Evaluation of immunosuppression induced by metronidazole in Balb/c mice and human peripheral blood lymphocytes

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Abstract

The immunomodulatory effect of metronidazole (MTZ), a nitroimidazole drug used as an antiprotozoal and antibacterial agent, was investigated using Balb/c mice and human peripheral blood lymphocytes. For in vivo studies, mice were divided into six groups, six animals per group, group I received vehicle alone while the other groups (II–VI) received intraperitoneal injections of MTZ (14, 28, 42, 57, and 114 mg/kg) respectively. For in vitro studies different concentrations of MTZ (5, 10, 50, and 200 μg/ml) were used. MTZ showed a significant decrease in the percentage of circulating neutrophils and monocytes and an increase in the percentage of circulating lymphocytes. The relative weights of spleen as well as the relative body weight gain also decreased. Detectable changes were seen in the histology of spleen and thymus. Splenic plaque-forming cells (PFC), hemagglutination (HA) titer to sheep red blood cells (SRBC), spleenocytes and human peripheral blood lymphocytes proliferation (MLR) were markedly suppressed by MTZ treatment as compared to control group. MTZ also induced a significant decrease in delayed-type hypersensitivity (DTH) reaction, phagocytic activity (assessed by phagocytic capacity and phagocytic index) as well as TNF-α secretion by peritoneal macrophages. These observations indicate that MTZ significantly induced immunosuppression in mice and in human peripheral blood lymphocytes.

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KEYWORDS
Metronidazole;
Immunotoxicity;
Human peripheral blood lymphocytes;
Balb/c mice

1. Introduction

Nitroheterocyclic chemicals have a wide variety of applications, ranging from food preservatives to antibiotics. 5-nitroimidazoles are a well-established group of antiprotozoan and antibacterial agents. They have a
heterocyclic structure consisting of an imidazole-based nucleus with a nitro group, NO2, in position 5. Metronidazole (MTZ) [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] is one of these chemotherapeutic agents that have been used in treatment of ailments caused by anaerobic bacteria and certain anaerobic protozoa, such as Trichomonas vaginalis, Entamoeba histolytica, and Giardia lamblia. Also, 5-nitroimidazoles are generally administered before various surgery operations [1].

All investigational new drugs should be evaluated for the potential to produce immunosuppression [2]. This is generally accomplished in repeat-dose toxicology studies using standard clinical and anatomic pathology methods. According to the American National Toxicology Program (NTP) and the Chemical Industry Institute of Toxicology (USA), a flexible approach to immunotoxicity assessment was designed. The assays would be applied to chemicals and drugs that demonstrate suspicious structure-activity relations and pharmacokinetic properties that suggest potential immunotoxicity. Among the indicators of immunotoxicity is the induction of neoplasia [3, 4].

Since the introduction of MTZ into clinical applications, its effectiveness in the treatment of various diseases was counterbalanced by the increasing evidence of its mutagenicity, carcinogenicity, and embryolethality. MTZ induced a reversible bone marrow depression and effects on male fertility [4, 5]. Some of its metabolites have been shown to be mutagenic in certain bacterial test systems. In addition, it has been observed that MTZ induces DNA single-strand breaks in the lymphocytes of patients on standard doses of the drug [6–8].

Toxicological investigations following long-term administration of high doses of some 5-nitroimidazole derivatives to rats and mice showed induction of various tumors. Therefore, the potential immunosuppressive effects of nitroheterocyclic compounds should be considered when these agents are evaluated in the laboratory or used in the clinic [9, 10]. On the other hand, heavy research have been started in order to study the significance of using antibiotics for their immunosuppressive side effect [11, 12].

MTZ is marketed by various generic manufacturers. In Jordan, it was found that MTZ was widely used by the Jordanian population during the year 2004, and it was estimated that only 43% of MTZ units sold was prescribed properly during the year 2004 [13]. MTZ is highly and repeatedly prescribed because of reifications, improper prescriptions and automedication. In Jordan, patients as well as specialists reported MTZ-induced side effects such as gastritis, nausea, and vomiting (unpublished observations) but documented immunotoxic and neurotoxic effects have also been reported [14–17].

