Original Article

Influence of morpholine on changes in kidney tissue and white blood cells of NMRI male Albino mice

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Abstract

Background: Morpholine is a toxic substance used in industry and agriculture and can be absorbed into the body through ingestion, inhalation, and the skin. The present study aimed to assess the effect of morpholine and physiological serum ingestion on qualitative and quantitative characteristics of white blood cells and kidney tissue of white male mice.

Materials and Methods: In the present study, 40 adult NMRI male Albino mice were placed in 4 groups; control group, physiological serum (sham) group, treatment group A [fed with 300 mg/kg of 1 ml of the prepared solution (0.009 ml morpholine + 0.091 ml of distilled water) per day for 15 days], and group B (the same volume of morpholine and physiological serum). After weighing, anesthesia, and blood sampling, all considered parameters were measured using MOTIC software. In addition, macroscopic and microscopic studies were conducted on prepared slides and obtained data were analyzed using SPSS software.

Results: In group A, reduced thickness of the outer cortex of the kidney (proximal convoluted tubule), increased thickness of the inner cortex (Malpighian body's, distal convoluted tubule), and reduced external medulla (Henle's loop) were significant compared to the control group and sham group. However, no significant difference was found among the groups with regard to the internal medulla (collecting pipes). Moreover, the kidney gained weight compared to the whole body. Changes in white blood cells in group A were significant compared to group B.

Conclusions: Stress morpholine causes changes in blood parameters, increased filtration, decreased reabsorption and absorption, weight loss, inflammation, hyperemia, urinary tract reconstruction and resulted polyuria. However, these impacts reduced via physiological serum.

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Keywords: Morpholine, White Blood Cells, Kidney

Introduction

Morpholine (C_4H_9NO), also called diethylene imidoxide, is a cyclic amine II which belongs to aromatic hydrocarbons (1); figure 1 shows its structure.

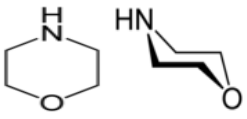

Figure 1: The chemical structure of Morpholine

Morpholine ca is produced through 3 techniques; mixture of ammonia, diethylene glycol and hydrogen, dehydration of ethylene amine with sulfuric acid, and heating di (chloroethylene) ether oxide and ammonia (2). It is a colorless liquid with an ammonia-like odor that is dissolved in water and other materials such as ethanol, benzene, and acetone (3).

Morpholine derivatives and salts are used in the plastics industry, as an anti-corrosion and rustproof factor in the protection of various

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metals and as a wax and polishing material. It is a strong alkaline that rapidly absorbs humidity and evaporates into the air (4). Morpholine is used in cosmetics as an additive to hair dye, conditioners, and shampoos (2), and as a disinfectant of water (5). To preserve fruits for a longer time and to process food, morpholine is used in waxes, wrapping coatings, and can be found in device's vapors (6, 7). Morpholine is also a good solvent for organic materials such as paints and resins in the paint industry (1, 8).

Morpholine is a cheap and readily available alkaline catalyst (9). It is used in agriculture as a pesticide, bactericide, herbicide, and fungicide, such as nitrophenyl derivatives which are composed of sulfur and morpholine and are used against powdery mildew on plants (10).

Morpholine decomposition was approved through colorimetric testing and production of ammonia (11). The substance has toxic properties which affects digestive, respiratory, and cutaneous systems (12). In the urine of guinea pig, the metabolite of N-methyl-morpholine-N was found (13). If morpholine is absorbed through the skin, it will destroy the skin tissue, cause irritation, redness, and pain in nails (14). The results of continuous exposure of the eyes to morpholine vapor include inflammation, irritation, itch, and tear secretion (15). Short-term use of morpholine on laboratory animals showed that the neutral and undiluted morpholine compound definitely causes death, and diluted compositions cause death, severe necrosis, and burning and inflammation of the skin. The use of morpholine for 5-15 minutes caused severe necrosis on rabbits' skin (16). In a study, after oral treatment with N-nitrosomorpholine, the carcinogenic properties of this substance were studied in rats, mice, hamsters, and various types of fish (17). As a result, liver and lung cancer were reported in mice, liver and kidney cancer in rats, and liver cancer in hamsters (17). In gavage with morpholine palmitate, about 7% of morpholine was excreted in stool (7, 17). This substance spreads in all body tissues (18) and has damaging and destructive effects on many tissues.

Blood containing red, white blood cells (WBCs), (RBCs), and platelets that are About 45% blood volume(19, 20). Based on the form of the nucleus and the type of granules in the cytoplasm, RBCs are divided into granulocytes and agranulocytes (21). Quantitative analysis of WBCs (leukocyte) is performed as a relative or an absolute count, and qualitative analysis is conducted through examination of cell morphology disorders and functions. Leukocytosis refers to an increase in the number of WBCs due to severe physical activities, anoxia, pregnancy, parturition, and stress (22).

