EXPERIMENTAL STUDY

Dinuclear ruthenium(II) Schiff base complex: a first *in vivo* study in Swiss albino mice

Muzika V[1*], Custovic S[1*], Alicelebic S[1], Cosovic E[1], Zahirovic A[2], Kahrovic E[2]

University of Sarajevo, Faculty of Science, Department of Chemistry, Sarajevo, Bosnia and Herzegovina. **emira_kahrovic@yahoo.com**

ABSTRACT

OBJECTIVES: Dinuclear ruthenium(II) Schiff base complex was selected for *in vivo* study among many other novel metal-based compounds, because of its previously proved *in vitro* anticancer and antibacterial properties. The aim was to investigate the potential toxicity of this compound in animal model through biochemical and histopathological assessment.

METHODS: Adult Swiss albino mice of both sexes were divided into high-dose and low-dose group that received a single intraperitoneal dose of ruthenium complex (175 mg/kg and 25 mg/kg, respectively) and one control group (vehicle only). After a follow-up period of 14 days, animals were sacrificed to obtain blood samples and organs.

RESULTS: The test compound was well tolerated in a low-dose group and did not cause any mortality. The histological findings and serum biochemistry suggested a reversible character of alterations found in vital organs of this group. However, in the high-dose group, adverse effects were more severe and indicated dose and gender-related toxicity.

CONCLUSION: Mild side effects found in a low-dose group together with excellent *in vitro* properties, made dinuclear ruthenium(II) Schiff base complex a promising candidate for further investigation and development as anticancer and antimicrobial agent *(Tab. 4, Fig. 6, Ref. 32)*. Text in PDF *www.elis.sk.*

KEY WORDS: ruthenium compounds, mice, toxicity tests, histology.

Introduction

The need for production of new drugs is a permanent and global challenge. The ruthenium complexes attract considerable attention over recent decades because of their unique characteristics, ranging from the chemical (ligand exchange, accessible oxidation states) to biological (transportation pathways, activation, and selectivity) (1, 2). Ruthenium complexes are investigated as both, potential anticancer and antimicrobial agents and a combination of those two properties is desirable in patients treated for cancer because of their increased susceptibility to infections (1, 3–6). Ruthenium complexes showed an improved selectivity and reduced side effects in comparison to platinum compounds, widely used in clinical practice (1–4, 7). The main target for ruthenium agents in the biological systems is still not elucidated. Even though these agents bind to DNA molecule, some authors demonstrated DNA-independent antitumor mechanisms (4, 7). Among ruthenium complexes that proved high *in vitro* and *in vivo* antitumor activity, NAMI-A, KP1019 and NKP1339 have entered human clinical trials (4, 7). These compounds showed a significant cytotoxicity, antimetastatic effects and a low general toxicity (7).

Dinuclear ruthenium(II) Schiff base complex prepared by Kahrovic et al (2) is one of the first ruthenium compounds having phenol oxygen from Schiff base as bridging ligand. *In vitro* studies proved that this complex has a strong anticancer activity against different cancer cell lines and is even more potent than cisplatin. Also, it exhibits antibacterial activity against Gram positive bacteria (8). Nowadays ruthenium complexes, having good anticancer and antimicrobial activities, are reported daily, but all of them are not good candidates for *in vivo* investigations. At least three substantial reasons candidate Ru(II) Schiff base complex of *N*-(2-pyridyl)-5-chlorosalicylideneimine, over hundreds of other ruthenium(II) complexes, as promising agent for *in vivo* testing: (i) *in vitro* experiments showed that the complex binds DNA in a groove with moderate binding affinity (binding constant 3.82×10^4 M^{-1}), suggesting lower risk of genotoxicity; (ii) contrary to the classic chemotherapeutic drugs, very strong *in vitro* anticancer activity of this complex is not DNA dependent, implying that other biological pathways might be disrupted by the complex, which is especially important in fighting the metastases; (iii) strong antimicrobial activity of the complex, which is better or comparable to vancomycin (2, 8).

