Electronic Physician (ISSN: 2008-5842) http://www.ephysician.ir
October 2015, Volume: 7, Issue: 6, Pages: 1412-1422, DOI: 10.14661/1412

The protective effect of lemon fruit extract on histopathological changes induced in small intestines and pancreas of male mice by cyclophosphamide

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Type of article: Original

Abstract

Introduction: Cyclophosphamide (CP) is alkylating agent and the most commonly used chemotherapeutic drug for various types of cancer; it causes severe toxicity. The aim of the research was to assess the protective effect of lemon fruit extract (LFE) against the side effects of the anti-cancer drug "cyclophosphamide" (CP).

Methods: This experimental study was conducted in 2015. Thirty male mice were divided into six groups: group A (control): intraperitoneal injection of saline, group B: oral LFE (10ml/kg), group C: intraperitoneal injection of CP (10 mg/kg), group D: intraperitoneal injection of CP (20 mg/kg), group E: intraperitoneal injection of CP (10 mg/kg) and oral LFE (10 ml/kg), and group F: intraperitoneal injection of CP (20 mg/kg) and oral LFE (10 ml/kg). All groups were treated daily for five consecutive days.

Results: The results of the group treated with the drug C and D was that, in their intestines, the effect was uneven between a severe to a sharp effect, and there was a lack of dense connective tissue and its collagen fibers and fat cells, the intestinal glands or crypt of Lieberkühn appeared few in number and distorted in composition when compared with control A, as the pancreas appeared divided into several lobes containing small numbers of pancreatic Acini, padded with secretory pyramid-shaped cells, although some of them appeared exaggerated. While treatment in group E and F resulted in the intestines and pancreas appearing to be semi-normal; regarding the pancreas, it showed an observed improvement more than the response of the intestines.

Conclusion: The results support the protective effect of lemon fruit extract against CP-induced intestinal and pancreatic injury.

Keywords: cyclophosphamide, lemon fruit, histopathological changes, intestines, pancreas, mice

1. Introduction

Cyclophosphamide, $C_7H_{15}Cl_2N_2O_2P$, is an anti-cancer drug used in the treatment of cancer tumors, and it is a part of cancer chemotherapy drugs. Because of its ability to destroy cancer cells and eliminate them and as a Systemic treatment due to the move of the treatment to organs and tissues of the body and thus eliminate all cancer cells wherever they present. CP is classified among drugs with a direct impact on the molecular structure of (DNA) of cancer cells within the category (nitrogen mustard alkylating agent) that adds alkyl set to DNA, which prevents replication of DNA and cancer cell proliferation process (1). CP also is used in the treatment of autoimmune diseases, such as rheumatoid arthritis, lupus erythematosus, vasculitis, scleroderma, and Hodgkin's disease, but scientific studies and experiments revealed that the treatment with CP caused occurrence of a lot of side effects which have negative influence on the lives of therapists by patients as it leads to the development of secondary tumors in different regions of the body especially breast, lung, and bladder (2). It activated the chromosomal aberrations, micronucleus formation and genetic mutations in somatic cells, CP induced cellular toxicity, genotoxicity and mutagenic effects (3). However, CP requires metabolic activation by the hepatic cytochrome P450 system (4). Metabolic conversion of CP leads to the formation of cytotoxic metabolites, acrolein and phosphormide mustard (5). Phosphormide mustard is believed to have anti-tumor effects, whereas, acrolein may be responsible for toxic side effects, including cell death, apoptosis, oncosis, and necrosis (6). These metabolites caused inhibition of

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Received: August 19, 2015, Accepted: September 28, 2015, Published: October 19, 2015
iThenticate screening: October 02, 2015, English editing: October 06, 2015, Quality control: October 15, 2015