Lymphocytosis is the abnormal increase in the number of lymphocytes in the blood which is diagnosed through blood count in clinical conditions (23). Qualitative changes also occur; for example, in viral infections and immune and stress responses, abnormal and enlarged lymphocytes are observed with abnormal morphology and ample cytoplasm, which are swollen during poisoning and their nucleus and cytoplasm is destroyed (24). Neutrophils are the most important polymorphonuclear granulocytes that have a high motility and phagocytosis (25).

The urinary system consists of 2 kidneys, ureters, bladder, and urethra. Kidneys filter blood, regulate fluids and regulate the electrolyte balance (26, 27). In the anatomic section of the kidneys, there are two parts including the cortex and medulla. The cortex consists of the outer cortex (proximal tubules) and the inner cortex (Malpighi grains and distal tubules), and the medulla is divided into inner (loop of Henle) and outer sections (collection tube). The kidney has about 1-4 million nephrons (28, 29).

Morpholine has a wide range of functions in daily life. Moreover, contact with it through oral, respiratory, and cutaneous systems can have devastating physiological and behavior effects on humans and animals. However, since no study focusing on the effects of renal

parameters was found, the aim of this study was to examine the blood and kidneys.

Materials and Methods

In this research, data were obtained from 40 white male Albino NMRI-strain mice (Pasteur Institute of Iran) with an average weight of 28 g. Each group of 5 mice in a cage was randomly divided into the control group, physiological serum group (sham), and treatment groups A and B.

(1) Control group: This group underwent normal conditions for 15 days. Enough water and food were supplied. They were fed with plete, a fast food purchased from Tehran Pars Animal Food Company, and bottles of water were filled with urban drinking water. Cages were cleaned once a week. The mice were kept in a suitable temperature of about 22 ± 2 °C and on a 12-hour lightness-darkness cycle.

(2) Physiological serum group or sham: This group was fed with 0.1 ml of the physiological serum once per day for 15 days in the form of gavage.

(3) Treatment group A: This group was fed with 0.1 ml of morpholine solved in distilled water (0.009 ml morpholine + 0.091 ml distilled water) proportional to 300 mg/kg of the body weight once per day for 15 days (7, 30).

(4) Treatment group B: This group was fed with 0.1 ml of morpholine dissolved in distilled water (0.009 ml morpholine + 0.091 ml distilled water) proportional to 300 mg/kg of the body weight once per day for 15 days along with physiological serum containing sodium electrolytes (147.5 meq/l), potassium (4 meq/l), calcium (4.5 meq/l), and chloride (156 meq/l) (20, 31).

These substances were inserted into the stomach of the mice with insulin syringes and gavage needles (no. 20) purchased from Razi Rad Company, Tehran, Iran. During the treatment, mice were weighed every day and gavage was performed at certain hours of the day.

At the end of each period of 15 days (18), after the dissection, in order to determine the blood parameters, blood drawing was performed from the right atrium of the heart, and WBC count was conducted using Neubauer slides (32). To evaluate the histology of the kidney sections, slides were prepared and hematoxylin and eosin staining performed. The preparation stages of the slides consisted of removal of tissue and numbering, dehydration ,clarification (Gzylvl used as a solvent intermediary between the wine-making process and forming tissue) fixation, molding, sectioning, fixation of sections on a slide, staining, separate paraffinization, hydration, and washing of slides in running water .

They were examined under the microscope to determine structural changes (33). Pictures were provided from blood and tissue samples using the microscope and the loop equipped with a digital camera. Then, the intended sections were measured using the motic advanced plus 2 software (Motic China Group Co., Ltd ,version 1)based on the unit of micrometer.

The analysis of data derived from the measurement of blood cells and the kidney tissue of the treatment groups (A and B) was conducted through applying one-way ANOVA. To assess the within-group differences and compare means, Tukey's test was used. The results were presented as Mean ± SEM (standard error of mean) and all P-values of less than 0.05 were considered significant. Excel and Motic software programs were used to draw the charts and measure the size of the cells, respectively.

Results

Quantitative and Qualitative Variations of WBCs

In treatment group A, the number of WBCs per cubic millimeter of blood increased. In treatment group B, the mean number increased from 4700 to 4670 WBCs per cubic millimeter of blood. The mean number of WBCs in the control group and the physiological serum group was 4400. Observations of blood spread indicated that the percentage of small lymphocytes in treatment group A and

treatment group B increased from 22% to 27% and 33%, respectively. The percentage of medium lymphocytes decreased. In treatment group A and treatment group B, this percentage decreased to 19% and 17%, respectively. In the control group and the physiological serum group, this percentage was 25% and 24%, respectively. In treatment group A and treatment group B, large lymphocytes decreased from 22%. In the control group and the physiological serum group, large lymphocytes decreased to 20% and 19%, respectively.

