J. Egypt. Soc. Toxicol.
(Vol. 39: 49-56 July. 2008)
WWW.jest.eg.net

HISTOLOGICAL STUDY ON THE EFFECT OF THYMOQUINONE -AN IMMUNOMODULATOR- ON DUODENUM AND SUPERIOR MESENTERIC LYMPH NODES OF CORTISONE TREATED RATS

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ABSTRACT

Thymoquinone (TQ) was demonstrated as the major active component of *Nigella sativa* seed oil. It was reported that thymoquinone has an anti-inflammatory and immune-stimulatory effects. On the other hand, cortisone is one of the steroid hormones that used as an anti-inflammatory and immune-suppressor drug. In the present study, the effects of both thymoquinone and cortisone were studied on the histological structure of both duodenum and superior mesenteric lymph nodes of rats. It was noticed that there was an increase of the duodenum mucosal mucus-secreting cells and some nuclear changes in the nuclei of the Brunner's gland cells. There was an increase in number of macrophages, plasma cells and lymphocytes in the superior mesenteric lymph nodes. These findings were discussed according the anti-inflammatory and the immune-stimulatory effects of thymoquinone as a point of view, and the anti-inflammatory and immune-suppressing effects of cortisone on another point of view. It was concluded that thymoquinone has an *anti-inflammatory* and *immune stimulatory* effects on rat duodenum and superior mesenteric lymph nodes. However, more investigations are still in need to recommend the replacement of this natural ingredient instead of cortisone with its side effects.

Key words: Thymoquinone – Histology – Duodenum – Superior mesenteric lymph node – Cortisone – Rats – *Nigella sativa*.

INTRODUCTION

The story of "immunomodulators" - or the biological response modifiers - and their possibilities for serious diseases management has opened a wide area of intense interest both for laboratory workers and clinicians. There are many agents either synthetic or natural can affect immune system in many aspects, most of these compounds possess side effects which limit their uses in therapeutic field (Sharma and Reddy, 1987). It is evident that a number of these substances (chemicals and drugs) act by covalent binding with critical macromolecules, thereby inhibiting cell proliferation or otherwise interfering with cellular functions (Dean *et al.,* 1979).

However, it is believed to promote positive health and maintain organism resistance against infection by re-establishing body equilibrium and conditioning the body tissues and organs (Dash, 1978).

Nowadays, the broad commercial production of *Nigella sativa* seeds oil by the pharmaceutical companies and its use for treating many diseases, improving health and increasing body immunity, has acted as a great stimulus to research on its role in the body as an immuno-stimulant (Hedaya, 1995 and Abdel-Ghany *et al.,* 1996).

One particular chemical entity found in *Nigella sativa* is thymoquinone. Mahfouz and El-Dakhakhny, (1960) reported the identification of "a polymer of the active principle" and named it nigellone. This component was separated from the essential oil of the seed. Three years later, El-Dakhakhny, (1963) isolated a crystalline substance from the essential oil, which he identified as "*thymoquinone*". Ghosheh *et al.* (1999) demonstrated that this component is the major constituent of *Nigella sativa*. Its anti-neoplastic activity has recently given a significant amount of attention. Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) was reported to be an active principle of *Nigella sativa* seed oil in a concentration range of 18% to 24% (Houghton *et al.,* 1995). *In vivo* studies, it has been reported that thymoquinone has

antitumoral properties in rats (Badary, 1999 and Badary *et al.,* 1997) and antioxidant potential (Milos *et al.,* 2000). Moreover, thymoquinone has been shown to have a remarkable impact on the suppression of doxorubicin-induced renal diseases in rats (Badary *et al.,* 2000 and Al-Shabanah *et al.,* 1998). Thus, all the pharmacological properties mentioned above render the analysis of thymoquinone structure relevant in order to enhance the understanding of its bioactivity.

Polysorbate 20 (commercially also known as Tween 20) - that used as a solvent for thymoquinone - is a polysorbate surfactant whose stability allows it to be used as a detergent and emulsifier in a number of domestic, scientific, and pharmacological applications (Ayorinde *et al.,* 2000).

Duodenum is a hollow jointed tube connecting the stomach to the jejunum. It is the first and shortest part of the small intestine where most chemical digestion takes place. Its lymphatic vessels follow the arteries in a retrograde fashion. Efferent lymphatic vessels pass ultimately from the duodenal lymph nodes into the celiac lymph nodes (Roitt *et al.,* 1993).