DNA, RNA, and protein synthesis and rapid death of divided cells by modification and cross linkage of purine bases in DNA or alkylating nucleophilic sites in DNA, RNA, and proteins, such as –COOH , -NH_2, -SH, and OH_2 (2). Previous studies reported that CP generated reactive oxygen species (ROS), like the hydroxyl radical, hydrogen peroxide, and superoxide anion, and further suppresses the liver's and intestine's antioxidant defense mechanisms (6-8). The mucosa is easily damaged by chemotherapy due to the fact that the small intestinal mucosa renews itself rapidly (9, 10). However, high doses of anticancer drugs can lead to many clinical problems by damaging the intestinal mucosa. These problems include bacterial translocation, diarrhea, and dyskinesia (11). CP has some major toxic side effects, including hematopoietic depression, gastrointestinal toxicity, and hemorrhagic cystitis (12). It also induced severe inflammation of the gastrointestinal tract in mice (13), intestinal mucosal injury in rats (14), and intestinal toxicity in mice (8, 15). In a study conducted by Reddy (16), it was concluded that the predominant immunolabeling of caspase-3 in intra-islet macrophages during cyclophosphamide-accelerated diabetes in the NOD mouse suggested that apoptosis of macrophages may be an important mechanism for its elimination. Even during heightened beta cell loss, the absence of caspase-3 immunolabeling in most beta cells indicates that they are rapidly eliminated following their death during insulin-dependent diabetes mellitus. The 3-week and 12-week old male NOD mice had apoptotic beta cells in the section of the pancreas that was harvested. This was observed from 8 h through 14 days after a single intraperitoneal injection of cyclophosphamide (17). Some studies have suggested using antioxidant agents during chemotherapy due to their ability to reduce cellular toxicity and damage in DNA in normal tissues as a result of this treatment (18); they also create scavenger of free radicals, preventing it from interacting with cellular molecules of DNA (19). The easiest means of getting such protection is in the exposure to natural inhibitors, especially through food (20, 21), so, increasing trends emerged in recent years to find natural elements present in food or in other natural sources that possess protective effects against environmental mutagens/carcinogens and endogenous mutagens (19). Myrcene is present in lemon grass (Cymbopogoncitratus), and it is a very potent analgesic substance and might be an alternative to the already-available analgesic drugs. There was no indication for induced cytotoxicity. However, myrcene reduced the sister chromatid exchanges (SCEs)-inducing effect of cyclophosphamide in human lymphocytes in a dose-dependent manner and also reduced the toxic and mutagenic effect of cyclophosphamide in V79 cells. The in vitro results showed that myrcene is not mutagenic in mammalian cells; rather, it has anti-mutagenic properties (22). The aberrant migration of leukocytes is thought to contribute to the pathogenesis of inflammatory bowel disease (IBD). Lemon grass, which is a natural herb that contains citral, inhibits the formation of retinoic acid, thereby suppressing the lymphocyte expression of gut-homing molecules. Thus, in chronic ileitis, lemon grass can be used to counter the excess migration of leukocytes to the inflamed intestine. The action of lemon grass is attributed to its ability to decrease then migration of lymphocytes by inhibiting beta 7-expression. Thus, it is useful for the therapeutic treatment of IBD (23). The compound limonene, which exists in citrus fruit, has anti-bacterial, anti-viral, anti-feedant, anti-nociceptive, anti-inflammatory, and anti-carcinogenic activities. However, its low bioavailability limits its clinical use. Thus, it is very important to determine the mechanism by which it is absorbed in the intestines and to formulate a dosage form to enhance the clinical use of limonene (24). Potential candidates for the treatment of cancer include chemicals that can interfere with the metabolism of reactive oxygen species. It has been found that the glucosides of citrus limonene can inhibit the endogenous generation of reactive oxygen species. Limonene in combination with cyclophosphamide was not found to inhibit the cyclophosphamide-induced tumor regression through a reduced mitotic index of tumor xenograft cells when compared to treatment with cyclophosphamide alone. Both in vitro and in vivo results suggested that limonene could be beneficial for breast cancer patients undergoing chemotherapy (25). Lemonmyrtle has been assessed for its potential cytoprotective properties. As measured by the cellular antioxidant activity (CAA) assay, all native herbs exhibited greater cellular antioxidant activity than bay leaf. They also reduced the hydrogen peroxide (H_2O_2)-induced death of hepatocellular carcinoma and the proliferation of cancer cells in the colon, stomach, and bladder. The results indicated that there was no direct DNA damage to adenocarcinoma cells in the colon as a result of treatment with lemon extracts (26). In general, flavonoids have very good antioxidant activity and can be used to treat many chronic diseases, including diabetes mellitus. At the concentrations that were tested (5-20 μM), flavonoids showed no cytotoxic effects. Also, the tests indicated that they had no negative insulinotropic effects (27). Lemon verbena (Aloysiatriphylla) is an herbal tea that is consumed by many people. It contains significant amounts of polyphenols, such as flavone diglucuronides and phenylpropanoid glycosides. The infusion of lemon verbena in rats provides significant beneficial effects against dextran sodium sulfate (DSS)-induced inflammation of the colon. These results showed that colonic inflammation did not affect the intestinal absorption and urinary excretion of lemon verbena flavone diglucuronides (28). The present work was conducted to study the protective effects of lemon fruit extract on histopathological changes induced in small intestines and pancreas of male mice by the anticancer drug, cyclophosphamide.

