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Histological and Immunohistochemical Study of the Protective Effect of Virgin Coconut Oil on Cyclophosphamide-induced Immunotoxicity of the Spleen and Peyer's Patches

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Abstract---Introduction: Virgin coconut oil (VCO) is an edible oil extracted from the meat of mature coconuts. It is also known for its health and therapeutic benefits, mainly attributed to its polyphenols

and medium-chain fatty acid contents the immunomodulatory effects of VCO have not been extensively investigated. The aim of the present study was to investigate the effects of VCO on cyclophosphamide (CY)-induced immunotoxicity of peripheral lymphoid tissues. **Methods:** Forty Wistar rats were divided into five groups of eight rats each. Group 1 served as the normal control, while the other groups were all given 10 mg/kg of CY orally once daily for 4 weeks. Group 2 received CY served as the negative control. Group 3 rats were treated with levamisole (LMS) for 6 weeks, while Groups 4A and 4B were given VCO at 10 mL/kg and 15 mL/kg, respectively, for 6 weeks. The spleen and ileum sections were subjected to routine histological examination and immunohistochemical evaluation for T and B lymphocytes. **Results:** Histologically, the spleen and Peyer's patches (PPs) of the ileum exhibited a significant reduction in the lymphoid cellularity following daily administration of 10 mg/kg CY for 4 weeks.

Keywords---cyclophosphamide, immunotoxicity, levamisole, Peyer's patches, spleen, virgin coconut oil.

Introduction

Immunodeficiency or immunosuppression is a pathological condition in which the function of the immune system is weakened. The normally operative immune system is important to producing appropriate adaptive immune responses, namely the humoral and cell-mediated immune responses. When an individual's immune response is depressed, that person is said to be immunocompromised and is susceptible to opportunistic infections and probably malignancies (1). Immunosuppressive therapy has been commonly used since the beginning of the 20th century to treat autoimmune disorders and to avoid organ transplant rejection and graft-versus-host diseases (2). Cyclophosphamide (CY) is one of the most harmful alkylating agents that can induce oxidative stress due to the overproduction of reactive oxygen species (ROS) (3). The use of CY and other cytotoxic chemotherapeutic drugs is concomitant with immunotoxicity that can raise the danger of immunosuppression-related complications in patients treated with these drugs, induce undesirable side effects in multiple organ systems, and lead to histological changes in lymphoid tissues (4). The occurrence of these side effects results in reduced immunity, emotional distress, negative quality of life, and reduced compliance with treatment regimens (5).

The lymphoid tissues, which are the primary sites for differentiation and proliferation of cells of the immune system, are among the first affected by ROS-mediated injury, predominantly during treatment with chemotherapeutic drugs (6,7). Injury to these tissues often causes the individual to become immunocompromised and predisposed to contract opportunistic infections. The synchronous administration of antioxidants during chemotherapeutic treatment remains controversial, and considerable scientific arguments regarding this issue have continued into the current decade 8. Antioxidant supplementation during a specific treatment with chemotherapy and/or radiotherapy has potential benefits

since it can decrease the occurrence of side effects and their severity, increase a patient's survival rate, and promote a tumor response (9).

Modulation of the immune system by various medicinal plants and their products has become an accepted supplementary therapeutic approach. As a result, several medicinal plant extracts and their immunomodulation of immune responses have become an adjunct to conventional immunosuppressive therapies for a variety of disease conditions due to their potential benefits in reducing or overcoming the adverse effects 10. One such natural product is virgin coconut oil (VCO). Presently, VCO is achieving extensive acceptance in the scientific and medical community. VCO has been shown to possess antihypertensive, antimicrobial, and anti-inflammatory properties 11. Additionally, VCO has been clinically established to be an effective moisturizing agent for patients with atopic dermatitis due to its anti-inflammatory and anti-infective activity, as well as being a protective skin barrier 12. Currently, clinical trials are underway to investigate the cardiovascular protective effect, quality of life enhancement, and other beneficial health effects of VCO and other plant-derived antioxidants (13,14).

Recently, the authors demonstrated that supplementation with 10 mL/kg and 15 mL/kg of VCO to adult rats treated with CY showed evidence of restoration for both the thymus lymphoid architecture and the total white cell counts, absolute lymphocyte counts, and plasma globulin levels, as compared to rats treated with CY alone¹⁵. The present histological and immunohistochemical study was conducted to investigate the protective effects of VCO against CY-induced degenerative changes in the spleen and Peyer's patches (PPs) of Wistar rats.