The percentage of neutrophils in treatment groups A and B reduced to 6% and 10%, respectively, while in the control group and the physiological serum group, this percentage was 21%. Figure 2 depicts the percentage of increase or decrease in small, medium, large, and degenerated lymphocytes, neutrophils, and other cells in the four groups.

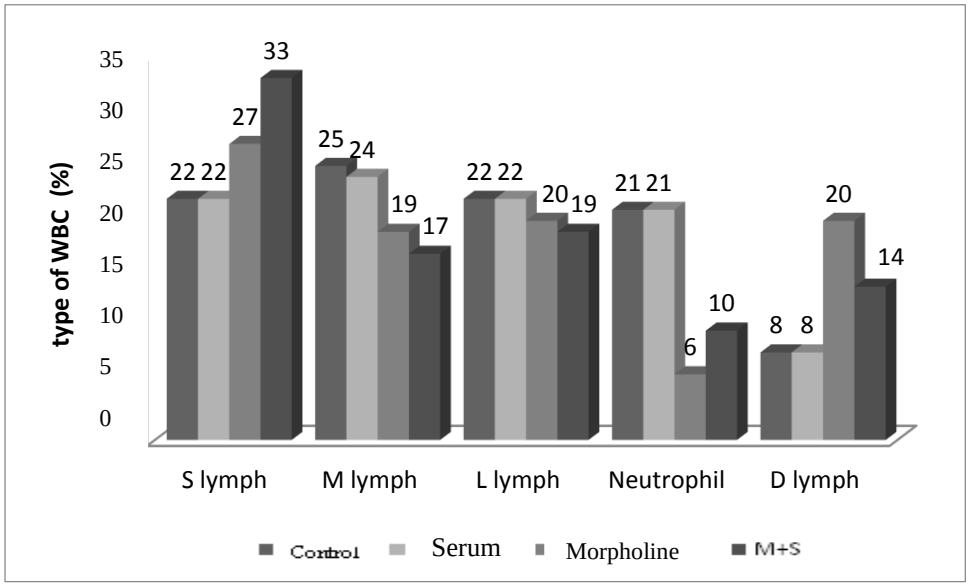
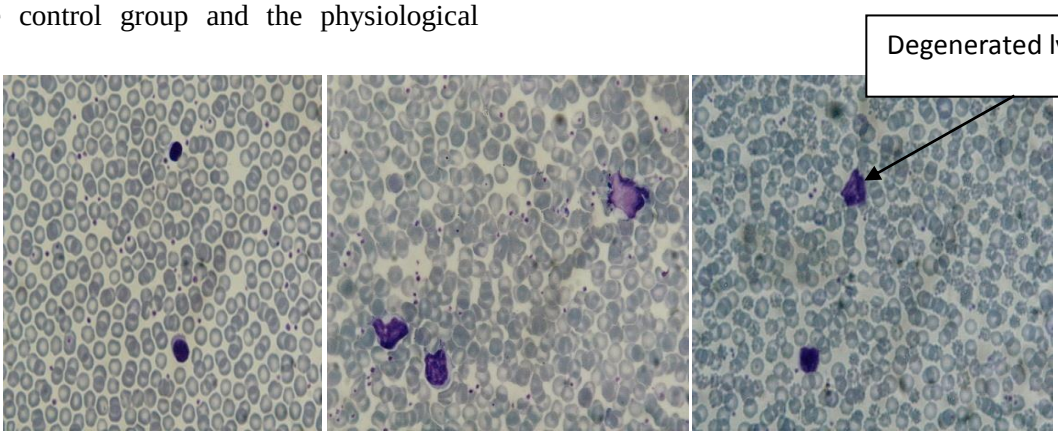


Figure 2: Comparison of different types of WBC in the 4 groups

According to figure 2, the number of degenerated lymphocytes in treatment group A significantly increased to 20%, while in treatment group B, this number was 14% and in the control group and the physiological serum group, it was 8%. Figure 3 represents changes in lymphocytes in these three experimental groups (the control group and the serum physiologic group were alike).



A B C

Figure 3: Infected and degenerated lymphocyte in A) control group (non-participation), B) treatment group A, and C) treatment group B (× 1000)

Variations in Renal Parameters

Studies performed on the tissue sections illustrate a significant reduction in the mean thickness of the outer cortex (proximal tubules) in treatment groups A and B in comparison with the control and physiological serum groups ($P < 0.05$). They also showed increase of the mean thickness of the inner cortex (Malpighi grains and distal tubules) in treatment groups A and B in comparison with the control and physiological serum groups (Figure 4).