The evaluation of toxicity is undoubtedly one of the most important steps in the evaluation process of potential therapeutic agent. Having proven its potency *in vitro* and based on the facts

[1]University of Sarajevo, Medical Faculty, Department of Histology and Embryology, Sarajevo, Bosnia and Herzegovina, and [2]University of Sarajevo, Faculty of Science, Department of Chemistry, Sarajevo, Bosnia and Herzegovina
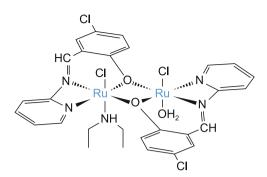
*These authors contributed equally to this work.

Address for correspondence: E. Kahrovic, Zmaja od Bosne 33–35, 71000 Sarajevo, Bosnia and Herzegovina.
Phone: +387.33.279910

Fig. 1. Structural formula of ruthenium (II) Schiff base complex [Ru$_2$L$_2$Cl$_2$(Et$_2$NH)(H$_2$O)].

mentioned above, the dinuclear ruthenium(II) Schiff base complex was selected for the first *in vivo* study to assess its safety profile.

The study was designed as a single-dose toxicity study in adult Swiss albino mice of both sexes. The aim of this study was to identify the target organs through assessment of biochemical and histopathological parameters.

Materials and methods

Dinuclear ruthenium(II) Schiff base complex

The dinuclear ruthenium(II) Schiff base complex [Ru$_2$L$_2$Cl$_2$(Et$_2$NH)(H$_2$O)], where L is *N*-(2-pyridyl)-5-chlorosalicylideneimine (Fig. 1) was prepared according to previously reported procedure (2). The purity of the compound was confirmed by infrared spectrum and elemental analysis.

The ruthenium(II) Schiff base complex is a dark green powder highly soluble in dimethylsulfoxide (DMSO). Fresh solutions of the complex were prepared each time prior the administration by dissolving an appropriate amount of the complex in DMSO and then diluting it with water to the required concentrations. The pH value of thus prepared solutions was in the range 5.20–5.50. The level of DMSO was kept below 20 %.

Animals

Adult (8–10 weeks old), Swiss albino mice (22.5–31.8g) of both sexes were obtained from the Faculty of Veterinary Medicine, University of Sarajevo. Only the nulliparous females were included. The mice were housed in polypropylene cages under a 12-hour light-dark cycle, temperature 23 ± 2 °C. They were fed with standard food pellets and water *ad libitum*. One-week acclimatization was provided before the animals were used in the experiment.

All experimental procedures were approved by the Institutional Ethics Committee and conducted in accordance with internationally accepted principles for the care and use of laboratory animals.

Experimental design

The experiment was designed as a single-dose toxicity study with a follow-up period of 14 days. The mice were randomized and divided by gender into two experimental and one control group.

The high-dose experimental group (H) included 16 females and 16 males while low-dose experimental group (L) and the control group (C) consisted of 12 female and 12 male mice. The dose levels were selected in the light of data from the preliminary study (not published). Thus, the high dose level of 175 mg/kg, represented the maximum feasible dose while a low-dose of 25 mg/kg was selected as a multiple of the probable therapeutic dose. The control group received vehicle only (DMSO). The dosing volume was 10 ml/kg. Before and immediately after administration, food and water were withheld from mice.

In-life observations and measurements

After treatment, the mice were observed for signs of toxicity at intervals of 15, 30, 60, 120, 180 minutes. Further, the animals were observed daily. The potential effects on behaviour and physiological functions were assessed using modified primary observation (Irwin) test for rodents (9). Monitoring of the experimental animals was complemented using camera with recording option (StarCam Wi-Fi IP, w/night vision; Eye4 Software). Body weight was recorded every other day.

Biochemistry

Biochemical analysis was performed on blood samples collected on sacrifice by tail snipping, to avoid compromising of cardiac tissue histological assessment. The following biochemical parameters were recorded: ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase), creatinine and urea using an automated chemistry analyser. All blood samples were randomly divided for detection of liver and renal functional parameters because of the relatively small amount that was obtained.

