Formaldehyde Inhalation Induces Histological and Immunohistochemical Aberrations in the Spleen of the Male Albino Rats

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ABSTRACT

Formaldehyde (FA) is widely used in several industries and medical applications. Healthcare workers, anatomy instructors and medical students are considered as high-risk population. FA induces alterations in the immune system as immune turbulences and even immunosuppression that consequently increases the progression of cancer or allergies. The ongoing debates surrounding FA-induced spleen toxicity necessitate additional analysis. Therefore, we studied the probable toxic effects of FA and subsequent histological, immune-histochemical and morphometric changes on the spleen. Forty adult male albino rats were assigned to four groups; control group (I), groups (II, III, IV) were exposed to 10% FA inhalation for 18 weeks in different doses. Spleen specimens were processed and stained using hematoxylin & eosin, CD4, CD8 and Bcl-2 immuno-staining. All experimental groups showed thicker connective tissue capsules, congested dilated blood sinusoids. Group (II) revealed a significant disruption in the histological structure. Lymphoid cells were degenerated with pyknotic nuclei and vacuolated cytoplasm. Some follicles exhibited necrotic germinal centers. These findings were more noticeable in groups (III, IV). Groups (III, IV) presented sinusoidal hemorrhage and megakaryocytes infiltrations. The experimental groups showed an increase in the positive immune expression of CD4, CD8 and Bcl-2. White pulp and germinal center measurements were decreased in experimental groups. Groups (II, III) displayed increased marginal zone diameter, while group (IV) showed its decrease. Mantle layer and PALS diameters decreased in group (II), and then increased in groups (III, IV). FA can induce harmful effects on the spleen through modifying CD4, CD8 and Bcl-2 in dose-dependent manner.

INTRODUCTION

Formaldehyde (FA) is a colourless flammable reactive gaseous molecule, easily polymerised at normal room pressure and temperature. Its molecular mass of about 30.03 and has a characteristic strong irritating odour. FA is soluble in ethanol, diethyl ether and water-forming formalin (40%) (Monfared *et al.,* 2013; Asghar, 2016). FA is an abundant chemical causing occupational and indoor pollution (Zhao *et al*., 2021), which is widely used in several industries as construction constituents, furniture, cigarette smoking and chipboards (Leng *et al*., 2019). Other applications range from resins and adhesives for fibres insulation, textiles, paper, biocides, timber covers, leather, plastic and even cosmetics such as hair straightening products (Nikle *et al*., 2019).

Although many manufacturers significantly decreased the release of FA from these materials, prefabricated houses' indoor FA concentrations have not reduced by the same level (Pierce *et al*., 2016). Moreover, FA is illegally added as a preservative to milk, yogurt and cheese causing profound health issues (Hossain *et al*., 2016).

On the other hand, FA is extensively used in medical applications as a disinfectant and sterilizing agent and also as a tissue preservative for anatomy and pathology specimens (Bono *et al*., 2012). As it is easily vaporized and emitted into the surrounding working environment, it is counted as an occupational indoor pollutant (Norbäck *et al*., 2017). Healthcare workers dealing with FA in these laboratories are at greater risk than other individuals due to daily basic exposure to FA in higher amounts either via inhalation or direct contact (Zain *et al*., 2019). Moreover, anatomy instructors and medical students are considered a high-risk population in the dissection rooms (Raja and Sultana, 2012).

The workplace exposure level advised by the National Institute for Occupational Safety and Health (NIOSH) is 0.016 parts per million (ppm), equivalent to 16 parts per billion (ppb). In urban cities like Houston, Mexico City, and Cairo, concentrations exceeding 20 ppb have been documented (Zhang *et al*., 2009). Occupational and environmental FA exposure postulates a lot of questions about its adverse health hazards.

Early cohort studies (Pinkerton *et al*., 2004) and follow-up studies reinforce the relationship between myeloid leukaemia (Hauptmann *et al*., 2009), Hodgkin lymphoma (Beane Freeman *et al*., 2009) and FA exposure. Furthermore, the International Agency for Research on Cancer (IARC) classified FA as a Group I human carcinogen as it leads to nasopharyngeal carcinoma (Kleinnijenhuis *et al*., 2013).

Generally, FA not only exerts its side effects mostly at the site of first contact but also negatively affects every cell of the body by modifying the metabolism in various body organs (Kleinnijenhuis *et al*., 2013). FA triggers the inflammatory processes in the lower respiratory tract (Lino-dos-Santos-Franco *et al*., 2011) and induces histological changes in the placenta of mice (Monfared, 2014). Also, FA exposure may influence cellular immunity, as well as bronchial local immunity in rats (Sandikci *et al*., 2007). Formerly, Lan *et al*., (2015) demonstrated that FA induces chromosomal aneuploidy and hematotoxicity in bone marrow and functional disruption of the hematopoietic stem/progenitor cells (Ye *et al*., 2013; Wei *et al*., 2017).

Inhalation of FA increases the reactive oxygen species (ROS) levels, DNA-protein cross-linking, and malondialdehyde and decreases levels of antioxidant factors such as glutathione (GSH) in the lungs (Ye *et al*., 2013), spleen and uterus (Ibrahim *et al*., 2016; Wen *et al*., 2016). Following ingestion, FA is metabolized in erythrocytes and liver cells to formic acid methanol leading to cellular apoptosis and liver injury (Golalipour *et al*., 2009; Xu *et al*., 2017). These metabolites can also cross the blood-brain barrier and induce central nervous system toxicity (Arici *et al*., 2014). FA can rapidly diffuse into various tissues by forming highly water-soluble structures with macromolecules as proteins and nucleic acids, or with micromolecules as amino acids (Tesfaye *et al*., 2021).

Acute and chronic exposure to FA is linked to irritation of the nose, eyes, and throat (Takahashi *et al*., 2007) and several symptoms such as nausea, vomiting, poor appetite, loss of weight, erythema, confusion (Tesfaye *et al*., 2021), headache, dyspnea, and may lead to pulmonary oedema, pneumonitis (Kurose *et al*., 2004), atopy, asthma, developmental and reproductive disorders (Park *et al*., 2020). FA

decreases the production of immunoglobin from the spleen cells (Golalipour *et al*., 2008).

Recently, several works of literature reported that FA induces alterations in the immune system as immune turbulences and even immunosuppression that consequently increases the progression of cancer or allergies. Increasing the concentration of indoor FA increases the incidence of allergic asthma (Park *et al*., 2022). FA may affect different populations of the immune cells including T helper cells (CD4+), cytotoxic T cells (CD8+), and memory T cells (Park *et al*., 2022).

In rodents, FA induces enhancement in the T lymphocytes (CD4+, and CD8+) and subsequently its linked cytokines (Park *et al*., 2020). On the other hand, in human studies, FA decreased the percentages and number of total T cells (CD3+) (Facciabene *et al*., 2012) and CD8+ cells and increased B cells percentage, while the CD4+ percentage remained unchanged. However, Hosgood *et al*. (2013) in their work reported that the counts of natural killer cells, CD8+ and CD4+ declined in workers subjected to FA. The spleen is one of the most important lymphoid organs, situated in the left hypochondrium and epigastrium just below the thoracic cage in mammals including humans. It exerts various imperative roles in enhancing immunity and purification of blood from damaged and old red blood cells. Moreover, the splenic red pulp shares in the generation of about half of monocytes and lymphocytes, which are essential for phagocytosis, immune response and tissue repair. Additionally, the spleen acts as a blood reservoir (Lewis *et al*., 2019). In the splenic white pulp, antibodies are synthesized and antibody-coated bacteria and blood cells are removed from systemic circulation (Agbasi *et al*., 2015).

The lack of consensus and ongoing debates surrounding FA-induced spleen toxicity in previous studies indicate a need for additional analysis to comprehend its negative impacts on health. Further investigation into the effects of FA on the immune system mainly the spleen is warranted, along with a deeper exploration of the underlying mechanisms involved. Therefore, the main aim of this work was to study the probable toxic effects of FA and subsequent histological, immune-histochemical and morphometric changes in the spleen of male albino rats.

MATERIALS AND METHODS

Animals:

Forty adult male albino rats, between 6 to 7 weeks old, were acquired from Helwan Farm located in Cairo, Egypt. Every step of the experiment was conducted in accordance with the guidelines set by the Faculty of Medicine, Ain Shams University, Research Ethics Committee in Cairo, Egypt (FMASU, REC), adhering to the United States National Institute of Health's Guide for the Care and Use of Laboratory Animals (Code FMASU R 118/2023). The rats were housed and allowed to acclimate at the Medical Ain Shams Research Institute (MASRI) of the Faculty of Medicine, Ain Shams University. They were individually placed in sterile cages, with strict regulation of temperature, humidity, and a 12-hour light-dark cycle, while being provided unlimited access to food and water.

Chemicals:

Exposure: A 10% FA solution (manufactured by El NASR Pharmaceutical Chemicals Co., Egypt) was placed in containers containing cotton soaked in the solution, which was periodically replenished every hour. The animals were exposed to the same amount (50 ml) of 10% formalin solution, resulting in a concentration of 12 parts per million (ppm) (Zaki *et al*., 2018).

