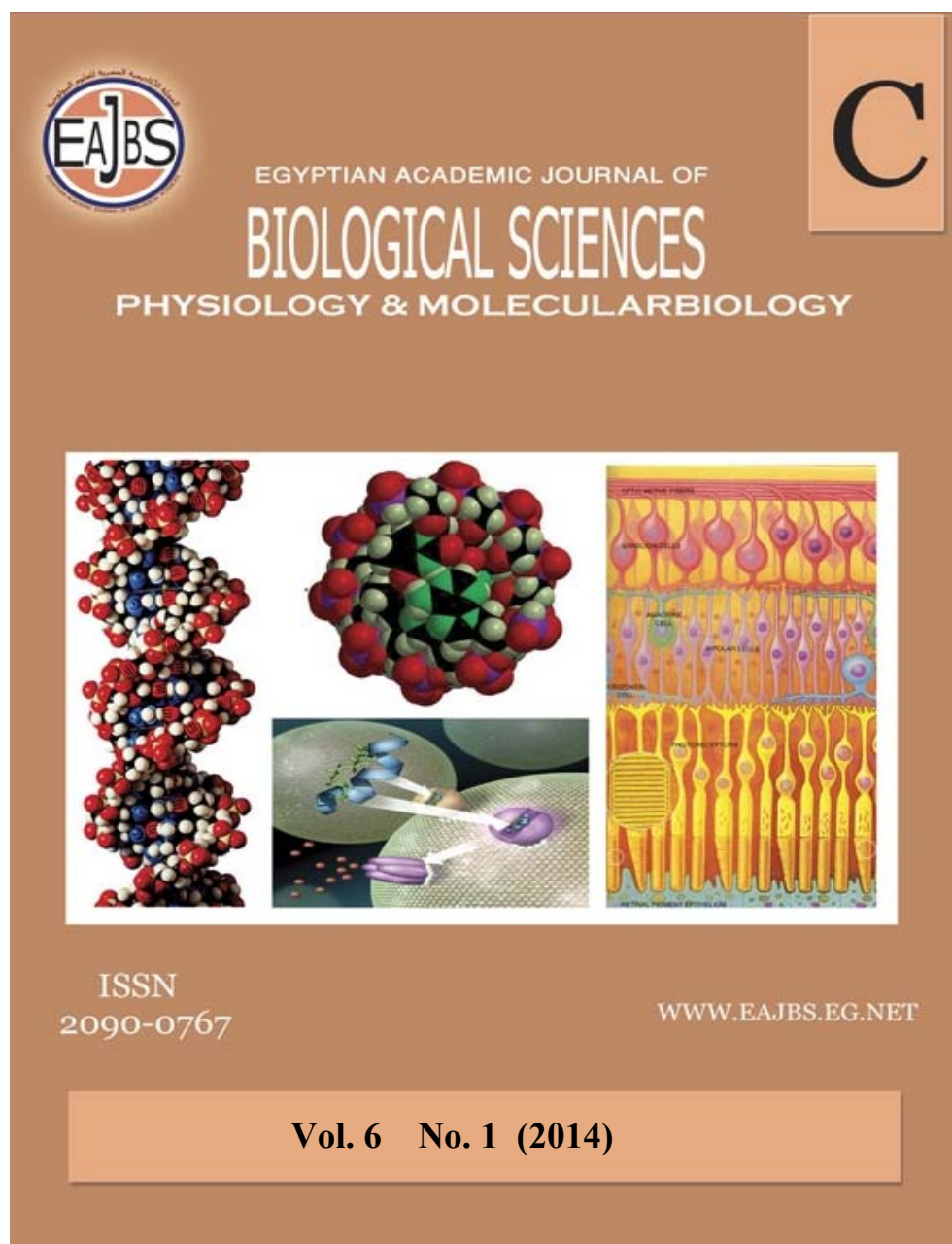


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Effect of soy isoflavones on some immunological parameters in ovariectomized female rats.