Lymph nodes are secondary - or peripheral - lymphoid organs formed of complex chambers through which the lymph flows to the blood. They form part of a body network, which filters antigens from the tissue fluid or lymph during its passage from the periphery to thoracic duct (Roitt *et al.,* 1993). The main function of lymph nodes is to trap antigens and cells containing antigens that flow into them via afferent lymphatics and to provide a site for clonal expansion of lymphoid cells recruited from the millions of cells that enter and leave via various routes. Superior mesenteric lymph nodes are numerous nodes located in the mesentery along the superior mesenteric artery, they receive lymph from the central mesenteric lymph nodes and drain into the intestinal lymph trunk. The medullary cords contain sessile B-lymphoblasts and plasma cells, which accumulate there after immune reactions (Gretz *et al.,* 1997).

Cortisone (17-hydroxy-11-dehydrocorticosterone, C21H28O5) is one of several steroid hormones secreted by the cortex of the adrenal gland and it is reduced to cortisol or corticosterone. These hormones are known as corticoids, they controlling sugar metabolism and the metaboliosm of minerals and water. Cortisol is the main hormone released by the body as a reaction to stress. It elevates blood pressure and suppresses the immune system (Ingle, 1950).

In this study, the histological structure of duodenum and superior mesentric lymph nodes of cortisone treated rats - after thymoquinone administration will be investigated.

MATERIAL AND METHODS

a- Preparation of TQ:

TQ was purchased from Sigma Chemical Company (St Louis, MO, USA), and reconstituted in Tween 20% at a concentration of 0.4mg/ml. This stock was stored at 4°C in 15ml centrifuge tubes wrapped in aluminum foil to avoid dimer formation.

b- Experimental design:

Albino male rats were grouped into seven groups. One group is normal, N (free injected), three groups are control and the other three groups were treated. Each group consisted of twenty four animals. The first control group (C_1) was injected intraperitonealy once every week for 4 weeks with 0.1 µ g/kg b.wt. of Phosphate Buffer Saline (PBS, PH=7.4) which is the solvent of cortisone, then it was injected intraperitonealy once every week for another 4 weeks with 0.1 µ g / kg b.wt. of Tween 20 which is the solvent of thymoquinone . The second control group (C_2) was injected intraperitonealy once every week for 4 weeks with 0.1 µ g/kg b.wt. of Tween 20, then it was injected intraperitonealy once every week for another 4 weeks with 0.1 µ g / kg b.wt. of PBS. The third control group (C_3) was injected intraperiteonaly once every week for 8 weeks with 0.05 µ g/kg b.wt. of PBS + 0.05 µg / kg b.wt. of Tween 20. The fourth group (treated) (T_1) was injected intraperitonealy once every week for 4 weeks with 0.1 µ g/kg b.wt. of cortisone solution, then, it was injected intraperitonealy once every week for another 4 weeks with 0.1 µ g / kg b.wt of thymoquinone solution. The fifth group (treated) (T_2) was injected intraperitonealy once every week for 4 weeks with 0.1 µ g/kg b. wt. of thymoquinone solution, then, it was injected intraperitonealy once every week for another 4 weeks with 0.1 µ g/kg b.wt. of cortisone solution . The six group (treated) (T_3) was injected intraperitonealy once every week for 8 weeks with 0.05 µg/kg b.wt. of cortisone solution + 0.05µg/kg b.wt. of thymoquinone solution (Table 1).

c- Collection of tissue samples:

At the end of each experimental period, animals were sacrificed and samples of duodenum and superior mesenteric lymph nodes were taken rapidly from each animal after dissection and fixed in Bouin's and formal saline fixatives. All samples were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in parablast. Sections of 5µm thickness were obtained and stained with the following stains:

(1) Haematoxylin and Eosin (H andE): for general histology of duodenum and superior mesenteric lymph nodes.

(2) Mallory's Triple stain: for collagenous fibers of duodenum and superior mesenteric lymph nodes.