Experimental Design:

Rats were randomly assigned to four groups (ten rats in each) as follows: The control group (Group I) was not exposed to any FA source for 18 weeks. Group (II) exposed to 10% FA inhalation

for 2 hours daily, twice weekly, for 18 weeks. Group (III) exposed to 10% FA for 4 hours per day, twice a week/18 weeks. Group (IV) exposed to 10% FA inhalation for 4 hours/ day, four times weekly for 18 weeks. All groups exposed to FA inhalation were housed in individual cages within a large, closed box with ventilation openings. Additionally, a beaker containing 10% FA was placed beside them (Asghar, 2016). At the end of the 18th week, the rats were euthanized, their heads were decapitated, and spleen specimens were obtained. For histopathological examination under a light microscope, the splenic tissue was cut into 3-5μm thick sections, processed, and subjected to staining using Haematoxylin & eosin (H&E), immunohistochemical staining for CD4, CD8 and Bcl-2 (AL-Aarajy *et al.*, 2023).

Immunohistochemical Staining:

1. CD4 & CD8 Immuno-Histochemical Staining:

Anti - CD4 monoclonal antibody (clone: 4B12, Dako. Denmark) was used to detect T helper cells. The antibody was provided in liquid form (1:50 dilution) and contained 0.015 mol/L sodium azide and stabilizing protein. To identify the cytotoxic T cells, a monoclonal mouse antibody against human CD8 (clone: C8144/B, Dako, Denmark) was employed and supplied in a liquid form with a stabilizing protein and 0.015 mol/L sodium azide buffer. Slides containing polyclonal rabbit antibodies were first deparaffinized, then rehydrated, and washed in tap water. They were subsequently immersed in a 3% hydrogen peroxide solution for 10 minutes to inhibit any internal peroxidase activity. Following this, sections were treated with 2% trypsin for 10 minutes. To prevent non-specific protein interactions, a blocking solution composed of phosphate-buffered saline (PBS) and 10% normal goat serum was used. The sections were treated with the primary anti-CD4 and anti-CD8 antibodies for thirty minutes. Subsequently, the sections underwent three 5-minute washes in the buffer before being exposed to the secondary antibodies at a 1:1000 dilution for an additional 30 minutes, followed by another round of washing. Following an additional 30-minute incubation with Vectastain ABC kits (Avidin-Biotin complex with horseradish peroxidase) and a subsequent 10-minute wash, the slides were treated with the substrate diaminobenzidine tetrahydrochloride (DAB) in distilled water for 5 to 10 minutes. They were then counterstained with Haematoxylin. This procedure was also used for negative control sections, except without the primary antibody. Rat spleen tissue served as the positive control for CD4 and CD8 staining (Rifaai and Baky, 2017).

2. Bcl-2 Immunohistochemical Staining:

The sections were deparaffinized using xylene and then underwent antigen retrieval in citrate buffer (pH 6.0) at 60 °C. Following this, the sections were exposed to 3% hydrogen peroxide to inhibit the endogenous activity of the peroxidase enzyme. Unwanted staining was prevented by using suitable serum. The primary antibodies for Bcl-2 were procured from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal primary antibodies were diluted in PBS at a 1:1000 ratio. The tissue sections were incubated with the primary antibodies at 4°C overnight then washed and treated with biotinylated secondary antibodies for an hour at room temperature. Subsequently, the immunostaining was identified using the ABC method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, California). DAB was used as the chromogen. Slides were counterstained with Haematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted with DPX (Ali *et al.,* 2023). Slides were treated with (DAB*)* as the chromogen. Lastly, slides were counterstained by using Haematoxylin followed by graded alcohol dehydration and then clearance in xylene.

Histomorphometric Study:

The measurements of the different parts of the white pulp; germinal centers, mantle zone, periarterial lymphoid sheaths (PALS) and the marginal zone were included in the study. In each group, measuring ten non-overlapping fields on slides from five distinct rats at a magnification of X200 was done (El-Bermawy and Salem, 2015; Ali *et al.*, 2023). In the examined groups, the following morphometric records were evaluated; the total surface area of the white pulp and germinal center in μm^2, and the diameters of the white pulp, mantle layer, PALS and marginal zone in μm (Golalipour *et al.*, 2008).

Quantitative Immunohistochemistry:

A quantitative assessment of the CD4, CD8, and Bcl-2 immunohistochemistry staining was performed. The assessment involved measuring ten non-overlapping fields on slides from five different rats in each group at a magnification of X200. The average percentage of immunoreactivity surface area for each splenic section was quantitatively determined in relation to the area of a known measuring frame (expressed as estimate area % /135.84 μm^2 frame) using Image J software version 1.50i, dated March 26, 2016, from the National Institutes of Health, USA (Ali *et al.*, 2021).

Statistical Analysis:

Statistical analyses were performed using SPSS software version 20 (SPSS Inc. Chicago, USA). One-way analysis of variance (ANOVA) was utilized, followed by the Bonferroni post hoc test for making multiple comparisons among the histomorphometric and quantitative data from the various groups under study. The results were presented as means ± standard deviation (SD), and statistical significance was considered for differences with a P-value of less than 0.05

RESULTS

Hx&E- stained sections:

1-Control Group (I): The sections of the control group stained with Hx&E displayed the typical histological architecture of the spleen, which includes two easily distinguishable compartments: the white pulp (lymphoid tissue) and the red pulp (vascular matrix), with marginal zones situated between them, all encased within a connective tissue capsule (Fig.1)**.** The white pulp consisted of lymphoid follicles connected to a lymphocyte sheath; the peri-arteriolar lymphatic sheath (PALS) encircling the central (eccentric) arteriole. The lymphoid follicles were made up of clusters of lymphocytes (mantle zone), with some follicles exhibiting a germinal center. A marginal zone of lightly stained lymphocytes wrapped the follicle, marking the boundary between the lymphoid follicle and the red pulp. The red pulp consisted of anastomosing splenic cords with blood-filled sinusoids interspersed among them (Figs. 2 & 3).

2-Experimental Groups (II, III and IV): The Hx&E-stained sections of all experimental groups showed relatively thicker connective tissue capsules as compared to the control group (Figs. 4, 7 & 10). In experimental group II, there was a significant disruption in the histological structure with a loss of distinction between the white and red pulps, and the marginal zone was indistinct and could not be clearly identified (Figs. 4 & 5). These findings were more noticeable in experimental groups (III) (Fig. 7) and (IV) (Fig. 11).

In experimental group (II), within the white pulp, lymphoid cells were degenerated with pyknotic nuclei and vacuolated cytoplasm. In some follicles, the germinal centers exhibited necrosis, while others lacked it (Figs. 4 & 6). Furthermore, the extent of necrosis was heightened in group (III) (Figs. 7, 8 & 9) and was notably severe in the experimental group (IV) (Figs. 11 & 12).

The red pulp was observed to be enlarged and exhibited significant congestion (Figs. 5, 6 & 7). All experimental groups displayed congested and dilated blood sinusoids (Figs. 6 & 9). Additionally, experimental groups III

and IV presented dilatation and haemorrhage of the sinusoids (Figs. 8 & 11). In group (IV), the marked congestion was clearly observed in the subcapsular region (Fig.12). Moreover, megakaryocyte cell infiltrations were frequently seen in both groups III and IV (Figs. 9 & 11).
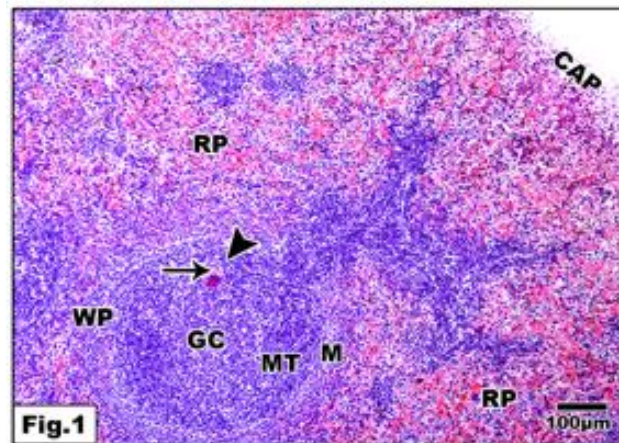

Fig. 1: Section of the control group (I) showing that the spleen is surrounded by connective tissue capsule (CAP). Under the capsule lies the two major components: the white pulp (WP) and the red pulp (RP). The white pulp lymphoid follicle (WP) is formed of various layers; pale germinal center (GC) surrounded by mantle (MT) and marginal (M) zones. Peripherally located central arteriole (arrow), surrounded by periarterial lymphatic sheath (arrowhead) were detected. (H&E, X 100).
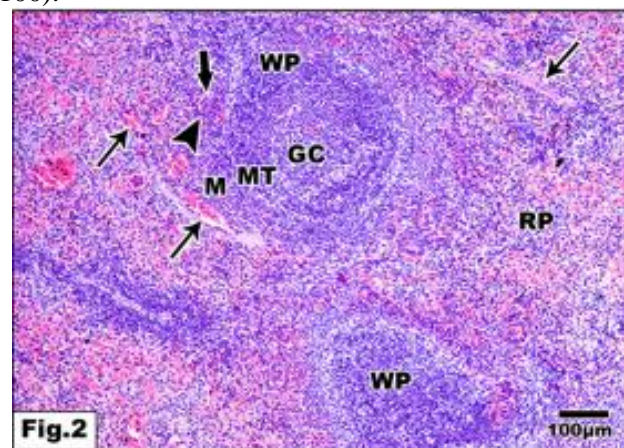