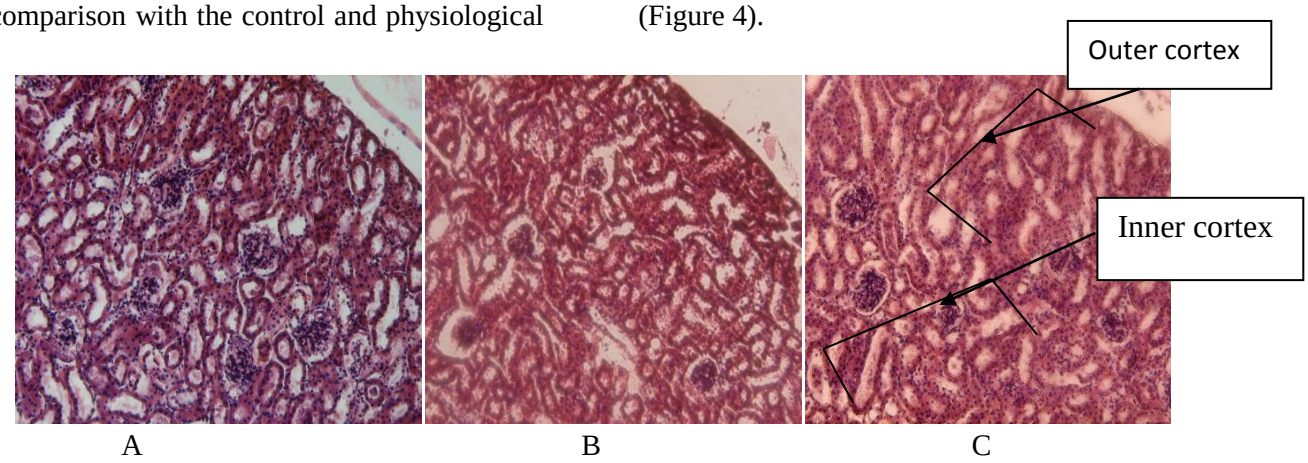


Figure 4: Renal cortex, A) Control group, B) Treatment group A, and C) Treatment group B (× 1000)

In the renal medulla, the mean thickness of the outer medulla (loop of Henle) significantly reduced in the treatment groups in comparison with the control and physiological serum groups ($P < 0.05$). Figure 5 illustrates these variations. The inner medulla (collection tube) in the experimental groups showed no significant variations. Results of examinations of the medulla and cortex tissue sections in the control group and the physiological serum group were the same.

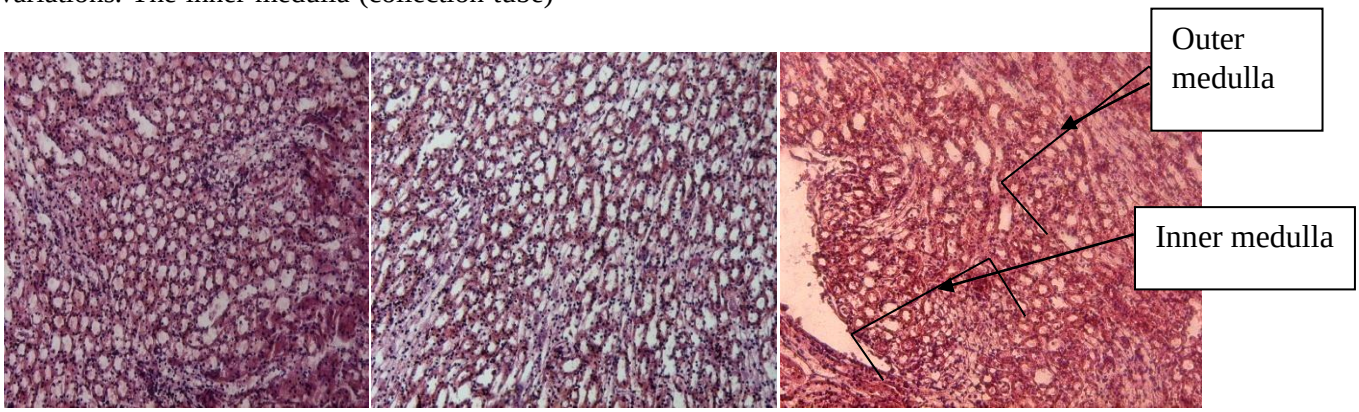


Figure 5: Renal medulla, A) Control group, B) Treatment group A, and C)Treatment group B (× 1000)

The properties of the slides containing proximal tubules were examined. These examinations revealed a significant reduction in the average cell wall thickness, and the total diameter and the inner diameter of the duct in the treatment groups compared with the control and physiological groups ($P < 0.05$) (Figure 6). Microscopic analysis of the kidney indicated that in Malpighi grains, the mean diameter of the glomerular network and Malpighi grains in both treatment groups reduced in comparison with the control group and the physiological group (Figure 7). However, a significant increase was observed in the mean area inside the Bowman's capsule and the thickness of the Bowman's capsule cortex ($P < 0.05$). Analysis of the features of distal tubules revealed that the average diameter of the tubule, the thickness of the wall, and the inner diameter of the tubule in treatment groups A and B increased significantly in comparison with the control

and the psychological serum groups (P < 0.05) (Figure 8).