Table (1): Doses and durations of different experimental groups

Dose / Group	0.1 µg/kg PBS	0.1 µg/kg Tween 20	0.05 µg/kg PBS + 0.05 µg/kg Tween 20	0.1 µg/kg Cortisone in PBS	0.1 µg/kg *Thymoquinone* in Tween 20	0.05 µg/kg Cortisone + 0.05 µg/kg Thymoquinone
Normal (N)	———	———	———	———	———	———
Group I C_1	W_1 -W_4	W_5 -W_8	———	———	———	———
Group II C_2	W_5 - W_8	W_1 -W_4	———	———	———	———
Group III C_3	———	———	W_1 - W_8	———	———	———
Group IV T_1	———	———	———	W_1 - W_4	W_5 - W_8	———
Group V T_2	———	———	———	W_5 - W_8	W_1 - W_4	———
Group VI T_3	———	———	———	———	———	W_1 - W_8

PBS: Phosphate Buffer Saline, C: Control, T: Treated, W: Week

RESULTS

Duodenum:

Duodenum is formed normally of an inner lining known as the mucosa (M), a supportive layer termed the submucosa (SM), a muscular stratum (MS) and an outer covering, or serosa (S). Brunner's glands (BG), which secrete highly alkaline mucus, are located in the submucosa of the duodenum. This mucus modifies the pH of the chyme that passes into the duodenum, changing it from acidic to basic, and facilitates additional food breakdown. Specialized ducts enable the secretions of the Brunner's glands to enter the lumen of the duodenum (Fig. 1).

In the First control group (C_1) that was injected with 0.1 µg/kg *PBS* from week 1-4 and with 0.1 µg/kg *Tween 20* from week 5-8, no observable structural changes regarding the duodenal mucosal villi, however, Brunner's glands exhibited a moderate increase in the collagen and reticular fibers especially in the glandular central area while nuclei appeared extra-basal (Fig. 2). There was a slight localization for connective tissue fibers among different segments of Brunner's glands.

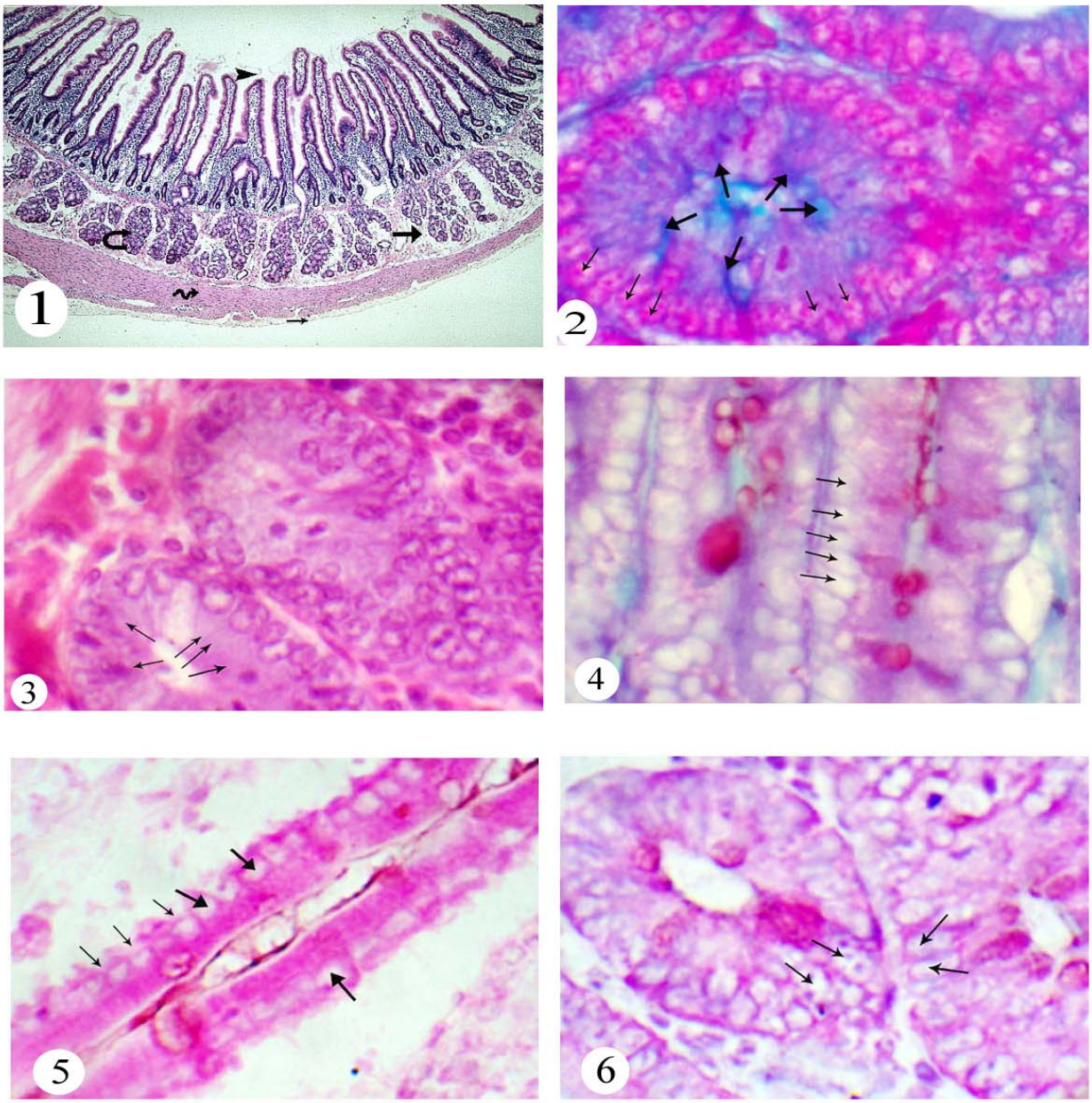
However, in the second control group (C_2) that was injected with 0.1 µg/kg *Tween 20* from week 1-4 and with 0.1 µg/kg *PBS* 20 from week 5-8, Brunner's glands exhibited just an increase of cytoplasm in the area between the basal nuclei and the glandular cell surfaces (Fig. 3). Duodenal mucosa does not express distinguishable changes.