Fig. 2: Section of the control group (I) showing intact both white (WP) and red pulps (RP), separated by marginal zone (M). The white pulp has follicle with pale germinal center (GC), mantle zone (MT) and a periarterial lymphatic sheath (arrowhead) surrounding central arteriole (Thick arrow). Intact thin-walled sinusoids (arrow) are also observed. (H&E, X 100).
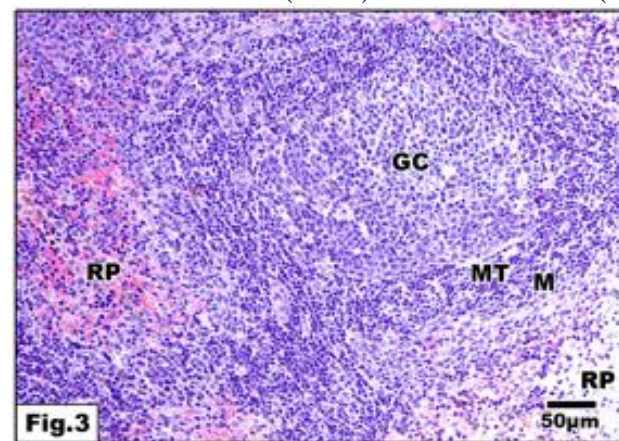

Fig. 3: Section of the control group (I) showing the different layers of lymphoid follicles of the white Pulp (WP) marginal zone (M), mantle zone (MZ), germinal center (GC) and red pulp (RP). (H&E, X 200).
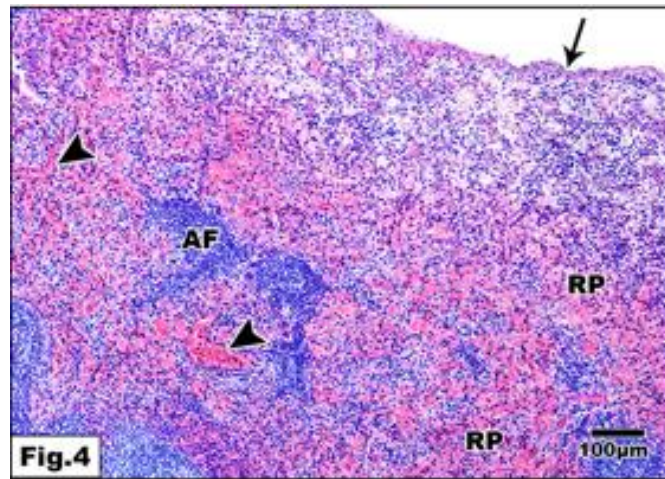
Fig.4: Section of group (II) showing loss of normal architecture of the spleen with no demarcation between white pulp and red pulp (RP). The white pulp exhibits small sized, atrophied follicles (AF). Congested blood sinusoids are detected (arrowhead) within the red pulp. The spleen is surrounded by a relatively thick capsule (arrow). (H&E, X 100).
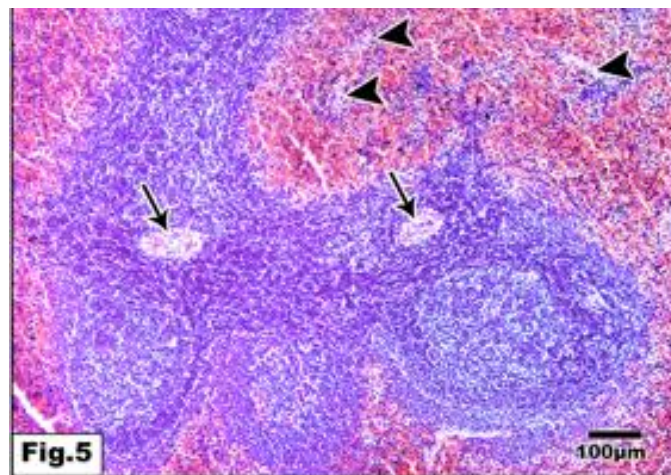
Fig.5: Section of the spleen of group (II) displays disorganization of the histological structure. The red pulp shows marked congestion of the blood sinusoids (arrowhead). Dilated congested thickened- wall central arterioles (arrow) are noticed in the white pulp. (H&E, X 100).
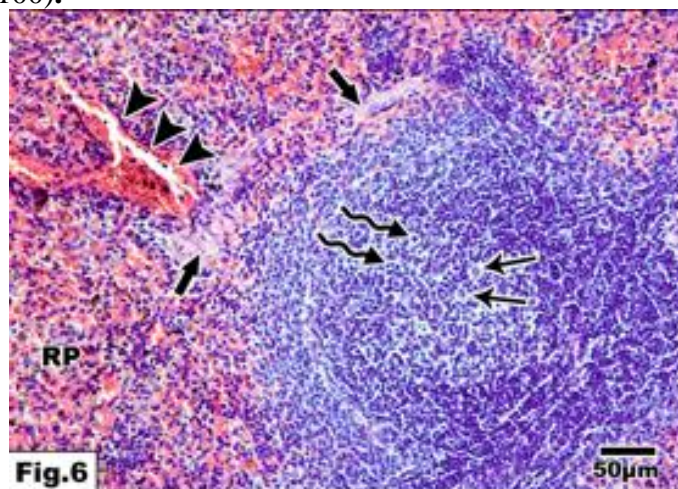
Fig.6: Section of the spleen of group (II) reveals congestion in the red pulp (RP) and dilated sinusoids (arrowheads). Additionally, necrosis is observed at the core of lymphoid follicles, where lymphocytes display vacuolated cytoplasm(arrow) and pyknotic nuclei (wavy arrow). Areas of degeneration are observed (thick arrow). (H&E, X 200).
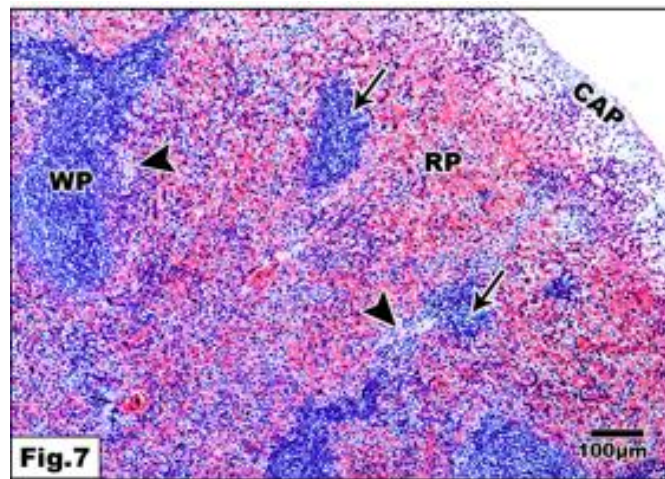
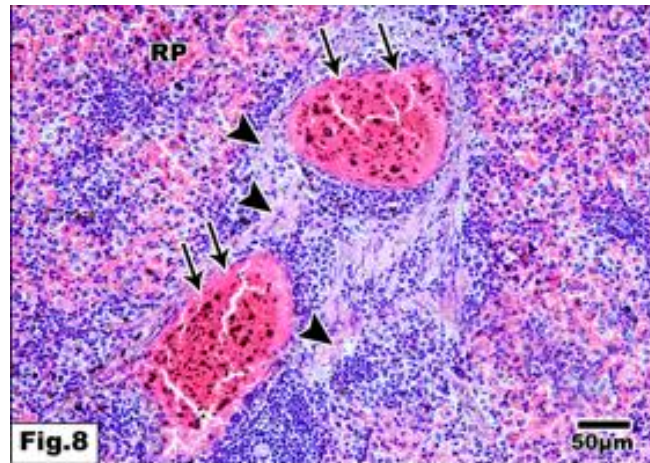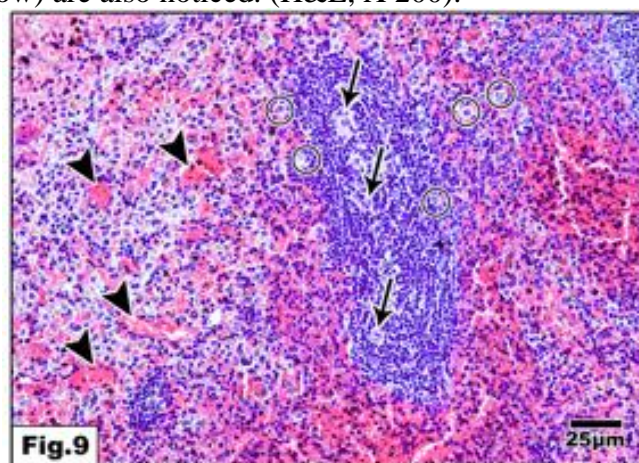**Fig.7:** Section from the experimental group (III) exhibits a greater disruption in the typical structure of the spleen, characterized by the absence of the usual demarcation between the white pulp (WP) and red pulp (RP). There is pronounced congestion in the red pulp, accompanied by shrinkage (arrow) and necrosis (arrowhead) of the distorted white pulp. Marked thickening of the covering capsule is also observed (CAP). (H&E, X 100).