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ABSTRACT

Soy Isoflavones have estrogenic activity and are widely used in human and animal diets. They have many useful activities *in vitro* and *in vivo*. However, Evidence is emerging that dietary isoflavones have an effect on immune system. The objectives of this study was to determine the effect of soy isoflavones on some cellular immunological parameters including through estimation of their effect on daily food intake, daily body weight gain, splenic and thymic weights, total and differential leukocytic count (TLC & DLC), IL-6 and histopathology of both thymus and spleen. A total of 30 ovariectomized female Albino rats were divided into three groups (10 females / group). Control group (C) received phytoestrogen-free casein-based diet, low soy phytoestrogens group (LF) received low phytoestrogens diet containing (7% soybeans) and high soy phytoestrogens (HF) group received high phytoestrogens diet containing (26% soybeans) for 30 days. The results revealed that dietary phytoestrogens didn't alter daily food intake while reduced daily body weight gain significantly ($P < 0.05$) in HF group than LF and control group. Splenic relative weight showed non-significant difference between groups while Thymus relative weight was significantly ($P < 0.05$) reduced in LF and HF groups than control in a dose depending manner. Total leukocytic count was significantly ($P < 0.05$) increased in LF and HF groups than control while DLC showed non-significant difference among groups. Interleukin-6 was significantly ($P < 0.05$) reduced in both treated groups than control. The histopathological studies of treated groups showed decreased white pulp (WP) area and cellularity with reduced number of lymphocytes especially in HF group with depletion in medullary area of thymus in LF group and apoptotic lymphocytes in HF group. These findings show that dietary phytoestrogens interfere with cellular mediated immunity in ovariectomized female rats, so they could alleviate autoimmune diseases manifestations.

INTRODUCTION

The immune system is a highly complex network of organs, cells and chemicals that provides a physical and functional barrier against invading pathogens.

It is composed of: 1) a non-specific system which includes skin, mucous membranes and polymorpho-nuclear leukocytes, including the neutrophils, basophils and eosinophils. And 2) the specific immune system, which relies on lymphocytes and sophisticated chemical communication to provide ongoing immunity to specific antigens. There are also numerous lymphoid organs essential for the functioning of the immune response including the thymus and bone marrow, which are the primary lymphoid organs where lymphocytes are generated and the spleen, adenoid, tonsils, appendix and lymph nodes which are the secondary organs and are where the adaptive immune response is initiated. The strength of immunological defense is dependent on both number and function of the immune cells (Janeway *et al.*, 1999).

Immune responses may be broadly divided into two categories: cell mediated immunity and humoral immunity. (Mosmann and Coffman, 1989). Immune variables included lymphocyte subsets, cytokine production, and markers of inflammation and oxidative damage (Ryan-Borchers *et al.*, 2006).

Inflammation is one of the first responses of the immune system to infection (Kawai and Akira, 2006). Inflammation is produced by eicosanoids and cytokines, which are released by injured or infected cells. Common cytokines include interleukins that are responsible for communication between white blood cells; chemokines that promote chemotaxis; and interferons that have anti-viral effects, such as shutting down protein synthesis in the host cell (Le Y *et al.*, 2004). These cytokines and other chemicals recruit immune cells to the site of infection and promote healing of any damaged tissue following the removal of pathogens (Martin and Leibovich, 2005).

Autoimmune disease as a clinical syndrome caused by the activation of T cells or B cells, or both. Classification of

autoimmune disease that distinguishes diseases caused by generalized defects in lymphocyte selection or homeostasis from those caused by aberrant responses to particular antigens (Davidson, *et al.*, 2001).

Female sex hormones, estrogens, play a role in the etiology and course of chronic inflammatory diseases (Olsen and Kovacs 1996 and Pfeilschifter *et al.* 2002) and ovariectomy (OVX) increases lymphopoiesis (Soung *et al.* 2004). There is now a large body of evidence suggesting that the decline in ovarian function with menopause is associated with spontaneous increases in proinflammatory cytokines (Pfeilschifter *et al.* 2002) and increase incidence of autoimmune diseases (Farage *et al.* 2012). Isoflavones, subclass of phytoestrogens, are found in many food stuffs especially soybean where they are found in high concentration (Lee *et al.*, 2003). They are known to bind to the mammalian oestrogen receptor and induce different physiological changes in mammalian models, including steroidogenesis and cell proliferation (Santini *et al.* 2009 and van Duursen *et al.*, 2013). This hormonal mechanism explains, in part, how isoflavones protect against Inflammation (Droke *et al.* 2007). Also dietary soy isoflavones may influence the differentiation, signalling and actions of numerous cells of the immune system as estrogen receptors (ER) have been identified on many cell types including lymphocytes and antigen presenting cells (Curran *et al.*, 2004).