The third control group (C_3), however, that was injected with a mixture of 0.05 µg/kg *PBS* and 0.05 µg/kg *Tween 20* from week 1-8, neither the duodenal mucosal villi, nor the duodenal Brunner's glands exhibited obvious changes.

In the fourth group (T_1) that was injected with 0.1 µg/kg *Cortisone in PBS* from week 1-4 and with 0.1 µg/kg *thymoquinone in Tween 20* from week 5-8 (Fig. 4), there is an obvious increase in the duodenal mucosal mucus-secreting cells but the connective tissue elements appear almost normal.

On the other hand, the duodenal mucosal mucus-secreting cells are slightly increased with some superficial degenerations in the fifth group (T_2) that was injected with 0.1 µg/kg *thymoquinone in Tween 20* from week 1-4 and with 0.1 µg/kg *Cortisone in PBS* from week 5-8, (Fig. 5).

Moreover, in the sixth group (T_3) that was injected with a mixture of 0.05 µg/kg *Cortisone in PBS* and 0.05 µg/kg *thymoquinone in Tween 20* from week 1-8 (Fig. 6), there are nuclear changes as dissociation of the fibrillar and granular components of the nuclei of Brunner's gland cells.



Duodenum:

Fig. (1): Normal histological structure of rat duodenum, H and E stain. Duodenal layers are: an inner lining mucosa (M), submucosa (SM), a muscularis stratum (MS), an outer serosa (S) and Brunner's gland (BG). X 200

