Acta Veterinaria-Beograd 2022, 72 (2), 235-255
UDK: 615.214.24.07
DOI: 10.2478/acve-2022-0019

Research article

EFFECTS OF DIAZEPAM ON HEMATOLOGICAL AND HISTOLOGICAL PARAMETERS IN RATS / *IN VIVO* AND UNBIASED STEREOLOGICAL INVESTIGATION

Jovana GRAHOVAC, Milenka IVANKOVIĆ, Radoslav DEKIĆ, Smiljana PARAŠ*

University of Banja Luka, Faculty of Natural Sciences and Mathematics, Department of Physiology, Mladena Stojanovića 2, Banja Luka, Bosnia and Herzegovina

(Received 10 March, Accepted 24 May 2022)

Diazepam-based drugs are widely used today in human treatment. Diazepam may be a primary drug aimed at treating neurological diseases or an associated drug in the treatment of other diseases in the purpose of symptomatic therapy. The sedative effect of diazepam characterizes it as a drug that people usually use on their own and without a doctor's supervision. Directly, but also through influencing the nervous system, diazepam disrupts proper functioning of all body organs. The purpose of this paper was to examine the effects of diazepam on blood and cytohistological parameters of rats in an *in vivo* experiment. Mallory-Azan and immunochistochemical staining methods BLX-CX and Survivin tissues of liver, kidney and spleen of rats were used to achieve the set goal. Cytometric analysis of rats detected cells in apoptosis and measurements of stereological parameters were made using a system according to Cavalier's principle. Results of analysis of hematological and histological parameters indicate a detrimental effect of diazepam on blood parameters, as well as on structure and functioning of the liver, kidneys and spleen of rats. This paper is a foundation for further detailed scientific research with the aim of elucidating all harmful effects that diazepam has on all organs in the body of rats. This data could serve as a starting point for future studies in clinical pharmacology on therapeutic protocols for usage of diazepam-based sedatives.

Keywords: diazepam, hematological, immunochistochemical staining, kidney, liver, spleen

INTRODUCTION

Fast-paced lifestyles, problems and stress are causing people to increasingly resort to the use of various sedatives. Most common symptoms of stress are feelings of tension, anxiety, irritability, trepidation and insomnia [1]. Organic compounds were introduced in the 1950s as sedatives for human use. Benzodiazepine drugs are still used today worldwide for sedation, pain relief, as anxiolytics, muscle relaxants, anticonvulsants, hypnotics, and for inducing short-term amnesia [2,3]. The sedative

*Corresponding author: e-mail: smiljana.paras@pmf.unibl.org

effect of benzodiazepines occurs as a consequence of its sedative effect on the central nervous system (CNS) in which it selectively acts on polysynaptic pathways by stimulating the inhibitory action of neurotransmitter γ-aminobutyric acid (GABA) [4]. Benzodiazepines act as depressants on the central nervous system since they facilitate binding of GABA to various GABA receptors throughout the central nervous system [5]. GABA receptor is a chloride channel of complex structure and benzodiazepines are responsible for increasing the frequency of the open state of this receptor channel. Increased GABA receptor activity causes a decrease in nerve activity at specific sites in the brain [6,7]. According to literature data there are harmful effects of diazepam shown by hematological, biochemical and histological parameters of organs [8,9]. Long-term application of diazepam may affect the development of diabetes, Alzheimer's disease, cardiopathy, liver toxicity, inappropriate renal metabolism or impaired endocrine function in humans [10]. Although useful, with a wide range of effects, these drugs have become one of the leading public health problems nowadays due to their irrational usage. Directly but also through their influence on the CNS, diazepam disrupts the proper functioning of all organs [11]. In order to obtain detailed information on the effect of this drug, kidneys and spleen were also analyzed in addition to blood parameters and stereological parameters of the liver. These three organs were selected for analysis of the effect of diazepam on metabolic status in rats. A wide range of diazepam doses affects changes in the structure of rat´s liver tissue. Its prolonged usage has an effect on blockage of blood capillaries, due to disturbed circulation of metabolites. It also affects hepatocyte degeneration, appearance of microvesicles and necrosis in the liver tissue, resulting in liver toxicity [12]. Diazepam impairs rat kidneys on the histological level, leading to irreversibile, such as irreversible damage to cells in renal glomeruli and tubules, as well as rupture and blockage of blood capillaries. All the above mentioned leads to impaired kidney function [13]. Diazepam is highly concentrated in the spleen after its oral administration to rats. Long-term usage of diazepam leads to damage of structure and cells in the white pulp of the spleen [14]. The aim of this study was to monitor the effects of diazepam (Bensedin®) on hematological, biochemical and histological parameters of three organs in Wistar rats. Our study is important because helps to define the exact dose and period of application of this drug in order to enter the protocols for safest possible treatments. In addition, there should be an appeal against uncontrolled and unsupervised use of diazepam-based drugs.

MATERIAL AND METHODS

Experimental design:

The experiment was conducted on the premises of the Faculty of Natural Sciences and Mathematics, University of Banja Luka. The study lasted 15 days and Wistar rats were used in the experiment: a total of 10 males divided randomly into two experimental groups of five rats each. All animal procedures were in compliance with Directive

2010/63 / EU on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee on Animal Experiments at the Faculty of Mathematics and Science University of Banja Luka, No. 01-9-192.2 / 20, Bosnia and Herzegovina. The first group of five test animals was the control group, which was watered with water at the same time when to the treated group diazepam was given. The second group of five animals was the treated group, which was treated with diazepam solution (Bensedin®, tablets of 2 mg, Galenika ad, Belgrade, Republic of Serbia). Rats from the treated group were watered with diazepam solution in concentration of 0.2 mg/kg body weight. Water solution of diazepam was made by dissolving one tablet (2 mg) of Bensedin® in 50 ml of destillited water. In order to facilitate the application of the drug, diazepam tablets were dissolved in destillited water using a magnetic stirrer with a heater. Diazepam solution prepared in our experiment had a concentration of 0.04 mg drug per one milliliter of destilled water and it was administered via oral intubation using a 5 ml pipette. Body weights of all experimental animals were measured prior to daily treatment with diazepam. All units from both experimental groups were housed in controlled lighting conditions (12 h in the light - 12 h in the dark), 22 ° C temperature and humidity. All rats from our experiment were fed standardized pelleted feed for experimental animals and water was consumed *ad libitum*.

Haematological and biochemical analysis

Blood samples were taken a day after the last treatment from all ten rats. Anesthesia was performed with an intramuscular administration of ketamine (Ketaminol 10, Intervet, 100 ml / mg) at a concentration of 50 mg / kg, to which a saline solution was added in a ratio of 1:10. Blood samples were taken about 10 minutes after administering the anesthetic by puncturing the heart with a sterile needle. Blood samples of all rats was collected in standardized blood-testing tubes containing an anticoagulant (K3EDTA). Blood for analysis, with a mean volume of about 5 ml, was decanted and centrifuged at 10,000 rpm (Laboratory Centrifuge LD-5 Electronic). Blood samples were analyzed using hematology counters Animal Blood Counter, abc ™ and Cobas Integra 400 Roche. The devices measured the following hematological parameters: number of red blood cells (RWC), mean erythrocytes volume (MCV), mean erythrocytes hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cells distribution width (RCD), hemoglobin (HG), hematocrit (HC), white blood cells (WBC), lymphocytes (LYM), monocytes (MON), granulocytes (NEUT-neutrophil, EOS-eosinophil, BAS-basophil), platelet count (PLC), and mean platelet volume (PLV). The parameters of blood biochemical analysis measured with a biochemical analyzer Reflovet® Plus and Cobas Integra 400 Roche were: total protein, triglycerides, cholesterol, glucose, creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT)), glutamate pyruvate transferase (GPT), gamma glutamate transferase (GGT), pancreas lipase,

urea, potassium, sodium and calcium. Both devices displayed parameter values to two decimal places.

