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Original Research Article

High Doses Of Ivermectin Cause Toxic Effects After Shortterm Oral Administration in Rats

Key words

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Abstract: The anthelmintic macrocyclic lactones (MLs) are the most important endectocides in modern pharmacotherapy of parasitic infections. However, during the COVID 19 pandemic, ivermectin was used in humans against infection with the SARS-CoV-2 virus in doses significantly higher than the approved antiparasitic doses. This kind of application was created solely on the basis of *in vitro* tests, and is not officially approved in any country in the world. Therefore, we conducted a study on rats treated orally with 0.6, 1.2, 2.4 and 4.8 mg/kg of ivermectin for 5 days. Based on our investigation, ivermectin at the doses used in humans against the SARS-Co-2 virus (3, 6, 12 and 24 times higher than the antiparasitic dose 0.2mg/kg), causes changes in red blood cell counts and increases the levels of liver enzymes without visible clinical symptoms. Histopathological changes were recorded in the liver, kidneys and testicles of rats, and the highest dose tested led to bleeding in the brain tissue. Obviously, ivermectin somewhat increases concentration of the enzyme P-450 isoform 3A4, whose substrate it is, but the highest tested dose reduces its concentration in plasma to the control level. Notably, the concentrations of ivermectin recorded in plasma of treated rats, indicate that even high doses do not reach the *in vitro* IC50 value of ivermectin for SARS-CoV-2 reported in the literature. On the other hand, the concentrations of ivermectin in the brain approach the values that can manifest extremely toxic effects described in humans.

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Introduction

The anthelmintic macrocyclic lactones (MLs) are the most important endectocides in modern pharmacotherapy of parasitic infections. This large group of hydrophobic, structurally related compounds are widely used in animals and humans (1). Therapeutic doses of ivermectin in veterinary medicine range from 0.2-0.3mg/kg, with a second administration after one to two weeks. In human medicine, antiparasitic doses of ivermectin approved by the FDA and EMA range from 0.15 to 0.4 mg/kg of body weight, depending on the type of parasite and repeated twice. The avermectins are often referred to as endectocides because of their activity against both endo- and ectoparasites, have received considerable interest from the agricultural chemical industry. Thay have extremely high activity against arachnoid and nematode pests, low toxicity in mammals, generally benign environmental characteristics, and a unique mode of action. The median lethal oral dose (LD_{50}) of ivermectin for rats (male and female) ranges from 42.8 to 52.8 mg/kg (2).

The mechanism of antiparasitic action of ivermectin is the activation or positive allosteric modulation of invertebrate specific glutamate-gated chloride channels, with a secondary inhibitory action on $GABA_A$ receptors. In mammals, ivermectin can also inhibit GABA-ergic neurotransmission by promoting GABA release and acting as a GABA receptor agonist when exhibits neurotoxic activity. Most likely, ivermectin causes neurotoxic disorders by acting on GABA-dependent as well as GABA-independent chloride channels (3), showing the ability to exert its effect on both central

and peripheral GABA-ergic structures (4, 5). At ten times therapeutic doses, ivermectin does not show any obvious visible clinical symptoms of neurotoxicity, but it disrupts integration of the CNS and motor coordination in the Rota-rod test (4).

The potential neurotoxic activity of ivermectin depends, in part, on the absorptionextrusion activity of the drug in the gastrointestinal tract/blood-brain barrier, which is regulated by multiple transport systems (P-gp, MRP, ABCB1 and other ABC transporters). With ivermectin, severe adverse effects in the central nervous system have been observed in various vertebrates due to the absence or functional deficiency of P-gp (6). Due to the wide spectrum of clinical use and common simultaneous use with other drugs used for the treatment of various diseases, there is a strong possibility of toxic and/or clinically significant drug-drug interactions. The consequences of a toxic drug-drug interaction that can occur during simultaneous treatment with ivermectin with other drugs depends on the metabolic and pharmacokinetic properties of both ivermectin and the simultaneously administered drugs.

Furthermore, it depends on their interaction with enzymes that metabolize the drug (cytochrome P450) and drug transporters involved in the metabolic pathways of ivermectin. Interactions could therefore result in changes in the activity of enzymes involved in drug metabolism and/or transporters, potentially leading to altered responses to drug therapy or significant side effects/toxic effects (7).

Several *in vitro* tests and several human clinical studies were conducted during the COVID-19 pandemic to test the effectiveness of ivermectin against the SARS-CoV-2 virus. The expectation that ivermectin will exert an antiviral effect possibly arose from the previous use of antimalarials (chloroquine and hydroxychloroquine) in some treatment protocols against COVID-19 infection, together with the fact that ivermectin acts *in vitro* on the causative agent of malaria, the protozoan *Plasmodium falciparum*. Unfortunately, none of the many studies demonstrated therapeutic efficacy; the doses used in clinical trials often showed toxicity. The doses of ivermectin used ranged from 0.6 to 2 mg/kg, given orally (usually for 5 days), and the studies were frequently interrupted due to the severe condition of the patients, which was attributed to the effects of ivermectin (8).

Based on data from the Oregon State Poison Center (USA) published in the New England Journal of Medicine, the doFses of ivermectin taken by people hospitalized at the center during the pandemic were up to 1.8 mg/kg, orally, while the therapy usually lasted 5-7 days. Confusion, ataxia, convulsions and hypotension were reported in hospitalized patients (9).

The toxicity of ivermectin administered orally over a 5-day period has not published to date. All ivermectin toxicity studies refer to classic acute, subacute and chronic toxicity protocols. We decided to examine potential toxic effects of the high doses of ivermectin that were applied against the SARS-CoV-2 virus and to determine the plasma and brain concentrations of ivermectin in treated rats. Also, it is important to compare these *in vivo* concentrations of ivermectin with the concentrations tested *in vitro* against the SARS-CoV-2 virus.

