



Special Issue:

Veterinary Medicine between Sustainable Development and Public Health to Confront Global Changes

Influence of Administration and Withdrawal of Soy Phytoestrogens on Immune Perturbations of Ovariectomized Females Wistar Rats

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Abstract | Soy phytoestrogens play substantial role in estrogen replacement. The present research explored whether soy phytoestrogens mitigate cell mediated immunity perturbations caused by ovariectomy besides, the influence of 1 month of their dietary withdrawal on the same parameters. Therefore, forty-two female Albino rats were subjected to ovariectomy then divided into six groups. Experiment I: 3 groups (7 each); group A, served as control, received based casein diet, group B, fed low dietary soy (6.6%) for one month, and group C, fed high dietary soy (26.41%) for one month. Experiment II: Group D, control group (WC), rats were given based casein diet for two months, group E withdrawal low (WL) and group F withdrawal high (WH), received low (6.6%) and high soy (26.41%) for one month then transferred to a casein-based diet for another month. Body weight, feed intake, leukocytes count, lymphocytes transformation (LTT), tumor necrosis factor-alpha (TNF- α) and interleukins were assayed. Moreover, resistin, nitric oxide (NO), C-reactive protein (CRP), antinuclear antibodies (ANA) levels and cyclooxygenase-2 (COX-2) were estimated. Soy phytoestrogens diminished body weight gain ($P < 0.05$), promoted total leukocytes count ($P < 0.05$), abridged ($P < 0.05$) the levels of interleukins, NO, CRP, resistin, ANA and COX-2 than control. Withdrawal of phytoestrogens in experiment II still keeping the reduced ($P < 0.05$) interleukins, resistin, CRP, ANA. Dietary soy phytoestrogens mitigated estrogen depletion immunological perturbations in ovariectomized females. This effect could not be reversed even after one month from their withdrawal from diet due to the persistence of their immune modulatory effects. The soy phytoestrogens inclusion into diet ameliorates cellular immune response due to estrogen depletion. The ubiquitousness of phytoestrogens in the human or animal diets and fluctuation or alteration of their intakes or withdrawal propose their feature to human or animal health especially immune response that could be influenced on the rat study model and authorize further research.

Keywords: Cytokines, Inflammatory Markers, Phytoestrogens, Soy, Withdrawal

Received | September 25, 2024; **Accepted** | October 16, 2024; **Published** | November 06, 2024

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Citation | Alkhatib SN, Ghoneim RM, Tag HM, Tantawy HM, Abdelrazek HMA (2024). Influence of administration and withdrawal of soy phytoestrogens on immune perturbations of ovariectomized females Wistar rats. Adv. Anim. Vet. Sci. 12(s1): 320-338.

DOI | <https://dx.doi.org/10.17582/journal.aavs/2024/12.s1.320.338>

ISSN (Online) | 2307-8316; **ISSN (Print)** | 2309-3331



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The immune system is an extremely intricate grid composed of cells, organs, and chemicals which serves as both a functional and physical fence counter to attacking pathogens (Ca, 2001). Immune responses are generally categorized into two types; humoral immunity and cell-mediated immunity (Mosmann and Coffman, 1989). Key immunological parameters include cytokines production, lymphocytes subsets, markers of oxidative stress and inflammation (Ryan-Borchers *et al.*, 2006).

The earliest responses to infection is inflammation (Kawai and Akira, 2006). It is triggered by the release of cytokines and eicosanoids from infected or injured cells. Interleukins are the utmost common cytokines which facilitate interplay between chemokines and white blood cells (Le *et al.*, 2004). The cytokines and further chemicals help attract immunological cells to the site of the infection and support tissue healing once pathogens are cleared (Martin and Leibovich, 2005).

Autoimmune diseases are conditions characterized clinically by the instigation of B cells, T cells, or both, without an ongoing infection or identifiable cause. These diseases can be classified based on whether they arise from generalized lymphocyte selection defects or abnormal responses to specific antigens (Davidson and Diamond, 2001).

Adipocytes release adipocytokines, which comprise various new and biologically active substances such as resistin, leptin, visfatin, adiponectin beside some cytokines like interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- α), possibly produced by inflammatory cells infiltrating fat tissue. The later substances can take action on the immunological cells, causing localized and systemic inflammatory response (Tilg and Moschen, 2006). Additionally, interactions between lymphocytes and adipocytes contribute to immune regulation (Fantuzzi, 2005).

Epidemiological and immunological studies indicate that female sex hormones influence the development and progression of chronic inflammatory disease, and ovariectomy has been shown to increase lymphopoiesis (Abdelrazek *et al.*, 2019). There is substantial evidence that the waning in ovarian functionality during menopause is linked to unprompted upsurges in proinflammatory cytokines (Malutan *et al.*, 2014) and a higher frequency of autoimmune diseases occurrence (Farage *et al.*, 2012). Consequently, hormone replacement therapy (HRT) can help mitigate the negative effects associated with menopause and estrogen deficiency (Cameron *et al.*, 2024).