Histological analysis

After 15 days of experimental treatments all 10 rats were decapitated in ketamine anesthesia. Three organs (liver, kidney and spleen) from all rats were taken for analysis. The organs taken for for histological analysis were immersed in Bouin's solution for 24h; for better adsorption, were further cut into smaller piceses and again immersed into fresh Buoin's solution. Samples of tissues of all three organs were embedded in paraffin, and were cut in a frontal plane on a Leica rotary Microtome RM 2165 (Leica Mycrosystems, Wetzlar, Germany), in 5 μm thick serial sections and stained with hematoxylin-eosin (H&E) and Mallory-Azan (all stains by Merck KGaA, Darmstadt, Germany). Apoptotic cells were detected with cytometric analysis, using immunochistochemical staining methods BLX-CX and Survivin. Briefly, after deparaffinization in xyline and rehydration through decreasing concentrations of ethanol, slides were immersed in citrate buffer (pH 6.5). Antigens were detected using a microwave oven at 98 °C for 20 minutes, slides were incubated in 3% hydrogen peroxide for 10 minutes at room temperature to block endogenous peroxidase activity. The sections were then incubated with primary rabbit antibodies to glucagon (1:75; DakoCitomation) at 4 °C overnight in a humid atmosphere. Negative control staining slides were incubated in absence of primary antibodies. The slides were washed in paraformaldehyde solution and chromogen was developed for 5 minutes using liquid 3,3'-diaminobenzidine; slides were then stained with Mayer's hematoxylin, dehydrated, and mounted with Canadian examination balm (Canada balsam CAS 8007-47-4, Merck).

Stereological analysis

Qualitative histology analysis of the tissue slides was performed while using the light microscope Leica DM8000 microscope with a MEGA VIEW camera and software system for digital image transfer, and magnification of 40X, 50X, 100X, 250X and 400X. Measurements were made using a stereo-universal test system according to Cavalier's principle, by using 16.0 point-counting system (MBF software system Application Suite 3.0.0., MBF Bioscience, Williston, VT, USA), with P2 spacing grid at maximum 400X magnification [15]. The parameters of stereological analysis were: volume density of tissues or cells, number of cells, numerical density of cells, cell surface, surface area of nuclei of cells that make up the analyzed tissue, nucleocytoplasmic ratio and mitotic and apoptotic index of cells. Stained tissue sections of the liver, kidney and spleen were used to measure the number and volume density (Vv) of all cells. Volume density was calculated through the following formula: $Vv=Pf/Pt$ (mm^0), where Pf represents the number of the desired phase on the test system and Pt represents the total number of points of the test system [16]. The following principle was used for the determination of the number of matches on the test system of all the cell and nuclei were marked

as reference point: the numerical density (Nv) of all cells was calculated based on the cell count (Q) in the volume of analyzed tissues (Vo), the volume of analyzed tissue was evaluated as the product of the number of counted frames (ΣPi), the space of counted frame (a=25002), and the height factor (h) in the histological section, as presented in the following formula [17]:

$$Nv = \frac{Q}{Vo} = \frac{Q}{\sum_{i=1}^{n} Pi \times a \times h} \ (mm^{-3})$$

MBF software system was used to measure the surface and volume of all cells and nuclei, by the thickness of their diameters. Measuring the surface of cells and their nuclei enabled the measurement of the cells, cytoplasm, and nuclei volumes. The ratio between the cells´ nuclei and their cytoplasm volume was used to determine the nucleocytoplasmic ratio (NCR). Mitotic index was determined at the ratio between the number of cells in mitosis and the total number of cells in 30 visible optical fields per slide, with maximum 200X magnification. Apoptotic index was determined at ratio between the number of cells in apoptosis and total number of cells in 30 visible optical fields per slide, with maximum 200X magnification.

Statistical analysis

In our study mean and standard deviation (mean ± SD) were calculated for all analyzed hematological, biochemical and stereological parameters. All results obtained in the study were statistically processed using software package OriginPro, a Microsoft Windows program, 10.5.91.46291/2018, USA. Statistical differences between control and treated groups were obtained using Dunnett's test.

RESULTS

Hematology

Haematological parameters are an indicator of changes in the physiological state of a rat's organism caused by diazepam treatment (Table 1).

Mean values of hematological parameters of control and treated groups of rats are within reference values according to Sharp and Vilano (2013) [18]. Based on the calculation of differences between values using Dunnett's test, it was found that there are statistically significant differences between hematological parameters: erythrocyte count ($p = 0.128$), neutrophil count ($p = 0.113$), eosinophil count ($p = 0.096$) and basophil count ($p = 0.122$) in the treated group compared to the control group. All other values of hematological parameters decreased in the group of rats treated with diazepam compared to the control group.

Blood biochemistry

Biochemical parameters of the blood are indicators of changes in the metabolic state of a rat's organism caused by diazepam treatment (Table 2).

Table 1. Results of blood analysis; values are shown as mean±SD (*p<0.05, Dunnett's test)

Parameters	Control group (n=5)	Treated group (n=5)	Reference range
Red blood cells (10^6/mm^3)	9.25±1.84	7.47±1.30*	7.27-9.65
Mean cell volume (μm^3)	54.11±2.28	53.90±1.29	48.9-57.9
Mean cell hemoglobin (pg)	17.35±1.09	17.67±1.23	17.1-20.4
Mean corpuscular hemoglobin concentration (g/dl)	31.31±1.12	32.52±1.87	29.9-37.5
Red blood cells distribution width (%)	15.45±2.48	14.93±1.87	11.1-18.2
Hemoglobin (g/dl)	17.02±1.98	12.26±2.45	13.7-17.6
Hematocrit (%)	50.85±10.30	40.05±7.21	39.6-52.5
White blood cells (10^3/mm^3)	4.65±1.25	3.38±0.65	1.96-8.25
Lymphocytes (10^3/mm^3)	3.18±0.88	2.57±0.16	1.41-7.11
% lymphocytes of WBC (%)	68.86±15.78	76.09±17.45	55.6-86.3
Monocytes (10^3/mm^3)	0.21±0.07	0.17±0.04	0.03-0.38
% monocytes of WBC (%)	4.55±1.32	5.18±1.97	0.8-8.8
Neutrophil granulocytes (10^3/mm^3)	1.12±0.09	0.60±0.09*	0.22-1.57
% neutrophil gran. of WBC(%)	24.15±3.72	17.73±2.25	6.2-26.7
Eosinophil granulocytes (10^3/mm^3)	0.10±0.01	0.03±0.01*	0.01-0.16
% eosinophil gran. of WBC (%)	2.13±0.02	0.81±0.08	0.2-3.5
Basophil granulocytes (10^3/mm^3)	0.014±0.002	0.006±0.002*	0-0.05
% basophil gran. of WBC (%)	0.31±0.07	0.19±0.06	0-0.8
Platelet count (10^3/mm^3)	648.17±343.44	534.92±345.94	538-1177
Mean platelet volume (μm^3)	11.42±2.83	11.16±2.48	6.2-9.4

NOTE: reference values taken from [18], *statistical significant

Table 2. Results of analysis of biochemical parameters; the values are shown as mean±SD (*p< 0.05; Dunnett's test)

Parameters	Control group (n=5)	Treated group (n=5)	Reference range
Total protein (mg/dL)	6.12±0.98	6.86±1.12	5.2-7.1
Triglycerides – mg/dL	39.15±8.61	48.24±7.15	8.7-60.7
Cholesterol mg/dL	42.79±5.63	51.11±7.29	14.4-87.6
Glucose – mg/dl	117.52±18.74	90.55±19.35	62.4-201.8
Creatinin- mg/dl	0.51±0.14	0.76±0.11*	0.2-0.9
Total Bilirubin - mg/dl	0.17±0.09	0.12±0.07	0.05-0.19
Alkaline Phosphatase - U/l	133.62±22.18	74.41±22.23*	62-230
Aspartate Aminotransferase - U/l	64.58±12.8	32.22±8.09*	74-143
Alanine Aminotransferase - U/l	34.28±8.95	19.66±3.08*	18-45
Glutamate Piruvate Transferase - U/l	47.53±16.93	59.59±16.98	24-55
Gama Glutamate Transferase - U/l	3.29±1.56	2.97±0.28	2-12
Pancreas Lipase – mmol/l	11.75±2.26	5.24±1.23*	1-17
Urea – mmol/l	17.26±1.65	15.12±2.14	12.3-24.6
Potassium – mmol/l	3.11±0.38	7.34±0.99*	3.82-5.55
Sodium – mmol/l	135.45±17.85	119.55±15.36	164.25-110.15
Calcium – mmol/l	1.95±0.44	1.27±0.16	1.05-2.34

NOTE: reference values taken from [19], *statistical significant

The mean values of biochemical parameters from the blood of rats of the control and treated groups are within reference values according to Giknis and Clifford (1995) [19]. Based on the calculation of the differences between values using Dunnett's test, it was found that there are statistically significant differences between biochemical parameters: creatinine ($p = 0.015$), alkaline phosphatase ($p = 0.045$), aspartate aminotransferase ($p = 0.052$), alanine aminotransferase ($p = 0.109$), pancreas lipase ($p = 0.124$) and potassium ($p = 0.077$) in the treated group compared to the control group. All other biochemical parameters of rat blood changed their values insignificantly in the diazepam-treated group compared to the control group.