Material and methods

Housing of rats and study design

A total of 30 adult Wistar male rats (140-150 g), 7 months old used in the present study were obtained from the Military Medical Academy breeding farm, Belgrade, Serbia. The study was performed on male rats due to data from the literature on the potential toxic effect of ivermectin on testicular function. On the other hand, according to Oregon Poison Center data, the toxic effects of ivermectin were more often recorded in male patients during the COVID-19 pandemic (9, 10).

After arriving at the facility, the animals were acclimated 10 days before the start of the study. Rats were housed in the groups of 6 in home cages on autoclaved wood shavings bedding under standard conditions: temperature of 21±1 ºC, relative humidity of 55-60 % and 12/12 h light/dark cycle. Food and water were provided *ad libitum*. Bedding (wood shavings) and water bottles were changed daily under strict, aseptic conditions. All cages and implements were washed in a mechanical washer and autoclaved prior to entry into the room.

Rats were randomly divided into 5 groups, each group consisting of 6 rats per dose of ivermectin (0.6, 1.2, 2.4, and 4.8 mg/kg), in addition to a control group. Each day at 10:00 am animals were treated with the assigned dose of ivermectin, while control animals were treated with the same volume of solvent. Ivermectin treatment lasted for 5 days. Ivermectin was dissolved in propylene glycol and applied orally through a rigid gastric tube in a volume of 0.1 ml/100 g of body weight.

At the end of five-day treatment (24 hours after last administration of ivermectin) rats were anesthetized with thiopentone sodium 35mg/kg applied intravenously in the tail vein and the blood sampling procedure was undertaken.

All experimental procedures in the study were approved by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia - Veterinary Directorate (permit N°323-07-11564/2022-05/4), according to the Serbian Animal Welfare Protection Law, and Directive 2010/63/ EU.

Assessment of the coordination and balance

The Rota-rod test was used to investigate the potential CNS effects of ivermectin. This test measures the ability of rats to maintain balance on a rotating rod. The testing was performed on the Rota-rod apparatus under software control (ElUnit, Serbia). Prior to the investigation, animals were trained for 5 days to remain 5 min on the rod rotating at constant speed of 15 rpm. Thirty (n=30) rats able to balance on a rotating rod for 5 minutes without falling were selected for the study. The effect of increasing doses of ivermectin on the integrity of motor coordination was assessed based on the ability of rats to stay on rotated rod for 5 min without falling. Testing was carried out every day 30 minutes after administration of ivermectin or solvent (control group).

Hematological and biochemical analyses

Blood sampling was performed 24 hours after the last ivermectin administration. A blood samples were taken by cardiac puncture, and then the rats were euthanized by decapitation. Blood was collected in tubes with anticoagulant (Ethylenediaminetetraacetic acid) and serum tubes. From the blood samples with anticoagulant, the parameters of the blood count were determined in the 5-part veterinary hematology analyzer Mindray BC-500 Vet immediately after sampling. After separating the serum, the concentration of total proteins (TP), urea, creatinine, and triglycerides (TG) were measured. We also measured the enzymatic activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CT) and gamma glutamyl transferase (gamma-GT) in an automatic biochemical analyzer (Mindray BC-240). These analyzes were performed immediately after sampling.

Cytochrome P450 concentration was determined in the rat serum, because ivermectin is metabolized *in vivo* and *in vitro* by cytochrome P450 enzymes through C-hydroxylation and O-demethylation reactions. Given that most of the metabolism of ivermectin is carried out via the cytochrome P450 3A4, its concentration was determined by Enzyme-Linked Immunosorbent Assay (ELISA) using the double sandwich ELISA method according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA). Serum samples for determination of cytochrome P450 concentration were frozen at -20°C immediately after sampling.

Liquid chromatography-tandem mass spectrometry assay of ivermectin in rat serum and brain tissue

Ivermectin concentrations were determined after Whelan and al. (11), with the following modifications. For chromatographic separation Kinetex® (Phenomenex, Torrance, CA, USA) column (100×2.1 mm, 2 µm) was used coupled with Shimadzu 8040 (Shimadzu Corporation, Kyoto, Japan) LC-MS/MS system explained by Simunovic et al. (12). Extraction was performed using 20 ml of acetonitrile/acetone (5:1) mixture, centrifugation, filtration and subsequent evaporation under nitrogen at 50°C. Reconstitution was performed using 1 ml of acetonitrile after which extract was transferred to HPLC vial. Validation of the method was performed according to European Regulation 2002/657/EC. Calculated limits of quantification for samples of brain and plasma were 1 µg/kg and 0.5 µg/l, respectively. For quantification, calibration standards were prepared by spiking blank samples with ivermectin working solution.

Gross and histopathological examination of rat tissues

After decapitation, a gross and histopathological examination of the organs and tissues of the rats was performed. Concurrently, tissue samples of the brain, liver, kidneys and testicles were taken for histopathological analysis. Tissue samples were fixed in 10% buffered formalin. After standard processing in an automated tissue processor, tissue samples were embedded in paraffin blocks and 5µm sections were stained with hematoxylin and eosin (HE). The results of histochemical staining were analyzed by light microscopy (BX51, Olympus Optical, Japan).

Images were taken with Olympus Color View III® digital camera.

Substances and drugs

Substances and drugs used in this study were: Ivermectin (Sigma-Aldrich, St. Louis, USA), Thiopental Panpharma (Panpharma, France).