Methodology

Virgin coconut oil (VCO)

VCO was acquired from the Malaysian Agricultural Research and Development Institute (MARDI) (Selangor, Malaysia). It was produced through the cold pressed method using MARDI's technology¹⁶. The white meat of the coconut was scraped and mixed with a sufficient quantity of high purity water. The drenched scraped meat was then mechanically compressed to yield coconut milk. The coconut milk was left in a sterile dish at room temperature for 24 hours. Following which, the oil layer on the top was removed and centrifuged. This step allowed for sedimentation of fine particles and clarification of the oil.

Cyclophosphamide (CY)

Cyclophosphamide monohydrate was purchased from Merck Sdn. Bhd. One gram of CY was dissolved in 1 L of sterile water for injection and placed on a magnetic stirrer for 5 minutes to produce a 1 mg/mL solution. The solution was preserved in the refrigerator at -2 to -4°C.

Levamisole (LMS)

Levamisole HCl was purchased from Asia Foresight-Care Group Limited & Xi'an Wango Biopharma Co., LTD. A solution of levamisole (LMS) (1 mg/mL) was prepared by dissolving 1 g of LMS HCl in 1 L of sterile water and mixing on

magnetic stirrer for 5 minutes. The produced solution was stored in the refrigerator at -2 to -4°C .

Animals & experimental design

Forty rats were randomly divided into five groups. Each group consisted of 8 rats with an approximately equal mean body weight of 160–170 g. Two rats were housed in each cage under standard experimental conditions consisting of a temperature of 25°C and a 12-hour light and 12-hour dark cycle each day. The rats were fed with rat chow and distilled water ad libitum. The rats were acclimatized for seven days prior to the experiment. Group 1, which served as the normal control, was given distilled water by oral gavage at 5 mL/kg once daily. Group 2, which served as the negative control, received CY at 10 mg/kg/day via gavage once daily for 4 weeks. Group 3 received CY at 10 mg/kg/day for 4 weeks and LMS at 3 mg/kg/day for 6 weeks. Group 4 rats were subdivided into two subgroups, namely 4a and 4b. Each subgroup received CY at 10 mg/kg/day for 4 weeks and VCO for 6 weeks at two different doses, 10 mL/kg for rats in Group 4a and 15 mL/kg for rats in Group 4b. The summary of this study protocol is illustrated in the flowchart of the study below (Figure 1). Subsequently, at the end of the experimental period, all rats were sacrificed, and the spleen and ileum (containing PPs) were rapidly collected and fixed in 10% formal saline for 72 hours at room temperature and processed for light microscopy. Sections of 5 μm thick sections were stained with hematoxylin and eosin (H&E) for immunohistochemical staining of T and B lymphocytes. All data of PP counts in various rat groups were analyzed using the Student's t-test and ANOVA. A p value of <0.05 was considered to be statistically significant.

Immunohistochemistry for CD3 and CD20

With optimum concentrations of primary antibodies and optimum incubation time, rabbit polyclonal antiCD3 and antiCD20 antibodies bind to the CD3 surface antigen of rat T-lymphocytes and the CD20 surface antigen of rat B lymphocytes, respectively. For chromogenic recognition, the primary antibodies derived from rabbit serum are conjugated to enzymes, namely horseradish peroxidase, which converts 3, 3'-diaminobenzidine (DAB) into brown end-products. The immunohistochemical staining was done according to the method of Fritschy (17).

Slide analysis

Detailed microscopic examinations of spleen and PP tissue sections were conducted in accordance with the best practice parameter for the routine pathology assessment of the lymphoid tissues by the Society of Toxicological Pathology 18. In brief, any microscopic alteration in the lymphoid tissue architecture, which holds a specific function, was described. Images that showed substantial histological alterations from the control group were examined and analyzed by two qualified histopathologists who were blinded to the study groups.

Results

Histology of the spleen

Group 1: water only: Splenic sections of rats from Group 1 showed normal splenic architecture surrounded by a connective tissue capsule. At low magnification, the parenchyma consisted of white pulp and red pulp (Figure 2A). The components of white pulp, e.g., follicles, periarteriolar lymphatic sheath (PALS), and the marginal zone, were present (Figure 2B). Germinal centers were observed in the follicles. Splenic cords and venous sinuses of the red pulp were very well illustrated. CD3-positive lymphocytes, indicating T cells, were seen in the PALS, with scattered positivity in the lymphoid follicles and the red pulp (Figure 2C). The B cells, as indicated by CD20 positivity, were seen mostly in the lymphoid follicle areas (Figure 2D).