Soy and products derived from soy are being consumed in increasing quantities by humans, laboratory and food animals (Brown & Setchell, 2001 and Court & Freeman, 2002). All this emphasize the need to more fully understand the actions of the isoflavones to prevent age and estrogen depletion related chronic inflammation through estimation of their effect on daily

food intake, daily body weight gain, thymic and splenic weights, total and differential leukocytic count, IL-6 and histopathology of both thymus and spleen.

MATERIALS AND METHODS

Animal care:

Thirty female Albino rats aged 14 weeks old and mean weight 135.9 ± 8.9 g were purchased from Lab animal house, Faculty of veterinary Medicine, Suez Canal University. They were housed in cages (5 females in each) under standard laboratory conditions. They were kept at room temperature ($28 \pm 2^\circ\text{C}$) under natural day light rhythm two weeks prior surgical interference to ovariectomy with free access to casein based diet and tap water. Experiments were carried out according to the criteria outlined by Faculty of Sciences, Suez Canal University.

Ovariectomy

Thirty female Albino rats (5 month age) weighing approximately 195.9 ± 8.1 g were anaesthetized by diethyl ether inhalation anaesthesia. Ovariectomy was preceded by a midline dorsal skin incision, 3 cm long, approximately half way between the middle of the back and the base of the tail. Incision of the muscles was made at linea Alba. The ovary was found, surrounded by a variable amount of fat after accessing to peritoneal cavity. The blood vessels were ligated at the connection between the Fallopian tube then the uterine horn was cut and the ovary moved out. Suturing to muscle layer then to skin was performed by simple continuous suture using vicryl 4/0 (Lasota and Danowska-Klonowska 2004). Animals were given broad spectrum antibiotic (amoxicillin, 10 mg/kg) for 3 successive days after ovariectomy and continued on casein based diet.

Experimental groups

After 3 weeks from ovariectomy, the ovariectomized female rats were divided randomly into three groups: Control group (C), $n = 10$, they were fed on casein based

diet, low phytoestrogens group (LF), $n = 10$, received low phytoestrogens diet (7% soybeans) and high phytoestrogens group (HF), $n = 10$, received high phytoestrogens diet (26% soybeans). All diets were formulated to fulfill all the nutritional requirements of adult rat (Table 1) according to NRC (1995) and were offered for 30 days. Daily food intake and body weight gain were recorded.

Blood and tissue sampling

At the end of experiment, the rats were fasted overnight and weighed then blood samples were collected from retro orbital venous plexus on EDTA tubes for total & differential leukocytic count, lithium heparin tubes for lymphocytes transformation test and plain tubes for serum IL-6 assay. Plain tubes were kept 15 min then centrifuged immediately at 3000 rpm for 20 minutes. The serum separated from plain tubes were collected and stored at -20°C till estimation of IL-6.

Animals were sacrificed under effect of light anaesthesia for obtaining blood. Thymus and spleen were dissected and weighed and the relative weight of them was calculated in relation to body weight as follow:

Relative thymic weight = $[\text{thymus weight (g)} / \text{body weight (g)}] \times 100$

Relative splenic weight = $[\text{spleen weight (g)} / \text{body weight (g)}] \times 100$

Total and differential leukocytic count (TLC & DLC):

Blood samples were collected from retro orbital venous plexus of ovariectomized female rats at the end of experimental period on EDTA containing tubes and placed immediately on ice for total leukocyte counts (TLC). Leukocytes were counted and the percentage and absolute values for each type of white cells were calculated according to (Feldman, *et al.*, 2000).

Interlukin-6 assay

Interlukin-6 levels were determined using Specific rat ELISA kit (IB49706, IBL Co. USA). These parameters were

estimated according to manufacturer instructions. gradually dehydrated and embedded in paraffin; 5- μ m sections were stained with

Histopathology:

Spleen and thymus were fixed in 10% neutral buffered formalin. They were histopathological examination (Bancroft and Gamble, 2007).

