 1000 mg/kg.

According to the US National Toxicology Program, it is important that the loss in body weight by the surviving animals not exceed 10% of the initial body weight [17]. The body weight changes serve as a sensitive indication of the general health status of animal [18, 19]. In the present study, no significant difference could be observed in the body weight of MTZ and MTZ-Ms treated rats.

In addition to weight gain, the determination of food and water consumption may well indicate intoxication in animals [20, 21]. A significant reduction in food and body weight could be observed in the groups treated with MTZ-I. Although such alterations are generally reported as unspecific signs of toxicity, when one considers MTZ, MTZ-Ms, and MTZ-I, these alterations can be correlated to the molecular structure. As no alterations in food consumption and body weight occurred when MTZ and MTZ-Ms, which are very similar in structure to MTZ-I, were applied, it is quite possible that the presence of iodine moiety played a significant role in the toxicity of this analog. As further proof to this fact, Jenkins and Hidiroglou [22] reported a reduction in weight gain and food consumption in calves treated with iodide compounds.

Daily clinical observations, as well as the final observations (end point), were of utmost importance [17, 20]. No behavioral changes could be observed in the groups treated with MTZ and MTZ-Ms. However, the animals treated with MTZ-I became aggressive throughout the experiment period, which was mainly noticed by their vocal outbursts during drug administration.

In the biochemical evaluation of all the drugs studied, dose-dependent changes were observed for the same parameters, although these changes presented no specific clinical correlation.

No significant differences could be observed in the groups treated with MTZ-I when evaluating blood parameters. However, significant increases in the number of leukocytes, lymphocytes, and red blood cells could be observed at all dose levels in the groups treated with MTZ and MTZ-Ms. The lymphocyte increase could also be observed after treatment with MTZ in both preclinical and clinical studies [23]. Elizondo *et al.* [24] observed an increase in the rate of lymphocyte proliferation kinetics, indicating a possible immunostimulatory effect in patients after treatment with MTZ. Moreover, prior studies have reported an increase of lymphocyte proliferation occurring concurrently with chromosomal aberrations and DNA breakage, leading altered cells to apoptosis [25]. This finding suggests that the increase in lymphocyte proliferation may well be a compensatory mechanism due to damage in cell DNA. The fact that this response did not occur in

the MTZ-I treatment may well indicate the greater toxicity of this drug.

By contrast, it has been reported that human lymphocyte stimulation is suppressed by MTZ in *ex vivo* cell culture assays. In addition, Mohammad *et al.* [26] reported immunosuppression induced by MTZ in mice and human peripheral blood (*in vitro*). The authors added that they had observed a decrease in delayed-type hypersensitivity reactions, phagocitic activity, as well as TNF-alpha secretion. However, different forms of administration (intraperitoneal), lower doses, and a shorter period of drug administration were employed in the *in vivo* assay described by these authors. Therefore, it is possible that the effect of MTZ in the immune system can be affected by these parameters. In the present study, the immunostimulation may well be a compensatory defense mechanism of organisms used to combat the subacute toxicity of MTZ and MTZ-Ms. However, further studies on this matter are warranted.

From the pathological point of view, the present study indicated signs of toxicity manifested by cellular hyperplastic proliferalion (hyperplasia) in the intestinal epithelium of groups treated with MTZ-Ms and MTZ. It is well established that most common metronidazole side-effects are gastrointestinal problems [27].

According to Hartwell and Kastan [28] and Elledge [29] cells harboring DNA damage are either blocked at well-defined cell cycle stages, called “checkpoints”, for repair, or are completely purged from subsequent division by apoptotic cell death. In the present study, apoptotic cells in the white pulp of the spleen were detected. The relation between hyperplasia in the intestine and the apoptotic process needs to be better assessed, as it could be due to drug treatment or to a secondary effect in intestinal lymphoid tissue by altered gut flora [30].

In previous studies, MTZ-I was active against strains of *H pylori* that are resistant to MTZ [6]. However, in the present study, it proved to have a different toxicity profile of MTZ, as there were differences in clinical observation, as well as in pathological findings. At the same doses, MTZ showed more favorable toxicological profile than MTZ-I. Therefore, further studies are necessary to identify the target organs and possible mechanisms of MTZ-I toxicity.

The present study showed similar toxicity profile of MTZ and MTZ-Ms. In addition, the same target organs and similar pathological alterations were identified which suggest similar mechanisms of toxicity. The higher used dose of MTZ-Ms ensured that this analog may be used in higher doses or at least the same dose than MTZ without increasing toxicity.

MTZ (600 mg/kg) and MTZ-Ms (1000 mg/kg) induced a decrease in testis weight, as well as the destruction of germinative cells of the male reproductive apparatus. Deleterious effects in reproductive male tissues have previously been reported in several studies using MTZ [31, 32, 33, 34]. Described alterations included a decrease in the weight of the testis, epididymides, and accessory sexual organs [32], sperm cell morphology [32], testicular degeneration [33], and the inhibition of spermatogenesis at the primary spermatocyte stage [31, 32].

All in all, pathological findings described are in accordance with those reported by Khalil *et al.* [34], who noticed lesions in the intestine, spleen, and testis of Tilapia zilli treated with MTZ.

In conclusion, MTZ presented a more favorable toxicological profile as compared to MTZ-I at the same doses. Lower doses of MTZ-I may be useful according to previous study [6]; however, further studies are warranted to identify the mechanisms of toxicity of this analog. MTZ-Ms presented a toxicity profile similar to MTZ and may be useful as an additional antibacterial/antiprotozoal drug in higher doses.

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