Gross observation and histopathology

At the end of the experiment, all mice were euthanized with ketamine and the necropsy was performed. Observations were made of the abdominal and thoracic cavity, as well as *in situ* of each organ's position, shape, colour and presence of any gross lesions. The heart, kidney, liver, spleen and ovaries/testes were carefully dissected out and weighted. Relative weight of organs

Tab. 1. Histopathology grading scale.

Numerical grade	Severity	Morphological criteria for grading	Percentage (%) of the changed tissue per slice
1	Minimal	Histological changes are inconspicuous, minor and infrequent	0–10
2	Mild	Histological change is noticeable but not a prominent feature of the tissue	11–20
3	Moderate	Histological change is prominent feature of the tissue	21–40
4	Marked	Histological change is the main characteristic of the tissue	41–100

(except testes) was calculated by dividing absolute organ weight with body weight.

Representative samples of organs were than fixed in 10% buffered formalin. Paraffin-embedded tissue blocks were cut into 5 micrometres thick sections and stained with haematoxylin and eosin (HE). Histological examination of slides was made using the light microscope with digital camera (Eclipse E400, Nikon) with 40x, 100x and 400x magnification, in a blinded fashion.

Severity grading scale (Tab. 1) proposed by Shackelford et al. (10), was used for evaluation of alterations found in all harvested organs.

Statistical analysis

Statistical calculations were performed using Stat Soft Statistica 13.2. and Microsoft Excel 2016 software package. The data were expressed as the mean ± standard deviation (SD) or median with interquartile range, depending on underlying distribution of the data. Comparisons were made using adequate parametric or non-parametric test (ANOVA and Kruskal–Wallis test, respectively) and p-value less than 0.05 was considered statistically significant.
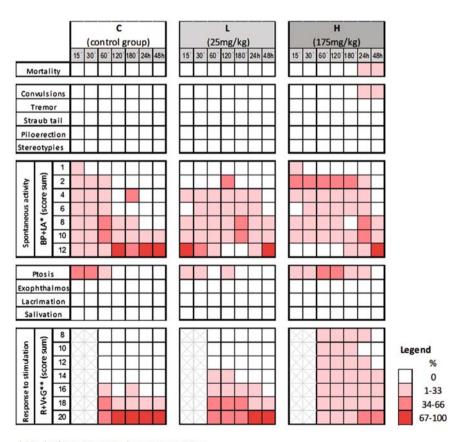
Results

In-life observations and measurements
Clinical signs and observations

No changes of the fur, skin and mucous membranes were recorded, as well as in orifices and eyes of animals in all the groups. Summary of the clinical signs and observations is presented in the Figure 2. Lower scores for the response to stimulation were registered in the high-dose group of animals that exhibited an incomplete recovery even 48 hours after treatment. All behavioural changes were more conspicuous among males of the high-dose group.

Body weight

Until the end of experiment, all animals gained body weight even though the relative increase was twice as high in the control group (9.81 %) as it was in high-dose group (4.95 %) (Fig. 3). When compared to the baseline, significant decrease in body weight (–6.67 %) was noted on the third day only in the high-dose treated group.

* BP = body position; LA = locomotor activity.
**R = reactivity to touch; V = visual placing response; G = grip strength.

Fig. 2. Behavioural and physiological measures of mice assessed using modified primary observation (Irwin) test. C – control group (n = 24), L – low-dose group (n = 24), H – high-dose group (n = 32).

Tab. 2. Mean/median value of biochemical parameters for control group (C), low-dose (L) and high-dose (H) groups of mice.

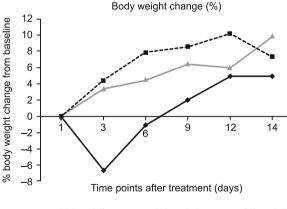| Parameter/group	C (control)	L (25 mg/kg)	H (175 mg/kg)
ALT (IU/L)	141.50±44.88 (n=12)	120.5 (104.50–139.00) (n=12)	134.98±66.81 (n=11)
AST (IU/L)	182.5 (118.00–186.50) (n=12)	172.67±35.86** (n=12)	134.11±41.45 (n=11)
ALP (IU/L)	171.92±41.83 (n=12)	126.21±22.03* (n=12)	161.05±45.21* (n=11)
Urea (mg/dL)	7.80±2.35 (n=12)	7.65±1.06 (n=12)	7.12±1.08 (n=12)
Creatinine (μmol/L)	39.49±10.65 (n=12)	41.73±11.22 (n=12)	41.64±9.12 (n=12)

The data are expressed as the mean ± SD or median with interquartile range (IQR, 25th – 75th percentile). * statistically significant result compared to control group ($p <$ 0.05); ** statistically significant result comparing low-dose and high-dose group ($p < 0.05$).