Table 1: Experimental diet composition of control, low and high dietary isoflavones for ovariectomized female rats.

INGREDIENTS	CONTROL (1) %	Low dietary isoflavones (2) %	High dietary isoflavones (3) %
YELLOW CORN	40.59	35.04	35.04
CORN GLUTEN	15.00	11.82	-
SOYBEAN*	-	6.60	26.41
CASEIN	5.00	5.00	5.00
SUCROSE	22.43	23.08	22.32
STARCH	7.63	9.08	4.16
CELLULOSE	1.30	1.10	0.17
CORN OIL	5.00	-	-
SOYBEAN OIL	-	5.00	5.00
GROUND LIMESTONE	1.02	1.00	1.04
DICALCIUM PHOSPHATE	0.34	0.31	-
COMMON SALT	0.13	0.13	0.13
PREMIX**	0.30	0.30	0.30
METHIONINE	0.30	0.33	0.43
LYSINE	0.26	1.16	-
TRYPTOPHAN	0.70	0.05	-
TOTAL	100.00	100.00	100.00

*Soybean was autoclaved at 110^oc for 30 minutes according to (Westfall and Hauge, 1948) to inactivate trypsin inhibitor, tannins, saponins, phytate, protease inhibitors, lectins and goitrogens.

**Premix produced by Muvco. Supplied per kilogram diet: 12.000 and 2.000 IU of vitamin A and D3 respectively; 10 g vitamin E, 1 g vitamin K, 0.005 g vitamin B2, 0.0015 vitamin B6, 10 g pantothenic acid, 0.02 niacin, 0.6 gm choline chloride, 0.03g iron, 0.06 g manganese, 0.004 g copper, 0.05 gm zinc, 1 mg vitamin B1, 0.001 mg vitamin B12, 1 mg folic acid, 0.05 mg biotin, 0.3 mg iodine, 0.1 mg cobalt and 0.01 mg selenium.

Statistical analysis:

Statistics were calculated with SPSS for windows version 20.0, the means value obtained in the different groups were compared by one way ANOVA followed by post hoc test. All results were expressed as mean values \pm SE and significance was defined as $P < 0.05$ (Field, 2000).

RESULTS

The performed experiment demonstrated that soy isoflavones didn't alter food intake in all groups (Fig. 1) while daily body weight gain was significantly ($P < 0.05$) lower in HF group than LF and control groups with values 1.23 ± 0.13 versus 2.39 ± 0.13 and 1.95 ± 0.07 g/ day, respectively. Low

dietary isoflavones group showed significant ($P < 0.05$) elevation in daily weight gain than control and HF groups (Fig. 2).

Thymus relative weight showed a significant ($P < 0.05$) reduction in both isoflavones treated (LF and HF) groups than control while splenic relative weight showed non-significant difference among the three groups (Table 2).

Dietary Isoflavones significantly increased TLC in both LF and HF groups than control while differential leukocytic count showed non-significant difference between all groups. Interleukin-6 levels revealed a significant ($P < 0.05$) reduction in LF and HF groups of ovariectomized female rats than control group (Fig 3).

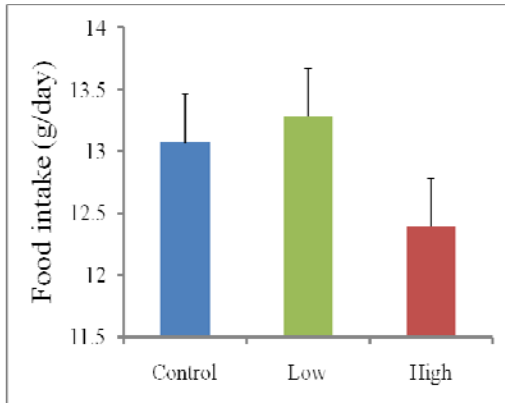


Fig. 1: Effect of dietary isoflavones on food intake in ovariectomized female rats.