In the present study we investigated the effect of MTZ on immune system cells and tissues in two models. Functional assays included screening for changes in percent body weight gain, organ body weight ratio, IgM plaque-forming cell (PFC) and human and mouse lymphocyte proliferation including mixed lymphocyte response (MLR), thymus, lymph nodes and spleen histopathology, delayed-type hypersensitivity (DTH) and peripheral blood cells parameters. The non specific immunity was also assessed by in vitro peritoneal macrophage phagocytosis as well as TNF-α production.

2. Materials and methods

2.1. Mice

Specific pathogen-free inbred female Balb/c mice (6–8 weeks old; 19–21 g weight) were housed in polystyrene cages in an air-conditioned room. All animals were maintained at laboratory diet and tap water ad libitum. Animals were acclimatized for 1 week in the lab prior to usage. For the MLR stimulator spleenocytes, male albino rats were used. The experiments were conducted according to the principles given in the guide for the care and use of laboratory animals issued by the University of Jordan.

2.2. Chemicals

Metronidazole intravenous infusion (500 mg/dl) was purchased from B. Braun Melsungen AG (Germany). Bovine serum albumin (BSA), Guinea Pig complement, Hank’s balanced salt solution (HBSS), Histopaque-1077, phosphate buffered saline (PBS), and RPMI-1640 medium were purchased from Sigma (USA). Mitomycin C was obtained from BDH Biochemicals (England). MTT nonradioactive cell proliferation kit was obtained from Promega (USA). Sandwich ELISA kits for quantitation of TNF-α were purchased from Euroclone (Italy).

2.3. Dose and exposure schedules

The mice were divided into six groups (I–VI), each of six animals. Group I (control group) received phosphate buffered saline (PBS). Groups II–VI (treatment groups) received 0.2 ml of the indicated doses of MTZ. Animals were treated with MTZ in PBS intraperitoneally (i.p.) for 14 consecutive days. The drug doses were 0.5×, 1.0×, 1.5×, 2.0× and 4.0× of the human therapeutic dose, and were equal to 14, 28, 42, 57, and 114 mg/kg respectively. Mice were bled and sacrificed by cervical dislocation 2 h after the administration of the last drug dose. For in vitro lymphocytes and spleenocytes proliferation, or phagocytosis experiments, final well concentrations of MTZ of 0, 5, 10, 50, and 200 μg/ml were used [8, 17–20].

2.4. Organ and body weight

The weight of animals was recorded at the beginning of experiments with MTZ and 2 h after the last treatment, and then the percent gain in body weight was calculated. Mice were sacrificed by cervical dislocation, spleen, kidney, liver, four lymph nodes (two inguinal and two axillary) and thymus were removed, weighed, and placed in sterile plastic tissue culture plates then 3 ml of RPMI-1640 medium were added to each plate before storage at 4 °C for further processing. For all organs, connective and adipose tissue were removed before weighing [3, 10].

2.5. Preparation of single cell suspension (SCS)

To disrupt mononuclear cells out of spleens, each spleen was teased between the frosted edges of two sterile microscopic slides, in a sterile tissue culture dish containing 10 ml complete medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine). The released cells were collected from the tissue plates and were transferred to a sterile centrifuge tube. The tubes were centrifuged at 1200 rpm for 7 min at 4 °C in a swinging bucket refrigerated centrifuge (Hitech, Germany). Supernatant was discarded, and the pellet was resuspended in 3 ml of RBC lysing buffer containing 0.83% NH4Cl in 100 mM Tris buffer, pH 7.4 (Sigma) and kept at room temperature for 3 min. The lysed RBCs were washed out 3 times in 10 ml of RPMI-1640 medium by centrifugation at 1200 rpm for 3 min using refrigerated
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centrifuge. Viable cells were resuspended in 1 ml RPMI-1640 medium and counted using Neubauer hemocytometer. The cell viability was determined by trypan blue exclusion test [21,22].