Tab. 3. Mean relative organ weight changes[a] 14 days after single intraperitoneal application of dinuclear ruthenium(II) Schiff base complex in mice of both sexes.

Organ/Group		Male		Female	
		Low–dose	High–dose	Low–dose	High–dose
heart	% change	–	−15.70	–	–
	incidence	–	8/11	–	–
kidney (left)	% change	–	−13.10	–	–
	incidence	–	10/11	–	–
kidney (right)	% change	−11.99	−12.44	–	–
	incidence	12/12	11/11	–	–
liver	% change	–	+17.90**	+11.44	+15.96
	incidence	–	9/11	12/12	16/16
spleen	% change	+14.09	+42.11	+16.52	+37.07*
	incidence	5/12	10/11	9/12	15/16
testis (left)	% change	–	+ 10.03	NA	NA
	incidence	–	9/11		
testis (right)	% change	–	+ 9.16	NA	NA
	incidence	–	8/11		
ovary+bursa (left)	% change	NA	NA	−31.43*	−28.00*
	incidence			10/12	16/16
ovary+bursa (right)	% change	NA	NA	−10.28	–
	incidence			8/12	–

[a] Mean compound treated group value compared to the mean control group value; NA – not applicable; * compared to control group ($p < 0.05$); ** comparing low-dose and high-dose group ($p < 0.05$); „–" the values less than 10 %.

Fig. 3. Changes in body weight (%) from initial body weight for mice in high-dose (H), low-dose (L) and control (C) group, during 14-days experimental period.

Mortality

Mortality appeared only among male mice (5/16; 31.25 %) (Fig. 2). The death was preceded by a few hours of decreased activity followed by completely flattened body position. The con-vulsions appeared just a few minutes before death, according to the camera recordings.

Biochemistry

Serum biochemical parameters showed variations among the tested groups of animals as shown in Table 2. All values, except serum creatinine concentration, tended to be lower in the experimental groups. The alterations were statistically insignificant, except for ALP and AST levels.

Only in female mice of the low-dose group, ALT level (157.75 ± 63.37) and serum urea concentration (6.98 ± 0.89 mg/dL) were significantly decreased in comparison to corresponding control group (ALT 173.50 ± 35.18; urea 9.40 ± 2.36 mg/dL).

Gross observation and histopathology

Organ weights

The mean relative organ weights from high and low-dose treated animals were compared with the mean control group values and then stratified by gender (Tab. 3). Changes of less than 10 % were considered to have a small biological impact (11) and thus are not presented, except for testes.
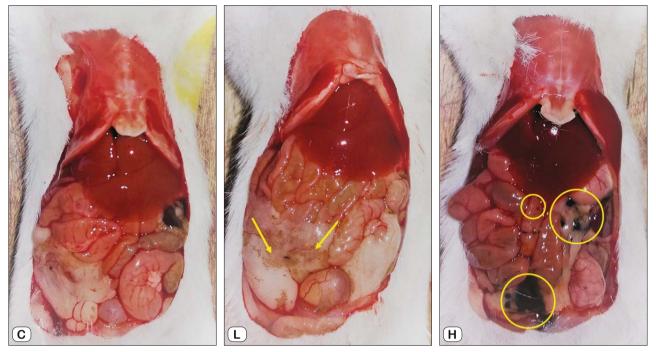
Fig. 4. Appearance of the abdominal organs on necropsy. Normal finding in the control group (C), diffuse and minute precipitate in the low-dose group (L), rough, multifocal, dark-green precipitate in the high-dose group (H).

The increase in relative liver weight was substantial among females, while the mean relative kidney weight was decreased in males of both treated groups. Spleen was enlarged in both sexes in a dose-related manner.