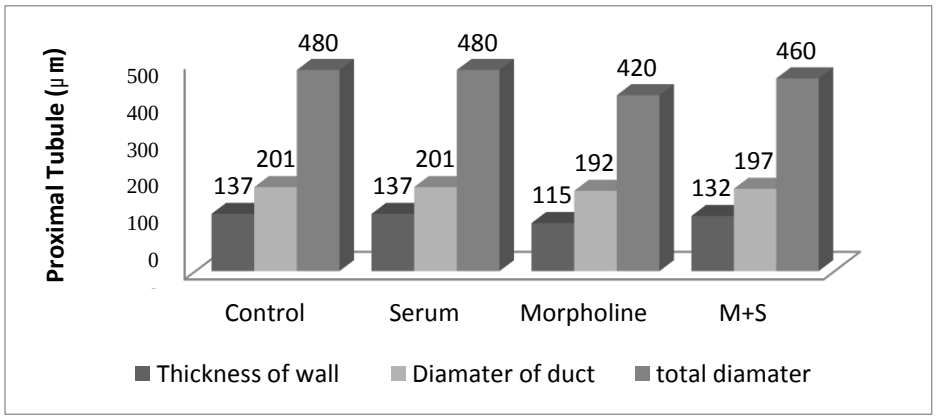


Figure 6: Changes in proximal tubule in the outer cortex

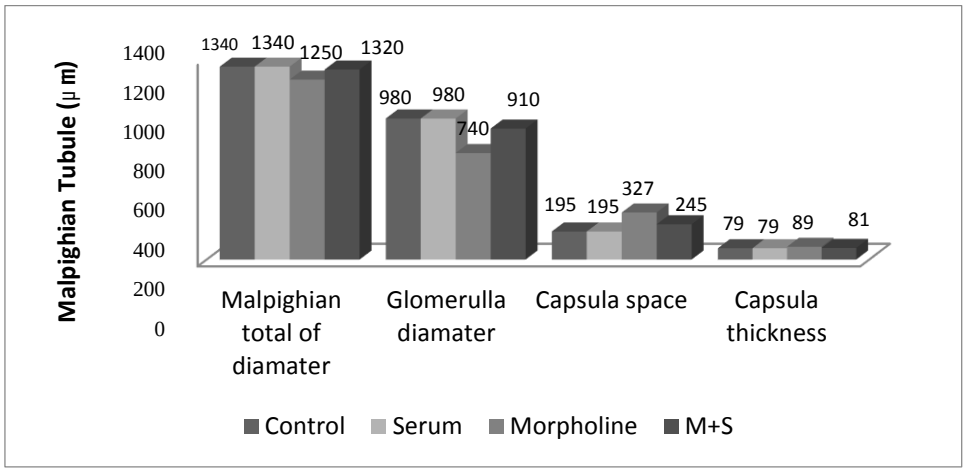


Figure 7: Changes in Malpighi tubule in the inner cortex

A significant decrease in the mean thickness of the Henle wall and increase in Henle loop was observed in the treatment groups in comparison to the other two groups (P < 0.05) (Figure 9).