2.6. Lymphocyte proliferation assay (mixed lymphocyte response, MLR)

Total viable stimulator cells (S), from three albino rat spleens, were counted, their concentrations were adjusted to 6×10⁶ cells/ml and inactivated by mitomycin C by incubating the stimulator cells with 25 μg/ml mitomycin C at 37 °C for 1 h. At the end of the incubation period, cells were washed three times with RPMI-1640 to remove traces of mitomycin C. For each treated group, 4×10⁶ cells/ml suspensions from each mouse spleen were prepared (R), 0.1 ml of MTZ concentrations were added so that the final well concentrations were 0, 5, 10, 50, and 200 μg/ml, respectively, then 0.1 ml of stimulator cells were mixed with equal volume of complete RPMI-1640 (S+M) or with equal volume of responder mouse spleenocyte cells (R+S). Also 0.1 ml of responder cells was mixed with equal volume of RPMI-1640 medium (R+M). This procedure was repeated in triplicates for every indicated MTZ concentration. Plates were then incubated in a CO₂ incubator (Binder, Germany) at 37 °C, 95% humidity, and 5% CO₂ for 72 h. At the end of the incubation period, the number of viable cells was determined using the trypan blue exclusion test for each well. Colorimetric determination of cell proliferation was done using cell titer 96 nonradioactive cell proliferation assay kit. Briefly, to each well, 10% of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium; 5 mg/ml] was added and incubated at 37 °C in 5% CO₂ incubator for 4 h. Then, the blue formazan precipitate was dissolved in acidic isopropanol, and its optical density was measured at 570 nm using a plate reader (Biorad, Japan). Then the percent difference between the proliferation of cells in the mixed cultures (R+S) as compared with the (R+M) cultures was calculated using the following formula [23]:

\[
\text{Proliferation} = \frac{|(R + S) - (R + M)|}{(R + M)} \times 100\%
\]

2.7. Human lymphocytes function (human MLR)

The study was carried out using blood samples from two healthy nonsmoker male donors, aged 26 and 38 years under their written consent. Results of clinical routine laboratory analyses of the two donors were in the normal range, and the absence of exposure to known immunotoxicants was considered. Eight milliliters of fresh heparinized blood samples were placed into 50-ml conical centrifuge tubes, and 7 ml of phosphate buffered saline was added and mixed well. 3 ml of Histopaque-1077 solution were layered underneath 15 ml of blood/PBS mixture by placing the tip of the pipette containing the Histopaque-1077 at the bottom of the sample tube. The mixture was centrifuged for 30 min at 2000 rpm, at 18 °C to 20 °C [24].

Using a sterile pipette, the upper layer that contains the plasma and most of the platelets was removed. Then the mononuclear cell layer was transferred to another centrifuge tube using another pipette. The cells were then washed by adding excess Hank’s balanced salt solution (HBSS) (3 times the volume of the mononuclear cell layer) and centrifuged for 10 min at 1300 rpm, at 18 °C to 20 °C. Supernatant was removed and cells were resuspended in HBSS. The wash steps were repeated until most of the platelets were removed then the cells’ pellet was resuspended in complete RPMI-1640. Resuspended mononuclear cells were counted and viability was determined by trypan blue exclusion test.

Mixed lymphocyte reaction was then conducted as follows: total viable stimulator cells from the donors were counted and their concentrations were adjusted to 6×10⁶ cells/ml. Then 100 μl of both cell preparation were mixed with 100 μl of different concentrations of MTZ so that the final MTZ concentrations were 0, 5, 10, 50, and 200 μg/ml per well respectively in a 96-well u-bottom tissue culture plate (Greiner, Germany). This procedure was conducted in triplicates for every selected MTZ concentration. Plates were incubated in a CO₂ incubator (Binder, Germany) at 37 °C, 95% humidity, and 5% CO₂ for 72 h. The results were measured using MTT test [22,23,25,26].