2. Material and Methods

2.1. Materials

1) Cyclophosphamide is known commercially as Endoxan, and it was purchased from Baxter Oncology, Halle, Germany, and dissolved in saline solution.
2) Lemon fruit extracts: The lemon fruits were obtained from Jeddah market, Saudi Arabia. They were washed with distilled water, cut into small pieces, ground in a mixer (Moulinex type 753) and then introduced for the tested animals.

2.2. Animals

Swiss albino male mice (Musmusculus 2n = 40) MFI strain, 8-9 weeks old, weighting 30 ± 3 g, which were obtained from the animal house at the King Fahad Medical Center at King Abdulaziz University in Jeddah. The animals were housed in polyplastic cages with steel wire tops in an air conditioned room (22 ± 1 °C, 45-75% relative humidity) with 12 h light/12 h dark cycles. Food and water were provided ad libitum.

2.3. Methods

Thirty male mice were divided into six groups (each containing five mice) as follows: group A(control): intraperitoneal injection of saline, group B: oral LFE (10ml/kg), group C: intraperitoneal injection of CP (10 mg/kg), group D: intraperitoneal injection of CP (20 mg/kg), group E: intraperitoneal injection of CP (10 mg/kg) and oral LFE (10 ml/kg), group F: intraperitoneal injection of CP (20 mg/kg) and oral LFE (10 ml/kg).

2.4. Animal treatment

CP was introduced by intraperitoneal injection as recommended by Anton (29). LFE was given by oral intubation as reported by Sakr et al. (26). All groups were treated daily for five consecutive days (30). The mice were killed 24 h after the last dose.

2.5. Tissue procurement

Upon the injection, the mice were killed and dissected, and the intestines and pancreas were used for histological examination.

2.6. Histology

The tissues were fixed overnight in 10% buffered neutral formalin processed to paraffin wax, sectioned at 5 mM, and stained with haematoxylin and eosin (H+E) (Mallory , 1900) for examination by light microscopy (Olympus BX51).

3. Results

3.1. Group A and Group B

Control mice (group A), Figure 1 and Figure 2, demonstrated normal histological findings in both tissues (intestine and pancreas). In lemon fruit extract mice (group B), the intestines were so similar to the control, while, in a few areas, the end of the vertical villi were degenerated (Figure 3). The pancreas was almost normal as in the control group (Figure 4).

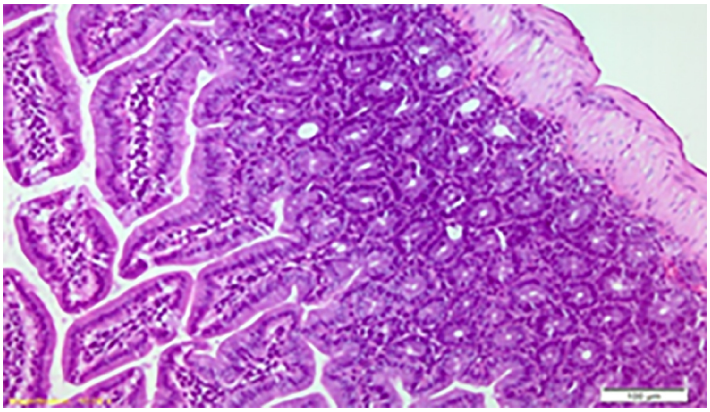

Figure 1. Transverse section in the intestine of a mouse of control group showing normal structure H&E x200

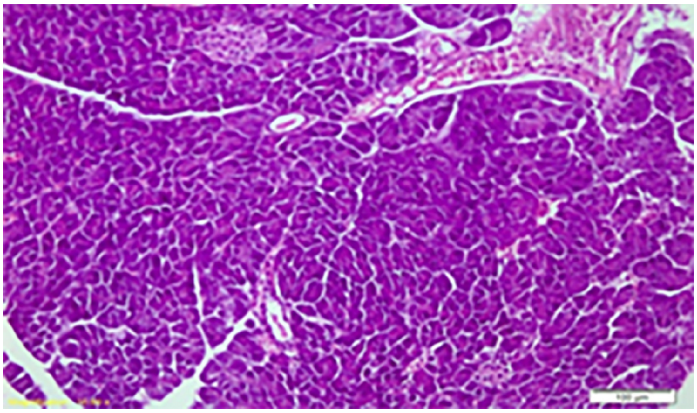

Figure 2. Transverse section in the pancreas of a mouse of control group showing normal structure H&E x200