Estrogens can control the proinflammatory signals/

pathways of the cellular immune system. Estrogens provoke this effect via estrogen receptor-alpha, (ER α), estrogen receptor-beta, (ER β) and G-protein coupled membrane receptors (Harding and Heaton, 2022). They can suppress the inflammatory signal via conquest of many proinflammatory cytokines through blocking NF- κ B signaling through ER β that occupies the transcription sites of NF- κ B or via induction of NF- κ B inhibitor expression (Cvoro *et al.*, 2008; Wira *et al.*, 2010). Moreover, estrogens have been demonstrated to abridge the toll like receptors downstream signaling cascade. The later events lead to reduction in pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α) and C- reactive protein (CRP) (Harding and Heaton, 2022). Among pro-inflammatory genes NF- κ B induced array; IL-6 and TNF- α from M1 cells are existed (Wang *et al.*, 2014) where NF- κ B activation binds to DNA promoter of the 2 genes initiating their expression (Collart *et al.*, 1990; Galien *et al.*, 1996). IL-6 manufacture promotes the expression of acute-phase reactants from liver, including CRP (Ridker *et al.*, 2002). Therefore, estrogens depletion provoked an inflammatory state (Harding and Heaton, 2022).

A subclass of phytoestrogens, isoflavones, are existed in various foods, particularly soybeans, where they are present in high concentrations (Gómez-Zorita *et al.*, 2020). Isoflavones bind to mammalian estrogen receptors, inducing various physiological effects, including steroid production and cell proliferation (Lecomte *et al.*, 2017). This hormonal machinery partly elucidates how isoflavones guard against age-related diseases (Steinberg *et al.*, 2003) and inflammation (Bernatoniene *et al.*, 2021). Additionally, soy isoflavones inclusion in diet might affect the discrepancy, signaling, and activity of various immune cells, as estradiol receptors have been detected on various types of cells, including antigen-presenting cells and lymphocytes (Curran *et al.*, 2004).

Soybeans administration in diet have substantial levels of daidzein and genistein and equol which is daidzein metabolite via the action of gut flora. The later phytoestrogens resemble to 17 β -estradiol and they can dimerize the conventional estrogen receptors; ER α , and ER β . Phytoestrogens have superior affinity for ER β than ER α (Cooke *et al.*, 2006). Both subtypes of estrogen receptors exist in immune system and perform myriads of functions therefore, soy phytoestrogens can modulate cellular and humoral immunity via binding these receptors in various immune cells and provoking expression of several related transcription factors (Kovats, 2015). Ovariectomy is a state of estrogen depletion that mimic menopause (Marková *et al.*, 2024). Ovariectomy and estrogen depletion are associated with exaggerated immune response (cellular or humoral), oxidative stress and

metabolic perturbations (Mohamad *et al.*, 2020). The usage of phytoestrogens as selective estrogen receptors modulator (SERM) could compensate the absence or depletion of natural estrogens (Oseni *et al.*, 2008). Phytoestrogens can down regulate CD4⁺ mediated humoral immune response in a dose dependent manner in ovariectomized mice. Moreover, they can downregulate CD8⁺ and CD4⁺ mediated cellular immune response beside thymic atrophy and thymocytes depletion (Yellayi *et al.*, 2002, 2003). In addition, phytoestrogens could promote antioxidant system and suppress adipokines induced by ovariectomy that influence cellular immune response in ovariectomized rats (Abdelrazek *et al.*, 2019).

This work aimed to elucidate the role of soy phytoestrogens in mitigating ovariectomy induced cellular mediated immune alterations to be further applied clinically on postmenopausal women or estrogen-deficient individuals. We hypothesized that soy phytoestrogens as SERM could mitigate estrogen depletion in ovariectomized rat model. Moreover, we hypothesized that dietary phytoestrogens withdrawal for 1 moth could reverse their immune modulatory effect. Thereby, the specific objectives were to determine leukocytes count, cytokines, CRP, antinuclear antibody (ANA), cyclooxygenase-2 (COX-2), nitric oxide (NO) and resistin with evaluation of thymic and splenic histoarchitecture.

MATERIALS AND METHODS

ANIMALS

Forty-two Wistar female albino rats (107-115g), aged 9 weeks were got from National Research Center, Dokki, Giza, Egypt. Rats were kept to acclimate for two weeks prior to the experiment commencement. The female rats were fed with casein-based diet (contains zero level of isoflavones) according to Abdelrazek *et al.* (2019) and *ad libitum* tap water.