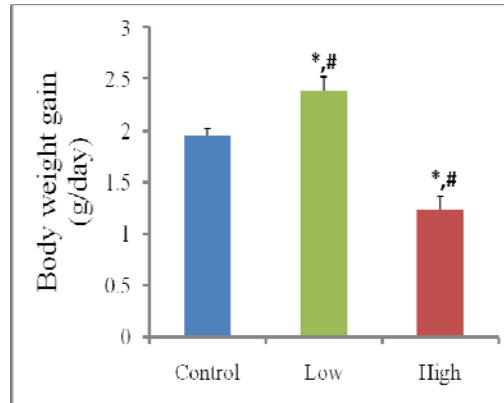


Fig. 2: Effect of dietary isoflavones on body weight gain in ovariectomized female rats. (*) represents a significant ($p < 0.05$) difference between the control and treated groups. (#) represents a significant ($p < 0.05$) difference between low and high dietary isoflavones concentrations.

Table 2: Effect of dietary isoflavones thymus relative weight / g, spleen relative weigh /g, Total leukocytic count/ μ l and IL-6 Pg/ ml in ovariectomized female rats.

Parameter	Control	Treated groups	
		LF	HF
Thymus relative weight / g	0.257±0.024	0.268±0.018 [#]	0.187±0.012 ^{*#}
Spleen relative weight / g	0.382±0.021	0.358±0.016	0.399±0.015
TLC/ μ l	12.600±1.307x10 [#]	16.350±658.063 x10 ^{*#}	19.125±0.780 x10 ^{*#}
IL-6 (Pg/ml)	18.488±0.503	14.784±0.238 ^{*#}	10.222±0.145 ^{*#}

(*) represents a significant ($P < 0.05$) difference between the control and treated groups.
 (#) represents a significant ($P < 0.05$) difference between low and high dietary isoflavones concentrations.

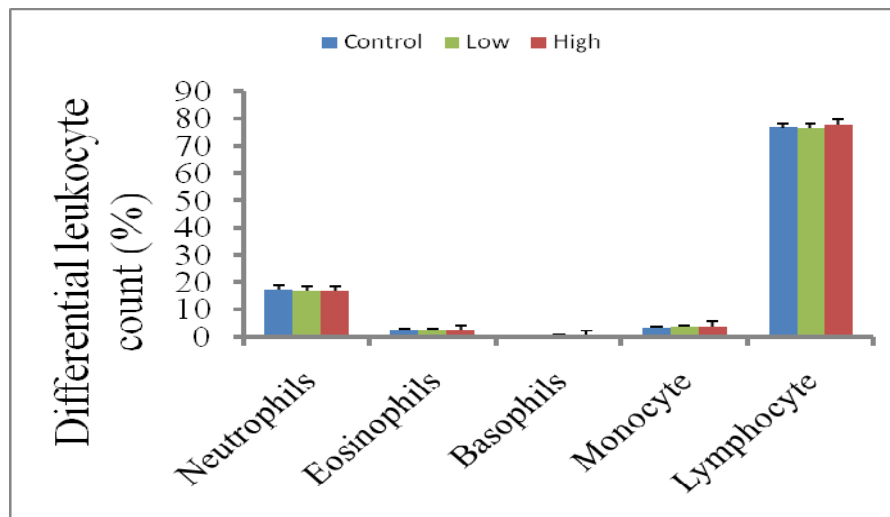


Fig. 3: Effect of dietary isoflavones on DLC in ovariectomized female rats.

Histopathological sections revealed normal splenic (Fig. 4A) and thymic architecture (Fig. 4D) in control group while LF group showed decrease in white

pulp (WP) area and cellularity with decrease in red pulp (RP) area and cellularity (Fig. 4B). Splens from high soy isoflavones group showed

reduced number of lymphocytes in follicular areas of the white pulp (WP) and the PALS (Fig. 4C). Thymus histopathology indicate depletion in medullary area of LF group (Fig. 4E). Thymic tissue of HF group showed apoptosis in lymphocytes (Fig. 4F).

DISCUSSION

Soy and products derived from soy, such as soy protein and isoflavone supplements, are being consumed in increasing quantities by humans and animals. Despite increasing number of studies, there is still a long way to a firm knowledge on the biological potency of dietary soy isoflavones and their effect on immune system. In current study the potential estrogenic effects of dietary soy isoflavones on cellular immune response which is assessed by determination of relative lymphoid organs weight (thymus and spleen), differential and total leukocyte count, IL-6 assay and histopathological examination of thymus and spleen.