Fig.8: Section from experimental group (III) displays significant congestion in the red pulp (RP), along with shrinkage and necrosis (arrowhead) of the white pulp, which appears notably distorted. Moreover, severe congestion, dilation, and haemorrhage of the sinusoids (arrow) are also noticed. (H&E, X 200).

Fig.9: Section from experimental group (III) showing marked congestion and dilatation of the blood sinusoids within the red pulp (arrowhead). Lymphoid cells are degenerated with pyknotic nuclei and vacuolated cytoplasm (arrow). Megakaryocytes cells (circle) are frequently seen. (H&E, X 400).
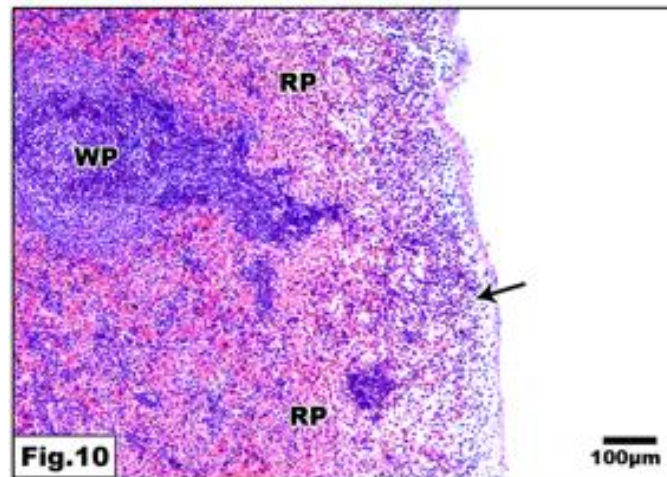
Fig.10: Section of experimental group (IV) displays marked thickening of the capsule (arrow) with severe necroses of the white pulp (WP) and marked congestion of the red pulp (RP). (H&E, X 100).
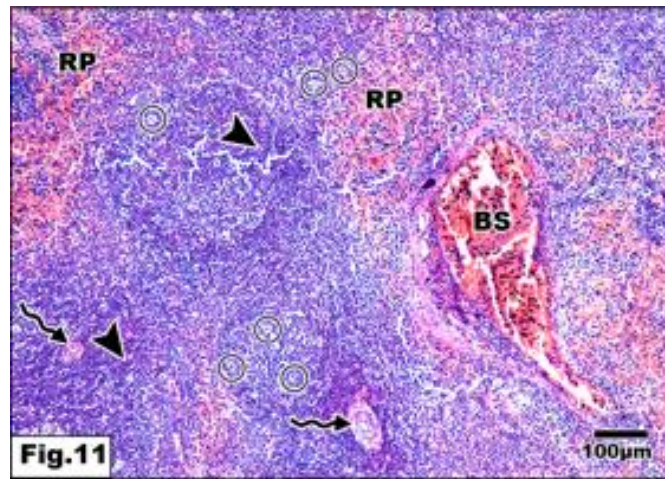
Fig.11: Section of experimental group (IV) exhibits obvious disruption of the normal boundary between the white and red pulps. Marked congestion in the red pulp (RP), severe degeneration of lymphoid cells in the white pulp with pyknotic nuclei (arrowhead) and enlarged central arterioles with thickened walls (wavy arrow) are detected. Additionally, there is severe congestion, dilation, and haemorrhage in the sinusoids (BS). Megakaryocytes are also observed (circle). (H&E, X 100).
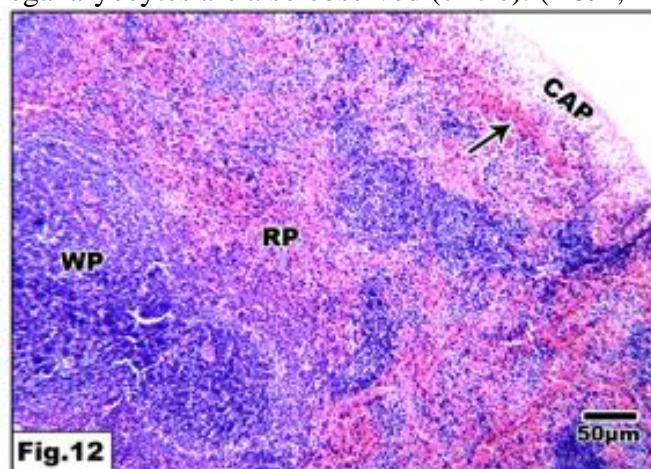
**Fig.12:** Section of experimental group (IV) displays marked congestion of the red pulp (RP) with severely distorted necrotic white pulps (WP) with pyknotic nuclei. Thickened capsule (CAP) and marked subcapsular congestion are detected (arrow). (H&E, X 200).

CD4 Immunohistochemical-Stained Sections:

The control group's sections stained with CD4 immunohistochemistry demonstrated a heightened expression of CD4-positive immune cells mainly in the red pulp with a few dispersed throughout the germinal center of the white pulp (Fig. 13 A).

The CD4 immunohistochemical-stained sections of experimental groups, showed more enhancement in the intensity of the immunoexpression within the red pulps, however the atrophic white pulps demonstrated less expression (Figs. 13 B, C & D).
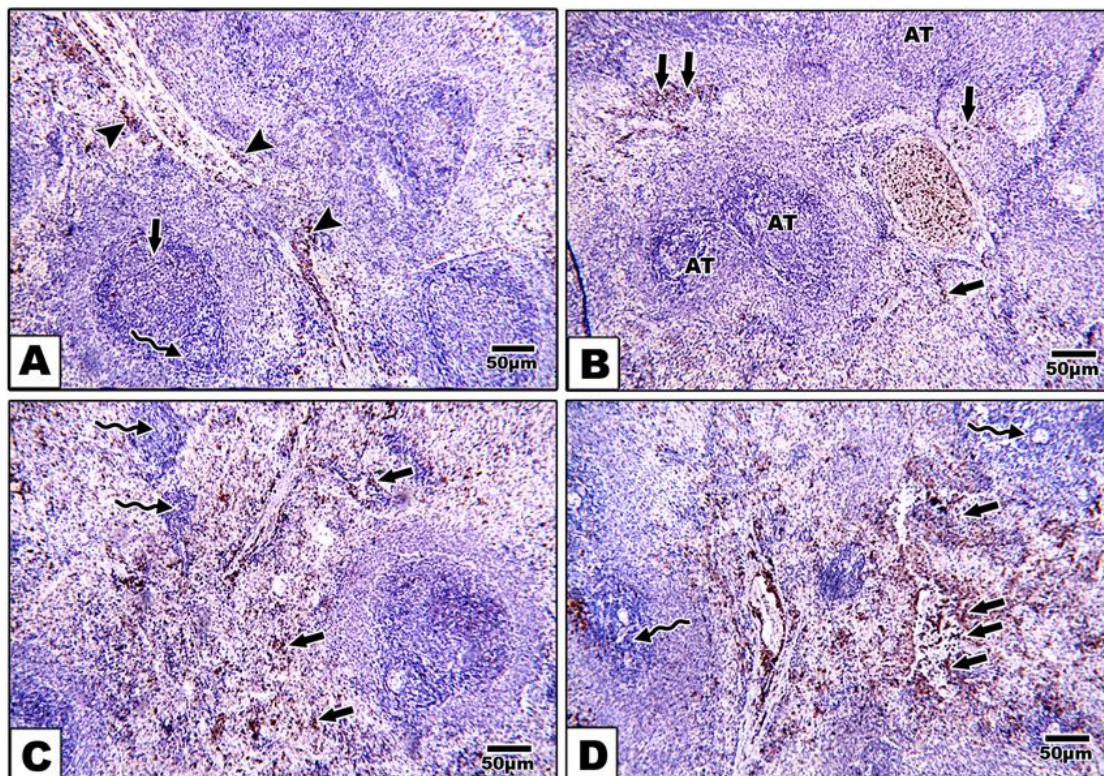
Fig. 13 A: CD4 immunohistochemical stained section of control group reveals immune-expression of CD4 positive cells predominantly located within the red pulp cords (arrowheads) and the peri-arteriolar lymphatic sheath (wavy arrow), with a few cells dispersed in the germinal center of the white pulp (thick arrow). (CD4 immune-expression, X 200). **B:** CD4 immunohistochemical stained section of the experimental group (II) exhibits mild increase in the CD4 immune-expression (arrow) within the red pulp when compared to control group. Decreased expression in the atrophic white pulps (AT) is also observed. (CD4 immune-expression, X 200).**C:** CD4 immunohistochemical stained section of the experimental group (III) displays moderate increase in the CD4 immune-expression (arrow) particularly within the red pulp as compared to control group and decreased expression in the atrophic white pulp (wavy arrow). (CD4 immune-expression, X 200). **D:** CD4 immune-stained section of the experimental group (IV) shows marked increase in the CD4 immune-expression (arrow) within the red pulp as compared to the control group and decreased expression in the atrophic white pulp (wavy arrow). (CD4 immune-expression, X 200).