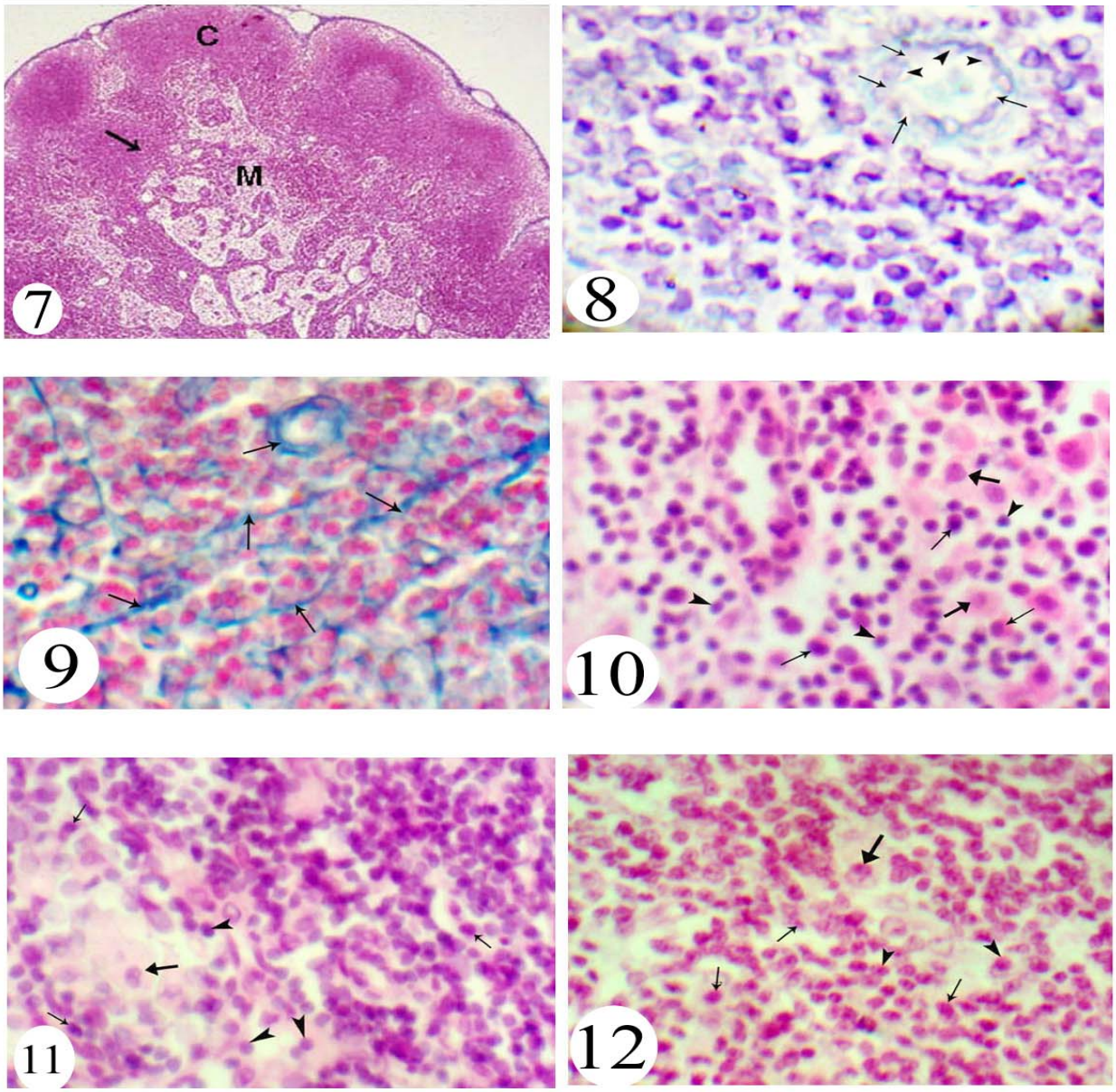
Fig. (2): Brunner's gland central area with a moderate increase in collagen and reticular fibers (Large arrows) among cells with extra-basal nuclei (Small arrows) as shown in the first control group, C_1 with Mallory's stain. X 400

Fig. (3): Second control group, C_2, Brunner's gland cells exhibited an increase of cytoplasm (Arrows) between the basal nuclei and the glandular cell surfaces. H and E stain, X 400

Fig. (4): An obvious increase in the mucus-secreting cells (Arrows) of the duodenal mucosa of rats of the treated group, T_1 stained with Mallory's stain. X 400

Fig. (5): Slight increase in the mucus-secreting cells (Large arrows) with some superficial degenerations (Small arrows) of the duodenal mucosa of rats of the treated group, T_2 stained with H and E stain. X 400.

Fig. (6): Nuclei of Brunner's gland cells of rats of the sixth group, T_3 showed nuclear dissociation of their fibrillar and granular components (Arrows). H and E, X400



Superior mesenteric lymph nodes:

Figure (7): Normal histological structure of rat superior mesenteric lymph nodes with cortex (C); medulla (M); lymphoid cells (LCs) and reticular matrix. H & E, X 200

Figure (8): In the first control group, C_1, leukocytes emigrate through the intercellular gaps (Arrows) among the endothelial cells (Arrow heads) of a blood vessel (BV) toward the interstitial spaces. Mallory‹s, X 400

Figure (9): A moderate increase of reticular fibers (Arrows) among leukocytes of rats of the second control group, C_2 stained with Mallory‹s stain. X 400

Figure (10): Superior mesenteric lymph nodes of rats of the treated group, T_1 exhibited an increase in number of macrophages (Large arrows); plasma cells (Small arrows) and lymphocytes (Arrow heads). H & E, X 400

Figure (11): Macrophages (Large arrows); and plasma cells (Small arrows) decreased but lymphocytes (Arrow heads) increased in the superior mesenteric lymph nodes of rats of the treated group, T_2. H & E, X 400