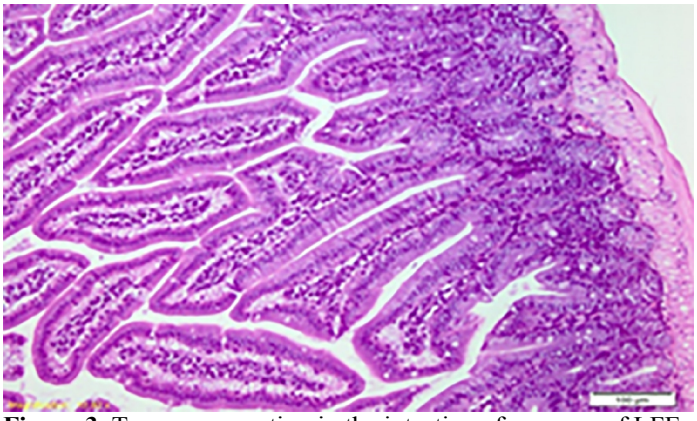

Figure 3. Transverse section in the intestine of a mouse of LFE group H&E x200

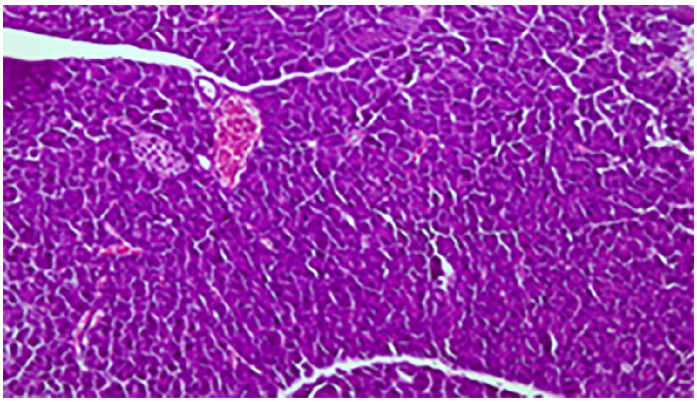

Figure 4. Transverse section in the pancreas of a mouse of LFE group H&E x200

3.2. Group C

3.2.1. Intestine

The effect was between hard to severe. There was a notable reduction in the size of all layers. The muscular layer of intestines smooth muscles appeared to be a narrow tape of circular muscles followed by a small layer of longitudinal muscles. It looked deformed so that one could not discern its fusiform shape. It was also unorganized with its nuclei scattered and with the absence of the connective tissue that separates the two layers. Concerning the sub-mucosal layer, there was no thick connective tissue with what it contains of collagen fibers and adipocytes, as it is in the control, there was a reduction in the duodenum tubal glandules number as well as being severely disfigured; as the glandule cavity was gone with some of them getting darker and others pale, they lost their normal structure, their lining cells were ball-shaped with grainy cytoplasm and central winding wall nucleus with chromatin on its outer edge. Some of them were completely degenerated, which explains the absence of the glandule cavity for it was filled

with cellular debris and as a result for the cells bulge with the absence and rarity of the collective tissue that forms the submucosal layer. Some muscular fibers also were found away from the mucosal muscles; at the same time, it was observed that the connective tissue was rare in the mucosal layer as well as the spread of lymphocytes in it and the intervillous space. The gastric glands or crypts of Liberkuhn were few and disfigured as most of its cells were degenerated; while vertical villi were short and compressed with no brush border and sticking together. They also lost the gaps between them as a result of the degenerated and necrosis of their cells; the cells that appeared to be with scattered nucleuses and wide spaces making it hard to distinguish them from the widely-spread goblet cells with no nuclei (Figure 5).

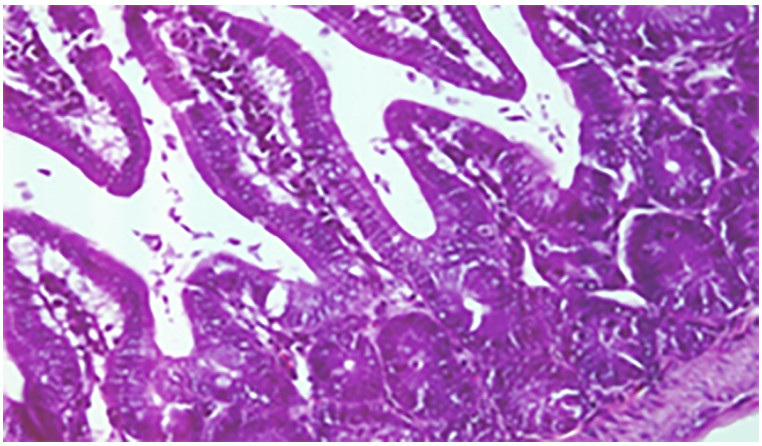

Figure 5. Transverse section in the intestine of a mouse of CP group (10 mg/kg) H&E x400