Group 2: A splenic section of Group 2 rats (10 mg/kg of CY) once daily for 4 weeks) revealed abnormal architecture. The trabeculae appeared to be thickened (Figure 2E). At low magnification, the follicles were markedly diminished in size and number. There were many focal hemorrhagic areas in the red and white pulps, as evidenced by the presence of erythrocytes. The venous sinuses were congested. At medium and high magnifications in the white pulp, there was an overall reduction in lymphoid cellularity, particularly in the follicles and the marginal zone. The germinal centers were barely seen, the wall of the central artery was thickened and hyalinized (Figure 2F), and the marginal sinus widened. In the splenic red pulp, there was a marked depletion in lymphocytes with congestion of the venous sinuses. Macrophages were found to be more prominent and widespread throughout the red pulp (Figure 2F). CD3-positive lymphocytes (T cells) were depleted in the PALS follicles and the red pulp as compared to Group 1 (Figure 2G). Also, there was a reduction in the number of CD20-positive B cells in the follicle regions (Figure 2H).

Group 3: CY and LMS: The spleen of rats that received CY and extended treatment of LMS showed a significant restoration in microscopic architecture. At low magnification, the spleen showed an increase in cellularity and the size of the follicle and marginal zone; the germinal centers were present and more prominent, and there were less focal hemorrhagic areas in the red pulp as compared to the CY group (Figure 3A). At high magnification, the germinal centers were present and the thickness of the central artery was noted to be normal (Figure 3B). The population of CD3-positive T cells in the PALS area was noted to be more than that in the CY group. Scattered CD3-positive T cells were seen in the follicle areas and the red pulp (Figure 3C). Increased population of CD20-positive lymphocytes (B cells) in the follicles with scattered positivity in the PALS was noted as compared to the CY group (Figure 3D).

Group 4A: CY and low dose VCO: The splenic sections of Group 4A rats that received CY and very low dose of VCO (10 mL/kg for 6 weeks) showed increased cellularity in the white pulp and red pulp. At low and medium magnifications, the sizes of the follicles and the lymphocyte cellularity of the white pulp were markedly increased as compared to the CY group (Figure 3E). There was evidence of germinal center generation, as indicated by the presence of mitotic figures and

follicular dendritic cells in the white pulp (Figure 3F). The cellularity of the red pulp increased, and there were focal hemorrhagic areas. An increased CD3-positive cell (T lymphocytes) population in the PALS, marginal zones, and red pulp regions was observed as compared to the CY group (Figure 3G). The lymphoid follicles and marginal zones showed increased positivity (Figure 3H).

Group 4B: CY and High Dose VCO: The spleen of rats administered with CY and a high dose of VCO (15 mL/kg) for 6 weeks showed normal capsule and trabeculae. There was a marked increase in lymphoid cellularity in the white and red pulps, including the marginal zones (Figure 4A, 4B). The congestion in the sinusoids was markedly reduced as compared to the CY group (Figure 4A). An increased CD3-positive T cell population in the PALS was noted. There were also increased CD3-positive T cells in the lymphoid follicles and red pulp as compared to groups 2, 3, and 4A (Figure 4C). The number of CD20-positive B cells increased in the follicle regions (Figure 4D).

Histology of Peyer's patches (PPs)

Group 1: water only: The PPs of the terminal ileum from the control group showed normal microscopic architecture. At low magnification, the section was composed of sub-mucosal lymphoid aggregations. The PPs contained follicles with a germinal center, surrounded by interfollicular regions (Figure 4E). There were mitotic figures seen in the germinal center. The apical region of the PP projected into the lumen and was lined by the follicle-associated epithelium. The follicle-associated epithelium was separated from the corona of the follicle by a sub-epithelial dome. The interfollicular regions contained densely packed, small lymphocytes. Small blood vessels, representing the high endothelial venules, were also delineated (Figure 4F). Tingible-body macrophages were seen throughout the follicles. CD3-positive T lymphocytes were seen in the interfollicular regions. Also, scattered CD3-positive T cells were seen in the follicles and lamina propria area (Figure 4G). Strong CD20 positivity was mainly observed in the follicular regions (Figure 4H).