Statistical analysis

All values are expressed as mean ± standard error of the mean (mean ± SE). Unless stated otherwise, results were tested using the one-way ANOVA or t-test, and differences were considered significant at $p<0.05$. Furthermore, linear regression was used to examine doseresponse relationship. All data was analyzed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

Results

General Observation

During the five-day treatment, rats were continuously observed for clinical manifestations of health disorders. No symptoms were observed in any of the treated rats or in the control group. The rats in each group consumed food and water normally, their behavior was normal and with no observable difference between control and treated rats.

Table 1: Mean values (± SE) of monitored hematological parameters in rats after treatment

Parameters	Unit	Mean ± SE				
		Control (n=6)	Ivermectin 0.6 mg/kg (n=6)	Ivermectin 1.2 mg/kg (n=6)	Ivermectin 2.4 mg/kg (n=6)	Ivermectin 4.8 mg/kg (n=6)
WBC	10^9/l	6.08±1.10	5.92±0.61	6.57±1.24	7.90±1.25	5.55±0.92
RBC	10^{12}/l	6.57±0.11	7.71±0.12**+ (p=0.004) (p=0.041)	7.02±0.65	6.98±0.21	6.83±0.27
HGB	g/l	127.50±2.43	142.83±1.47**++ (p=0.0072) (p=0.0086)	126.66±11.92	127.83±4.46	125.83±3.97
HCT	%	38.55±0.83	41.63±0.46	37.23±3.29	37.83±1.23	38.71±1.16
MCV	fl	58.66±0.33	53.73±0.36*** (p=0.0004)	53.23±0.37** (p=0.0028)	54.15±0.37** (p=0.0065)	56.76±1.50
MCH	pg	19.45±0.07	18.36±0.13** (p=0.0015)	18.06±0.11*** (p=0.0002)	18.31±0.15** (p=0.0072)	18.40±0.44
MCHC	g/l	331.00±1.43	342.50±1.20**++ (p=0.0030 (p=0.0038)	339.16±3.11	338.16±2.02++ (p=0.0458)	327.33±2.87

*-Statistically significant difference compared to control; +- Statistically significant difference compared to ivermectin 4.8 mg/kg

Table 2: Mean values (± SE) of monitored biochemical parameters in rats after treatment

Parameters	Unit	Mean ± SE				
		Control (n=6)	Ivermectin 0.6 mg/kg (n=6)	Ivermectin 1.2 mg/kg (n=6)	Ivermectin 2.4 mg/kg (n=6)	Ivermectin 4.8 mg/kg (n=6)
ALT	U/l	57.63±3.25	94.26±3.55**** (p=0.0001)	90.86±3.75**** (p=0.0002)	97.90±5.81**** (p=0.0001)	93.45±5.84**** (p=0.0001)
AST	U/l	143.36±20.32+ (p=0.0284)	126.08±12.87++ (p=0.0084)	120.06±7.06+ (p=0.0116)	115.93±8.29++ (p=0.0065)	165.11±24.28
ALP	U/l	83.93±9.47	11.05±3.18****+++ (p<0.0001) (p=0.0005)	19.31±7.22****++ (p<0.0001) (p=0.0034)	19.60±3.19****++ (p<0.0001) (p=0.0036)	64.33±11.95
gamma - GT	U/l	1.38±0.13	1.68±0.20++++ (p<0.0001)	1.36±0.30++++ (p<0.0001)	2.06±0.17++ (p=0.0015)	5.20±1.68**** (p<0.0001)
TP	g/l	58.03±1.28	64.10±1.75	63.31±1.51	60.90±2.04	61.90±2.11
TG	mmol/l	0.40±0.04	0.59±0.06	0.56±0.09	0.55±0.05	0.89±0.17* (p=0.0128)
CREA	µmol/l	23.36±1.49	17.85±4.15+ (p=0.0232)	23.10±0.67	22.35±1.32	27.60±0.64
UREA	mmol/l	7.00±0.34	7.38±1.81	8.29±0.53	8.24±0.34	7.29±0.68

*-Statistically significant difference compared to control; +- Statistically significant difference compared to ivermectin 4.8 mg/kg

Table 3: Mean values (± SE) of ivermectin concentration in rat plasma and brain tissue

Parameters	Mean ± SE			
	Ivermectin 0.6 mg/kg	Ivermectin 1.2 mg/kg	Ivermectin 2.4 mg/kg	Ivermectin 4.8 mg/kg
Ivermectin concentration in the plasma (µg/L)	18.217±2.317** (P=0.0018)	32.717±4.671** (P=0.0046)	61.167±11.745* (P=0.0290)	165.317±46.450
Ivermectin concentration in the brain (µg/kg)	1.467±0.312*++ (P=0.0148) (P=0.0076)	3.783±0.930* (P=0.0149)	6.667±0.932* (P=0.0193)	33.033±6.313

*-Statistically significant difference compared to ivermectin 4.8 mg/kg; +- Statistically significant difference compared to ivermectin 2.4 mg/kg