3.2.2. Pancreas

It was divided into many lobes containing a few of pancreatic acinis lined with pyramid-shaped secretary cells; though some of them appeared to be distending as those cells resulted from the inflammation while some others were just fine with granules producing enzymes. In the upper part, there was a thin layer of connective tissue separating the lobes, though being less than the Control. Canals were disordered lined with decomposed vertical cells. The Langerhans cell were few, tiny, and degenerated; which lead to examining its acervate shape and discovering it contained spaces as a result of degeneration; especially the basic beta cells which compose the biggest part of Islets of Langerhans, around 60% as in control (Figure 6).

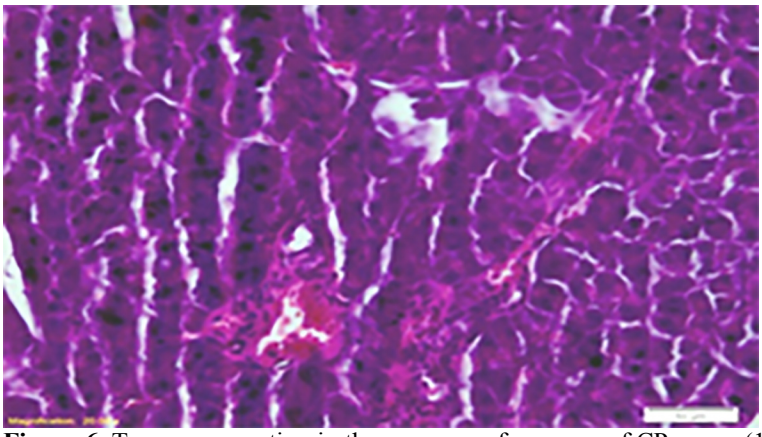

Figure 6. Transverse section in the pancreas of a mouse of CP group (10 mg/kg) H&E x200

3.3. Group D

3.3.1. Intestine

It was severely affected, where areas included the muscular layer was very thin. Also submucosal layer, a dense connective tissue as a thin layer between the duodenum glands that appeared to be accumulated for its higher number than the last one, while lower than the control; though some of them were with degenerated cells and others

were with bloated cells leading to narrowing the cavity. However, they still maintained the pyramid-shape and the basic nuclei and they were still based on a basement membrane with some degenerated cells through it. Some of them had their cells damaged and became a cellular mass with hemorrhage containing only red cells with no trace of any white ones. While the connective tissue of mucosal layer spreads as a thin layer inside the villi cavity, it contained many lymphocyte cells in a less rate than the last one. Crypts of Liberkuhn were darker stain and disfigured with the goblet cells in them. The villi were lined with columnar epithelium cells without brush border and that were hyperplasia that they became accumulated leading to the blocking some of the cavities. The goblet cells rate was near to normal (Figure 7).

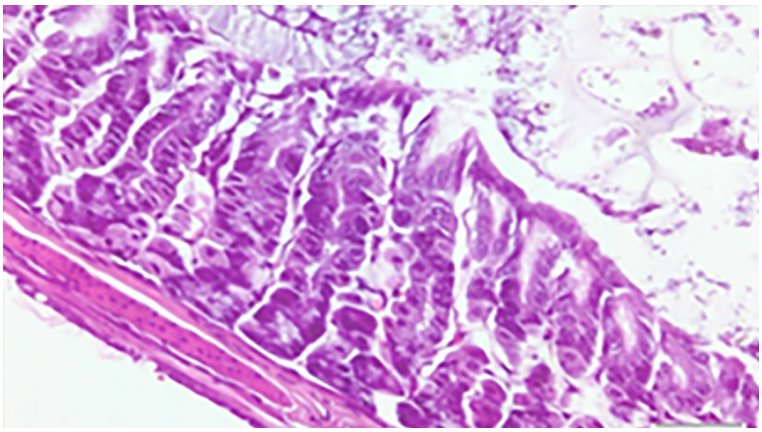

Figure 7. Transverse section in the intestine of a mouse of CP group (20 mg/kg) H&E x200

3.3.2. Pancreas

The drug affect was severe upon the pancreas. As the lobes production was not as obvious as that of the control; as it was like a scattered nuclei as a cellular blending. That may has been the result of the collective tissue absence; the tissue that goes through the pancreas diving it to lobes. There were also some indistinct acini; as their secretive cells lost their pyramid-shape; also, most of them were degenerated and became cellular debris within the tissue. Isles of Langerhans were degenerated and were replaced with gaps inside the tissue. There was also a notable degeneration of the secretive canals as well as a hemorrhage (Figure 8).