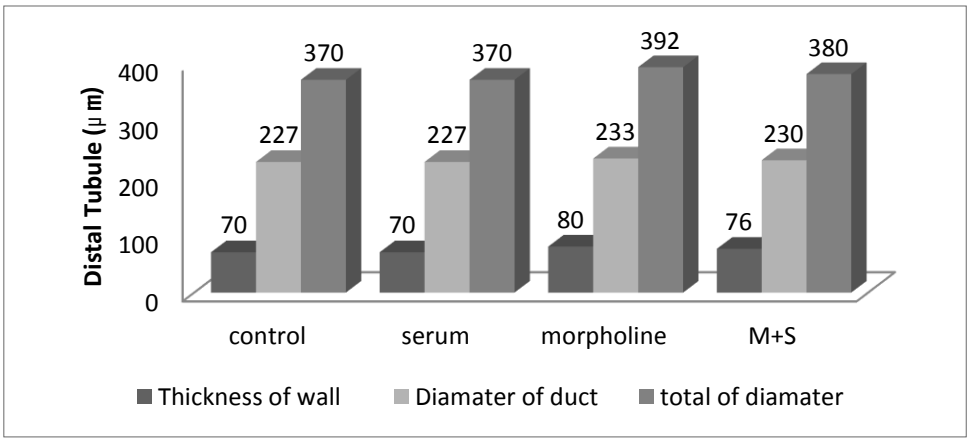


Figure 8: Changes in distal tubule in the inner cortex

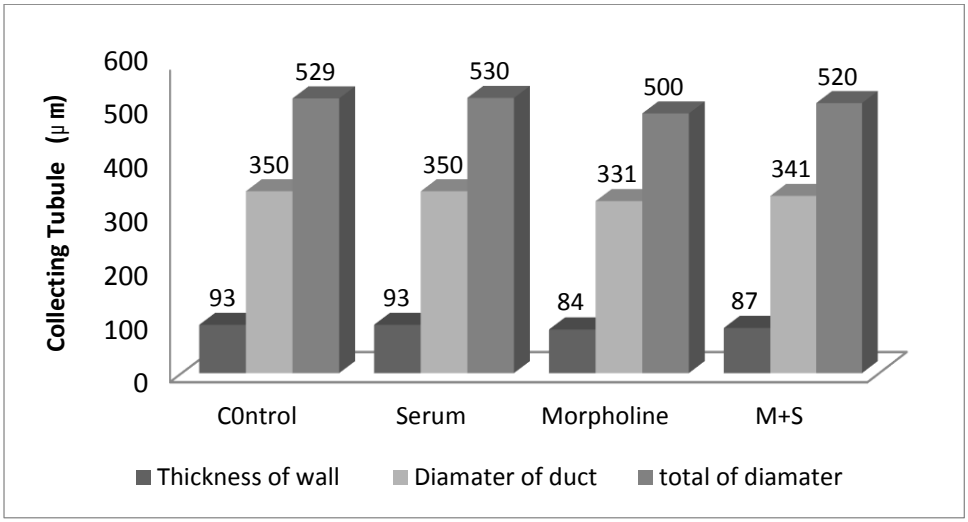


Figure 9: Changes in Henle tubule in the external medulla

In the case of collecting tubules, measures in figure 10 show that the average thickness of the wall, the diameter of duct, and the total thickness of the tubules in the treatment groups differed significantly from that of the control group and the physiological group ($P < 0.05$). The mean values of all measured parameters are provided in table 1.

Table 1: Mean parameters of kidney tissue (µm) in four experimental groups of male Albino NMRI white mice

Number	Mean ± SEM	Morpholine	Serum	Control	Kidney of parameters
40	2950.2 ± 0.09	2048.3 ± 0.08	3214.8 ± 0.11	3214.5 ± 0.03	Outer cortex thickness
40	5695.1 ± 0.08	6422.3 ± 0.05	5455.2 ± 0.09	5455.2 ± 0.07	Inner cortex thickness
40	17653.3 ± 1	17138.3 ± 0.09	18367.1 ± 0.08	18367.2 ± 0.09	Outer medulla thickness
40	25700.2 ± 0.1	25728.7 ± 0.09	25719.3 ± 0.08	25719.5 ± 0.06	Inner medulla thickness
40	1320.5 ± 0.12	1250.4 ± 0.05	1340.3 ± 0.05	1340.2 ± 0.09	Malpighian total of diameter
40	910.02 ± 0.14	740.02 ± 0.01	980.05 ± 0.26	980.07 ± 0.26	Glomerulla diameter
40	245.04 ± 0.02	327.04 ± 0.03	195 ± 0.02	195 ± 0.02	Capsula space
40	81.03 ± 0.05	89.04 ± 0.05	79.04 ± 0.03	79.08 ± 0.03	Capsula thickness
50	460.1 ± 0.1	420.5 ± 0.22	480.4 ± 0.15	480.2 ± 0.13	Proximal total diameter
50	197.1 ± 0.15	192.2 ± 0.13	201 ± 0.1	201 ± 0.2	Duct diameter
50	132.1 ± 0.2	115.3 ± 0.15	137 ± 0.25	137 ± 0.1	Thickness of wall
40	380.1 ± 0.15	392.1 ± 0.12	370.1 ± 0.09	370.1 ± 0.1	Distal total diameter
40	230.05 ± 0.03	233.05 ± 0.01	227 ± 0.02	227 ± 0.04	Duct diameter
40	76.1 ± 0.1	80.8 ± 0.13	70.4 ± 0.13	70.1 ± 0.1	Thickness of wall
50	240 ± 0.3	279.2 ± 0.25	230.1 ± 0.1	230.5 ± 0.13	Total diameter of Henle
50	180.04 ± 0.02	218.03 ± 0.21	169.05 ± 0.25	169.06 ± 0.26	Duct diameter
50	31 ± 0.12	29.06 ± 0.03	34 ± 0.1	34 ± 0.02	Thickness of wall
40	520 ± 0.13	500.2 ± 0.09	530.1 ± 0.13	529.9 ± 0.15	Collecting tubule total diameter
40	341.9 ± 0.13	331.2 ± 0.22	349.9 ± 0.2	350.1 ± 0.15	Duct diameter
40	87.08 ± 0.21	84.03 ± 0.04	93.08 ± 0.02	93.05 ± 0.2	Thickness of wall