Figure (12): In the treated group, T_3, lymphocytes (Arrow heads) are still more than plasma cells (Small arrows) and macrophages (Large arrows). H & E, X 400

Superior mesenteric lymph nodes:

Normal Lymph nodes are connective tissue bags filled with mobile cells organized into functional compartments by a meshwork of reticulin fibers ensheathed by fibroblastic reticular cells and supplied by a system of specialized blood vessels and nerves. The histological structure of the superior mesenteric lymph node exhibits a cortex (C) and medulla (M), which is distinguishable with small lymphoid cells (LCs) contained in the reticular matrix (RM). The superficial cortex contains a lymphatic sinus, macrophages rich zone and B-cell follicles. The deep cortex is a high traffic zone where migrant or re-circulating T- and B-lymphocytes enter from the blood. The cortex is organized into hemispheric lobules where the flat surfaces face the afferent lymph supply and the round central borders merge with stromal chords and sinuses to form the medulla (Fig. 7).

Regarding the first control group (C_1) that was injected with 0.1 µg/kg *PBS* from week 1-4 and with 0.1 µg/kg Tween 20 from week 5-8, (Fig. 8), a clear acute inflammation is observed in a cortical blood vessel. Increased permeability of the vessel is observed, which is accompanied by transduction of fluid through the intercellular gaps and the cytoplasm of the endothelial cells as well. Leukocytes emigrate through the intercellular gaps among the endothelial cells toward the chemotactic stimuli in the interstitial space.

In the second control group (C_2) that was injected with 0.1 µg/kg *Tween 20* from week 1-4 and with 0.1 µg/kg *PBS* 20 from week 5-8 (Fig. 9) superior mesenteric lymph nodes exhibited a moderate increase of reticular fibers among lymphoid cells.

In the third control group (C_3) that was injected with a mixture of 0.05 µg/kg *PBS* and 0.05 µg/kg *Tween 20* from week 1-8, no remarkable changes could be observed in the structure of the lymph nodes.

On the other hand, a clear stimulatory immune mechanism appeared in the fourth group (T_1) that was injected with 0.1 µg/kg *Cortisone in PBS* from week 1-4 and with 0.1 µg/kg *thymoquinone in Tween 20* from week 5-8, (Fig. 10). The superior mesenteric lymph node cells exhibited a marked increase in number of macrophages together with plasma cells and lymphocytes.

In Fig. 11, it is noticed that the number of macrophages and plasma cells decreased while the number of lymphocytes increased. These changes occurred in the fifth group (T_2) that was injected with 0.1 µg/kg *thymoquinone in Tween 20* from week 1-4 and with 0.1 µg/kg *Cortisone in PBS* from week 5-8.

However, almost normal structural pattern is observed in the sixth group (T_3) that was injected with a mixture of 0.05 µg/kg *Cortisone in PBS* and 0.05 µg/kg *thymoquinone in Tween 20* from week 1-8, (Fig. 12). There is a slight increase in lymphocytes with few macrophages and plasma cells.

DISCUSSION

Thymoquinone (TQ), the main active constituent of the volatile oil extracted from *Nigella sativa's* seeds, has been reported to have an *anti-inflammatory* and *immune stimulatory* effects, however, little is known about the factors and mechanisms underlying these effects (El- Gazzar *et al.*, 2006).

One of cortisone's effects on the body and a potentially harmful side effect when administered clinically, is the *suppression of the immune system* in spite of its *anti-inflammatory effects* (Woodward *et al.*, 1951).

Duodenum:

The injection with Tween 20 during the last four weeks of the experiment in C_1 caused a moderate increase in the connective tissues among Brunner's gland cells which may be considered as a defense mechanism against the solvent. This may coincide with the cytotoxic characters of Tween 20 as a detergent, in an agreement with Ayorendi *et al.* (2000).

The increased cytoplasm on the apices of the Brunner's gland cells that was noticed in $GIIC_2$ may be considered as a repair and healing response after PBS injection during the last four weeks. Serna *et al.* (2006) described similar findings after rat duodenum inflammation. This also could be attributed in this work as a repair or healing features against the chronic inflammation occurred due to injection of Tween 20 during the first four weeks.

The almost normal appearance of duodenum in C_3 may be due to that the animals of these groups were administered a mixture of PBS and Tween 20 allover the eight weeks of injections. It may be suggested that PBS as a buffer and regulatory solution minimized the inflammatory effects of Tween 20. Moreover, 8 weeks were long enough to allow duodenal cells to be repaired.