OVARECTOMY

In accordance with Lasota and Danowska-Klonowska (2004), ovariectomizing (OVX) was performed on the animals two weeks after their arrival while they were under the influence of 13 mg xylazine/kg and 87 mg ketamine/kg anesthesia. Amoxicillin 10 mg/kg was orally given to the female rats for three consecutive days following ovariectomy. Additionally, the rats were provided with *ad libitum* diet based on casein and water, and they were kept on a natural day-light cycle. The Faculty of Veterinary Medicine at Suez Canal University Committee gave their approval for all of the animal research that were applied in accord with the protocols and procedures for animal care and use (protocol No. SCU-VET-REC 2024040).

EXPERIMENTAL DESIGN

Experiment I: Effect of dietary soy phytoestrogens on immunological parameters of OVX female albino rats: After 3 weeks from ovariectomy, the OVX female rats were aligned randomly without selection according to their size, weight, age and any other criteria into three groups: Group A, was the control group, n= 7, they were fed on based casein diet (contains zero levels of genistein and daidzein) for one month, Group B, low group, n= 7, received low dietary contents of soy phytoestrogens 6.60 % (contained 400 µg/g genistein and 195 µg/g daidzein, respectively) for one month, and Group C, high group, n= 7, received high dietary contents of soy phytoestrogens 26.41% (contained 1500 µg/g genistein and 800 µg/g daidzein, respectively) for one month. All diets were formulated to fulfill all the nutritional requirements for adult rats according to NRC (1995) as mentioned by Tag *et al.* (2014) as shown in Table 1. The choice of soy phytoestrogens doses 6.60% and 26.41% covered the human exposure range especially for Asian people who may consume 20 to 50 g of soybeans daily (Adlercreutz *et al.*, 1991).

Experiment II: Influence of dietary soy phytoestrogens withdrawal on immune related parameters of OVX female albino rats.

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

Ingredients	Control (%)	Phytoestrogens	
		Low (%)	High (%)
Corn gluten	15.00	11.82	-
Yellow corn	40.59	35.04	35.04
Soybean	-	6.60	26.41
Sucrose	22.43	23.08	22.32
Casein	5.00	5.00	5.00
Starch	7.63	9.08	4.16
Corn oil	5.00	-	-
Soybean oil	-	5.00	5.00
Cellulose	1.30	1.10	0.17
Ground limestone	1.02	1.00	1.04
Common salt	0.13	0.13	0.13
Dicalcium phosphate	0.34	0.31	-
Premix	0.30	0.30	0.30
Lysine	0.26	1.16	-
Methionine	0.30	0.33	0.43
Tryptophan	0.70	0.05	-
Total	100.00	100.00	100.00

Three weeks after undergoing ovariectomy, an overall of 21 rats were aligned randomly without selection according to their size, weight, age and any other criteria into three groups:

Group D, the withdrawal control group (WC) consisting of 7 rats, which were fed a control diet for two months; Group E, the withdrawal low group (WL) consisting of 7 rats, which received a diet with low levels of isoflavones (6.6%) for one month and then switched to a control diet for another month, and Group F, the withdrawal high group (WH) consisting of 7 rats, which received a diet with high levels of soy (26.41%) for one month and then switched to a control diet for another month.

DAILY WEIGHT GAIN, FOOD INTAKE, AND RELATIVE LYMPHOID ORGANS WEIGHT

The daily food consumption of all rats in each cohort was ascertained. The difference between two successive body weights of each rodent at a one-week interval was used to calculate the daily weight gain of OVX rats, which was then divide by seven. Seven female rodents from each group were sacrificed through decapitation under tetrahydrofuran anesthesia after 30 days of treatment in experiment I and 60 days in experiment II. The spleen and thymus were weighed then calculation of their relative weights was done following this formula:

$$\text{Relative splenic or thymic weight} = (\text{spleen or thymus weight (g)} / \text{body weight (g)}) \times 100$$

BLOOD SAMPLING

Three blood samples were obtained at termination of the experimental periods under tetrahydrofuran anesthesia. Blood was drawn from the retro-orbital plexus using capillary tubes and placed in three different types of collection tubes: Ethylene-diamine-tetra-acetic acid (EDTA) tubes, lithium heparin tubes, and plain tubes. The EDTA tubes were used for total and differential leukocytes count, the lithium heparinized tubes for the lymphocytes transformation test (LTT), and the plain tubes to get sera. The blood was first allowed to clot for 15 minutes and then refrigerated for three hours. Afterwards, they were centrifuged at 3000 rpm for 20 minutes to separate the sera, then collected and kept at -20°C until analysis of immunological parameters.