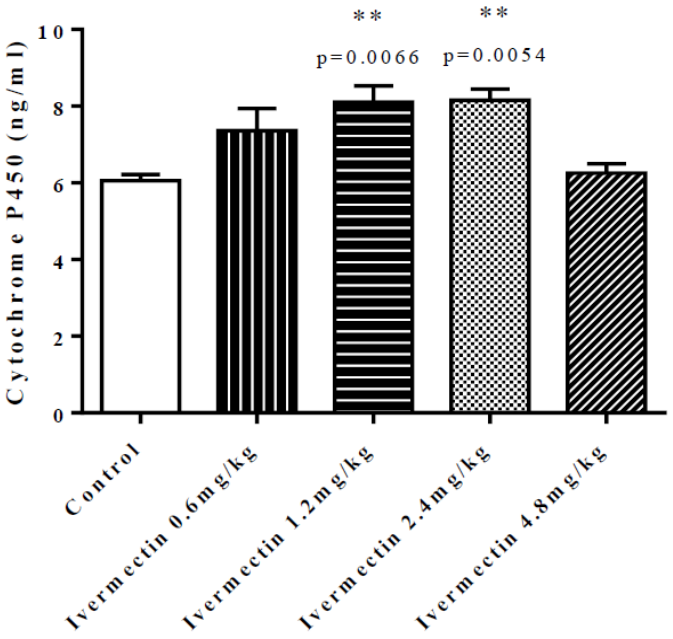

Figure 1: Cytochrome P450 concentration in rat plasma after 5 days treatment with increasing doses of ivermectin

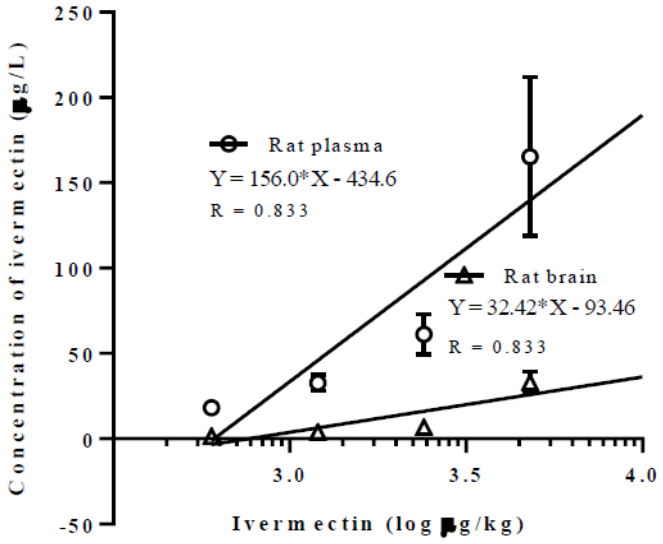

Figure 2: Linear regression analysis of dose-dependent increase in ivermectin concentration in plasma and brain of rats

Assessment of the coordination and balance

The Rota-rod test has been used to assess motor coordination and balance alterations in rodents (13). None of the treated or control rats fell off the rotating rod during the test. Also, none of the tested rats displayed visible signs of CNS depression, the righting reflex was fully preserved, walking on a flat static surface (after tail pinch test) was normal and all of the tested animals responded normally to external stimulation (approach response and touch response test).

Hematological and biochemical analyses

Determination of hematological parameters showed that the five-day treatment of rats with increasing doses of ivermectin (0.6-4.8 mg/kg) did not significantly affect the total number of leukocytes, but a dose of 0.6 mg/kg significantly increased the red blood cell count. In the other rats treated with ivermectin, there was an apparent increase in the number of erythrocytes compared to controls, but not to a significant value (Table 1). As with the number of erythrocytes, the concentration of hemoglobin in rats treated with 0.6 mg/kg was significantly increased compared to the controls. In the other treated groups of rats, this value did not differ either within groups or compared to control results (Table 1). Hematocrit was not significantly different in control and treated rats. However, MCV was reduced in all treated rats and reached a significant level in rats treated with ivermectin doses of 0.6, 1.2 and 2.4 mg/kg. In all ivermectin treated rats the MCH value was significantly reduced compared to the untreated control group (Table 1). However, the value of MCHC increased significantly only in rats treated with the lowest dose of ivermectin (0.6 mg/kg), while in other treated animals it was indistinguishable from control values.

In all rats treated for 5 days with ivermectin, a significant increase in the value of ALT was recorded, while the value of this enzyme did not differ between groups. On the other hand, the AST concentration increased only in rats treated with the highest tested dose of ivermectin. ALP was significantly reduced in rats treated with 0.6, 1.2 and 2.4 mg/kg, while in rats that received the highest dose (4.8 mg/kg), the value of ALP was only slightly reduced compared to the control. Gamma GT levels were higher than control in rats treated with 0.6 and 2.4 mg/kg, but significantly higher only in rats treated with the highest dose of ivermectin (4.8 mg/kg). Other biochemical parameters TP, TG, Creatinine and Urea did not differ significantly, both in relation to the control and between the treated groups (Table 2).

Ivermectin is extensively metabolized by cytochrome P450 enzymes (P450s, CYP) both *in vivo* and *in vitro* (7), therefore it was particularly important to examine the activity of this enzyme. The concentration of CYP in the plasma of treated rats increased proportionally with the dose of ivermectin administered (0.6, 1.2 and 2.4 mg/kg). From control 6.05±0.16 ng/ml, to 7.36±0.57, 8.11±0.43 and 8.15±0.30 ng/ml However, in rats that received the highest dose of ivermectin (4.8 mg/kg), the value of CYP remained similar to the control level (6.26±0.23 ng/ml) (Figure 1).

Liquid chromatography-tandem mass spectrometry assay of ivermectin in rat serum and brain tissue

We considered it important to determine ivermectin concentrations in plasma and brain of rats after the 5 daily treatments various oral doses of ivermectin. The selected doses used in humans at the time of COVID-19 infection were many times higher than the recommended antiparasitic dose of ivermectin (3, 6, 12 and 24x). Table 3 shows the mean values of the results. The concentration of ivermectin in plasma and brain tissue after 5 days treatment was proportional to the administered dose of the drug. Linear regression analysis shows that the increase in ivermectin concentrations in plasma and brain tissue is dose-dependent (Y=156.0*X-434.6, r=0.8338 and Y=32.42*X-93.46 r=0.7359) (Figure 2). Each two-fold increase in the dose of ivermectin (from 0.6 to 1.2 and from 1.2 to 2.4 mg/kg) produced an almost two-fold increase in plasma and brain drug concentrations. However, after administration of a dose of 4.8 mg/kg, the concentration recorded in the plasma was 2.5 times higher, while in the brain, almost 5 times higher than in rats that received twice the lower dose (2.4 mg/kg) (Table 3).