Group 2: CY only: PP sections from group 2 rats treated with CY showed depletion of lymphoid cellularity in the follicles and interfollicular regions as compared to group 1. The subendothelial dome appeared to be widened. There was a marked reduction in the size of the PP, with diminished germinal centers (Figure 5A). At high magnification, the blood vessels appeared congested, with an increased number of macrophages noted in the interfollicular regions (Figure 5B). CD3-positive T cells were seen in the interfollicular regions and lamina propria (Figure 5C), while CD20-positive cells were seen in the diminished lymphoid follicles (Figure 5D).

Group 3: CY and LMS: Conversely, the PPs of rats receiving CY and treatment with LMS showed restoration of PP cellularity and architecture. The cellularity increased (Figure 5E, F), and the blood vessels appeared to be less congested as compared to the CY group. At high magnification, the size of the germinal centers was markedly increased as compared to group 2 (Figure 5F). CD3 positivity was seen in the interfollicular region within the follicles and lamina propria (Figure 5G). The section exhibited scattered moderate positivity with CD20 (Figure 5H).

Group 4A: CY and low dose VCO: The PPs of rats in group 4A (CY and low dose of VCO (10 mL/kg for 6 weeks)) showed improvement in cellularity as compared to CY group. At low magnification, the size of the follicles generally increased, with narrowing of the sub-endothelial dome. The germinal center was very well differentiated and developed (Figure 6A). Restoration of PP cellularity was better viewed at medium and high magnifications (Figure 6B, C). CD3-positive T cells were seen in the interfollicular region (Figure 6C), while increased CD20 positivity was observed in the follicles and germinal centers (Figure 6E, D)

Group 4B: CY and high dose VCO: The PPs of rats in group 4B (CY and high dose of VCO (15 mL/kg for 6 weeks)) showed marked histological restoration. At low and medium magnifications, the follicle size and cellularity were markedly increased as compared to other treatment groups (Figure 6E, F). Increased CD3 and CD 20 positivity were seen in the inter-follicular and follicular regions, respectively, as compared Group 4A (Figure 6G). The follicles were positive for CD20-positive B cells (Figure 6H).

Peyer's patches (PP) count

The terminal ileum of Group 1 rats showed the highest mean PP count (6.5 ± 0.8), while Group 2 showed the lowest (3.2 ± 1.2). Generally, rats that received CY showed a significant reduction in PP count, except in rats who received CY and 15 mL/kg of VCO (4.8 ± 0.5) as compared to normal control rats.

Discussion

The present histological and immunohistochemical study has demonstrated that VCO had preventative effects against CY-induced histological changes in lymphoid tissues of Wistar rats. In the spleen, the VCO treated groups displayed increased lymphocytic cellularity in the white pulps when compared to the CY group. The PPs of the group administered VCO displayed increased follicle size, cellularity, and germinal centers in contrast to the group administered CY. These histological findings were further documented by the increase in the PP number. In the present study, CY was used to induce immunotoxicity due to its evident efficiency in doing so from previous studies on experimental animals and humans (19,20). Immunotoxicity reactions induced by CY often manifest themselves as immunosuppression. This immunosuppressive effect of CY has long been considered problematic in treating patients with cancer 21. This effect has been closely linked to the production of free radicals that induce oxidative stress and lead to injury of healthy and highly proliferative cells and tissues, particularly the lymphoid tissues that serve an immune-protective function (22). Subsequent to the immunocompromised state, these patients are liable to numerous infections that are concomitant with a higher rate of mortality (1).

The accumulating evidence from the literature powerfully confirms the importance of histologic modification in primary and secondary lymphoid tissues of rodents following CY administration (6,23). Moreover, LMS has been reported to display nonspecific immune stimulation on both cellular and humoral immunity and, hence, it has been applied as a positive control treatment in a number of immunotoxicity studies (24,25). Certain naturally extracted remedies that possess

high antioxidant characteristics were shown to prompt protective effects on CY-induced myelo-suppression and cytotoxicity in lymphoid tissues^{25,26}. As such, in the present investigation, we evaluated the protective effects of VCO against the adverse histological changes of lymphoid tissues of rats administered with CY. Changes in the body weight, full blood count, serum protein levels, serum alanine aminotransferase levels, and renal function tests were evaluated in our study (the authors, not yet published data), in accordance with the standard basic immunotoxicity study recommended by the Society of Toxicologic Pathology (STP) (18).