2.8. Determination of the hematological parameters

Blood was collected from the retro-orbital plexus of each mouse before being sacrificed using heparinized capillary tubes. 0.2 ml of blood was collected in a sterile (K₃-EDTA) anticoagulated tubes (Minicollect®, Greiner, Germany) so that the blood to anticoagulant ratio was 1:0.075 v/v. Routine hematological parameters were assessed including hemoglobin content, packed cell volume percent (PCV%), red blood cell (RBC) count, and white blood cell (WBC) count. A blood film was stained with Gimsa and Wright for each treated animal, slides were observed under light microscope (Labomed, USA) using oil immersion. At least 200 cells were studied for differential count.

2.9. Histopathological examination

At day 14 and 2 h after the last dose, all groups of mice were sacrificed by cervical dislocation. Thymus (TM), spleen and four lymph nodes (2 axillary and 2 inguinal) of each mouse were then collected, fixed in 10% formalin and sectioned. 5-μm-thick sections were stained with Hematoxylin–Eosin (H–E). Histopathological changes in these organs were observed under light microscope and scored according to the degrees of changes as described by Hassan et al. [27].

2.10. Delayed-type hypersensitivity response (DTH)

Delayed-type hypersensitivity response (DTH) was determined for treatment and control groups using the method of Bin-Hafeez et al. (2003) with some modification [28]. On the 9th day of the treatment, animals were immunized i.p. with 1×10⁸ sheep red blood cells (SRBCs). After five days of immunization (day thirteen of experiment), all animals were again challenged with a booster dose of 1×10⁸/50 μl SRBCs in the left hind footpad [26]. The right hind footpad was injected with the same volume of PBS to serve as trauma control for nonspecific swelling. Increase in left footpad volume was measured 24 h after the challenge with SRBCs and the difference between left and right hind footpad volumes was calculated using digital plethysmometer (Hugo Basile, Italy).

2.11. IgM plaque-forming cell assay (PFC)

The primary IgM response to SRBC was assessed as follows: All mice were immunized by i.p. injection of 50 μl antigen (5×10⁹ SRBC in PBS) 4 days before the end of the experiment, and the number of spleen plaque-forming cells was counted in duplicates on day 14 as described by the slide technique of Cunningham and Szenberg [29]. The spleen single cell suspension of 1×10⁶ cells/ml was prepared in RPMI-1640 culture medium. For PFC assay, the SRBCs were prepared at a cell density of 5×10⁶ cells/ml in PBS. One milliliter of SRBC in medium along with 0.5 ml of diluted guinea pig complements (1:10 diluted with normal saline) were added to 1 ml of spleen suspension. Cunningham chambers were prepared using glass slides and double-sided adhesive tape (Scotch Brand, St. Paul, MN). The chambers were loaded with a known volume of assay mixture, sealed with tissue grade wax and incubated at 37 °C for 1 h. The plaques were counted under a light microscope (Labomed, USA). Results were reported as the number of PFC per spleen.

2.12. Phagocytosis study

To collect resident peritoneal cells, three mice were sacrificed by cervical dislocation and wetted with 70% alcohol to sterilize the whole body area. A midline incision was made under sterile conditions. The abdominal skin was retracted with forceps to expose the intact
peritoneal wall. A 10-ml syringe with 19-G needle was filled with harvest medium cRPMI-5. Ten milliliter of harvest medium was injected into each mouse peritoneum. The same syringe and needle were used to withdraw peritoneal fluid slowly. Fluid recovery of 8 ml/mouse was achieved. This procedure was repeated three times per mouse, and then peritoneal fluid was pooled in 50-ml polypropylene centrifuge tubes on ice. A 20 μl sample was removed and cells were counted using a hemocytometer. Cell concentration was 3×10⁶/ml. All the harvested cells were mixed and the concentration was then adjusted to 4×10⁶/ml by centrifugation.