Histology and Stereology

Histological analysis of the liver, kidney and spleen tissues of rats treated with diazepam shows changes in the architecture and blood supply of their cytological elements in relation to the control group. Stereological analysis of these organs of treated and control rats detects a change in the mean values of stereological parameters. Liver, kidneys and spleen tissues of diayepam treated rats lack necrosis, cysts, cell loss or severe leucocyte inflammation.

Liver tissue: Histological and stereological analysis of the effects of diazepam on liver tissue included the measurement of 16 stereological parameters in both treated and control rats (Table 3).

Table 3. Stereological parameters of the liver tissue of the control and experimental group of rats; values are shown as mean±SD (*$p<0.05$; t-test)

Parameters	Control group (n=5)	Treated group (n=5)
Volume density of hepatocytes (mm^0)	0.615±0.048	0.671±0.058*
Volume density of capillary sinusoids (mm^0)	0.129±0.016	0.171±0.019
Volume density of connective tissue (mm^0)	0.109±0.011	0.125±0.014
Number of hepatocytes	274938.6±21189.4	343273.8±29015.2*
Numerical density of hepatocytes (mm^{-3})	32245.5±4571.8	38201.7±4133.2*
Surface area of hepatocytes (μm^2)	152.4±3.7	147.3±3.5
Surface area of hepatic nuclei (μm^2)	46.3±1.9	49.2±2.3
NCR of hepatocytes	0.355±0.034	0.389±0.039
Mitotic index of hepatocytes	1.41±0.18	2.05±0.37
Apoptotic index of hepatocytes	0.12±0.01	1.55±0.25*
Number of connective tissue cells	114252.4±10203.6	12248.5±11326.5
Numerical density of connective tissue cells (mm^{-3})	21453.7±2581.2	23096.2±2959.5
Surface area of connective tissue cells (μm^2)	107.6±9.7	110.6±9.1
Number of capillary endothelial cells	125984.3±11326.9	140342.7±12099.9
Numerical density of capillary endothelial cells (mm^{-3})	14725.4±2186.3	21783.2±2366.4*
Surface area of capillary endothelial cells (μm^2)	82.5±9.2	85.9±10.4

Volume densities of hepatocytes, sinusoidal capillaries and connective tissues showed increased values in livers of diazepam-treated rats compared to control. In our experiments increase in hepatocyte volume density was statistically significant ($p = 0.027$), while increase in the value of this parameter in sinusoidal capillaries and connective tissues was not statistically significant. Liver tissue of diazepam-treated rats showed small changes compared to control group of rats. The changes were mostly reflected in increasing number of hepatocytes and erythrocytes in the liver of treated rats compared to control (Figure 1).

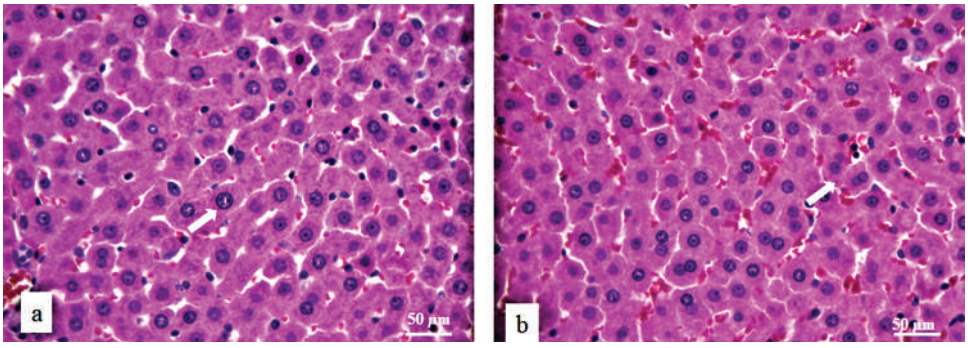

Figure 1. Micrographs of the histological cross-section of the rats' liver, H&E, magnification 100X. Hepatocytes (white arrows) and eritrocytes, and hepatocytes in mitosis; control **(a)** and diazepam-treated rats' tissue **(b)**

The number ($p = 0.015$) and numerical density ($p = 0.019$) of hepatocytes demonstrate a statistically significant increase in rats treated orally with diazepam compared to the control group. Also, numerical density of sinusoidal capillary epithelial cells showed a statistically significant increase ($p = 0.036$) in the liver of diazepam - treated rats compared to control rats. Reason for this increase is increased vascularization and increased number of erythrocytes in the liver tissue of treated rats. In addition to the increase in the number of hepatocytes and increase in their mitotic index, their number in apoptosis in treated rats is also increased (Figures 2 and 3). The increase in apoptosis index of hepatocytes in treated rats was statistically significant ($p = 0.044$).

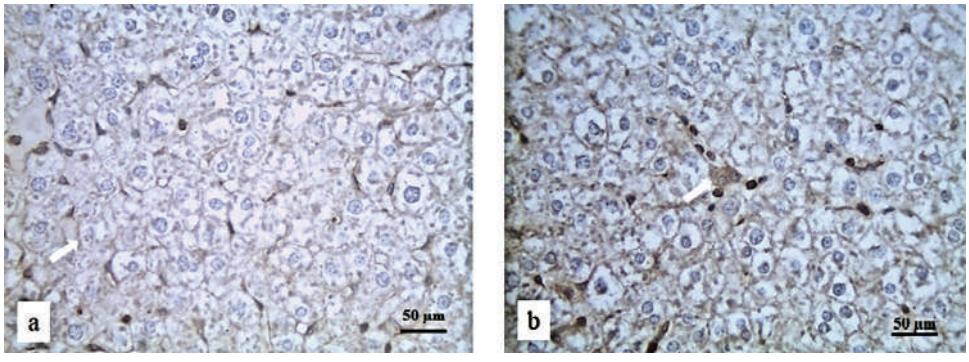

Figure 2. Micrographs of the histological cross-section of the rats´ liver, BLX-CX, magnification 100X. Apoptotic hepatocytes (white arrows); control **(a)** and diazepam-treated rats' tissue **(b)**

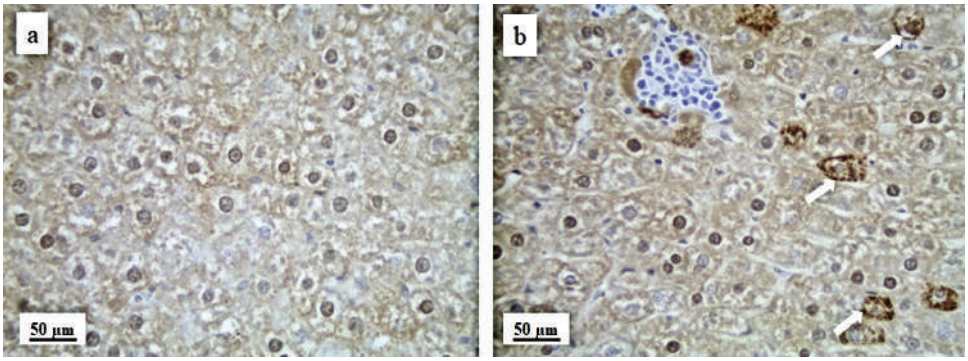

Figure 3. Micrographs of the histological cross-section of the rats´ liver, Survivin, magnification 100X. Detection of apoptotic hepatocytes (white arrows); control **(a)** and diazepam-treated rats' tissue **(b)**

Kidney tissue: All kidney tissue of rats treated with diazepam orally showed greatest changes in epithelial cells of collecting ducts and capillary sinuses. For this reason, 17 stereological parameters of kidney tissues of both treated and control rats were analyzed (Table 4).