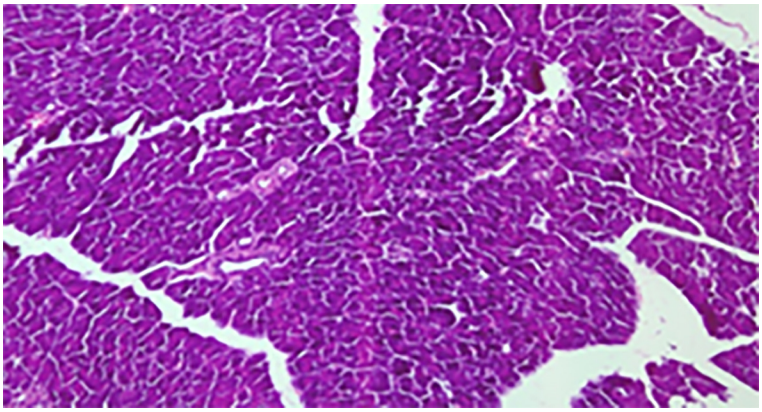

Figure 8. Transverse section in the pancreas of a mouse of CP group (20 mg/kg) H&E x200

3.4. Group E

Intestines were almost normal except the effect on some villi and crypts still degenerated of their cells (Figure 9). The pancreas was normal except that the number and the size of Isles of Langerhans and its cells were less than those of the control group (Figure 10).

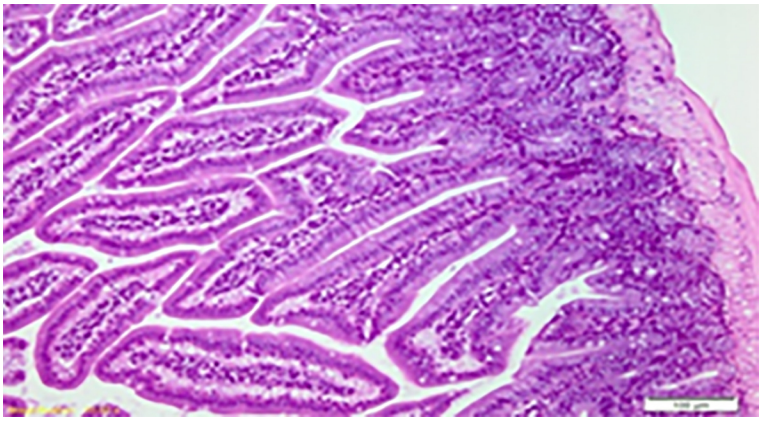

Figure 9. Transverse section in the intestine of a mouse of CP (10 mg/kg) +LFE (10 ml/kg) H&E x400

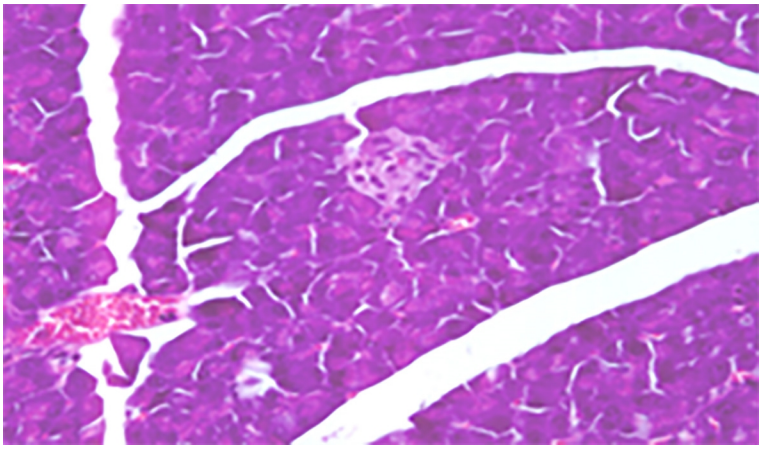

Figure 10. Transverse section in the pancreas of a mouse of CP (10 mg/kg) +LFE (10 ml/kg)

3.5. Group F

Though the lower severity impact, the intestines were still affected by the drug. That was apparent in the retraction and decomposition of the cells, the connective tissue reduction, and the disfiguration of the Liberkuhncrypt as well as the muscular layer (Figure 11). However, for the pancreas, it was improved more than the intestines; but still, it suffers from degeneration and necrosis of many of the pancreatic lobes and the cells of Langerhans (Figure 12).