The splenic histology of the rats of the CY group revealed a prominent reduction in the cellularity of the white pulp, including both the B and T cell areas, while the PP exhibited reduction in the follicle size as well as cellularity, with diminished germinal centers. These observations in the spleen and PP have been documented by earlier studies, demonstrating that CY selectively suppressed proliferation and activation of B cells, T regulatory cells, and NK cells and that B cell repopulation in lymphoid tissues would take a considerable amount of time (27,28). However, B lymphocytes in the spleen and PP appeared to be more sensitive to the action of CY, as determined by the reduced follicle size and inhibition of germinal center development in rodents 29. Also, the PP number was found to be significantly decreased, which is consistent with earlier findings that showed significant degeneration of PP following CY treatment (26,30).

In the current study, VCO supplementation at 10 mL/kg and 15 mL/kg to the CY-treated rats was concomitant with improvement in the histologic structure of the spleen and PP as compared to the group only treated with CY. These favorable effects on lymphoid organs were comparable to those in the group of rats treated with LMS at 3 mg/kg. In fact, the histological improvement in rats receiving the highest dose of VCO could be considered more pronounced than in the group treated with LMS. The protective effects of VCO against the histological changes of CY could be closely ascribed to its polyphenols content since polyphenols have been identified as powerful naturally existing antioxidants (13,31). Another factor that may contribute to the histologic improvement is the medium-chain fatty acid composition of VCO, which has been shown to have immunomodulatory effects, this is an area that deserves further investigation (32).

Conclusion

In conclusion, this study demonstrated the immune-protective effects of VCO against lymphocytotoxicity induced by CY in rat lymphoid tissues. The administration of VCO significantly restored the microscopic architecture of the spleen and PPs in a dose-dependent manner. These effects were comparable if not superior to that of LMS.

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Statement of ethics

The study protocol was revised and approved by the Institutional Animal Care and Use Committee (IACUC), at International Islamic University Malaysia (IIUM). The experiment was conducted according to the Guidelines for the Care and Use of Laboratory Animals of the Kulliyah of Medicine, IIUM.

Conflict of interest statement

The authors have no conflicts of interest to declare

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Author contribution section

All authors discussed the results and contributed to the final manuscript.