SEM: Standard error of mean

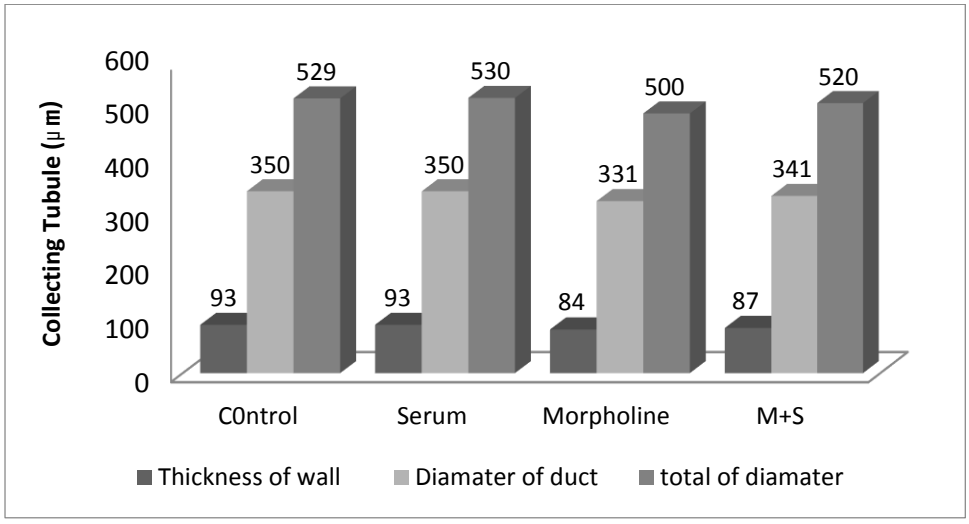


Figure 10: Changes in collecting tubule in the internal medulla

Variations in Kidney Weight

At the end of the 15-day period, the body weight of all the groups was measured. Average weight of the kidney seemed to have no variations. The average weight of the control group and the physiological serum group was 0.23 g. In treatment groups A and B, the average weight was 0.24 and 0.23 g, respectively (Figure 11). As a result, no variations seemed to occur. However, since treatment group A indicated a reduction in weight, it could be argued that the proportion of the weight of the kidneys to the overall weight of the body increased. This increase was significant in the treatment groups ($P < 0.05$).

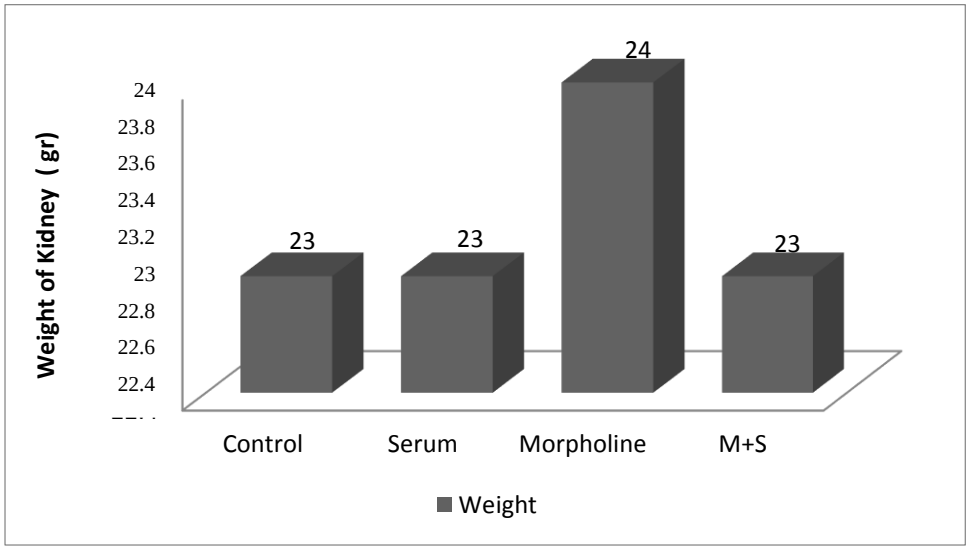


Figure 11: Comparison of kidney weight to body weight in Albino NMRI mice

Discussion

A previous study was conducted on mice, which were orally exposed to morpholine concentrations of 1 g/kg of body weight for 5 consecutive days in the form of morpholine oleic acid (18). The results indicated that the substance was found in various parts of their body (18). Oral use of morpholine in mice at the concentration of 2 g/kg of body weight caused nosebleed and gastric bleeding (20). The oral lethal dose of morpholine for mice (LD_{50}) was 525 mg/kg of body weight (7, 17). In a study by Van et al., 5 mM of morpholine labeled with carbon-14 by 435 mmol/kg was

intravenously injected to 6 groups of male New Zealand rabbits (34). Analyses of radioactive distribution after 30 minutes indicated that the highest concentrations were in the renal cortex (36 mmol/kg), lungs (1.5 mmol/kg), liver (7.4 mmol/kg), and blood (3.2 mmol/liter). In addition, the findings showed that morpholine was not connected to plasma proteins (34).