One gram of yeast (Saccharomyces cerevisiae, L-20 strain, identified by the manufacturer) was suspended in 50-ml RPMI-1640 medium in a 100-ml flask, incubated at 37 °C in an incubator for 15 min and its count was adjusted to 8×10⁷ yeast cell/ml. Then 100 μl of each of: the indicated MTZ concentrations, macrophage suspension, and yeast cell suspension were added and mixed in triplicates in a 96-well plates and incubated at 37 °C for 1 h with occasional shaking.

After incubation, 50 μl of this mixture was smeared on a glass slide, air dried and fixed with absolute methanol, and stained with Wright-Gimsa stain. The slides were observed under light microscope using oil immersion. At least 200 cells were counted and the phagocytic activity was calculated, as phagocytic index (PI) and phagocytic capacity (PC) using the following formula: PI=AB. Where A is the percentage of yeast-ingesting phagocytes and B is the number of yeast cells ingested per phagocyte, PC is the mean percentage of cells that engulfed ≥4 yeast cells[28].

2.13. Serum antibody titer: hemagglutination (HA) titer

Four days before ending the treatment, mice were immunized by i.p. injection of 5×10⁸ SRBCs in PBS intaperitonially. At the end of experiment (day 14), blood was collected from the retro-orbital plexus of each mouse before being sacrificed by cervical dislocation. 0.2 ml of blood was collected in a sterile silica gel-containing plain tubes (Minicollect®, Greiner, Germany), centrifuged for 10 min at 300 rpm then sera were serially diluted (in doubling dilutions) in PBS and placed in the wells of a U-shape 96-microtiter plates. Aliquots (25 μl) of twofold diluted sera in PBS were challenged with 25 μl of 1% v/v SRBCs suspension and mixed. The plates were incubated at 37 °C for 1 h, and then observed for hemagglutination. The log₂ highest dilution giving hemagglutination was taken as the antibody titer[27,28].

2.14. TNF-α quantitation by sandwich ELISA

For this purpose, murine TNF-α kit (mTNF-α), (Euroclone Life Science, Italy) was used. 100 μl of supernatant of the previously described macrophage cell culture were pipetted directly onto wells. The wells were then incubated in dark for 2 h at room temperature (18–25 ºC). At the end of the incubation period the wells were aspirated and washed 5 times with wash buffer, 50 μl of the biotinylated anti-mTNF-α were added to each well, plates were covered and incubated for 1 h at room temperature. At the end of the incubation period the washing step was repeated as indicated above and 100 μl horse radish peroxidase, that was prepared just before

![Figure 1](image1.png) Percent increase in body weight in groups of mice receiving daily i.p. injection of the indicated doses of MTZ for 14 days. Data are means± SD (n=6). *p<0.05 **p<0.01 when compared to control animals.

![Figure 2](image2.png) Relative spleen weight (% body weight) in groups of mice receiving daily i.p. injection of the indicated doses of MTZ for 14 days. Data are means±SD (n=6). **p<0.01 when compared to control animals.

![Figure 3](image3.png) The effect of increasing concentrations of MTZ on the mixed lymphocyte reaction (MLR) of spleenocytes from healthy Balb/c female mice and albino rat placed in RPMI-1640 medium in vitro. The color of reaction product was detected at 570 nm wavelength using (MTT) test. Data are means±SD. Experiments were performed by triplicates, *p<0.05 **p<0.01 and when compared to control wells.

![Figure 4](image4.png) Effect of MTZ on the proliferation of two unrelated human peripheral blood lymphocytes in vitro treated with the indicated concentrations of MTZ in RPMI-1640 medium. The color of reaction product was detected at 570 nm wavelength using (MTT) test. Data are means±SD. Experiments were performed by triplicates, *p<0.05 and **p<0.01 when compared to control wells.
were made using GraphPad Prism 4.03 statistical software package. Treatment with MTZ caused a significant decrease in body weight gain at doses 28, 42, 57, and 114 mg/kg, but there was no significant differences. A difference was considered significant at the conventional level of significance of 0.05 (p<0.05) when compared to control.