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Appendix

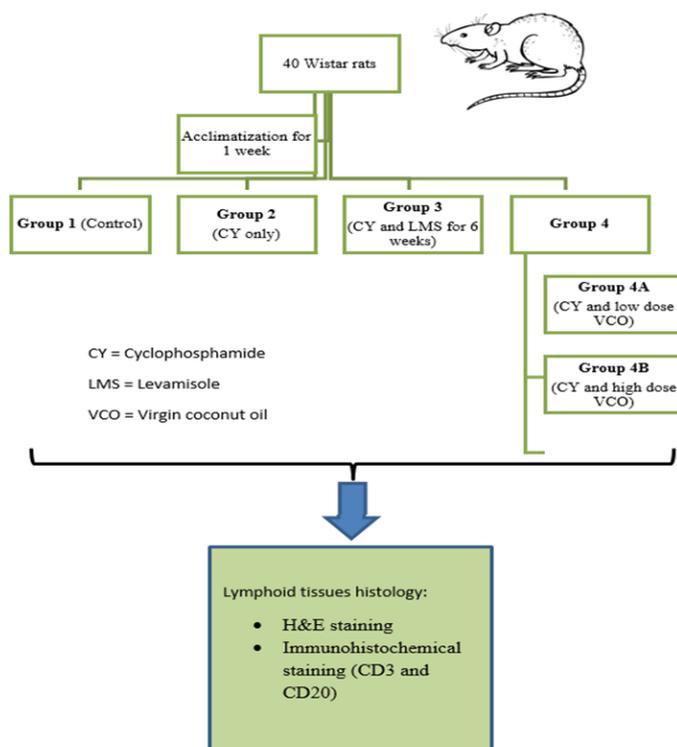


Figure 1. Flowchart of the Study

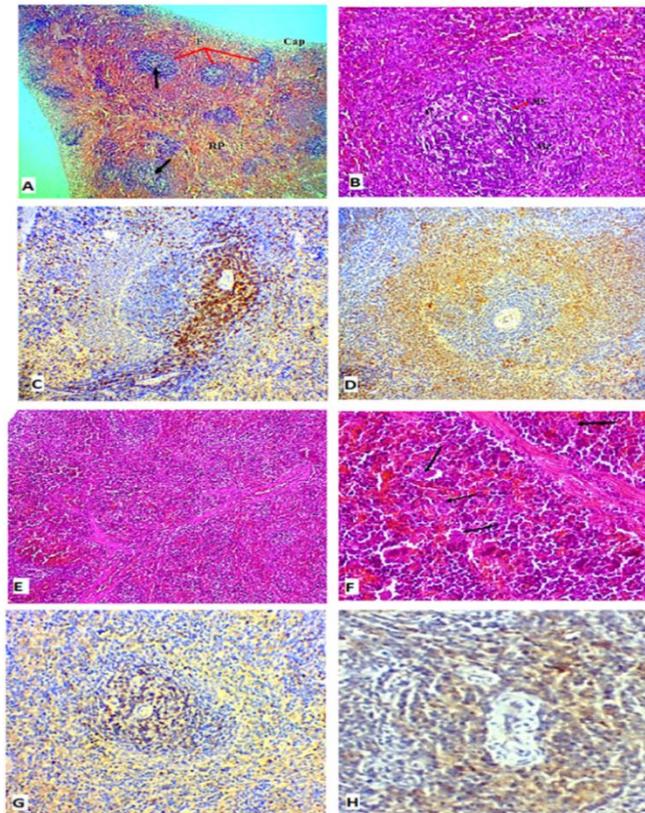


Figure 2. G1 group: Spleen of a normal rat. A) H&E×4. B) H&E ×20. C) CD3 immunohistochemical staining ×10. D) CD20 immunohistochemical staining ×10. Well-developed follicles (F) of the white pulp, central artery (black arrows), red pulp (RP), capsule (Cap), marginal zone (MZ), periarteriolar lymphatic sheath (PALS) germinal centers (GC), marginal sinus (MS) and central artery (CA). G2 group: Spleen of rats that received 10 mg/kg of CY only. E) Thickening of splenic trabeculae, and free erythrocytes (hemorrhage) in the spleen tissues with distended and congested venous sinuses; H&E×10. F) Thickening and hyalinization of trabeculae. There were focal hemorrhagic areas with congested blood vessels, hyalinization, and lymphocyte depletion. Macrophages were more prominent in the red pulp (arrows); H&E ×40. G) CD3; immunohistochemical staining ×10. H) CD20; immunohistochemical staining ×10.