Table 4. Stereological parameters of the kidney tissue of the control and experimental group of rats, values are shown as mean±SD (*p<0.05; t-test)

Parameter	Control group (n=5)	Treated group (n=5)
Volume density of collecting ductus epithelial cells (mm^0)	0.406±0.051	0.325±0.041*
Volume density of blood sinusoids (mm^0)	0.233±0.036	0.312±0.053*
Volume density of connective tissue (mm^0)	0.097±0.010	0.086±0.009
Volume density of glomeruli (mm^0)	0.216±0.025	0.194±0.022
Number of collecting ductus epithelial cells	193890.1±20546.3	125749.2±15702.3*
Numerical density of collecting ductus' epithelial cells (mm^{-3})	18843.3±2834.6	10952.5±2349.7*
Surface area of collecting ductus epithelial cells (μm^2)	177.4±18.5	168.2±18.1
Surface area of collecting ductus' epithelial cells nuclei (μm^2)	60.5±6.9	69.4±7.7
NCR of collecting ductus epithelial cells	0.311±0.032	0.362±0.037
Number of connective tissue cells	158318.9±12497.5	168429.5±13852.6
Numerical density of connective tissue cells (mm^{-3})	23052.8±2015.5	24654.7±2249.3
Surface area of connective tissue cells (μm^2)	111.4±10.4	108.6±10.1
Number of capillary endothelial cells	122497.6±10488.5	204857.5±18357.2*
Numerical density of capillary endothelial cells (mm^{-3})	25112.5±3549.8	36424.8±4795.1*
Surface area of capillary endothelial cells (μm^2)	79.6±9.5	81.2±10.4
Surface area of glomeruli (μm^2)	3956.3±445.3	3128.4±414.9
Bowman's space	44.4±5.6	46.1±6.7

Volume density of epithelial cells of collecting ducts showed a statistically significant ($p = 0.012$) decrease in the treated rats compared to the control animals. Same parameter

of capillary sinuses demonstrated a statistically significant (p = 0.015) increase in its values in the kidney tissues of rats treated with diazepam compared to control (Figure 4).

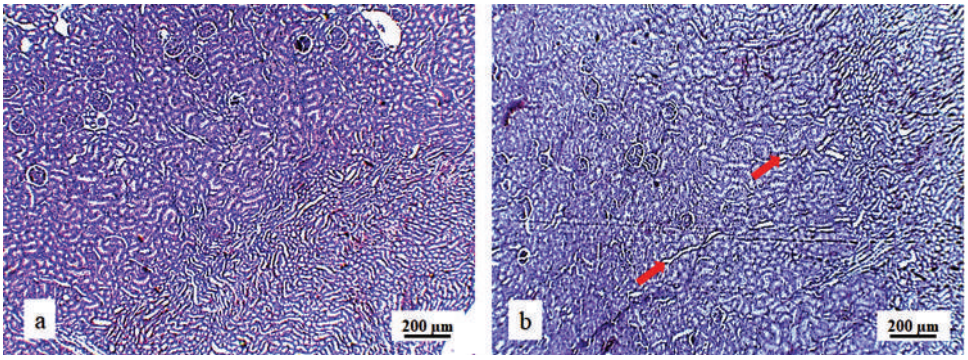

Figure 4. Micrographs of the histological cross-section of the rats´ kidney, Mallory-Azan, magnification 40X. Increased volume density of collecting ducts' epithelial cells (red arrows); control **(a)** and diazepam-treated rats' tissue **(b)**.

Volume densities of connective tissue and glomerulus decreased their values in kidney tissues of diazepam – treated rats (Table 4) and this decrease wasn´t statistically significant. The number (p = 0.039) and numerical density (p = 0.033) of epithelial cells of renal collecting ducts show a statistically significant reduction in diazepam-treated rats compared to control rats (Figure 5).

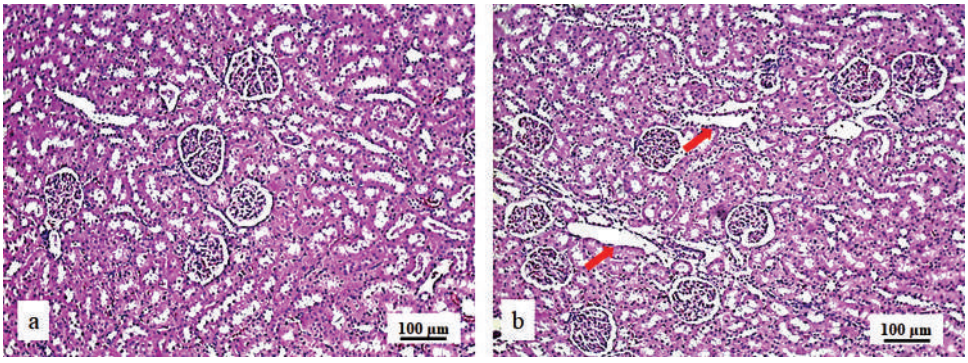

Figure 5. Micrographs of the histological cross-section of the rats´ kidney, H&E, magnification 50X. Dilatation of collecting dusts (red arrows); control **(a)** and diazepam-treated rats' tissue **(b)**.

In the kidney tissues of treated rats, there was a statistically insignificant increase in the number and numerical density of connective cells compared to control rats. In our study a statistically significant increase in the number (p = 0.028) and numerical density (p = 0.025) of endothelial cells of capillaries was found in the kidneys of treated rats. The surface area of glomeruli decreased, while the surface area of Bowman's capsule increased on cross-sections of kidney tissue of rats exposed to diazepam compared to control ones, and this increase was not statistically significant (Figure 6).

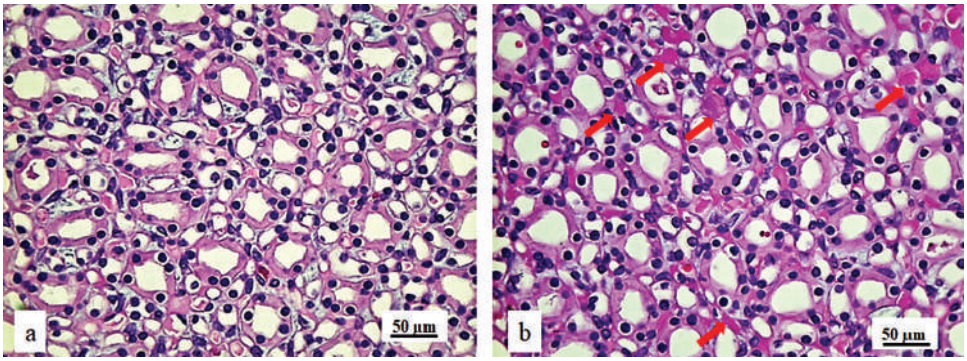

Figure 6. Micrographs of the histological cross-section of the rats´ kidney, BLX-CX, magnification 100X. Decrease in collecting dusts epithelial cells, increase in capillary endothelial cells (red arrows), and number of eritrocytes; control **(a)** and diazepam-treated rats' tissue **(b)**

Spleen tissue: Tissue of the spleen in rats treated with diazepam orally showed slight changes compared to the tissues in the control group of animals. Therefore, a total of 20 stereological parameters of spleen tissue of both treated and control rats were analyzed (Table 5).