Gross and histopathological examination analysis of rat tissues

Gross pathology changes in the internal organs of rats treated with ivermectin were not observed. However, histopathological changes were noted in the majority of treated rats. Focal mononuclear infiltration of liver tissue was observed in rats of all treated groups, but intracellular edema and vacuolar degeneration of hepatocytes (Figure 3a) were more frequent in rats treated with the two highest doses of ivermectin, 2.4 and 4.8 mg/kg. Focal necrosis of hepatocytes and focal calcification were detected in the liver tissue of rats that received 4.8 g/kg of ivermectin. Tubular dilatation, intracellular edema and interstitial hemorrhage were observed in the kidney tissue of all treated rats. Glomerular sclerosis was observed in rats treated with ivermectin in doses of 1.2, 2.4 and 4.8 mg/kg (Figure 3b). Changes in brain tissue were found only in rats receiving the highest

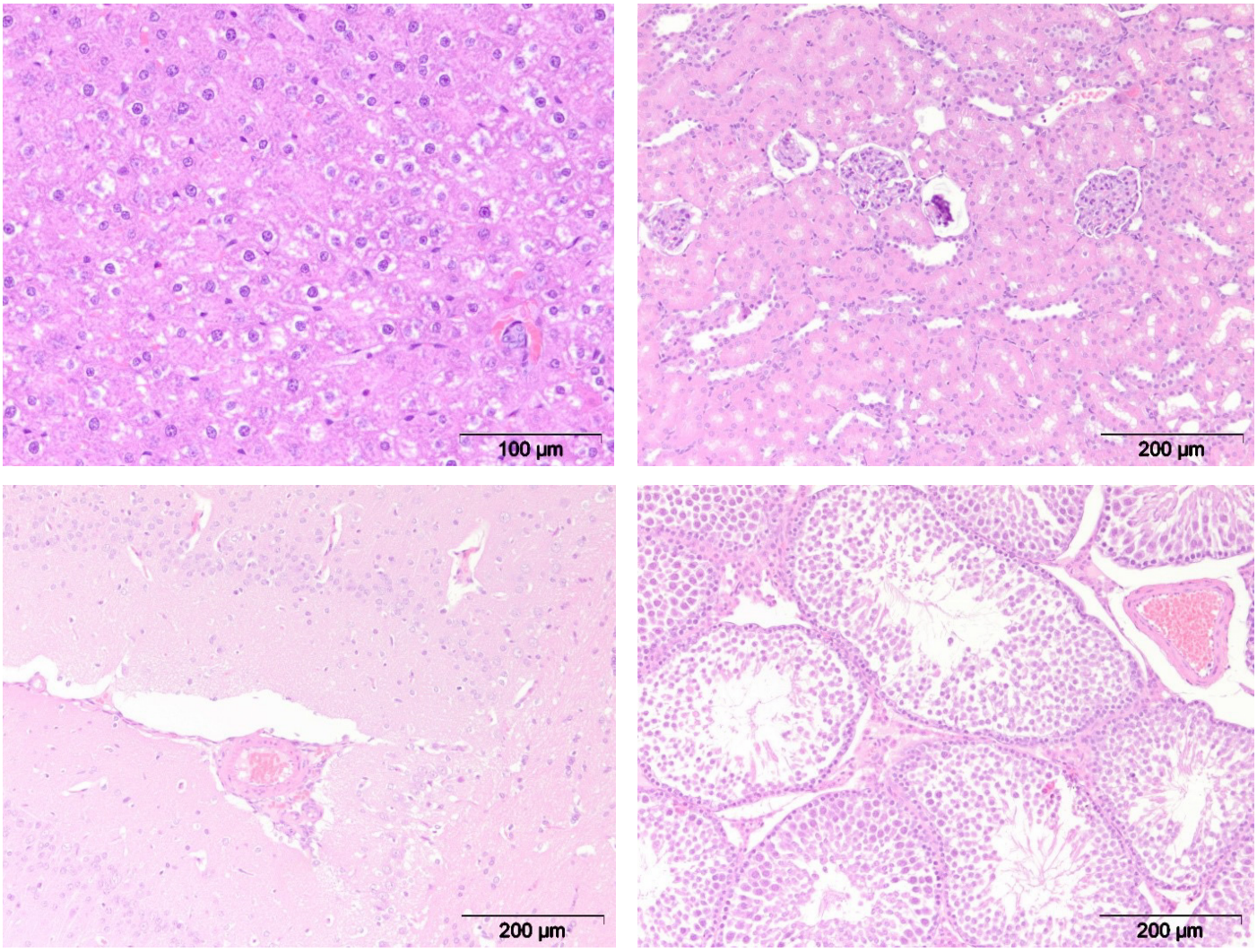

Figure 3: a) Intracellular edema and vacuolar degeneration of hepatocytes; b) Glomerular sclerosis 121 in the kidneys of rats; c) Focal and perivascular bleeding in the rat brain; d) Degeneration of the 122 epithelium of the seminiferous tubules of the testicles

tested dose of ivermectin (4.8 mg/kg). Focal bleeding as well as perivascular bleeding was observed (Figure 3c). Also, histopathological changes in testicular tissue were detected only in rats treated with the highest dose of ivermectin. These changes included epithelial degeneration of the seminiferous tubules of the testes and the epithelium of the epididymis (Figure 3d), reduction and absence of spermatogenesis was also observed.