Exposure of ovariectomized female rats to dietary soy isoflavones doesn't affect daily feed intake in this study. These results are in agreement with those of Weber *et al.* (1999; 2001), and disagree with those of Lephart *et al.* (2004); Cave *et al.* (2007) and Kishida *et al.* (2008). While daily body weight gain was significantly ($P < 0.05$) decreased in high dietary soy isoflavones-fed rats than control group. These results are generally consistent with those reported by studies of Awoniyi *et al.* (1998); Atanassova *et al.* (2000); Lephart *et al.* (2001) and Zhang *et al.* (2009). The decrease in body weight gain in spite of absence of change in food consumption may be due to loss of large amount of ingested food as energy lost during the increased locomotor activity (as observed in the current study) (Weber *et al.*, 2001).

Splenic weight in this study was not affected by dietary soy isoflavones treatment. These results are similar to

those obtained by Schoenroth *et al.* (2004); Zhao *et al.* (2005); Chan *et al.* (2009); Sakai *et al.* (2010) and Kakehashi *et al.* (2012). Current study revealed significant ($p < 0.05$) decrease in thymic relative weights in high dietary soy isoflavones group than low dietary soy isoflavones and control groups, these results are in agreement with Yellayi *et al.* (2002) and Zhao *et al.* (2006), while they disagreed with those of Klein *et al.* (2002); Zhao *et al.* (2005); Kakehashi *et al.* (2012) and Nishide *et al.* (2013). This reduction in thymic weight could be attributed to the apoptotic effect of genistein on thymocytes, as observed here, through two possible mechanisms; estrogen receptors (ERs) and non-ER-mediated mechanisms. Where thymus expresses both ERs; ER- α and ER- β (Yellayi *et al.*, 2002). Isoflavones especially genistein has affinity for both receptor subtypes due to physiochemical similarity between isoflavones and estradiol (Sakai and Kogiso, 2008). It is not surprising that exposure to dietary isoflavones mimics estrogen's hormone action as it acts as estradiol and the estrogen treatment was reported to induce thymic atrophy and immune suppression in developed rodents (Kohen *et al.*, 1998; Olsen and Kovacs, 1996). Moreover, isoflavones as genistein effects on thymus by non ER dependent mechanism that confirmed by (Yellayi *et al.*, 2003) who blocked thymic ERs by the ER antagonist ICI 182,780 with genistein treatment and the reduction in thymic weight remains obvious. The non ERs dependent mechanisms could involve effects of isoflavones on protein tyrosine kinases and/or topoisomerase II, which have been shown to be inhibited in thymocytes and other cell types by high genistein concentrations in vitro (Essex, 1996; Mustelin *et al.*, 1990).

The dietary soy isoflavones significantly ($P < 0.05$) increased total leukocyte count (TLC) in both low and high groups than control group. These

results are in agreement with those of Genlin *et al.* (2002); Zhao *et al.* (2005); Abbès *et al.* (2006); Soung *et al.* (2006) and Banik *et al.* (2013), while disagree with Huang *et al.* (2005) and Gredel *et al.* (2008). Differential leukocyte count (DLC) in this study didn't affected by dietary soy isoflavones treatment. These results are similar to those of Zhao *et al.* (2005), while disagree with Soung *et al.* (2004); Abbès *et al.* (2006); Cave *et al.* (2007) and Banik *et al.* (2013). This action could be accord to Ugochukwu *et al.* (2008); estrogen may down regulate the expression of adhesion and chemokine molecules in response to inflammation in many animals. Therefore it may be one cause behind the increase in the number of TLC after prolonged genistein (a phytoestrogen) administration. Researchers have also reported that estrogen treatment alters the recruitment and adhesion of leukocytes to the endothelium, which was induced by inflammation promoters that offer a possible mechanism by which estrogen exert an anti-inflammatory effect. These effects of estrogens were due to aiming at the interaction of monocytes with the vascular endothelium (Nilsson, 2007).