Necropsy

The appearance (Fig. 4) and the incidence of precipitation on peritoneal lining and adhesions among abdominal organs was different in high-dose and low-dose group of animals (Tab. 4). Thoracic cavity was devoid of precipitation, but pericardial and thymic fat appeared oedematous. The cranial mediastinal lymph nodes appeared enlarged and dark in the high-dose treated group and thus were taken out for the further histopathological evaluation.

The finding of bilaterally oedematous and soft testes was noted in all mice (11/11) in high-dose group and in only one mouse in low-dose (1/12) and the control group (1/12).

All other gross findings were incidental and routinely observed in Swiss albino mice of this age.

Histopathology

Compound-related changes were noted in multiple organs in animals of both experimental groups while the control group exhibited generally normal histological features (Fig. 5).

Myocardium of both experimental groups did not show any visible morphological alterations. Kidney and liver were affected in a dose-related manner, where the liver in females and kidneys in males seemed more altered. Changes were graded as marked in the high-dose group and minimal in low-dose group of animals.

The most prominent findings in the kidney of the high-dose group included narrowed Bowman's space with glomerular shrinkage and focal tubular hyperplasia and hypertrophy. Tubule cells exhibited basophilia accompanied with marked karyomegaly and unusual chromatin pattern ("empty nuclei"). There was no evidence of necrosis. Renal interstitial tissue was reduced.

Hepatocytes appeared enlarged and vacuolated, while their trabecular arrangement was irregular and zonation less prominent. Cytoplasmic vacuolation appeared coarser in high-dose in comparison to the low-dose group. Similarly, to the renal tubular epithelium, hepatocytes showed "empty nuclei". The nuclei of sinusoidal lining cells were prominent and hyperchromatic.

Spleen sections revealed foci of haematopoiesis that were more prominent in the high-dose group.

Testes of low-dose group revealed mostly regular seminiferous tubules and interstitium. Mildly widened interstitium and eosinophilic masses in the lumen of seminiferous tubules, along with a frequent finding of atypical residual bodies represented the features of the testis histology in the high-dose group. Ovarian follicles were mainly preserved in a low-dose group. Thickened

Tab. 4. Incidence of necropsy findings in the low-dose and high-dose group of animals.

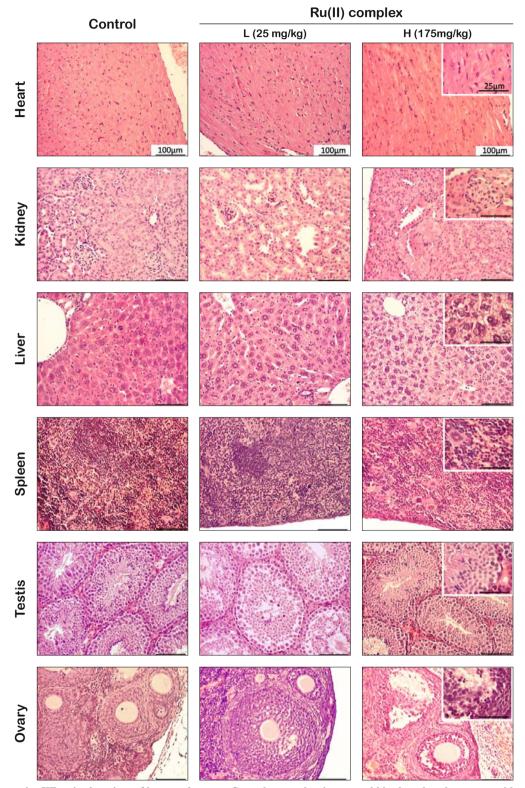| Group		precipitate		adhesions	
		+	–	+	–
L (25 mg/kg)	Male	9/12	3/12	0/12	12/12
	Female	10/12	2/12	2/12	10/12
H (175 mg/kg)	Male	10/11	1/11	10/11	1/11
	Female	14/16	2/16	5/16	11/16