Discussion

In this study, we examined the toxic effects of high oral doses of ivermectin after five days of administration in rats. The most commonly applied antiparasitic dose of ivermectin in human and veterinary medicine is 0.2 mg/kg of body weight (14,15). Based on data from the Oregon Poison Center (9), we applied oral doses of ivermectin of 0.6, 1.2, 2.4, and 4.8 mg/kg, which is 3, 6, 12, and 24 times higher than the therapeutic antiparasitic dose. During the fiveday treatment with various oral doses of ivermectin, no clinical symptoms of poisoning were noted. The oral LD_{50} values of ivermectin in rats are reported to be in the range of 42.8-52.8 mg/kg (2) and 10-50 mg/kg (16). In our study, rats received 3, 6, 12, or 24 mg/kg of ivermectin for a total of 5 days, which means that rats treated with the two highest doses received nearly ¼ and ½ the LD_{50} of ivermectin, but did not show any clinical symptoms. In addition, the Rota rod test did not show any disruption of CNS integration as a consequence of the neurotoxic effect of ivermectin even at these at high concentrations.

Hematological analyses indicated no changes in the number of leukocytes in the white blood cell differential count. Hematocrit values and hemoglobin concentrations were unchanged compared to control group, except in rats treated with ivermectin at a dose of 0.6 mg/kg where the hemoglobin concentration was significantly higher compared to the control. We emphasize that the number of erythrocytes was slightly increased in all treated rats, while MCV and MCH values were lower than in controls (Table 1). This finding indicates microcytosis in treated rats, which could be a consequence of ivermectin treatment, but this effect is not dose-dependent (at the concentrations tested) and did not differ between groups in relation to the dose of ivermectin received.

The analysis of biochemical parameters showed a significant increase in the level of ALT (not dose-dependent) and a significant dose-dependent increase in the level of gamma-GT (Table 2). The increase in the value of these two enzymes, which indicates liver tissue damage, even at the lowest test dose of ivermectin, is in agreement with the histopathological changes in the liver that we observed. These results are in in agreement with the hepatic disorders (hepatitis, hepatocellular injury, cholestasis, increased alanine aminotransferase and/or aspartate aminotransferase levels, abnormal liver function tests) observed in humans who received ivermectin as therapy against the SARS-CoV-2 (16). However, our results are not in agreement with the research of Dong et al. (18) where 14 daily intraperitoneal application of ivermectin in doses of 100 to 380 mg/kg surprisingly, did not lead to changes in liver enzyme values. On the contrary, when Wistar rats were treated intraperitoneally 4 times a week for 21 days in doses of 0.4 mg/kg and 4 mg/kg of body weight, an increase in ALT, GGT and AST values was recorded (19). These results are consistent with our findings. Remarkably, in our investigation, we noted histopathological changes in the kidneys of treated rats, despite the absence of increased concentrations of creatinine and urea in the blood. There are published data that ivermectin can cause glomerular and tubular dysfunctions in humans, determined after 5 days of treatment against onchocerciasis (20). Similar changes in the kidneys of rabbits were recorded by GabAllh et al. (21). In rabbits treated once a week with ivermectin 0.8 mg/kg for 8 weeks, congestion of renal blood vessels was recorded. The renal tubules showed severe degeneration as evidenced by vacuolation of cytoplasm, necrosis and desquamation of affected epithelium. The highest dose of ivermectin in our study caused degenerative changes in testicular tissue. Similar changes in the testicles of rabbits were described GabAllh et al. (21). In our study, histopathological changes in the brain including focal bleeding as well as perivascular bleeding were observed only in rats treated with 4.8 mg/kg of ivermectin. Degenerative changes in the rabbit brain after administration of therapeutic and double therapeutic doses for 8 weeks were also described by GabAllh et al. (21).

It was particularly important to examine cytochrome P-450 concentrations in rats treated with increasing doses of ivermectin administered in humans during the COVID-19 pandemic. Ivermectin is extensively metabolized by human liver microsomes by cytochrome P-450. The predominant isoform responsible for the biotransformation of this compound in the liver is cytochrome P-450 3A4 (7). Our results show that doses of ivermectin 3, 6, and 12 times higher than the therapeutic dose, lead to a dose-dependent increase in plasma concentrations of P-450 3A4 (Figure 1). However, a dose 24 times higher than the therapeutic did not increase serum enzyme concentrations further. Ivermectin is known to be both a substrate for and inhibitor of human P-450 enzymes (7). Based on our results, it is obvious that in very high doses, ivermectin did not cause an increase in the concentration of this enzyme, it remains at the control level which leads to an elevation in the concentrations of ivermectin in plasma and tissues.