Dietary soy isoflavones significantly ($p < 0.05$) reduced serum IL-6 in both low and high dietary soy isoflavones groups than control group. These results are coincided with those of Jiang *et al.* (2008); Hong *et al.* (2009) and Abdelkarem *et al.* (2011), while disagreed with Azadbakht *et al.* (2007); Hong *et al.* (2008) and Tara (2009). The suppressing effect of isoflavones could be due their selective binding to ERs that impedes TNF- α induction of IL-6 through prevention of c-rel and, to a lesser extent, RelA proteins binding to the NF- κ B site of the IL-6 promoter (Galien and Garcia, 1997). Also isoflavones reduce protein tyrosine kinase activity (Gredel *et al.*, 2008) that is associated with the stimulation of TNF- α and IL-6 production in response

to introduction of lipopolysaccharides in murine macrophages (Beaty *et al.*, 1994 and Geng *et al.*, 1993). Also the inhibitory effect of isoflavones could be attributed to antioxidant effect of them and their metabolites via the regulation of the ROS pathway. Another explanation to this effect is that, dietary soy isoflavones decreased body weight gain and adiposity as demonstrated in this study and so decreased IL-6 (Bastard *et al.*, 2000) that proposed to be produced from adipose tissue as one of adipokines (Mohamed-Ali *et al.*, 1997).

CONCLUSION

Soy Isoflavones have a modulating effect to cellular immune system through decreasing thymus weight, increasing TLC, and decreasing IL-6. These compounds would be used as replacement therapy in absence or deficiency of endogenous estrogen. Also they could alleviate autoimmune diseases manifestations.

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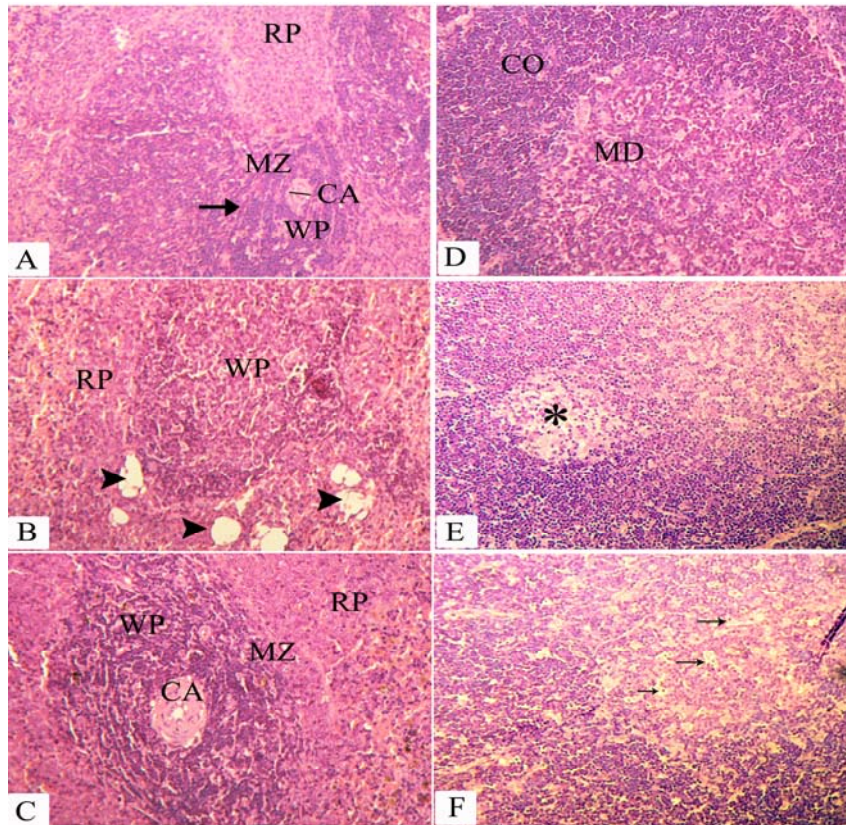


Fig. 4: Normal spleen from a control rat is depicted in Figure (A) arrow indicates the PALS region of the spleen surrounding the central artery (CA). This is the darkest region of the white pulp (WP) due to the presence of predominately small lymphocytes. (MZ) indicates the marginal zone that surrounds the PALS. Splenic tissue from rat treated with low dose of phytoestrogen in figure (B) had a diagnosis of decreased white pulp (WP) area and cellularity and decreased red pulp (RP) area and cellularity, headarrows indicated small and localized region of lipidosis. In spleen from rat treated with high dose of phytoestrogen (C) follicular areas of the white pulp (WP) and the PALS has a reduced number of lymphocytes. Normal thymus from a control rat is displayed in Figure (D) Thymus lobule consists of peripheral cortex (CO) composed of lymphocytes and a medulla (MD) lacking lymphocytes but containing glandular tissue. A qualitative assessment would indicate depletion in medullary area (*) of low dose treated phytoestrogen group in Figure (E). thymic tissue of high dose treated group (F) showed apoptotic lymphocytes (arrows).