### Data are means ±SD (n=6). (PVC; packed cell volume, WBC; white blood cells, MTZ; metronidazole.)

### 2.15. Statistical analysis

Data were presented as means ±SD of the indicated number of experiments. Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett's posttest to check for significant differences. A difference was considered significant at the conventional level of significance of 0.05 (p<0.05). All analyses were made using GraphPad Prism 4.03 statistical software package.

### 3. Results

#### 3.1. Effect of MTZ on organ and body weight ratio

None of the doses of MTZ showed mortality in any treated group. Treatment with MTZ caused a significant decrease in body weight gain at doses 28, 42, 57, and 114 mg/kg, but there was no effect at the dose of 14 mg/kg (Fig. 1). All doses of MTZ caused a decrease in the relative weight of spleen (Fig. 2). No effects were observed in the thymus, liver, kidneys and lymph nodes relative weights at any dose when compared with the control group (data not shown).

### Table 1 The effect of MTZ on hematological parameters of different treatment groups of female Balb/c mice

<table>
<thead>
<tr>
<th>MTZ (mg/kg)</th>
<th>RBCs parameters</th>
<th>WBCs parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RBCs count (×10^6/mm³)</td>
<td>PCV (%)</td>
</tr>
<tr>
<td>Control</td>
<td>9.83 ± 0.85</td>
<td>48.66 ± 3.92</td>
</tr>
<tr>
<td>14.0</td>
<td>9.76 ± 1.11</td>
<td>48.33 ± 3.55</td>
</tr>
<tr>
<td>28.0</td>
<td>8.74 ± 1.40</td>
<td>48.66 ± 1.86</td>
</tr>
<tr>
<td>42.0</td>
<td>8.65 ± 0.67</td>
<td>47.33 ± 2.73</td>
</tr>
<tr>
<td>57.0</td>
<td>9.61 ± 1.23</td>
<td>46.86 ± 3.77</td>
</tr>
<tr>
<td>114.0</td>
<td>8.93 ± 0.48</td>
<td>47.16 ± 4.91</td>
</tr>
</tbody>
</table>

Data are means ±SD (n=6). (PVC; packed cell volume, WBC; white blood cells, MTZ; metronidazole.)

#### 3.2. Effect of MTZ on murine lymphocyte proliferation assay (mixed lymphocyte reaction, MLR)

The results depicted in Fig. 3 show that MTZ at high doses (50 and 200 µg/ml) had a marked inhibitory effect on lymphoproliferative assay assessed by MTT test. Other doses 5 and 10 µg/ml showed no effect.

#### 3.3. Effect of MTZ on human lymphocytes proliferation (human MLR)

Metronidazole in concentrations of 10, 50 and 200 µg/ml caused concentration-dependent inhibition of the human peripheral blood lymphocytes proliferation (Fig. 4).

#### 3.4. Effect of MTZ on hematological parameters

Hematological examination of the peripheral blood revealed that mice treated with MTZ showed a significant (p<0.05) decrease in erythrocytes counts at the doses 28 and 114 mg/kg. Moreover, MTZ significantly decreased the percentage of neutrophils at 57 and 114 mg/kg. The percentage of peripheral blood lymphocytes was significantly increased at doses of 14, 28, 57, and 114 mg/kg. On the other hand, the percentage of circulating peripheral blood monocytes was significantly decreased at doses of 14, 42, 57, and 114 mg/kg. No significant changes in other hematological parameters were seen at all the indicated doses of MTZ (Table 1).

#### 3.5. Effect of MTZ on histopathology of spleen and thymus

Light microscopic examinations of lymph nodes did not show any significant differences between MTZ-treated and control groups (data not shown). Atrophy in spleen white pulp was observed at the doses of 42, 57, and 114 mg/dl (Table 2 and Fig. 5). Other doses caused minimal histopathological changes. On the other hand, our study showed a significant decrease in thymus medullary tissue at doses of 28, 42, 57, and 114 mg/dl (Table 3 and Fig. 6).