Ivermectin concentrations recorded in the plasma of treated rats were compatible with the administered doses. A two-fold increase in the dose resulted in a two-fold increase in the concentration of the drug in the plasma. The highest dose tested showed an exception, with the plasma concentration being nearly three times higher compared to the preceding dose level. This result is in agreement with the finding that this highest dose of ivermectin does not increase

concentration of P-450 enzyme that metabolizes ivermectin and allows such an increase of drug level in plasma. On the other hand, tested doses 3, 6, 12, and 24 times higher than therapeutic, produced maximum plasma concentrations that were far lower than the *in vitro* IC50 value of ivermectin for SARS-CoV-2 virus. Caly et al. (22) reported that ivermectin inhibited SARS-CoV-2 *in vitro* causing a ~5000-fold reduction in viral RNA at 48h at concentrations of 5 μM. The concentration resulting in 50% inhibition (IC50) which they obtained, was 1750 μg/L, while we recorded a concentration of 165.317±46.450 μg/L, 24 hours after the last administration. Our results indicate that it is almost impossible to achieve an effective antiviral concentration of ivermectin, even a 24-fold higher than therapeutic dose produces a concentration in plasma 10 times lower than the IC50 for SARS-CoV-2. Furthermore, serious damage of the body is highly likely. Our findings are supported by the results of Buonfrata et al. (8). In order to treat SARS-CoV-2 infection, these authors administered ivermectin to humans orally in doses of 0.6 and 1.2 mg/kg, for 5 days. Serious side effects observed included visual impairment, abdominal pain, diarrhea, nausea & vomiting, arthralgia, dizziness, headache, and paranesthesia. However, log10 viral load reduction did not differ between untreated and ivermectin-treated humans.

The concentrations of ivermectin in the brain were consistent with the administered doses, and the observed increase in concentrations was directly proportional to the dose. Similar to plasma, doubling the dose produced a two-fold increase in the concentration of ivermectin in the brain, except at the highest dose tested (Table 3). The highest dose tested produced a fivefold increase in brain ivermectin concentrations compared to the previous dose. Such a high concentration of ivermectin in the brain is probably due to saturation of P-450 and the absence of increased activity of P-450 as well as insufficiency of the P-glycoprotein efflux transporters at the blood-brain barrier. However, the concentration of 33.033±6.313 μg/kg does not lead to clinical symptoms, but does damage brain tissue. This is in agreement with the data of Geyer et al. (23) who indicate that a therapeutic dose of ivermectin in mice 24 hours after p.o. administration produces a brain concentration of 2 μg/kg, but in MDR1-deficient knockout mice, the concentration of ivermectin was 127 μg/kg. Significant for comparison is the case of a man who received ivermectin p.o. and subcutaneously a dose of 12 mg against infection with *Strongyloides stercoralis*. Although the infection was significantly suppressed, the patient fell into a coma and died. The concentration of ivermectin detected postmortem in his brain was 30 μg/kg tissue and by excluding other potential causes of neurotoxicity, the authors of this clinical report suggest that ivermectin was the main cause (24). This concentration of ivermectin is lower than the concentration we detected, which obviously indicates that some people are more sensitive to the neurotoxic effects of ivermectin.

Conclusions

Ivermectin at the doses used in humans against the SARS-CoV-2 (3, 6, 12 and 24 times higher than the antiparasitic dose), causes changes in red blood cell counts and increases the levels of liver enzymes in treated rats, without visible clinical symptoms. Histopathological changes were recorded in the liver, kidneys and testicles of rats, and the highest dose tested led to bleeding in the brain tissue. Obviously, ivermectin somewhat stimulates the activity of the enzyme P-450 isoform 3A4, whose substrate it is, but the highest tested dose reduces its concentration in plasma to the control level. Notably, the concentrations of ivermectin recorded in plasma of treated rats, indicate that even high doses do not reach the *in vitro* IC50 value of ivermectin for SARS-CoV-2 reported in the literature. On the other hand, the concentrations of ivermectin in the brain approach the values that can manifest extremely toxic effects described in humans.

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References

1. Prichard R, Ménez C, Lespine A. Moxidectin and the avermectins: consanguinity but not identity. Int J Parasitol Drugs Drug Resist 2012; 2: 134–53. doi: 10.1016/j.ijpddr.2012.04.001

2. Siegmund OH, ed. The Merck veterinary manual. 5th ed. Rathway: Merck, 1979.

3. Trailović SM, Varagić VM. The effect of ivermectin on convulsions in rats produced by lidocaine and strychnine. Vet Res Commun 2007; 31(7): 863–72. doi: 10.1007/s11259-007-0050-3

4. Trailovic SM, Ivanovic SR, Varagić VM. Ivermectin effects on motor coordination and contractions of isolated rat diaphragm. Res Vet Sci 2011; 91(3): 426–33. doi: 10.1016/j.rvsc.2010.09.016

5. Trailovic SM, Nedeljkovic JT. Central and peripheral neurotoxic effects of ivermectin in rats. J Vet Med Sci 2011; 73(5): 591–99. doi: 10.1292/jvms.10-0424

6. Edwards G. Ivermectin: does P-glycoprotein play a role in neurotoxicity? Filaria J 2003; 2(Suppl. 1): S8. doi: 10.1186/1475-2883-2-S1-S8

7. Rendic SP. Metabolism and interactions of Ivermectin with human cytochrome P450 enzymes and drug transporters, possible adverse and toxic effects. Arch Toxicol 2021; 95(5):1535–46. doi: 10.1007/s00204-021-03025-z

8. Buonfrate D, Chesini F, Martini D, et al. High-dose ivermectin for early treatment of COVID-19 (COVER study): a randomised, double-blind, multicentre, phase II, dose-finding, proof-of-concept clinical trial. Int J Antimicrob Agents 2022; 59(2): 106516. doi: 10.1016/j.ijantimicag.2021.106516

9. Temple C, Hoang R, Hendrickson RG. Toxic Effects from Ivermectin Use Associated with Prevention and Treatment of Covid-19. N Engl J Med 2021; 385(23): 2197–8. doi: 10.1056/NEJMc2114907

10. Collins AB, Zhao L, Zhu Z, et al. Impact of COVID-19 on male fertility. Urology 2022; 164: 33–9. doi: 10.1016/j.urology.2021.12.025