CD8 Immunohistochemical-Stained Sections:

The spleen sections from the control group, stained for CD8 immunohistochemistry, exhibited a typical pattern of distribution with only a small number of cells dispersed throughout both the white and red pulps (Fig. 14 A).

In contrast, the sections from the experimental groups showed a seemingly increased presence of CD8-positive immune cells within the red pulp cords and the peri-arteriolar lymphatic sheath (PALS) of the spleen as compared to the control group (Figs. 14 B, C & D).
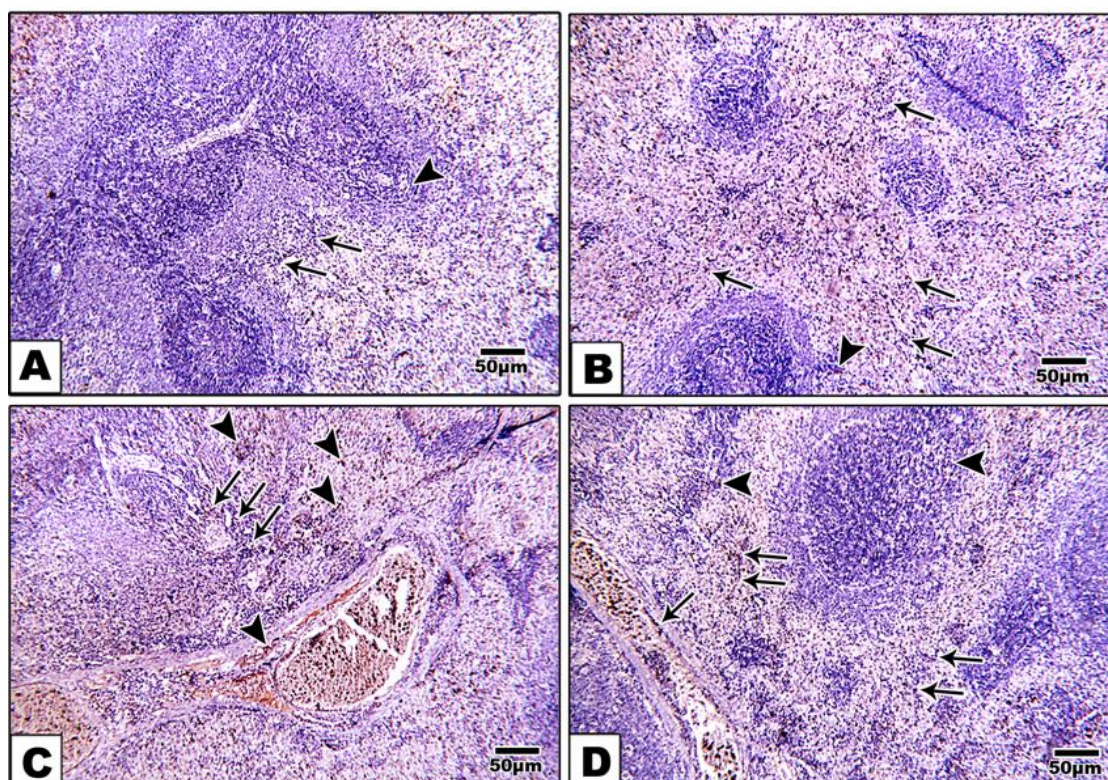
Fig 14 A: CD8 immune-stained section of the spleen from the control group (I) shows few positive cells in the PALS (arrowhead) and red pulp (arrow). (CD8 immune-expression, X 200). **B:** CD8 immune-stained section of the experimental group (II) exhibits mild increase in the immune-positive cells either in the PALS (arrowhead) or in the red pulp cords (arrow). (CD8 immune-expression, X 200). **C:** CD8 immune-stained section of the experimental group (III) displays moderate increase in the immune-expression of CD8 positive cells in the PALS (arrow) and in the red pulp cords (arrowheads). (CD8 immune-expression, X 200). **D:** CD8 immune-stained section of the experimental group (IV) displays increase in the immune-expression of CD8 positive cells in the PALS (arrowheads) and in the red pulp cords (arrow) as compared to the control group. (CD8 immune-expression, X 200).

Bcl-2 Immunohistochemical-Stained Sections:

Spleen sections stained for Bcl-2 in the control group displayed only a few cells scattered in the white or red pulps. However, the experimental groups showed a noticeable increase in the positive immune expression of Bcl-2 compared to the control group (Figs. 15 B, C & D).
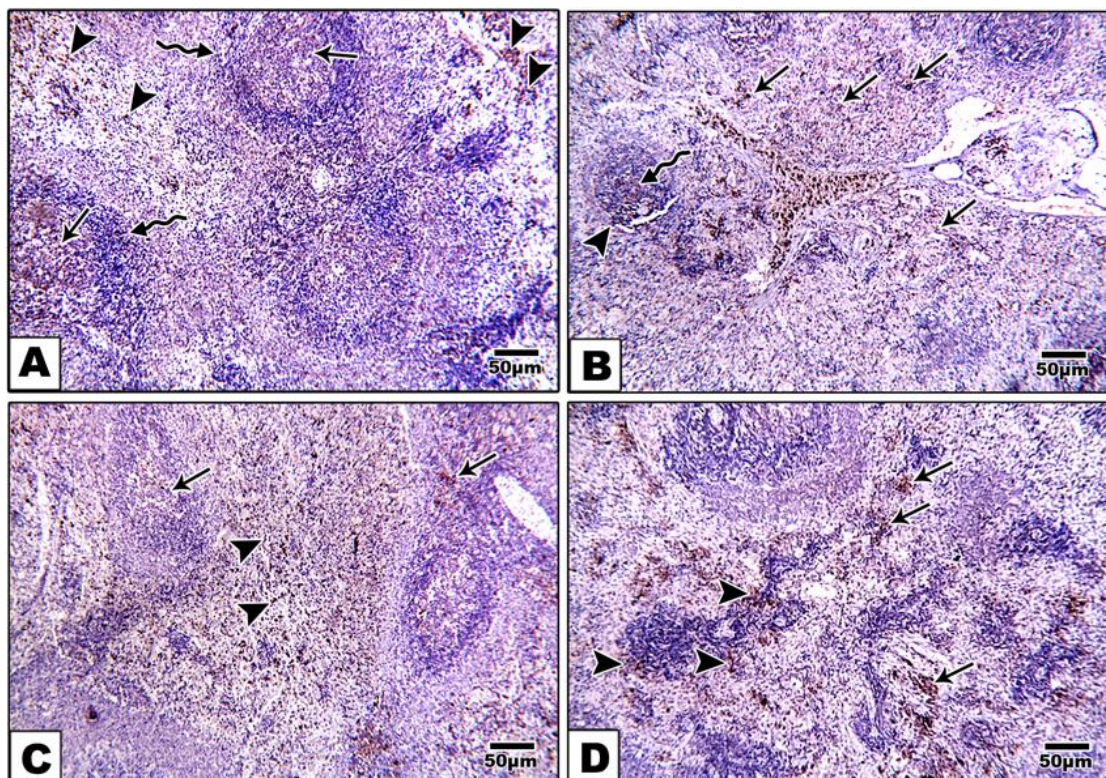
Fig.15 A: Section of the control group (I) shows apparently few positive immune-expression of Bcl-2 in the white pulp (arrow), marginal zone (wavy arrow) and red pulp (arrowhead). (Bcl-2 immune-expression, X 200). **B:** Section of the experimental group (II) shows mild positive immune expression of Bcl-2 in the white pulp (wavy arrow), marginal zone (arrowhead), red pulp (arrow). (Bcl-2 immune-expression, X 200).
C: Section of the experimental group (III) shows moderate positive immune expression of Bcl-2 in both the white pulp (arrow) and red pulp (arrowhead).
(Bcl-2 immune-expression, X 200). **D:** Section of the experimental group (IV) shows marked positive immune-expression of Bcl-2 in the white pulp (arrowhead) and red pulp (arrow) (Bcl-2 immune-expression, X 200).

Histomorphometric Study:

1-White Pulp Surface Area (μm^2): There was a statistically non-significant decrease in white pulp surface area in experimental groups (II & III) with a statistically significant decrease in group (IV) as compared to group (I) (Table 1, Fig.16).