#### 3.6. Effect of MTZ on DTH

All doses of MTZ suppressed the DTH response 24 h after secondary injection of antigen (SRBCs) in a dose-dependent manner (Fig. 7).
3.7. Effect of MTZ on IgM PFC

The dose of 14 mg/kg MTZ caused no significant effect on PFC response. Higher doses, however, caused a decrease in the number of lyse plagues per spleen for MTZ-treated animals (Fig. 8).

3.8. Effect of MTZ on phagocytic activity of peritoneal macrophages

The effect of MTZ on macrophage functions in vitro are presented in Figs. 9, 10, and 11. The drug at concentrations of 5, 10, 50, and 200 μg/ml caused significant decrease in phagocytic capacity, when the results were compared with control wells (0.0 MTZ concentrations). The latter two doses (50 and 200 μg/ml) also caused significant decrease in the phagocytic index.

3.9. Effect of MTZ on serum antibody titer: hemagglutination (HA) titer

As presented in Fig. 12, hemagglutination titer at the dose (14 mg/kg) showed significant inhibition (p<0.05) in the concentration of the anti-SRBCs antibodies expressed as antibody titer, whereas higher doses caused a larger inhibition (p<0.01) compared with the control (group I).

3.10. Effect of MTZ on TNF-α production by peritoneal macrophages

The concentration of TNF-α from the supernatant of the peritoneal macrophage culture was significantly (p<0.01) reduced by MTZ in a dose-dependent manner (Fig. 13).

4. Discussion

Despite the overt use of MTZ as an antibacterial and antiparasitic antibiotic in humans, there is little information about its potential influence on the immune system cellularity and function. MTZ has been shown to induce suppression in the bone marrow, a primary lymphoid organ, and to affect male fertility [4]. Furthermore, it has been observed that MTZ induces DNA single-strand breaks in the lymphocytes of patients on standard doses of the drug [3,8], therefore toxicity to the peripheral lymphoid organs is suspected.

Gastrointestinal absorption of MTZ after oral administration is very fast and is comparable to intraperitoneal administration. In the present experiments, MTZ decreased the gain in body weight of MTZ-treated mice in all used doses over 14 mg/kg. Furthermore, all the tested doses of MTZ decreased the relative weight of the spleen, and the doses 42, 57 and 114 mg/kg body weight induced atrophy in the white pulp of the spleen. In addition, the dose 28 mg/kg induced atrophy in the medulla of the thymus, suggesting a possible effect on the activation and differentiation of the T-lymphocytes, an effect that might explain the relative increase in the circulating lymphocytes (Table 1). We have shown that the highest two doses (57 and 114 mg/kg) of MTZ decreased the percentage of neutrophils and at the same time increased the percentage of the lymphocytes at the same level of significance (p<0.01), suggesting a suppression of the

Table 3 Effects of MTZ exposure on thymus histopathology in mice receiving (14, 28, 42, 57, and 114 mg/kg) as daily i.p. injections for 14 days

<table>
<thead>
<tr>
<th>Thymus</th>
<th>Treatment (mg/kg)</th>
<th>Medullary atrophy</th>
<th>Starry sky appearance</th>
<th>Capsule and trabecule changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>14.0</td>
<td>–</td>
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<td>–</td>
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<tr>
<td>28.0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>42.0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>57.0</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>114.0</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

−: no changes observed; +: minimal changes; ++: readily detectable changes.
phagocytosis defense mechanism by neutrophils, therefore bactericidal activity inhibition is suspected. The decrease in the spleen relative weight and in the atrophy of spleen white pulp and thymus medulla may be the reason behind relative lymphocytosis because of lymphocyte primary and secondary lymphoid tissue homing suppression, a conclusion which is consistent with the finding that MTZ inhibits leukocyte-endothelial cell adhesion in rat mesenteric venules [9]. Peripheral blood RBCs showed a decrease in number when MTZ was used in the highest dose which may be due to the suppression of the bone marrow [4]. These parameters form, in part, the first level of tests for immunotoxicity screening [1]. Since MTZ induced changes in these parameters, the results of the present work indicate that this drug causes modulation of the immune function.