11. Whelan M, Kinsella B, Furey A, et al. Determination of anthelmintic drug residues in milk using ultra high-performance liquid chromatography-tandem mass spectrometry with rapid polarity switching. J Chromatogr A 2010; 1217(27): 4612–22. doi: 10.1016/j.chroma.2010.05.007

12. Simunovic S, Jankovic S, Baltic T, et al. Histamine in canned and smoked fishery products sold in Serbia. Meat Technol 2019; 60(1): 8–16. doi: 10.18485/meattech.2019.60.1.2

13. Jones BJ, Roberts DJ. The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. J Pharm Pharmacol 1968; 20(4): 302–4. doi: 10.1111/j.2042-7158.1968.tb09743.x

14. Brunton LL, Chabner BA, Knollmann BC. Goodman and Gilman's the pharmacological basis of therapeutics. 12th ed. New York: McGraw-Hill Education, 2011: 1456-7.

15. Riviere JE, Papich MG. Veterinary pharmacology and therapeutics. 10th ed. Hoboken: Wiley-Blackwell, 2018: 1101–27.

16. Lankas GR, Gordon LR. Toxicology. In: Campbell WC, ed. Ivermectin and abamectin. New York: Springer, 1989: 89–112.

17. Oscanoa TJ, Amado J, Romero-Ortuno R, Carvajal A. Hepatic disorders associated with the use of Ivermectin for SARS-CoV-2 infection in adults: a pharmacovigilance study in VigiBase. Gastroenterol Hepatol Bed Bench. 2022; 15(4): 426–9. doi: 10.22037/ghfbb.v15i4.2383

18. Dong Z, Xing SY, Zhang JY, Zhou XZ. 14-Day repeated intraperitoneal toxicity test of ivermectin microemulsion injection in Wistar rats. Front Vet Sci 2020; 7: 598313. doi: 10.3389/fvets.2020.598313

19. Utu-Baku AB. Effect of therapeutic and toxic doses of ivermectin (Mectizan) on total serum proteins and hepatic enzymes of Wistar albino rats. Int J Biol Chem 2009; 3(4): 142–7. doi: 10.3923/ijbc.2009.142.147

20. Burchard GD, Kubica T, Tischendorf FW, Kruppa T, Brattig NW. Analysis of renal function in onchocerciasis patients before and after therapy. Am J Trop Med Hyg. 1999; 60(6): 980-6. doi: 10.4269/ajtmh.1999.60.980

21. GabAllh M, El-mashad A, Amin A, Darweish M. Pathological studies on effects of ivermectin on male and female rabbits. Benha Vet Med J 2017; 32(1): 104–12. doi: 10.21608/bvmj.2017.31162

22. Caly L, Druce JD, Catton MG, Jans DA, Wagstaff KM. The FDA-approved drug ivermectin inhibits the replication of SARS-CoV-2 in vitro. Antiviral Res 2020; 178: 104787. doi: 10.1016/j.antiviral.2020.104787

23. Geyer J, Gavrilova O, Petzinger E. Brain penetration of ivermectin and selamectin in mdr1a,b P-glycoprotein- and bcrp- deficient knockout mice. J Vet Pharmacol Ther 2009; 32(1): 87–96. doi: 10.1111/j.1365-2885.2008.01007.x

24. Westerloo van DJ, Landman GW, Prichard R, Lespine A, Visser LG. Persistent coma in Strongyloides hyperinfection syndrome associated with persistently increased ivermectin levels. Clin Infect Dis 2014; 58(1): 143–4. doi: 10.1093/cid/cit656

Veliki odmerki ivermektina povzročajo toksične učinke pri podganah po kratkotrajnem peroralnem dajanju

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Izvleček: Protiglivični makrociklični laktoni (ML) so najpomembnejše učinkovine v sodobni farmakoterapiji parazitskih okužb. Vendar so med pandemijo covida-19 pri ljudeh proti okužbi z virusom SARS-CoV-2 uporabljali bistveno višje odmerke ivermektina od odobrenih antiparazitičnih odmerkov. Takšna uporaba je bila ustvarjena izključno na podlagi testov *in vitro*, vendar ni bila uradno odobrena v nobeni državi na svetu. Zato smo izvedli študijo na podganah, ki smo jih 5 dni peroralno zdravili z 0,6, 1,2, 2,4 in 4,8 mg ivermektina na kg telesne teže. Naša preiskava je pokazala, da ivermektin v povišanih odmerkih, ki se uporabljajo pri ljudeh proti virusu SARS-CoV-2 (3-, 6-, 12- in 24-krat večjih od antiparazitskega odmerka 0,2 mg/kg), povzroča spremembe v številu eritrocitov in zvišuje raven jetrnih encimov brez vidnih kliničnih simptomov. Histopatološke spremembe smo zabeležili v jetrih, ledvicah in testisih podgan, največji testirani odmerek pa je povzročil krvavitev v možganskem tkivu. Znano je, da ivermektin kot substrat nekoliko poveča koncentracijo encima P-450 izoforma 3A4, vendar največji testirani odmerek zmanjša njegovo koncentracijo v plazmi na kontrolno raven. Koncentracije ivermektina, zabeležene v plazmi zdravljenih podgan, kažejo, da tudi visoki odmerki ne dosežejo *in vitro* vrednosti IC50 ivermektina za SARS-CoV2, ki je navedena v literaturi. Po drugi strani pa se koncentracije ivermektina v možganih približujejo vrednostim, ki lahko povzročijo izjemno toksične učinke, opisane pri ljudeh.

Ključne besede: ivermektin; toksičnost; SARS-CoV-2; citokrom P-450; P-gp; histopatološke spremembe; podgane