Table 5. Stereological parameters of the spleen tissue of the control and experimental group of rats, values are shown as mean±SD (*p<0.05; t-test)

Parameters	Control group (n=5)	Treated group (n=5)
Volume density of epithelial cells (mm^0)	0.335 ± 0.021	0.265±0.021
Volume density of lymphocytes (mm^0)	0.241 ± 0.034	0.356±0.033*
Volume density of all capillary cells (mm^0)	0.268 ± 0.039	0.294±0.042
Volume density of connective tissue (mm^0)	0.156 ± 0.032	0.085±0.029
Number of epithelial cells	215649.4 ± 19606.7	201473.9±21433.9
Numerical density of epithelial cells (mm^{-3})	25654.8 ± 3951.2	22324.4±3843.6
Surface area of epithelial cells (µm2)	136.6 ± 12.6	131.5±13.8
Surface area of epithelial cells' nuclei (μm^2)	74.8 ± 8.8	75.5±8.9
NCR of epithelial cells	0.266 ± 0.085	0.271±0.061
Mitotic index of epithelial cells	1.04±0.22	1.67±0.45
Apoptotic index of epithelial cells	0.08±0.02	1.23±0.18*
Number of lymphocytes	296548.3± 25508.6	414687.1±23641.3*
Numerical density of lymphocytes (mm^{-3})	30537.1 ± 4768.3	48885.4±4108.7*
Surface area of lymphocytes (μm^2)	82.6 ± 10.2	85.5±10.8
Number of capillary endothelial cells	154873.6 ± 11245.8	183794.2±14698.7
Numerical density of all capillary cells (mm^{-3})	23353.3± 2430.1	29937.7 ± 2655.1
Surface area of capillary endothelial cells (μm^2)	64.5 ± 9.2	66.8±8.7
Number of connective tissue cells	109513.5±12589.4	110249.8±11254.3
Numerical density of connective tissue cells (mm^{-3})	21468.3 ± 3642.1	24861.7±3394.5
Surface area of connective tissue cells (μm^2)	112.1 ± 12.9	109.5±11.6

Values of volume densities of splenic parenchyma epithelial cells, blood capillary epithelial cells, and spleen connective tissue in rats treated with diazepam compared to controls were not statistically significant. Only the value of volume density in spleen lymphocytes treated animals showed a statistically significant increase ($p = 0.028$) in compared to the control group. The increase in number ($p = 0.039$) and numerical density ($p = 0.035$) of lymphocytes in the spleen tissue was also statistically significant in rats treated with diazepam compared to control rats. The number and numerical density of splenic parenchyma epithelial cells decreased while same parameters in capillary epithelial cells increased (Figure 7).

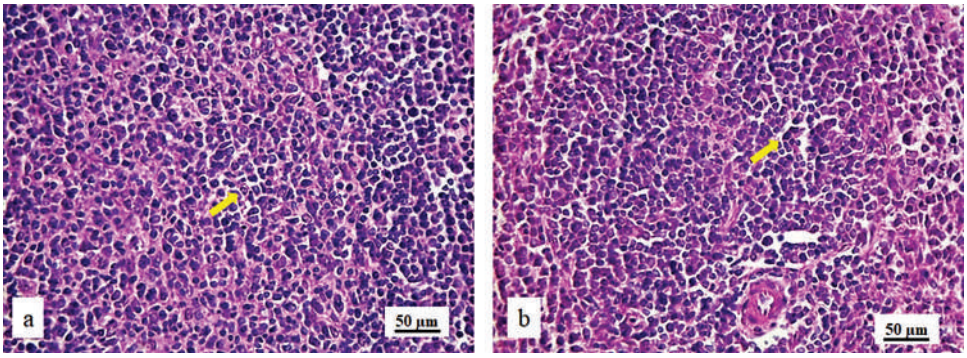

Figure 7. Micrographs of the histological cross-section of the rats´ spleen, H&E, magnification 100X. Lymphocytes (yellow arrows), connective and epithelial cells; control **(a)** and diazepam-treated rats' tissue **(b)**

The value of apoptotic index of epithelial cells in spleen parenchyma treated rats exhibited a statistically significant increase ($p = 0.009$) (Table 5). Apoptotic epithelial cells in the spleens of rats treated with diazepam are shown in Figure 8. Mitotic index in the parenchymal epithelial cells of spleen tissues of rats treated with diazepam was increased in relation to controls. Epithelial cells of splenic parenchyma divided and were more in apoptosis in the treated than control animals, which is a consequence of increased spleen function and increased immune response as a consequence of diazepam.

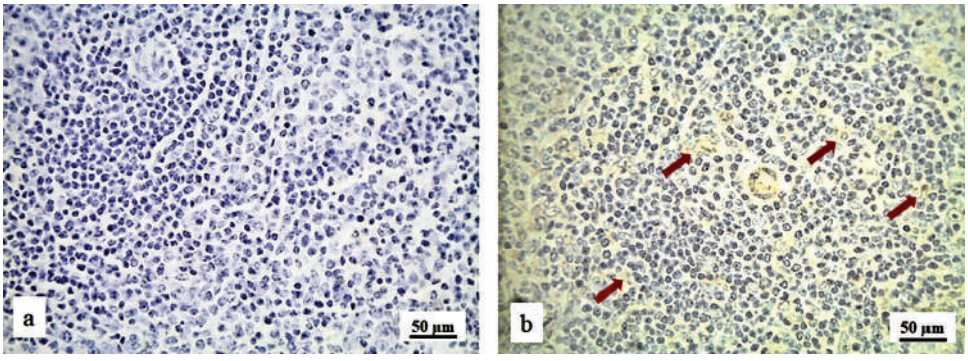

Figure 8. Micrographs of the histological cross-section of the rats´ spleen, Survivin, magnification 100X. Lymphocytes, epithelial cell in apoptosis (red arrows); control **(a)** and diazepam-treated rats' tissue **(b)**

DISCUSSION

In this study, oral treatment with diazepam at a dose of 0.2 mg / kg body weight had the effect of reducing the value of hematological parameters of rats. Decreased erythrocyte and leukocyte parameters are indicative of the depressant effect of diazepam on hematopoiesis and immunity of diazepam-treated rats. Literature data indicate that treatment of rats with diazepam may on one hand increase [20,21], on the other hand decrease [22,23] or not affect the change in the values of hematological parameters of rat blood [24,25]. Different results in literature are present due to usage of different doses and timeline of diazepam administration in experimental protocols. The results obtained in the study of Anacletus and Onyegeme-Okerenta, (2017) show a statistically significant decrease in the concentration of hemoglobin in the blood of rats treated with diazepam compared to control animals. Decrease in hemoglobin concentration is the same as in our study, although in the author's study, rats were treated with a much lower dose of diazepam of only 0.012 mg / kg body weight [26]. In the study of Nicholas et al. [27], the authors proved that diazapan affects the immune system of rats by reducing the number of inflammatory and infiltrating immune cells in the blood, as well as in the brain and liver tissue. Benzodiazapenes have a direct inhibitory effect on the proliferation and production of anti-inflammatory cytokines and activation of T lymphocytes isolated from lymph nodes. Finally, the authors revealed a reduction in the number of inflammatory cells: microglia, monocytes and macrophages in cerebellum, cerebrum and spinal cord of rats exposed to benzodiazapine treatment [27]. Oral diazepam treatment in rats had a significant effect on the changes in the values of biochemical parameters in our study. In our study the concentration of total blood proteins of rats increased in treated group as well as in the study of Nzor et al. [25] who treated rabbits with a dose of 0.0036 mg diazepam per 100 g body weight. In our study, the increase in triglycerides and cholesterol in blood of diazepam-treated rats compared to control group coincides with literature where photoperiodic rhythm of diazepam-treated rats was tested and leads to an increase in lipids and triglycerides [20]. According to literature, blood glucose levels in rats may decrease [25] or increase [20,22] after diazepam treatment. This difference in the results of the way diazepam acts on the concentration of glucose in the blood of rats is only a confirmation of fact that it acts systemically on the entire organism and not only on the CNS. Concentrations of bilirubin and urea were decreased in the blood of diazepam-treated rats in our study, due to decreased hematopoiesis. This result is in contrast to literature, which states that urea concentration remains unchanged after the action of diazepam [25]. Mean value of creatinine significantly ($p = 0.015$) increased in the blood of rats, which is an indicator of workload of kidneys of rats with diazepam in our study. Research conducted by Setiawan et al. (2016) [13] shows that, under the influence of diazepam, there was a significant increase in the concentration of creatinine [13] in the blood of rats, which isn´t consistent with our results. Diazepam, by the activity of its chemical composition, affects hemodynamic aspect of kidney cells, and can reduce blood pressure and blood flow to the kidneys.