The conclusion of an immunomodulatory role of MTZ is substantiated in the present work by further experiments. For example, MTZ caused significant toxicity for mice spleenocytes and human peripheral blood lymphocytes since 50 and 200 μg/ml of MTZ inhibited the proliferative capacity of mice spleenocytes. Human lymphocyte proliferation was more sensitive to MTZ since it was suppressed by doses of MTZ as low as 10 μg/ml, indicating higher sensitivity in humans. The results of the DTH to SRBCs experiments lend further support to the above conclusion since MTZ suppressed delayed-type hypersensitivity at all the used MTZ doses. Similar conclusions were reached by other researchers using mice sensitized on the abdomen with 2, 4-dinitro-1-fluorobenzene (DNFB) and subsequently challenged on ears with DNFB [35].

The decrease in humoral immunity response to MTZ was manifested by suppression of antibodies production in HA titer by all MTZ doses and confirmed by a decrease in the PFC
count. Spleen relative weight decrease and white pulp atrophy observed in our experiments, is a common finding following the administration of certain immunosuppressive drugs, and are accompanied by the decreased ability of the animal to produce antibody [2]. Phagocytosis and killing of invading microorganisms by macrophages constitute body's

Figure 9  Light microscopic slides of peritoneal macrophages stained with Wright–Gimsa stain showing the phagocytosis capacity changes. (A–D) the macrophage culture treated with different concentrations of MTZ (200, 50, 10, and 5 μg/ml) respectively. (E and F) show activity of macrophage phagocytic capacity in the control cell cultures (PBS-treated macrophages). (M; macrophage, SC; Saccharomyces cerevisiae).

Figure 10  Effect of MTZ on the non specific immunity as assessed by the phagocytic capacity (PC), (calculated as the mean percentage of macrophages that engulfed >4 yeast cells). Data are means±SD. *p<0.05 and **p<0.01 when compared to the control cells.

Figure 11  Effect of MTZ on the nonspecific immunity as assessed by the phagocytic index (calculated as PI = AB, where A is the percentage of yeast-ingesting phagocytes and B is the number of yeast cells ingested per phagocyte). Data are means±SD. *p<0.05 when compared to control cells.
primary line of defense against infections [28,32,34]. Macrophages are an integral part of the immune system, acting as phagocytic, microbicidal and tumoricidal effector cells. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of the immune response [36]. Our in vitro results show that all doses of MTZ suppressed macrophage phagocytic capacity, and the highest two doses (50, 200 μg/ml) suppressed the phagocytic index, an observation that demonstrates that the immunomodulation by MTZ is multifaceted. On the other hand, MTZ in vivo markedly decreased the percentage of peripheral blood monocytes in the animals treated with 14, 42, 57 and 114 mg/kg, suggesting that this drug at these doses has an immunosuppressive effect on the nonspecific immune function. The inhibitory effect of MTZ on macrophage phagocytic activity shown in the present study is supported by the data showing inhibition of macrophage TNF-α production (Fig. 13). Other researchers have found that the production of TNF-α by human peripheral blood mononuclear cells (PBMC) was also suppressed by MTZ [6], thus lending further support to the present work.

It has been shown that MTZ is intracellularly activated by reduction and the toxic effect of reduced intermediates bind to DNA leading to loss of helical structure, strand breakage, and impairment of DNA function [7]. The observed effects which are thought to be due to reduction of the nitro group to radicals and to other reactive metabolites are possibly the mechanisms of MTZ-induced primary and secondary lymphoid organs toxicity observed in the present experiments [37].

In conclusion, the present study shows that MTZ has an inhibitory effect on the humoral as well as cell-mediated immune responses. The results demonstrate an immunosuppressive effect of MTZ in mice as well as in human lymphocytes. The effectiveness of MTZ in the treatment of diseases should be counterbalanced by the increasing evidence of its immunotoxicity. Further studies should be considered to study the significance of using MTZ in patients under long-term treatment.

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