2-White Pulp Diameter (μm): A statistically insignificant decrease in the white pulp diameter of the experimental groups (II & III) was noticed when compared to group (I), meanwhile, group (IV) showed a statistically significant decrease in the white pulp diameter as compared to groups (I & II) (Table 1, Fig.16)

3-Germinal Center Surface Area (μm^2): Experimental groups (II, III & IV) exhibited a statistically insignificant

decrease in the germinal center surface area when compared to group (I) (Table 1, Fig.16).

4-Marginal Zone Diameter (μm): Experimental groups (II & III) displayed a statistically insignificant increase in the marginal zone diameter when compared to group (I). While group (IV) showed a statistically insignificant decrease in the marginal zone diameter as compared to other groups (Table 1, Fig.16).

5-Mantle Layer Diameter (μm): The experimental group (II) showed a statistically significant decrease in the mantle layer diameter as compared to group (I). Groups (III & IV) posed a statistically insignificant decrease as compared to group (I) with a statistically insignificant increase as compared to

group (II) in the mantle layer diameter (Table 1, Fig. 16).

6-PALS Diameter (μm): The experimental group (II) demonstrated a statistically insignificant decrease in the PALS diameter as compared to group (I).

However, an increase in the PALS diameter was observed in experimental groups (III) (insignificant) and (IV) (significant) as compared to group (I) (Table 1, Fig. 16).

Table 1: Different histomorphometric measurements of the studied groups.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)
White pulp surface area (μm^2)	276.02±133.2	193.98±115.89	135.34±165.83	118.89±70.49*
White pulp diameter (μm)	16.48±3.85	12.48±5.93	9.999±7.76	7.64±2.27*#
Germinal center surface area (μm^2)	28.48±22.39	26.02±26.02	17.15±9.88	12.03±7.06
Marginal zone diameter (μm)	2.996±0.349	3.03±0.673	3.79±0.89	2.85±1.34
Mantle layer diameter (μm)	4.68±1.52	2.93±0.76*	3.41±1.25	4.66±1.58
PALS diameter (μm)	1.16±0.30	1.05±0.28	1.17±0.35	2.32±0.895*

All results are expressed as mean ± SD. *P* value <0.05 is statistically significant; *compared to the control group (I), #compared to group (II). n = 10 (in each group).
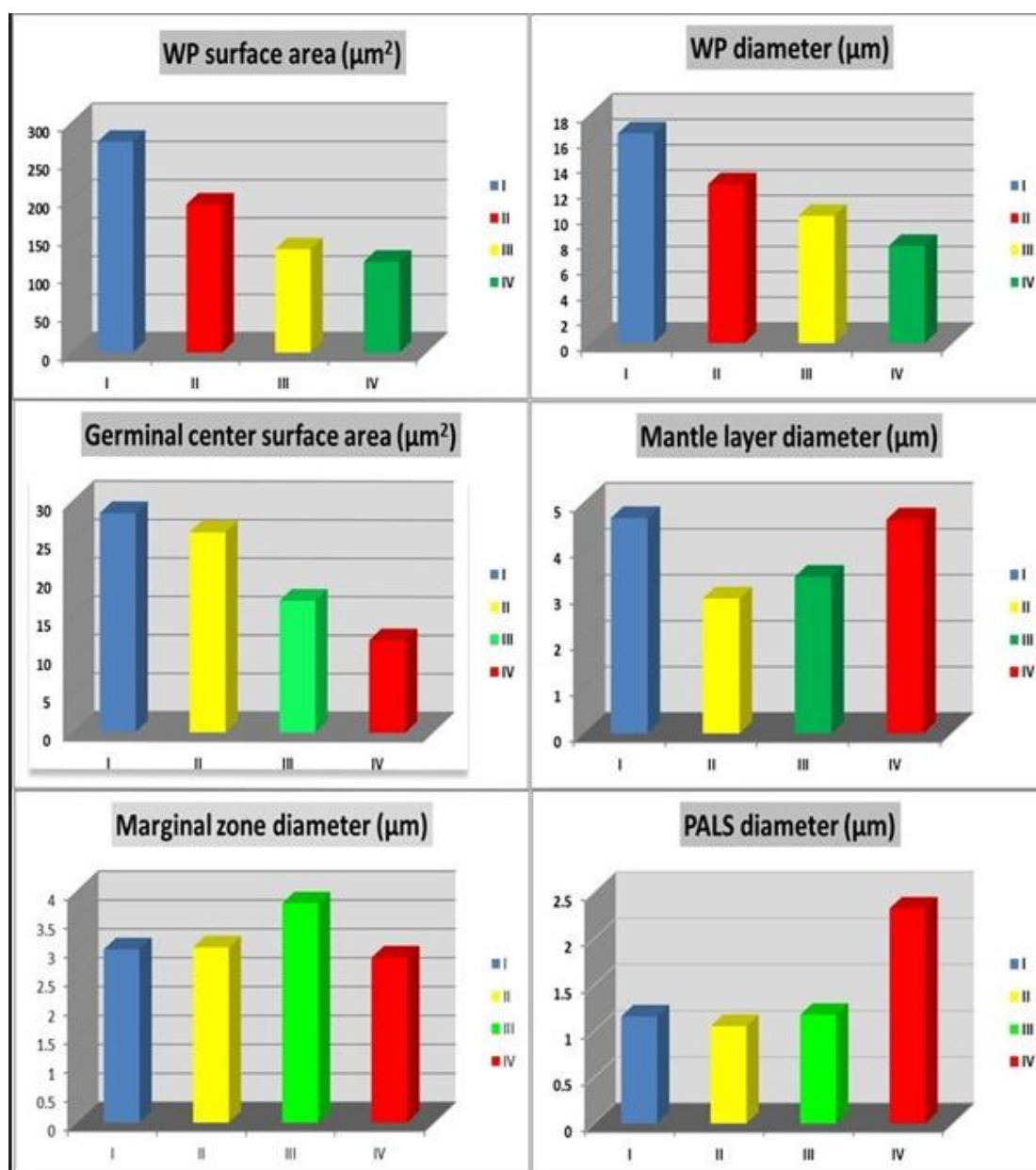
Fig. 16: A histogram showing the histomorphometric measurements observed in the studied groups.

Quantitative Immunohistochemistry:

1) **CD4 Immuno-Expression:** There was a statistically insignificant increase in surface area % of CD4 immuno-expression in group (II) as compared to group (I); meanwhile, groups (III & IV) displayed a statistically significant increase of it as compared to group (I) (Table 2, Fig. 17).

2) **CD8 Immuno- Expression:** A statistically significant increase in surface area % of CD8 immuno-expression in groups (II, III and IV) was perceived as compared to group (I); meanwhile, group (IV) displayed a statistically insignificant decrease as compared to groups (II and III) (Table 2, Fig.17).

3) **Bcl-2 Immuno- Expression:** Experimental groups (II and III) exhibited a statistically significant increase in surface area % of Bcl-2 immuno-expression as compared to group (I). Additionally, group (IV) revealed a statistically significant increase in surface area % of Bcl-2 immuno-expression as compared to groups (I & II) with a statistically insignificant increase of it when compared to group (III) (Table 2, Fig.17).

Table (2): Quantitative immunohistochemistry for surface area % of CD4, CD8 & Bcl-2 immuno-expression in the studied groups.

Parameter	Group (I)	Group (II)	Group (III)	Group (IV)
Mean surface area % of CD4	8.14+2.76	13.29+5.38	20.69+5.73*	32.27+6.54*
Mean surface area % of CD8	12.39+4.84	30.7+7.16*	30.71+9.55*	26.26+11.36*
Mean surface area % of Bcl-2	11.72+4.1	22.47+6.94*	28.82+7.56*	30.06+5.83*#

All results are expressed as mean ± SD. *P* value < 0.05 is statistically significant; *Compared to the control group (I), #Compared to group (II). n = 10 (in each group).
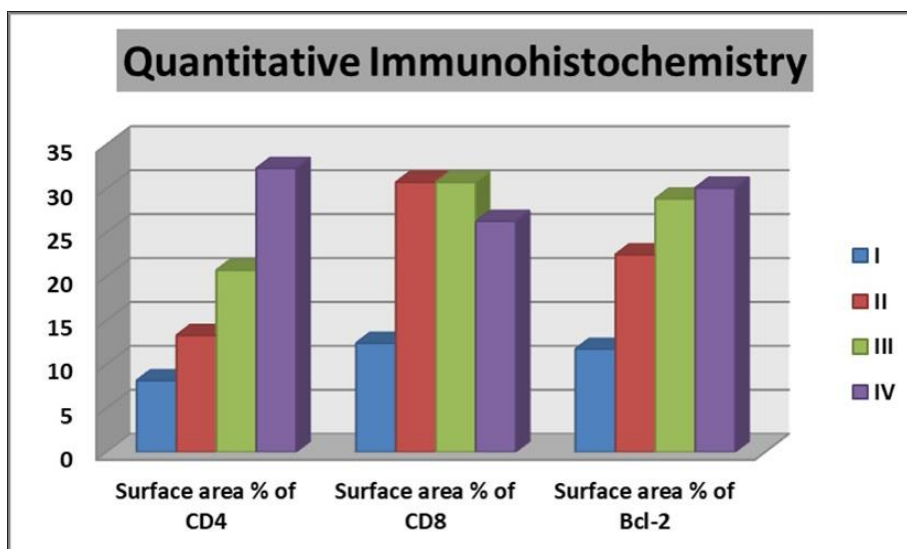
Fig. 17: A histogram showing the quantitative immunohistochemistry representing the mean surface area % of the applied immunohistochemical stains in the studied groups.