The obvious increase in the duodenal mucosal mucus-secreting cells may be considered as an immune stimulatory mechanism of thymoquinone since animals of this group (T_1) were treated with thymoquinone during the last four weeks after the first four weeks of cortisone injection. However,the immune stimulatory effects of thymoquinone was stated by El-Gazzar *et al.* (2006), and the increased mucus- secreting cells of the duodenal mucosa was considered as a promoted defense mechanism by Vinderola *et al.* (2007).

Meanwhile, the slight increase of mucus secreting cells together with the degenerative superficial cells in duodenum of animal of T_2 could be attributed to the anti-inflammatory effects of cortisone which is in agreement with Woodward *et al.* (1951). In this group the immune suppressor effects of cortisone -which was administered in the last four weeks- minimized the immune stimulatory effects of thymoquinone injected during the first four weeks.

The nuclear changes as dissociation of the fibrillar and granular components of the nuclei of Brunner's gland cells in the animals of T_3 may be explained from our point of view- partly, on the base of the anti-inflammatory effects of both cortisone (Woodward *et al.,* 1951) and thymoquinone (El-Gazzar *et al.,* 2006). This could also be attributed to the immune stimulatory effects of thymoquinone that was administered together with cortisone along the eight weeks of the experiment. This may generate two reverse effects: 1-immune-supressor effect of cortisone and 2-the immune stimulatory effect of thymoquinone on the cells at the same time.

Superior mesenteric lymph nodes:

The clear acute inflammation that was observed in a cortical blood vessel of the superior mesenteric lymph nodes of the animals of C_1 was described by Damjanov, (1996). However, the increased permeability of the vessel and the transduction of fluid through the intercellular gaps with the cytoplasm of the endothelial cells as well could be attributed to the effects of tween 20 as detergent during the last 4 weeks of the experiment (Ayorinde *et al*., 2000). Leukocytes emigrated through the intercellular gaps among the endothelial cells toward the chemotactic stimuli in the interstitial space could be related to acute inflammatory mechanism as previously stated by Damjanov, (1996).

In the superior mesenteric lymph nodes of the animals in the group C_2, the increase of reticular fibers among leukocytes with generally normal distribution of the node structural elements could be attributed to light effects of PBS that was injected during the last four weeks after Tween 20 administration.

Regarding the superior mesenteric lymph nodes of the animals of C_3 that exhibited no remarkable changes, this may be due to the lighter effects of Tween 20 as detergent when mixed with PBS and injected along the whole experimental period.

A clear immune stimulatory mechanism – as explained by El-Gazzar *et al.* (2006) - was observed in superior mesenteric lymph nodes of the animals of T_1. This mechanism exhibited as a marked increase in number of macrophages together with plasma cells and lymphocytes. Machrophages would act as phagocytic immune response and the plasma cells, which derived from B lymphocytes by secreting antibodies (Damjanov, 1996). This could be attributed to the immune stimulatory effects of thymoquinone that was given after cortisone administration.

In superior mesenteric lymph nodes of the animals of group T_2 the number of macrophages and plasma cells decreased with more lymphocytes. This could be explained according to the anti-inflammatory and immune suppressing effects of cortisone (Woodward *et al.,* 1951) that was administered during the last four weeks of the experiment after thymoquinone administration.

The normal structure of superior mesenteric lymph nodes of the animals of group T_3 with slight increase in lymphocytes and few macrophages and plasma cells may be attributed to the administration of the animals with a mixture of both immune stimulatory (thymoquinone) and immune suppressor (cortisone) ingredients allover the experiment. This might generate two opposite immune mechanisms which caused these findings.

Finally, these findings would be attributed to that the superior mesenteric lymph nodes are numerous nodes located in the mesentery along the superior mesenteric artery, they receive lymph from the central mesenteric lymph nodes and drain into the intestinal lymph trunk (Brian *el al.,* 2005). This also may be due to the reverse administration of both ingredients in the present experimental design.

In conclusion, we can conclude that thymoquinone , the main active constituent of the volatile oil extracted from *Nigella sativa's* seeds, has an anti-inflammatory and immune stimulatory effects in spite of little is still known about the factors and mechanisms underlying these effects. However, more investigations are still in need to recommend the replacement of this natural ingredient instead of cortisone with its side effects.

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