This condition can lead to ischemic kidney disease, which induces hypoxia in the renal cortex cells and elevated levels of creatinine in sera [28,29]. The biochemistry of rat blood sera in our short-term study shows that kidneys are under a heavy load due to effects of diazepam. Also, values of liver enzymes (ALP, AST, ALT and GGT) exhibited a statistically significant reduction in rats treated with diazepam, except for GPT, whose value increased. This is an indication that this drug directly affects the dynamics of liver enzyme synthesis. Literature data indicate that benzodiazepine sedatives may increase [11,14,30], significantly decrease [31] or not alter liver enzymes at all [32,33]. A significant reduction in pancreatic lipase concentration in our study in diazepame-treated rats is in accordance with the literature. Decreased pancreatic lipase concentration has been described as a consequence of decreased physical activity in rats due to the sedative effect of diazepam [34,35]. Concentration of potassium ions was ($p = 0.077$) increased, while concentrations of sodium and calcium ions decreased insignificantly in our study after treating rats with diazepam. An imbalance of these ions in the body of rats leads to dysfunction of the nervous tissue and heart function in form of arrhythmias, decreased heart rate and muscle contractility [36]. Once the effects of the drug wear off, a renewed imbalance in the functioning of electrolytes takes place, which leads to a change in signaling, structural, metabolic and regulatory proteins on membranes of nerve and muscle cells [37]. There are numerous but contradictory opinions in literature on how diazepam acts in a pathohistological sense on the tissues of liver, kidneys and spleen. Diazepam has a deleterious effect on nerve tissues, especially on nerve cells and nerve fibers because it increases the level of oxidative stress in them [38]. After treatment with diazepam the enzymatic activity of neurons that build every organ in the body of rats changes completely [39]. The liver tissue of treated rats showed a change in cytoarchitecture and vascularization due to effect of diazepam in our study. There was an increase in the number, mitotic index, numerical and volume density of hepatocytes in rats treated with diazepam. The reason for these changes in the hepatocytes is increased filtration of diazepam from the blood through the liver of rats. An intensive increase in surface area of nucleus and a statistically significant increase ($p<0.01$) in hepatocyte proliferation index in the livers of rats treated with diazepam were published. In study of G. H. El-Sokkary (2008) [40] rats were watered a dose of diazepam of 5 mg / kg body weight and this was characterized as a high dose. Rat liver often cannot metabolize the entire amount of the applied diazepam and thus accumulates it [41]. In our study, there was an increased liver neovascularisation after diazepam treatment, which was confirmed by an increase in the volume density of capillary sinus in the liver tissue. Also comes to statistically significant increase in the numerical density of capillary sinus epithelial cells and number of erythrocytes per unit of area in the liver tissue. Stimulation of hepatic blood flow in rats after treatment with sodium pentobarbital has been shown in Mohamed et al. (2020) [42]. These authors state that in addition to erythrocytes, the number of leukocytes in blood capillaries in the liver of treated rats is increased compared to control rats [42]. Increased liver vascularization is the cause of a potential diazepam intoxication of the rats' liver in our study. An indicator of this is a statistically

significant increase in hepatocyte apoptosis in treated group of rats ($p = 0.044$). Ilesanmi and Odewale [12] describe changes in histological parameters of liver rats after tramadol-treatment as mild hepatocyte necrosis and blockage of blood vessels without inflammation, which was not detected in our study. These authors explain the occurrence of clogged blood vessels in the liver as a consequence of an impaired lipid metabolism, primarily cholesterol in the blood of rats under the influence of diazepam [12]. Diazepam can lead to histopathological disorders in rabbit liver tissue, inflammatory processes, congestion of blood vessels, dilated sinusoids and vacuolar degeneration [25]. In our study utmost changes occur in the kidney epithelial collecting ducts cells of diazepam-treated rats. Volume density, number, area and numerical density of epithelial cells of collecting ducts in the kidney of rats treated with diazepam exhibited a statistically significant decrease. Diazepam induced decay in kidney epithelial collecting ducts cells and reduced capacity of kidney filtering tissue. The same data are stated by E. Bribes et al. [43], who state that benzodiazepines affect the process of excretion through kidneys and interfere with the process of glomerular filtration, reabsorption and tubular secretion. Large number of peripheral benzodiazepine receptors (PBRs), wich nephrons contain, are the cause for excretion of benzodiazepine through kidneys. Difficult excretion of diazepam through the kidneys is increased by its solubility in fats, because excretion of non-polar drug metabolites is delayed in comparison with water-soluble drugs. Based on the ability of kidneys to accumulate xenobiotic compounds, diazepam often accumulates in the glomeruli and proximal tubules. Such high concentrations of diazepam at the renal level exhibit a toxic effect and can often lead to kidney impairment [43]. In our experiment, diazepam induced a reduction of the surface area of glomeruli and increased Bowman's space in the glomeruli, thus changing its cytoarchitecture. Together with data that there was a statistically significant increase in the concentration of creatinine and total protein in sera of treated rats, this implies deterioration of the kidneys and impaired kidney activity. Data on the deterioration of the structure of renal glomeruli leading to an increase concentration of creatinine in sera of rats treated with tramadol are shown by Setiawan et al. [13]. The uncontrolled use of diazepam may lead to an increased risk of glomerulonephritis [44] which further leads to decreased immune capacity [45]. In diazepam-treated rats, there was an increase in the vascular network through the renal tissue in our study. There was a statistically significant increase in the values of blood sinusoids volume density, as well as the number and numerical density of capillary endothelial cells in the kidneys of treated rats. In our study the stereological evidence shows an increase in the surface area of capillary endothelial cells, a decrease in volume density of connective tissue and a decrease in surface area of connective cells. This indicates congestion to diazepam-treated renal tissue. Literature data confirm our results on increase in volume of blood capillaries in kidney tissue of diazepam-treated rats by the fact that there is an increase in the number of inflammatory cells, especially mononuclear cells and neutrophils [46]. Sayed et al. [47], explain their results of increased vascularization of kidneys and dilation of blood capillaries after diazepam treatment of rats by slowed metabolism

and water retention. They even state that blood vessels in the kidneys become congested due to protein accumulation [47]. This explanation is in agreement with our finding of elevated total protein concentrations in the blood of diazepam-treated rats. Moreover, Mousa [48], observed dilation of blood capillaries and increased renal vascularization in diazepam-treated rats in the direction of relaxation of muscle cells around the blood vessels, as well as an increased permeability of blood capillaries. The same authors state that higher doses of diazepam are toxic and accumulate in the rat kidney tissue [48]. In our study, diazepam treatment increased inflammation and lymphopoiesis in the rat spleen tissue. A statistically significant increase in volume density, number and numerical density of lymphocytes was at the expense of epithelial cells in the spleen. This change of values is a result of increased blood filtration and accumulation of lymphocytes [42]. Spleen cells remain unchanged despite the fact that free radicals from this sedative have been shown to damage cell membranes of epithelial cells of the spleen. On one hand, Mohame et al. [42] reports show that the spleen as an organ is invulnerable to damage after treatment with an excessive dose of sodium pentobarbital. On the other hand, Bartolomucci [49] has published results in which histopathological analysis of rats spleen, treated for eight weeks with 0.1 mg/kg diazepam, confirm excessive infiltration of lymphocytes in the red pulp and depletion of lymph tissue of white spleen pulp. All analysis of detection of diazepam confirms that it was most concentrated in the spleen, intestines and liver after its experimental application [14]. Stimulation of the spleen function in treated rats in our experiment was also confirmed by increased volume density of blood vessels, number, numerical density and surface area of capillary cells. Huemer et al. [50] state in their results that long-term treatment of rats with diazepam leads to production and retention of lymphocytes in the spleen, as well as obstruction of blood vessels. Congealing of blood vessels is caused by aggregations of lymphocytes which are proliferated and mobilized from white pulp and clinged between epithelial cells. Diazepam leads to an increase in percentage of T cytotoxic cells in the blood serum of rats [50]. Diazepam caused histological changes in the liver, kidneys and spleen of experimental rats making these organs adapt to changes in order to decrease the toxic effects. This lead to the increase in immune rensponse.