DISCUSSION

FA is a prevalent chemical extensively utilized across numerous industries (Zhao *et al*., 2021). Workers in specific occupational environments, including those involved in FA textile and resin production, medical laboratories and embalming, primarily encounter FA through inhalation or skin contact (McGwin *et al*., 2011). Additionally, the general population is regularly exposed to FA from various sources like building materials, cigarette smoke, and certain wood or fabric products (Nikle *et al*., 2019). The widespread exposure to FA in both work

and environmental settings prompts concerns regarding potential adverse health impacts. Lymphocytes are found in numerous organs, with the spleen, lymph nodes, and blood being particularly significant. The spleen, which receives roughly 5% to 10% of the cardiac output and holds about 15% of the body's lymphocytes, plays a crucial role in cell-mediated immunity (Cesta, 2006). The spleen is regarded as a key organ in immune responses, tasked with filtering out pathogens, foreign antigens, and irregular cells. This process is primarily carried out by T and B lymphocytes (Lewis *et al.*, 2019). Maintaining equilibrium within the immune system and the production of cytokines is critical. Disruption of this equilibrium can lead to health issues, including an increased risk of cancer development (Azad *et al.*, 2018).

In the current research, the Hx&E-stained sections of all the experimental groups displayed thicker connective tissue capsules. In experimental group II, there was a notable disturbance in the histological structure, resulting in a loss of differentiation between the white and red pulps, and the marginal zone was unclear and difficult to distinguish. These observations were even more pronounced in groups III and IV. In the experimental group (II), lymphoid cells within the white pulp showed signs of degeneration with condensed nuclei and vacuolated cytoplasm. Some follicles within the white pulp had necrotic germinal centers. Additionally, the degree of necrosis was dose-dependent and tended to be more obvious in group III and particularly severe in experimental group IV. The red pulp appeared enlarged and showed noticeable congestion. All experimental groups exhibited congested and widened blood sinusoids. Furthermore, experimental groups III and IV displayed dilatation and haemorrhage of the sinusoids. Additionally, both groups III and IV frequently showed the presence of megakaryocyte cell infiltration. These

results were consistent with those obtained by Golalipour *et al.* (2008) who demonstrated that FA inhalation induced remarkable morphometric alterations in the white pulp of rat spleens with subsequent immunosuppression. Moreover, increased capsular thickness, a significant decline in the lymphoid cell count and an increase in the megakaryocyte population were demonstrated by Monfared *et al.* (2013). In addition, FA exposure leads to dose-dependent enhanced lymphoproliferation ,increased megakaryocytes, and expansion of the germinal center within splenic corpuscles (Ge *et al.*, 2020). A study conducted by Abd-Elhakim *et al.* (2016) detected areas of necrosis within the splenic parenchyma together with alteration of the immunological responses. Similarly, histopathological examination of the spleen on exposure to FA showed disrupted structural organization, notably a marked decrease in the dimensions and cellular components of the lymphocytic follicles along with marginal zone distortion. Additionally, the splenic blood sinusoids exhibited congestion, particularly when exposed to high doses of FA *(El-Nagdy et al., 2020)*. Macroscopically, Asghar *et al.* (2016) found splenomegaly with obliterated margins in 0% of rats. Also, rats exposed to FA for shorter duration showed normal histological structure. However, in one group congested red pulp and reduction of white pulp surface area were noted.

FA not only strongly triggers inflammation in the lower respiratory tract but also adversely affects other organs (Lino-dos-Santos-Franco *et al.*, 2011). It has the ability to interact with molecules found in cell membranes, as well as those present in body tissues and fluids (proteins and DNA). Elevated concentrations of FA lead to protein precipitation, ultimately resulting in cell death (Golalipour *et al.*, 2008). As a result of these interactions, it can disrupt cellular functions (Kleinnijenhuis *et al.*, 2013). Research has demonstrated that animals exposed to FA experience

elevated levels of reactive oxygen species (ROS), reactive nitrogen species (RNS), DNA-protein cross-linking and malondialdehyde (MDA). Additionally, there is a reduction in glutathione (GSH) levels and other antioxidant enzymes both in the lungs and systemically including the spleen and uterus (Ye *et al*., 2013; Ibrahim *et al*., 2016; Wen *et al*., 2016). The oxidative stress resulting from lipid peroxidation and protein biotransformation in individuals exposed to FA recruits inflammatory cells and induces the inflammatory process (Jr *et al*., 2005).

In the existing investigation, histomorphometric studies revealed a statistically significant decrease in white pulp surface area and diameter in the experimental group (IV). However, the germinal center surface area exhibited a statistically insignificant decrease in experimental groups (II, III & IV). The Marginal zone diameter displayed a statistically insignificant increase in groups (II & III) and an insignificant decrease in group (IV). Experimental groups (II, III & IV) showed a statistically significant and insignificant decrease in the mantle layer diameter as compared to group (I). Additionally, the experimental group (II) demonstrated a statistically insignificant decrease in the PALS diameter. However, an increase in the PALS diameter was observed in experimental groups (III) (insignificant) and (IV) (significant).

Prolonged exposure to FA vapour increases the diameters of the germinal center and marginal zone as well as an increase in the germinal center area. However, it decreases the diameter of the mantle layer (Golalipour *et al*., 2008). FA can act as an antigen, triggering immune reactions, and may lead to increased multiplication and cellularity of immature B-cells resulting in larger secondary follicles with major germinal centers, and an expansion of the white pulp area and follicle diameters (increase in the germinal centers, marginal and mantle zones diameters) (Elmore, 2006; Lino dos Santos Franco *et al*., 2006).

In the current study, The CD4 and CD8 immunohistochemical-stained sections of experimental groups showed more enhancement in the intensity of the immunoexpression within the red pulps and PALS as compared to the control group. These results were confirmed by using quantitative immunohistochemistry; the surface area % of CD4 immuno-expression displayed an insignificant increase in group (II) and a significant increase in groups (III & IV) as compared to control. Moreover, a significant increase in surface area % of CD8 immuno-expression in groups (II, III and IV) was perceived as compared to group (I); meanwhile, group (IV) displayed a statistically insignificant decrease as compared to groups (II and III).

The spleen, as an organized lymphoid structure, is the site where adaptive immune responses begin. Within the spleen, the appropriate differentiation and development of various effector T cell subsets (Th1, Th2, Th17) and Treg cells occur, triggered by specific effector cytokines related to their lineage (Park *et al*., 2020). Recent research on both humans and animals has shown that exposure to FA has detrimental effects on the immune system. This is manifested by changes in the populations of various T cell types and alterations in the secretion of related cytokines (Wei *et al*., 2014).

Treg cells play essential roles during the priming and effector phases of innate and adaptive immune responses (Miyara and Sakaguchi, 2007). The potential mechanisms of suppression can be categorized into four main types: via inhibitory cytokines, cytolysis, metabolic disruption, and modulation of dendritic cell maturation or function (Park *et al*., 2020). FA exposure leads to a dose-dependent significant increase in the population of splenic CD4+, CD25+ Foxp3+ Treg cells (Park *et al*., 2020). Interestingly, the percentage of CD4+ helper T cells remained unaffected by FA exposure, which aligns with findings from previous studies involving both FA-

exposed humans and mouse models (Ye *et al*., 2005; Jung *et al*., 2007). However, FA exposure suppressed the expression of T helper cells-related genes and hence the synthesis of Th-1, Th-2, and Th-17 cells-related splenic cytokines in a dose-dependent manner (Wei *et al*., 2014; Park *et al*., 2020; Park *et al*., 2022). Moreover, in the lungs of rodents, FA inhalation suppressed Th1- and Th2-related cytokines, thereby decreasing airway inflammation and bronchial hyperresponsiveness (Li *et al*., 2017). El-Nagdy *et al*. (2020), in their 2020 research, examined the subsets of T-lymphocytes, B lymphocytes, NK cells, and the cytokine profiles of IL-10 and IFN-γ in the blood of adult male albino rats subjected to FA. The findings indicated that rats treated with FA exhibited a reduction in T-lymphocyte subsets relative to the control group, whereas B-lymphocytes were found to be elevated, particularly in rats exposed to higher doses of FA. In contrast to our results, El-Nagdy *et al*. (2020) reported that immunohistochemical staining demonstrated a notable reduction in CD4+ expression on lymphocytes within the spleen for FA-exposed groups in a dose-dependent way. This suggests an impact on the splenic role as a critical organ in lymphocyte production including CD4+ T helper cells (El-Nagdy *et al*., 2020). Aligning with these results, Hosgood *et al*. (2013); Zhang *et al*. (2010); and Li *et al*. (2013) observed a reduction in CD4+, CD8+, and natural killer (NK) cell counts in workers exposed to FA. Similarly, (Ye *et al*., 2005) noted a decline in T lymphocyte counts, particularly in the CD3+ and CD8+ subsets, among FA-exposed workers, while CD4+ cell counts remained unchanged.