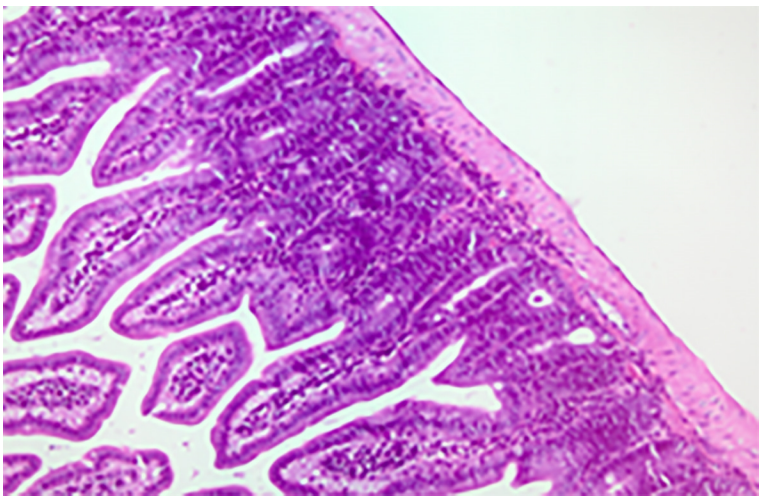

Figure 11. Transverse section in the intestine of a mouse of CP (20 mg/kg) +LFE (10 ml/kg) H&E x400

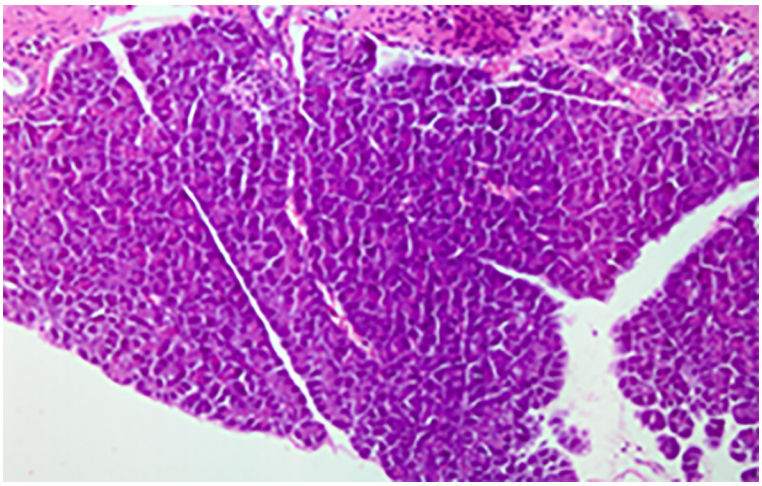

Figure 12. Transverse section in the pancreas of a mouse of CP (20 mg/kg) +LFE (10 ml/kg) H&E x400