Fig. 5. Representative HE stained sections of harvested organs. Control group showing normal histology, low-dose group with minimal and high-dose group with marked structural alterations in kidney, liver, spleen and reproductive organs.
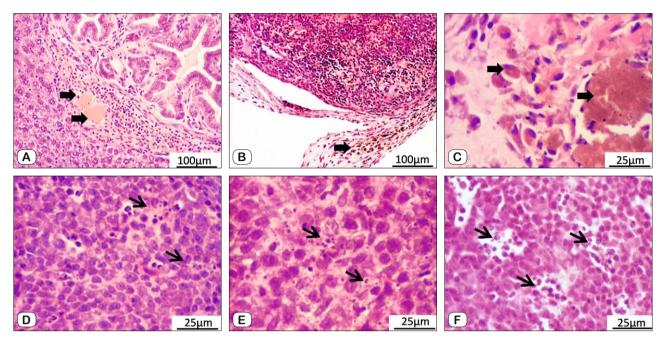
Fig. 6. Histological findings in the high-dose group of animals. The foci of yellow-brownish aggregation (thick arrow) accompanied with macrophages and other inflammatory cells in the obliterated liver interlobar fissure, near the gall bladder (A), in the thickened capsule of the spleen (B) and the wall of ovarian bursa (C). Apoptotic bodies and tangible body macrophages (arrows) in the germinative centre of the splenic lymphatic follicle (D), corpus luteum (E) and germinative centre of the cranial mediastinal lymph node (F).

bursa, hyperaemic ovarian blood vessels, rare primordial follicles and degenerating antral follicles characterised the ovaries of the high-dose treated females.

Thorough histological examination of thickened capsule (liver, spleen, ovarian bursa) revealed identical foci of macrophage aggregations with yellow-brownish cytoplasm, accompanied with other inflammatory cells (Fig. 6). Apoptotic bodies and tangible body macrophages were noted in germinative centres of the cranial mediastinal lymph nodes and white pulp of the spleen as well as in stratum granulosum of antral ovarian follicles and corpora lutea (Fig. 6).

Discussion

Among novel metal-based compounds, inspired by currently used platinum-containing drugs, ruthenium complexes appear promising. To our knowledge, this is the first *in vivo* study on dinuclear ruthenium(II) Schiff base complex. In previous *in vitro* studies, this complex showed significant anticancer and antibacterial activity (2, 8). Swiss albino mice of both sexes were treated with two doses of the compound to estimate the toxicity in otherwise healthy animals.

All animals of the control and low-dose group survived, while the mortality was recorded in the high-dose group, only among male mice, suggesting gender-related differences in reaction to the test substance. Generally, low-dose was well tolerated and 24 hours after treatment, the animals from this group showed the same behavioural pattern and appearance as the control group of animals, which is in accordance with the findings of other studies on acute ruthenium toxicity (5, 12). Lower scores on Irwin test up

to 48 hours after treatment and incomplete recovery were recorded in the high-dose group, more conspicuously among males. These findings correlated with a decreased food and water intake resulting in weight gain decrease, reaching the peak on the third day. Similarly, Guichard et al (13) observed prolonged recovery and significant weight loss by third day after a single intraperitoneal administration of novel ruthenium-based compound in high-dose treated mice. This can be explained by long compound's plasma half-life, which is about 100 hours (~3 days) for majority of ruthenium complexes (14). Contrary to our results, Grozav et al (5) found no significant body weight changes even in the high-dose treated animals. Discrepancy can be explained with species (rat vs. mice) and gender (females vs. males) selection.

The necropsy findings of adhesive peritonitis and precipitate in abdominal cavity, confirmed by the histological analysis, were less prominent or completely absent in the low-dose group in comparison to the high-dose group. Macrophages within inflammatory infiltrate contained yellow-brownish material indicative for the presence of the test substance, while sub-mesothelial thickening with an increased cellularity was similar to those described in other types of peritonitis (15). Peritoneal adhesions and fusion of liver lobes were more frequent among male mice in the high-dose group. This is supported by the evidence, that oestrogen inhibits macrophage activation and connective tissue deposition in murine model of peritonitis (16).

Myocardium of all treated animals exhibited a preserved histological appearance. This is in accordance with the findings of other authors, who reported no or mild cardiotoxicity induced by ruthenium complexes (17, 18).