These findings contrast with those of (Aydın *et al*., 2013), who found a marked increase in the absolute count of T-lymphocytes in FA-exposed workers. (Wei *et al*., 2014) suggested that the varying effects of FA on T-lymphocytes could be due to differences in the levels and duration of FA exposure. Ultimately, it is presumed that FA has an influence on T-lymphocytes, which in turn affects the immune response.

In the present work, the experimental groups showed a noticeable increase in the positive immune expression of Bcl-2 compared to the control group. Moreover, quantitatively, the Experimental groups (II and III) exhibited a statistically significant increase in surface area % of Bcl-2 immuno-expression as compared to group (I). Additionally, group (IV) revealed a statistically significant increase in surface area % of Bcl-2 immuno-expression as compared to groups (I & II) with a statistically insignificant increase when compared to group (III).

Bcl-2 family proteins implement numerous essential functions during the body's growth and performance (Campbell and Tait, 2018). One of the most important members of the family is the Bcl-2 protein. It has a molecular weight of 26 kilodaltons and is encoded by a gene on the 18th chromosome (Park *et al*., 2021).

Multiple studies have shown that gene regulation encoding Bcl-2 family proteins alters cancer (Kaloni *et al*., 2023). This overexpression can prevent cell death induced by different triggers, especially anticancer drugs. Irregularities in the expression of anti-apoptotic or pro-apoptotic BCL-2 proteins can contribute to the development, growth and progression of tumors and also render cancer cells resistant to treatment (Kaloni *et al*., 2023). Consequently, targeting Bcl-2 proteins could be valuable in diagnosing cancer and predicting outcomes when treating cancer with chemotherapy (Qian *et al*., 2022). By binding with BAX, the Bcl-2 protein can block apoptosis and promote cell survival by controlling calcium levels and providing antioxidant protection (Park *et al*., 2021). Moreover, it can suppress the functions of caspase-9, 3, 6, and 7 (Arbab *et al*., 2012), leading to the prevention of apoptosis, extending the

lifespan of cancer cells, and inducing the malignant conversion of cells (Qian *et al.*, 2022). It has been reported that the upsurge in (ROS) levels, along with the resulting genotoxicity, can lead to chromosomal injury in peripheral blood cells (Ladeira *et al.*, 2011; Santovito *et al.*, 2011). Moreover, it has been observed that highly exposed workers in pathology and anatomy laboratories exhibit a greater incidence of chromosomal damage resulting in micro-nucleated lymphocytes compared to normal persons. This disparity is believed to stem from an increased rate of chromosome loss, indicating that FA may cause abnormalities in the mitotic divisions and hence the development of cancer as leukaemia (Orsière *et al.*, 2006). (Zhang *et al.*, 2013). Previous studies examined the possible genotoxic carcinogenic impacts of FA in both rats (Gomaa *et al.*, 2012) and (El-Nagdy *et al.*, 2020) and humans by using Comet assay and detected a variable degree of DNA damage in the form of increased tail length (Gomaa *et al.*, 2012) and DNA hydroxymethyl mono-adducts and methylene crosslinks (Kawanishi *et al.*, 2014).

Conclusion:

The study highlighted in this research indicates that prolonged exposure to FA can lead to detrimental effects on the spleen, causing morphometric alterations, disrupted structural organization, and immunomodulation. These changes were dose-dependent and manifested through necrosis, congestion, and an increase in megakaryocytes in the spleen. Overall, the findings underscore the complex and multifaceted impact of FA exposure on immune responses, cellular functions, and potential implications for health, particularly in relation to cancer development and genetic damage. Further research is warranted to elucidate the mechanisms underlying FA-induced toxicity and develop strategies to mitigate its adverse effects on human health.

Limitations and the Need for Further Research:

The current study on FA's effects on the spleen, while thought-inspiring, has limitations. Most studies rely on animal models with high-dose exposures, making it difficult to generalize the findings directly to human health at lower exposure levels. Moreover, many studies focus on short-term exposures, and the long-term outcomes of chronic low-level exposure remain largely unclear. Furthermore, there's a lack of research on specific immune cell populations. A deeper understanding of which cell types are most susceptible is necessary. Also, the individual's susceptibility to FA exposure based on factors like genetics or pre-existing health conditions is unspecified. These are crucial questions that need to be addressed. It's important to pursue additional research to deepen our knowledge of how FA affects the spleen. It's vital to conduct studies where human subjects are exposed to FA at levels commonly found in residential and occupational environments, to observe its impact on spleen functionality and immune cells. Inquiries about whether such exposure could increase the risk of infections or autoimmune diseases require long-term studies involving human subjects. Additionally, exploring the exact mechanisms by which FA affects the spleen is still a query. Research should also investigate the vulnerability of various individuals to the harmful impacts of FA on the spleen. Through addressing these research gaps, we aim to achieve a clearer picture of the potential effects of FA exposure on the spleen and overall immune function.

Declarations:

Ethics Approval: The Ethics Committee for Animal Experimentation guidelines at Ain Shams University's College of Medicine approved the research protocol and the animal studies (Approval number: Code FMASU R 118/2023). The NIH Guide for the Care and Use of Laboratory Animals was followed

throughout the handling and scarification of the animals.

Conflict of Interest: The authors declare that they have no conflict of interest.

Author contribution: Dr. Sayed M. El-Sayed, Eyad M.T. Ali and Hesham I. Abdallah: Conceptualization, project administration, data analysis, investigation, validation, visualization, writing original draft, and writing-review and editing. Dr. Sayed M. El-Sayed and Eyad M.T. Ali: data curation, formal analysis, tabulation investigation, validation, visualization, writing and editing.

Data availability statement: Data related to this research can be obtained from the author based on appropriate request.

Funding Information: Self-funded.

Acknowledgment: The author is grateful to all laboratory technicians for their efforts and time.

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ARABIC SUMMARY

استنشاق الفورمالديهايد يستحث التغيرات النسيجية والنسيجية الكيميائية المناعية في طحال ذكور الجرذان البيضاء

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يستخدم الفورمالديهايد (FA) على نطاق واسع في العديد من الصناعات والتطبيقات الطبية، ويعتبر العاملون في مجال الرعاية الصحية ومدربو التشريح وطلاب الطب من الأشخاص الأكثر عرضة للخطر. يتسبب الفورمالديهايد في حدوث تغييرات في جهاز المناعة مثل الاضطرابات المناعية وايضا كبت المناعة مما يؤدي بالتالي إلى زيادة تطور السرطان أو الحساسية. تحتم المناقشات الجارية حول سمية الطحال الناجمة عن الفورمالديهايد تحليلا إضافيا. ولذلك، فقد قمنا بدراسة التأثيرات السامة المحتملة للفورمالديهايد والتغيرات النسيجية والكيميائية المناعية والشكلية اللاحقة على الطحال. تم تقسيم أربعين من الجرذان الذكور البيضاء البالغة إلى أربع مجموعات؛ المجموعة الضابطة الاولى، المجموعات التجريبية الثانية والثالثة والرابعة والتي تعرضت لاستنشاق 10% فورمالديهايد لمدة 18 أسبوع بجرعات متفاوتة. عولجت عينات الطحال وصبغت باستخدام الهيماتوكسيلين والأيوسين، والصبغ المناعي لـCD4 ، CD8 وBcl-2. ولقد أظهرت جميع المجموعات التجريبية ان النسيج الضام المغلف للطحال أصبح سمكًا أكثر مع وجود جيوب دموية محتقنه ومتوسعة، ولقد أظهرت المجموعة الثانية خللاً كبيراً في التركيب النسيجي، مع وجود تحلل بالخلايا اللمفاوية حيث ظهرت بنواة منكسة وسيتوبلازم مفرغ، وأظهرت بعض الاكياس مراكز جرثومية ميتة. وكانت هذه النتائج أكثر وضوحا في المجموعتين الثالثة والرابعة. بالإضافة إلى ذلك، قدمت المجموعتان الثالثة والرابعة نزيف الاوعية الجيوبية وتسلل الخلايا المكروية. أظهرت المجموعات التجريبية زيادة ملحوظة في التعبير المناعي الإيجابي لـ CD4 و CD8وBcl-2. انخفضت قياسات كلا من اللب الأبيض والمركز الجرثومي في المجموعات التجريبية، كما أظهرت المجموعتان (الثانية والثالثة) زيادة في قطر المنطقة الحدية، في حين أظهرت المجموعة (الرابعة) انخفاضا فيه. انخفضت أقطار طبقة الوشاح وPALS في المجموعة الثانية، ثم زادت في المجموعتين الثالثة والرابعة. ومن هنا يتضح أن الفورمالديهايد يسبب تأثيرات ضارة على الطحال من خلال تنظيم CD4 وCD8 وBcl-2 اعتمادا على الجرعة المعطاة.