CONCLUSIONS

The fast and efficient sedative effect of diazepam, on muscle and nerve cells, is the primary feature for its great therapeutic usage in humans and animals today. The dose and duration of diazepam therapy must be adjusted individually because it affects functioning of the entire organism. In this paper, we proved that diazepam had an effect on reducing blood cell production and lowering immunity in rats. Diazepam disrupted metabolism of lipids and blood sugar in rats after only fifteen days of usage. This is important because diazepam is used by patients who suffer from diabetes. Our treatment of rats with diazepam caused an imbalance in concentration of electrolytes

in the blood, which lead to a disruption of functioning of both nerve and heart tissue. The dose of diazepam in our experiment increased the processes of filtration and detoxification of rat blood through the liver, which was proven by a change in histological parameters and a decrease in the production of liver enzymes. In our study, diazepam has been shown to be a burdening factor on renal function, which is of particular importance for its use in patients with renal insufficiency. Biochemical parameters, indicators of kidney function, of rat blood as well as stereological analysis of kidney tissue show effects of diazepam on the reduction of capacity and deterioration of kidney tissue. Diazepam treatment of rats stimulated spleen functiontioning through increased lymphocyte infiltration and vascularization. Finally effect of diazepam on hematological and histological parameters is significant in the direction of workload of liver, kidneys and spleen in rats. Therefore, usage of diazepam must be strictly controlled by physician and must not be left to the free will of patients.

Acknowledgement

Authors thank the Ministry of Scientific and Research Development, Higher Education and Information Society of Republic of Srpska for the projects support (Contract numbers: 19/6-20/961-98/18 and 19/6-20/961-66/18).

Authors' contributions

MI has made substantial contributions to conception and design of experiment. JG was responsible for data collection, performed the haematological analysis and made substantial contributions to interpretation of data. SP was responsible for data collection performed the histological and stereological analysis and made substantial contributions to acquisition of data, analysis and interpretation of data. RD has been involved in drafting the manuscript or revising it critically for important intellectual content. All authors have given final approval of the version to be published and agree to be accountable for all aspects of the manuscript in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Sevastre-Berghian AC, Fägäräsan V, Toma VA, Baldea I, Olteanu D, Moldovan R, Decea N, Filip GA, Clichici SV: Curcumin Reverses the Diazepam-Induced Cognitive Impairment by Modulation of Oxidative Stress and ERK 1/2/NF-ϰB Pathway in Brain. Oxid Med Cell Longev 2017, 2017(3): 3037876.
2. Olfson M, King M, Schoenbaum M: Benzodiazepine use in the United States. JAMA Psychiatry 2015, 72(2):136–142.
3. Amorim CG, Araújo AN, Montenegro SM, Silva VL: Cyclodextrin-based potentiometric sensors for midazolam and diazepam. J Pharm Biomed Anal 2008, 48(4):1064–1069.
4. Šegrt Z, Đorđević S, Jaćević V, Kilibarda V, Vučinić S, Jović-Stošić J, Potrebić O, Perković-Vukčević N: Farmakodinamski i farmakokinetski efekti primene flumazenila i teofilina kod pacova akutno trovanih diazepamom. Vojnosanit Pregl 2009, 66(2):141-148.
5. Ashton H: The diagnosis and management of benzodiazepine dependence. Curr Opin Psychiatry 2005, 18(3):249–255.
6. Mimica N, Folnegović-Šmalc V, Uzun S, Rušinović M: Benzodiazepini: za i protiv. Med Psihofarmak 2002, 11(2):183–188.
7. Malcolm RJ: GABA Systems, Benzodiazepines, and Substance Dependence. J Clin Psychiatry 2003, 64(3):36-40.
8. Honeychurch KC, Hart JP: Electrochemical Detection of Benzodiazepines, Following Liquid Chromatography, for Applications in Pharmaceutical, Biomedical and Forensic Investigations. Insciences J 2014, 4(1):1-18.
9. Owen G, Smith THF, Agersborg HPK: Toxicity of some benzodiazepine compounds with CNS activity. Toxicol. Appl. Pharmacol 1970, 16(2):556–570.
10. Pomares FB, Funck T, Feier NA, Roy S, Daigle-Martel A, Ceko M, Narayanan N, Araujo D, Thiel A, Stikov N, Fitzcharles MA, Schweinhardt P: Histological Underpinnings of Grey Matter Changes in Fibromyalgia Investigated Using Multimodal Brain Imaging. J Soc Neurosci 2017, 37(5):1090–1101.
11. Anber ZNH, Fadhil AA, Anber SA: The biochemicl and histological effect of diazepam on the liver of albino male rats. Internat J Acad Res Reflec 2018, 6(3).
12. Ilesanmi OB, Odewale TT: Effect of classic soft drink Coca-Cola as a solvent in the administration of tramadol and diazepam on biochemical and histological changes in liver and kidney. Ukr J Nephrol Dial 2020, 3(67).
13. Setiawan PGM, Tunjung WAS, Nurhidayat L: Effect of diazepam on kidney function and histological structure of white rats kidney. J Biol Res 2016, 22(1):6.
14. Al-Rekabi AE, Al-Rumaidh SZ, Al-Fartosi KG: Effect of tramadol and diazepam on some biochemical parameters of male rats. J Nat Remed 2021, 21(12):1-12.
15. Nestorović N, Trifunović S, Ivana J, Manojlović-Stojanovski M, Ristić N, Filipović B, Šošić-Jurnjević B, Milošević V: Sex steroid application reverses changes in rat castration cells: Unbiased stereological analysis. Arch Biol Sci 2016, 68(4):821–828.
16. Paraš S, Janković O, Trišić D, Čolović B, Mitrović-Ajtić O, Dekić R, Soldatović I, Živković-Sandić M, Živković S, Jokanović V: Influence of nanostructured calcium aluminate and calcium silicate on the liver: histological and unbiased stereological analysis. Int Endod J 2019, 52(8):1162–1172.