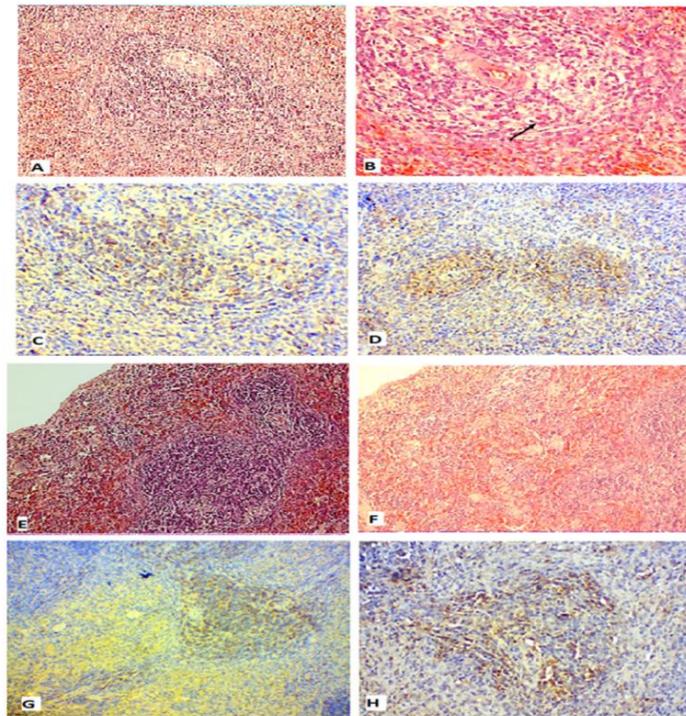


Figure 3. G3 group: The spleen of rats that received 10 mg/kg of CY for 4 weeks and 3 mg/kg of LMS once daily (orally) for 6 weeks. A) Normal architecture of spleen with normal white pulp; red pulp with few focal haemorrhagic areas; H&E $\times 20$. B) Tingible-body macrophages (arrow) were noted; H&E $\times 40$. C) CD3; immunohistochemical staining $\times 10$. D) CD20; immunohistochemical staining $\times 10$. G4 group: The spleen of rats that received 10 mg/kg of CY for 4 weeks and 10 mL/kg of VCO once daily (orally) for 6 weeks. E) Restoration of lymphocytes in the white pulp and the marginal zone. H&E $\times 20$. F) Restoration of lymphocytes in the red pulp. The red pulp contained focal haemorrhagic areas and macrophages. H&E $\times 20$. G) CD3; immunohistochemical staining $\times 10$. H) CD20 immunohistochemical staining $\times 40$.

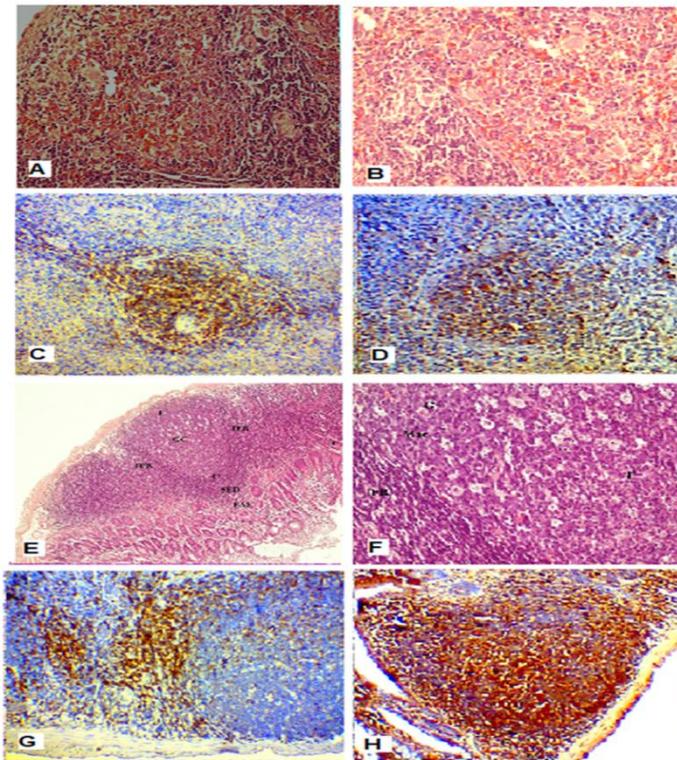


Figure 4. G5 group: The spleen of rats that received 10 mg/kg of CY for 4 weeks and 15 mL/kg of VCO once daily for 6 weeks. A) Normal splenic capsule. Restoration of lymphocytes in the white pulp and the marginal zone. The red pulp contained focal haemorrhagic areas. H&E $\times 20$. B) Macrophages were found in both the white and red pulps (arrows); H&E $\times 20$. C) CD3 immunohistochemical staining $\times 10$. D) CD20 immunohistochemical staining $\times 10$. G1 group: Peyer's patch (PP) of control rats. E) Normal histological architecture. Follicles (F), GC, interfollicular region (IFR), corona (C), subendothelial dome (SED), follicle-associated epithelium (FAE); H&E $\times 10$. F) Various stages of lymphocytes with conspicuous amount of cytoplasm. Numerous follicular dendritic cells seen in the germinal center (red arrows). Few tingible-body macrophages (Mac) were seen; H&E $\times 40$. G) CD3 immunohistochemical staining $\times 10$. H) CD20 immunohistochemical staining $\times 40$.