In this study, morpholine was also found to have various effects on WBCs and kidney tissue. The number of WBCs varies in mouse strains and has been reported between 3,000 and 14,200 per cubic millimeters (20). According to figure 2, the average increase in the number of WBCs (leukocytosis) in group B and group A were 6.8% and 5.9%, respectively. Moreover, their distribution in the blood of the mice of these two groups indicated an increase compared to that of the control and sham groups. In leukocytosis, marginal granulocytes enter the bloodstream as a result of severe physical activities, anoxia, pregnancy, parturition, and stress (21). In this study, morpholine was the stressor. Increase in the percentage of small mature lymphocytes was associated with the relative decrease of large and medium lymphocytes in the treatment groups. According to figure 3, increase in degenerated and destroyed lymphocytes in the treatment group indicated the destructive effect of morpholine on WBCs.

In this study, the percentage of degenerated and destroyed lymphocytes increased in the treatment groups, since lymphocytes become swollen due to stress and toxicity and their nucleus and cytoplasm are deteriorated (24). According to figure 3, the presence of unconventional types of blood cells belonging to the family of leukocytes in the treatment groups was significant. Regardless of degeneration of and infectious lymphocytes and neutrophils, the presence of progenitor cells of leukocytes was also considerable. The presence of monoblasts, promonocytes, lymphoblasts, and prolymphocytes leukocytes indicated the stimulatory effect of morpholine on the cells of the blood-generating centers, stimulating humoral immunity and their presence in peripheral blood before their maturation. The physiological serum had a reductive effect on harmful effects of morpholine (34).

In the kidney tissue, according to table 1, the following average variations could be observed. The proximal tubule in the outer cortex is highly important in reabsorption of water and salts. Reduction of the thickness of the cell wall indicated a reduction of the height of epithelial cells of the proximal wall as well as the reduction of their gland activity that will lead to reduction of water and salt absorption. However, significant differences between groups A and B in terms of these reductions indicated the harmful effects of morpholine on all parts of the outer cortex.

In the inner cortex, Malpighi grains are widely extended. According to figure 7, 17% and 5% increase in its thickness was observed in treatment groups A and B, respectively, which were significant compared to the control groups. The development of the inner cortex indicated the high renal filtration, and consequently, an increase in the filtration of blood water and salts which was associated with lower reabsorption (reduction of the outer cortex).

Malpighi grains are composed of Bowman's capsule and the capillary network and have a basic role in filtration and formation of urine. Their average diameter in treatment group A reduced. The glomerular network diameter also decreased. Increase in the space of Bowman's capsule in treatment group A was about 67%, a sign of formation of primary urine, which leads to the formation of additional urine. In group B, this increase was 25%. On the other hand, increased thickness of the Bowman's capsule cortex, 12.6% in group A and 2.5% in group B, was a sign of fluid leakage resistance of Bowman's capsule out of the urinary tract.

The distal tubules were more or less developed in the inner cortex. In figure 8, the increase in the overall diameter, inner diameter, and distal wall thickness was observed in parallel with

the increase of the outer cortex in group A and a lower increase in treatment group B. Distal tubules have a major role in the osmolality regulation and hormonal control of excretion and reabsorption of salts in connection with the organization of glomerulus.

The overall diameter of Henle's loop in groups A and B increased about 21.3% and 4.3%, respectively. The inner diameter of Henle's loop in groups A and B increased about 29% and 6.5%, respectively. Henle's loop wall thickness in groups A and B decreased 14.7% and 8.8%, respectively, indicating a reduction of the reabsorption of water and salts.

Collection tubules form the end part of the urinary tubules and have a minor role in reabsorption and secretion. In these tubules, a decrease in the overall diameter, inner diameter, and wall thickness was observed. In groups A and B, the decrease was, respectively, 5.6% and 1.8% for the overall diameter, 5.4% and 2.6% for the inner diameter, and 9.6% and 6.4% for the wall thickness of the tubules, indicating a decrease in the reabsorption activity.

Conclusion

Morpholine decomposes the structure of the kidneys, disturbs their general activity, increases the filtration in the primary capillary network, and decreases the reabsorption in the secondary capillary network. High levels of blood in the kidney and the resulting swelling in the renal parenchyma leads to an increase in volume and weight of the kidney. It stimulates hemogenerating centers and the immunity system and increases the number of blood cells, transmits immature leukocytes in the bloodstream, and causes some variations in the formulation of blood development. It also causes inflammation and affects metabolic mechanisms. The role of the physiological serum in neutralization of the toxic effects of morpholine was observed in all of the parameters under study.

Considering the effects observed in the present study, the use of morpholine should be limited or replaced through increasing supervision on its application and providing information for the stakeholders of the health sector and industries.

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Conflict of interest: Non declared

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