4. Discussion

In this study, we examined the histopathological status of these tissues to determine the toxic effect of CP and possible protective roles of LFE in intestine and pancreas of healthy male mice. CP, which is a cytotoxic alkylating agent, is used extensively as an anti-neoplastic agent in the treatment of various cancers; however, several adverse effects limit its full clinical utility. These effects include intestinal toxicity (14, 15). It is believed that free radicals generated in the tissues constitute the biochemical basis of the toxicity of CP. The mechanisms involved in CP's causing damage to the intestines are not well understood. However, CP treatment has been associated with the generation of free radicals and ROS, which produce oxidative stress (8, 14, 15). Free radicals can be scavenged by antioxidant substances. Various enzyme systems, such as SOD, CAT, and antioxidant substances such as reduced GSH, are involved in the antioxidant defense system of the human body (31). In addition, oxidative stress occurs when oxidative substances disturb the oxidant-antioxidant balance and cause oxidative damage to deoxyribonucleic acid, proteins, and lipids (32, 33). GSH is vitally important for protecting cells against oxidative injury. CP treatment can deplete the GSH level, which may be due to the direct conjugation of metabolites of CP, acrolein with GSH, producing oxidative stress (34). The depletion of GSH lowers the cells' defense against injury induced by free radicals, and necrotic cell death is the result (35). The results of our study clearly demonstrated that treatment with cyclophosphamide revealed several histopathological alterations in intestine of male mice such as severe damage to the lengths of the villi were markedly reduced and the crypt of Liberkuhn were few and disfigured as most of its cells were degenerated. These results are in agreement with many investigators who reported that the high dose for 10 days or acute single dose of CP treatment leads to intestinal injury. Sheeja and Kuttan (8) reported severe damage to the intestinal villi and the lengths of the villi were markedly reduced and the crypt architecture was largely destroyed in mice treated with 25 mg/kg for 10 consecutive days of CP. In the same treatment, Hamsa and Kuttan (15) found that the intestinal villi in CP treated group looked blunt, shortened, and eroded; crypts were non-uniform, fused, and the numbers were reduced. Mucous cell necrosis was observed. Owari et al. (14) reported that they treated rats with an acute single dose (300 mg/kg) of CP, causing mucosal atrophy, and the villous height and crypt depth were significantly decreased. The number of apoptotic cells appeared to increase and cell proliferation was markedly decreased following cyclophosphamide administration. However, the results of histological examination of the current study indicated that administration of low and high dose of CP for 5 consecutive days to the male mice caused a few of acinis in the pancreas and there was a thin layer of connective tissue separating the lobes. Canals were disordered lined with degenerated vertical cells. The Langerhans cells were few, tiny, and degenerated. Ready et al. (16) assured that using CP drug harmed the pancreas in diabetes patients, as the connective tissue obviously decreased in pancreas cells as well as apoptotic cells. Using the drug also led to nephrotoxicity, hepatic necrosis and the reduction of white blood cells and platelets in pancreas cancer patients. O'Brien et al. (17) noted that apoptotic beta cells were present within itself of Langerhans after a single dose of CP. There are several reports on the benefits of antioxidants in protecting intestinal tissues from deleterious effects of reactive oxygen species and other free radicals generated during CP treatment. It was found that andrographis paniculata extract reduces CP-induced intestinal toxicity (8) as well as ipomoea obscura (15) and glutamine prevent intestinal mucosal injury (14).

In the present study, it was shown that lemon fruit extract co-administration with cyclophosphamide was effective in protection of the intestines and pancreas. The positive effect of lemon might be because of its containing great deal of polyphenol, such as diglucuronides, flavones, and phenyl glycosides. Verbascoside and lemon are useful for colitis in mice (28). We know that the lemon extracts is anti-genetic mutants, antioxidant, good for colitis treatment, and decreases DNA disfiguring in mice. Lemon contains nutrients that have vital functions with anti-mutant effect. Lemon is considered a powerful antioxidant. It is also known for its ability to remove radicals and its effectiveness in cancer chemical treatment (36) for its ability to stop cellular toxicity and DNA damages (18) as it works as a radical scavenger, preventing them from interacting with DNA cellular molecules (19). Lemon also contains myrsine, which is an effective analgesic that can replace the other available analgesic drugs. This substance, when examined with cyclophosphamide, reduced the drug's effect in the induced human lymphatic cells. It also reduced the toxicity and effects of the cyclophosphamide upon cells. The lab results showed that myrsine had the characteristics of an anti-mutant substance (22). Lemon grass is a natural herb that contains citral, which suppresses lymphocyte expression of gut-homing molecules by inhibiting retinoic acid formation that lemon grass intake could ameliorate excess migration of leukocytes to the inflamed intestine in chronic ileitis, which leads to colon improved significantly (23). There was an improvement in pancreas and its canals after drinking lemon juice. Differences in values before and after drinking the juice were so significant whether in volunteers or patients (37). Limonene in combination with cyclophosphamide was not found to inhibit the cyclophosphamide-induced tumor regression through a reduced mitotic index of tumor xenograft cells when compared to treatment with cyclophosphamide alone. Both in vitro and in vivo results suggested that limonene could be beneficial for breast cancer patients undergoing chemotherapy (25). Lemon juice was considered a potent antioxidant as it contains citrate, flavonoids, vitamin E, vitamin C (38), and limonids (39). As reported, the ability of vitamin C as an anti-clastogeneic and anti-mutageneic agent (40) and as strong antioxidant agent (41) and all the aforementioned vital elements work as scavengers for free radicals and prevent it from destroying cells and tissues.

5. Conclusions

The findings of our study indicated that cyclophosphamide can adversely affect the intestine and pancreas tissues, while lemon fruit extract co-administration could effectively prevent these adverse effects and protect the intestine and pancreas.

Acknowledgments:
The authors are thankful to the King Fahad Medical Center at King Abdulaziz University for providing the animals that were used to conduct this research.

Conflict of Interest:
There is no conflict of interest to be declared.

Authors' contributions:
Both authors contributed to this project and article equally. Both authors read and approved the final manuscript.

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