ARABIC SUMMARY

تأثير ايزوفلافونات الصويا على بعض المعايير المناعية فى اناث الجرزان مستأصلة المبايض.

هند معروف تاج ، حكمت محمد طنطاوى ، رشا محمد غنيم

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ايزوفلافونات الصويا لديها نشاط استروجينى وتستخدم على نطاق واسع في الأنظمة الغذائية البشرية والحيوانية . كما أن لديهم العديد من الأنشطة المفيدة في التجارب المخبرية والحية . هناك العديد من الأدلة على أن الايزوفلافونات الغذائية لها تأثير على الجهاز المناعي. و من هنا كان هدف البحث تحديد تأثير ايزوفلافونات الصويا على المناعة الخلوية من خلال تقدير تأثيرها على استهلاك العلف اليومي ، و معدل زيادة الوزن اليومي، و وزن الطحال و الغدة التيموسية ، و مجموع عدد كرات الدم البيضاء الكلى و النوعى، و مستوى - Inteleukin 6 و عمل قطاعات نسجومرضية لكل من الطحال والغدة التيموسية. أجريت هذه الدراسة على 30 من اناث الجرزان مستأصلة المبايض و تم تقسيمهم الى ثلاث مجموعات (10 إناث / المجموعة)، المجموعة الضابطة (C) تلقت نظام غذائي قائم على الكازين (خالية من الاستروجينات النباتية) و مجموعة متوسطة الاستروجينات النباتية (LF) تلقت غذاء يحتوى على (7 ٪ فول الصويا) و مجموعة اخرى عالية الاستروجينات النباتية (HF) تلقت غذاء يحتوى على (27 ٪ فول الصويا) لمدة 30 يوما. أظهرت النتائج أن الاستروجينات النباتية الغذائية لم تؤثر على استهلاك العلف اليومي في حين انخفض كسب وزن الجسم يوميا معنويا فى المجموعة عالية الاستروجينات النباتية عن المجموعة متوسطة الاستروجينات النباتية و المجموعة الضابطة. لم تظهر النتائج اى تأثير للايزوفلافونات النباتية على أوزان الطحال بينما انخفض وزن الغدة التيموسية معنويا فى المجموعة عالية الاستروجينات النباتية و المجموعة متوسطة الاستروجينات النباتية و المجموعة الضابطة. ارتفع العدد الكلى لكرات الدم البيضاء معنويا فى المجموعة عالية الاستروجينات النباتية و المجموعة متوسطة الاستروجينات النباتية عن المجموعة الضابطة، بينما لم تسفر الدراسة عن فروق ملحوظة فى عدد كرات الدم البيضاء النوعى. اسفرت الدراسة عن نقص معنوى فى مستوى - Inteleukin 6 فى كل من فى المجموعة عالية الاستروجينات النباتية و المجموعة متوسطة الاستروجينات النباتية عن المجموعة الضابطة. وأظهرت الدراسات النسجومرضية نقص فى خلايا اللب البيض فى الطحال مع انخفاض عدد الخلايا الليمفاوية وخصوصا فى المجموعة عالية الاستروجينات النباتية مع نضوب فى منطقة نخاع فى الغدة التيموسية فى المجموعة متوسطة الاستروجينات النباتية بالاضافة الى تحلل فى الخلايا الليمفاوية خاصة فى المجموعة عالية الاستروجينات النباتية. هذه النتائج تشير الى أن الاستروجينات النباتية تثبط المناعة الخلوية فى اناث الجرزان مستأصلة المبايض و لهذا فمن الممكن استخدامها للحد من وطأة أمراض الجهاز المناعى الذاتية.