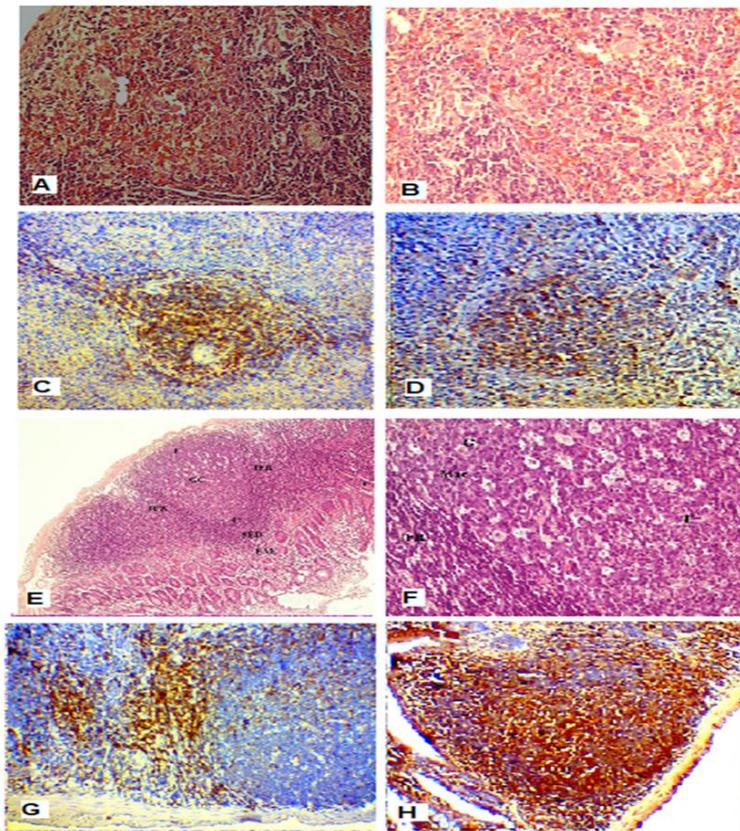


Figure 5. G2 group: PPs of rats that received 10 mg/kg of CY (orally) once daily for 4 weeks. A) Profound reduction in lymphoid cellularity of PPs, with decreased size of the follicles and germinal center (G), IFR, C, and SED; H&E $\times 20$. B) Numerous apoptotic bodies were seen in the germinal center. Macrophages were seen in the germinal center and interfollicular regions. High endothelial venules (HEV); H&E $\times 40$. C) CD3 immunohistochemical staining $\times 10$. D) CD20 immunohistochemical staining $\times 40$. G3 group: PP of a rat that received 10 mg/kg of CY once daily (orally) for 4 weeks and 3 mg/kg of LMS once daily (orally) for 6 weeks. E) Increase in the PP cellularity, follicular size, and its germinal center with congested blood vessels; H&E $\times 10$. F) Restoration of lymphoid cellularity in the follicle and decreased number of congested blood vessels as compared to Group 3A; H&E $\times 20$. G) CD3 immunohistochemical staining $\times 10$. H) CD20 immunohistochemical staining $\times 40$.

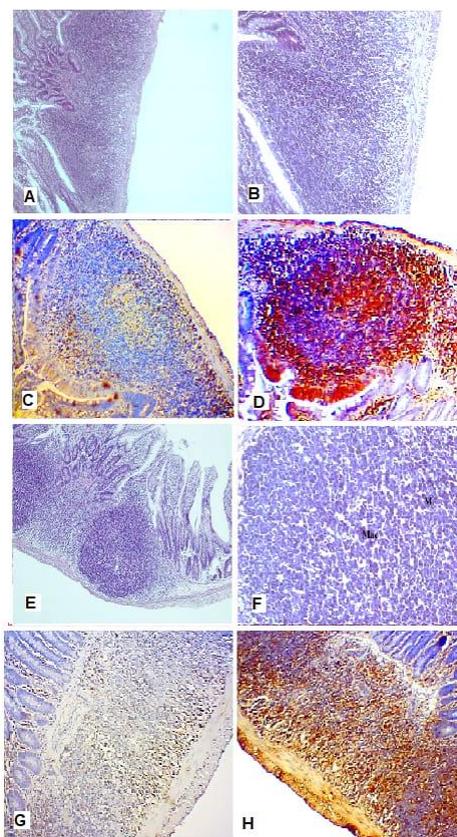


Figure 6. G4 group: PP of a rat receiving 10 mg/kg of CY once daily for 4 weeks and 10 mL/kg of VCO once daily for 6 weeks, showing improvement in the PP cellularity. Restoration of lymphocytes and the size of the follicle. A) H&E $\times 10$. B) H&E $\times 20$. C) CD3 immunohistochemical staining $\times 10$. D) CD20 immunohistochemical staining $\times 40$. G5 group: PP of a rat receiving 15 mg/kg of CY once daily for 4 weeks and 10 mL/kg of VCO once daily for 6 weeks, showing improvement in the PP cellularity. Restoration of lymphocytes and the size of the follicle and germinal center. E) H&E $\times 10$. F) H&E $\times 20$. G) CD3 immunohistochemical staining $\times 10$. H) CD20 immunohistochemical staining $\times 40$.