17. Santos M, Marcos R, Santos N, Malhão F, Monteiro RAF, Rocha E: An unbiased stereological study on subpopulations of rat liver macrophages and on their numerical relation with the hepatocytes and stellate cells. J Anat 2009, 214(5):744–751.
18. Sharp P, Villano JS: The Laboratory Rat. Routledge & CRC Press 2012.
19. Sasse EA: How to define and determine reference intervals in the clinical laboratory approved guideline. Wayne Pa NCCLS 1995.
20. Goel N, Bale TL: Organizational and Activational Effects of Testosterone on Masculinization of Female Physiological and Behavioral Stress Responses. Endocrinology 2008, 149(12):6399–6405.
21. Elusiyan CA, Faria ALG, Mendes AEQ, Silva IO, Martins JLR, Rosa DA, Pedrino GR, Costa EA, Ibrahim MA, Zjawiony JK, Fejemiroye JO: Involvement of the Benzodiazepine Site in the Anticonvulsant Activity of Tapinanthus globiferus against Pentylenetetrazole-induced Seizures in Mice. Planta Med 2020, 86(16):1204–1215.
22. Mohammad RA, Shukhi K, Lalin M, Zunayeed R, Mostafa A, Mohammad LM: Evaluation of some prescribed and over-the-counter drugs induced hemato-biochemical changes in mice. Asian J Med Biolog Res 2019, 5(4).
23. Kamel M: Neurochemical, hematological and behavioral alterations related to eszopiclone administration in rats. Slovenian Vet Res 2018, 55:41-50.
24. Osonuga I, Osonuga O, Osonuga A, Onadeko A, Osonuga A: Effect of artemether on hematological parameters of healthy and uninfected adult Wistar rats. Asian Pac J Trop Biomed 2012, 2(6):493–495.
25. Nzor JN, Uwakwe AA, Onuoha SC: Impact of benzodiazepines administration on selected biochemical parameters of albino Wistar rats (Rattus rattus). Egyptian Pharm J 2018, 17(1):40-47.
26. Anacletus FC, Onyegeme-Okerenta BM: Evaluation of the influence of therapeutic, prolonged and overdose intake of diazepam on hematological indices and liver emzyme markers of male Wistar rats. Res J Life Sci Bioinforma Pharm Chem Sci 2017, 2(6).
27. Fernández NH, Zanetti SR, Báez NS, Bibolini MJ, Bouzat C, Roth GA: Diazepam treatment reduces inflammatory cells and mediators in the central nervous system of rats with experimental autoimmune encephalomyelitis. J. Neuroimmunol 2017, 313:145–151.
28. Bellomo R, Kellum JA, Ronco C: Acute kidney injury. Lancet Lond Engl 2012, 380(9843):756–766.
29. Khajuria A, Tay C, Shi J, Zhao H, Ma D: Anesthetics attenuate ischemia–reperfusion induced renal injury: Effects and mechanisms. Acta Anaesthesiol Taiwan 2014, 52(4):176–184.
30. Vozarova B, Stefan N, Lindsay RS, Saremi A, Pratley RE, Bogardus C, Tataranni PA: High alanine aminotransferase is associated with decreased hepatic insulin sensitivity and predicts the development of type 2 diabetes. Diabetes 2002, 51(6):1889–1895.
31. Bian T, Corral P, Wang Y, Botello J, Kingston R, Daniels T, Salloum RG, Johnston E, Huo Z, Lu J, Liu AC, Xing C: Kava as a Clinical Nutrient: Promises and Challenges. Nutrients 2020, 12(10):3044.
32. De P, Pang T, Das G: Clinical Implications and Management of Sub Clinical Hyperthyroidism: A Review. Open J Endocr Metab Di 2012, 2(3):27–35.
33. Atici S, Cinel I, Cinel L, Doruk N, Eskandari G, Oral U: Liver and kidney toxicity in chronic use of opioids: an experimental long term treatment model. J Biosci 2005. 30(2):245–252.

34. Abed A, Minaiyan M, Safaei A, Taheri D: Effect of Diazepam on Severity of Acute Pancreatitis: Possible Involvement of Peripheral Benzodiazepine Receptors. ISRN Gastroenterol 2013, 54:1–6.
35. Akram M, Riaz M, Munir N, Akhter N, Zafar S, Jabeen F, Shariati MA, Akhtar N, Riaz Z, Altaf SH, Daniyal M, Zahir R, Khan FS: Chemical constituents, experimental and clinical pharmacology of Rosa damascena: a literature review. J Pharm Pharmacol 2020, 72(2):161–174.
36. Stevens CM, Rayani K, Singh G, Lotfalisalmasi B, Tieleman DP, Tibbits GF: Changes in the dynamics of the cardiac troponin C molecule explain the effects of Ca2+-sensitizing mutations. J Biol Chem 2017, 292(28):11915–11926.
37. Al-Abbasi FA, Kumar V, Anwar F: Biochemical and toxicological effect of diazepam in stress-induced cardiac dysfunctions. Toxicol Rep 2020, 7:788–794.
38. Griffin CE, Kaye AM, Bueno FR, Kaye AD: Benzodiazepine Pharmacology and Central Nervous System–Mediated Effects. Ochsner J 2013, 13(2):214–223.
39. Supasai S, Gonzalez EA, Rowland DJ, Hobson B, Bruun DA, Guignet MA, Soares S, Singh V, Wulff H, Saito N, Harvey DJ, Lein PJ: Acute administration of diazepam or midazolam minimally alters long-term neuropathological effects in the rat brain following acute intoxication with diisopropylfluorophosphate. Eur J Pharmacol 2020, 886:173538.
40. El-Sokkary HG: Melatonin and vitamin C administration ameliorate diazepam-induced oxidative stress and cell proliferation in the liver of rats. Cell Prolif 2008, 1:168-76.
41. Wang HJ, Benet LZ: Protein Binding and Hepatic Clearance: Re-Examining the Discrimination between Models of Hepatic Clearance with Diazepam in the Isolated Perfused Rat Liver Preparation. Drug Metab Dispos 2019, 47(12):1397–1402.
42. Mohame AS, Hosney M, Bassiony H, Hassanien SS, Soliman AM, Fahmy SR, Gaafar K: Sodium pentobarbital dosages for exsanguination affect biochemical, molecular and histological measurements in rats. Sci Rep 2020, 10(1):378.
43. Bribes E, Casellas P, Vidal H, Dussossoy D, Casellas D: Peripheral Benzodiazepine Receptor Mapping in Rat Kidney. Effects of Angiotensin II-Induced Hypertension. J Am Soc Nephrol 2002, 13(1):1–9.
44. Howse MLP, Bell GM: Drugs and toxins that damage the kidney. Med Baltimore 2007, 35(7):399–403.
45. Crowe AV, Howse M, Bell GM, Henry JA: Substance abuse and the kidney. QJM Int J Med 2000, 93(3):147–152.
46. Ali AH, Zinad KH: Histopathological changes and immunosuppression induce by diazepam in mice. Al-Qadisiyah J Vet Med Sci 2014, 13(1).
47. Sayed GM, Desoky N, El-Refaiy A, Abd-Elrahman IM, Nagy MH: Histological study on the stomach of immobilized-stressed albino rat and the curative role of diazepam. Egypt J Exp Biol Zool 2011, 7(2):153–161.
48. Mousa AM: Light and electron microscopic study on the effect of diazepam on the cardiac muscle of adult albino rat and the possible role of garlic. Egypt J Histol 2014, 37(1):102–111.
49. Bartolomucci A: Social stress, immune functions and disease in rodents. Front Neuroendocrinol 2007. 28(1):28–49.
50. Huemer HP, Lassnig C, Nowotny N, Irschick EU, Kitchen M, Pavlic M: Diazepam leads to enhanced severity of orthopoxvirus infection and immune suppression. Vaccine 2010, 28(38):6152–6158.

EFEKAT DIAZEPAMA NA HEMATOLOŠKE I HISTOLOŠKE PARAMETRE PACOVA / *IN VIVO* I NEPRISTRASNO STEREOLOŠKO ISTRAŽIVANJE

Jovana GRAHOVAC, Milenka IVANKOVIĆ, Radoslav DEKIĆ, Smiljana PARAŠ

Lekovi na bazi diazepama danas su u širokoj upotrebi u lečenju ljudi. Diazepam može da bude primarni lek usmeren za lečenje neuroloških oboljenja ili pridružen lek uključen za lečenje drugih oboljenja u cilju simptomatske terapije. Sedativni efekat diazepama karakteriše ga kao lek koji ljudi najčešće koriste samoinicijativno i bez nadzora lekara. Direktno ali i preko uticaja na nervni sistem diazepam remeti pravilno funkcionisanje svih organa u organizmu. Ideja ovog rada bila je da se ispita efekat diazepama na hematološke i citohistološke parametre pacova u *in vivo* eksperimentu. Za dostizanje projektovanog cilja korišćene su Mallory-Azan i imunohistohemijske bojenja BLX-CX i Survivin metodama tkiva jetri, bubrega i slezina pacova. Citometrijska analiza organa pacova u tretmanu detektovala je ćelije u apoptozi i određivanje vrednosti stereoloških parametara pomoću Kavalierovog principa. Rezultati analize hematoloških i histoloških parametara ukazuju na štetan efekat diazepama na parametre krvi kao i na arhitekturu jetre, bubrega i slezine pacova. Rad predstavlja osnovu za dalja detaljna naučna istraživanja sa ciljem osvetljavanja svih štetnih efekata koje diazepam ima na sve organe u organizmu pacova. Podaci bi mogli da posluže kao osnova za buduće studije iz kliničke farmakologije o terapijskim protokolima za upotrebu sedadativa na bazi diazepama.