The mean relative kidney weight was decreased in both experimental groups. Recorded decrease in the mean relative kidney weight in males compared to the corresponding control group was twice as high as the value found in females. The decrease in organ weight correlated with findings of reduced renal interstitial tissue and glomerular shrinkage, even though some tubules were dilated with hypertrophy and hyperplasia. Basophilia in tubular epithelium along with karyomegaly and nuclear crowding could represent a reparative response to previous injury (19). An early stage of chronic progressive nephropathy could not be excluded. Also, histological alterations of kidney were more pronounced in males. Previous studies in humans and animal models showed that the kidney is an organ with the most sex-biased gene expression resulting in higher probability of renal disease progression and higher incidence of end-stage renal disease in males (20). Similarly to Hu et al (21) and Cocchietto and Sava (22), our ruthenium complex induced renal injury limited to potentially reversible morphological changes without notable alterations in serum urea and creatinine level.

Significant increase in liver weight was detected in all treated females and only high-dose treated males. This was confirmed by histological findings of hepatocellular hypertrophy, vacuolization and capsule fibrosis. Hepatocellular hypertrophy is the main morphological feature of enzyme induction and is not necessarily followed with substantial changes in serum enzymes levels in rodents (23, 24). This is in accordance with results of liver enzyme assay in our study. However, lower values of liver enzymes in serum could be a sign of toxin-supressed enzyme synthesis or their altered clearance (25, 26). Hepatocyte cytoplasmic vacuolization, which we found in all treated animals, can be a sign of adaptive response, rather than a severe liver injury (27). Some authors (13, 18, 21) found similar alterations in liver of animals treated with different ruthenium complexes, while Grozav et al (5) and Wang et al (4) did not report any ruthenium-induced hepatotoxicity. Our results led us to the conclusion that female mice were more susceptible to liver injury. It is known that liver-enzyme induction shows sex-differences and drug-induced liver injury is more frequently found among females (25, 28).

Histological findings of increased extramedullary haematopoiesis in spleen and presence of extensive capsular fibrosis with adhesions were consisted with recorded increase in absolute and relative organ weight. Foci of apoptotic cells in germinal centres, suggesting lymphocyte depletion, were found in our study and were also reported by other authors (5, 22). Administration of platinum and some ruthenium complexes was associated with lymphotoxicity and myelotoxicity, while the affection of spleen represented adaptive response (4, 22, 29).

Application of test compound caused dose-related alterations of testes and ovaries. Increased testis weight correlated with the oedematous consistency found at necropsy and interstitial oedema was observed histopathologically. When accompanied by macroscopic or microscopic findings, the change of absolute testis weight of as little as 5 % is highly indicative for testicular toxicity (11). Abundant eosinophilic masses found in majority of the seminiferous tubules of high-dose treated animals, accompanied with a lack of mature sperm cells, were highly indicative for spermatids retention. Similarly to our results, the study of Ciftci et al (30) reported an increased testes weight and spermatid retention in mice treated with ruthenium complex. Spermatid retention is typically caused by reduced testosterone level that in turn is the result of interstitial oedema with short recovery period (30, 31).

Mean relative ovary weight was reduced in all treated females due to massive follicle atresia. Even though follicle and corpora lutea degeneration was more pronounced in high-dose group, hyperaemia and bursa fibrosis diminished ovary weight reduction detected in this group. To our knowledge, there is no data on ruthenium-induced ovarian toxicity, although chemotherapeutic agents are known for various side effects on reproductive organs, targeting oocytes or somatic cells (32).

Dinuclear ruthenium(II) Schiff base complex was well tolerated and did not cause any mortality among mice of both sexes when administered intraperitoneally in a low dose, as multiple of probable therapeutic dose. The incidence of peritoneal adhesions was negligible while histological findings and serum biochemical analysis suggested a reversible character of alterations found in vital organs of this group. However, adverse effects reported in the high-dose group were more severe, targeting different organs in male and female mice and indicating dose and gender-related toxicity. Also, irritation at the injection site caused by the maximum feasible dose, advice the future optimization of the compound's formulation.

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