TOTAL (TLC) AND DIFFERENTIAL LEUKOCYTIC (DLC) COUNTS

Blood samples collected on EDTA tubes were used to count leukocytes. The total leukocytes count was done manually using Neubauer chamber. Each type of white cells percentage was obtained after spreading on blood films that were stained via Giemsa stain according to Jain (1986). Both TLC and DLC were performed blindly by the same person for all groups.

LYMPHOCYTES TRANSFORMATION TEST (LTT)

Blood samples collected in lithium heparinized tubes were

promptly placed on cold packs and immediately transported to the laboratory for lymphocytes transformation assay. In summary, the isolated buffy coat was rinsed with RPMI-1640 medium (Cat. No. R8758, Sigma-Aldrich, Egypt) and the resulting sedimented lymphocytes were suspended in 1 mL of RPMI-1640 medium enriched fetal calf serum 10% (Cat. No. F2442, Sigma-Aldrich, Egypt) following the protocols of Boyum (1968) and Burrells and Well (1977). The quantification of viable lymphocytes per milliliter of RPMI medium was assessed using the method mentioned by Hudson and Hay (1980). The lymphocytic transformation assay was conducted using MTT staining techniques, as described by Abdelrazek *et al.* (2019).

SERUM TUMOR NECROSIS FACTOR-ALPHA (TNF-α) AND INTERLEUKINS LEVELS

The level of TNF-α in the serum was determined via a specific rat enzyme linked immunosorbent assay (ELISA) for TNF-α specific kit (Code No. 27194, IBL Co., Japan) with sensitivity 10.55 pg/mL. The levels of serum interleukin-2 (IL-2) and interleukin-6 (IL-6) were quantified using specific rat ELISA kits (BE45321 and IB49706, IBL Co. USA, respectively) the sensitivity were 11 pg/mL and 0.92 pg/mL, respectively. All of the later parameters were determined following the manufacturers' instructions.

SERUM RESISTIN HORMONE LEVEL

The rat specific resistin ELISA kit (Catalog Number RD391016200R, Biovendor Company, Czech Republic) was utilized to perform the serum resistin hormone assay following the provided instructions by the manufacturer. The sensitivity of the test was 0.05 ng/mL.

SERUM NITRIC OXIDE (NO), C-REACTIVE PROTEIN (CRP), ANTINUCLEAR ANTIBODIES (ANA) LEVELS AND CYCLOOXYGENASE-2 (COX-2)

Serum NO, CRP, ANA and COX-2 levels were measured using rat ELISA kits (Cat. No. ABIN457252, Antibodies online, Germany) with sensitivity 0.06 ng/mL, (Ref. No. EU59131, IBL Co., USA) with sensitivity 0.225 µg/mL, (Ref. No. EIA-3562, DRG Co., USA) and (Code No. 27187, IBL Co. Japan) with sensitivity 0.16 ng/mL, respectively. All were performed according to manufacturers' instructions.

HISTOPATHOLOGY

The rats were sacrificed, then a necropsy was performed on them, and a gross examination was performed on all of the organs and tissues. There was a 10% neutral buffered formalin solution used for spleen and thymus fixation. Subsequently, the specimens underwent a slow dehydration process and were subsequently embedded in paraffin. Histopathological examination was performed

by staining 5- μ m sections with hematoxylin and eosin (H & E) (Bancroft and Gamble, 2008). The examination was carried out by a pathologist who was blinded to treatment.

STATISTICAL ANALYSIS

Statistical analysis was performed using IBM-SPSS software (version 28.0 for Mac OS). Prior to conducting the analysis, the data were explored for normality using the Shapiro-Wilk test. Subsequently, one-way analysis of variance (ANOVA) was used to verify significant differences among the treated groups (Knapp, 2017). ANOVA indicated statistical significance ($p < 0.05$), Tukey's HSD test post-hoc test was conducted for further comparison to determine source of difference. Data was presented as mean $\pm$ SE.

RESULTS AND DISCUSSION

EXPERIMENT I

In experiment I, the impact of soy-derived phytoestrogens on food consumption, weight gain, and the ratios of spleen-to-body weight and thymus-to-body weight for ovariectomized female albino rats was illustrated in Table 2. The data was presented as the mean $\pm$ standard error (SE),

with confidence intervals (95%) and p-values determined through statistical analysis. The low-dose phytoestrogens treated- group exhibited a notable increase in weight gain, averaging 2.37 ± 0.12 g/day (95% CI: 2.13–2.61, $p = 0.03$), which was significantly higher than the control group at 1.93 ± 0.08 g/day (95% CI: 1.77–2.09) and the high-concentration group at 1.21 ± 0.11 g/day (95% CI: 1.00–1.42, $p = 0.01$). This suggests that the variations between the low-concentration group and the others are notably significant, indicating that a lower concentration of phytoestrogens has a more pronounced impact on weight gain. The analysis of thymus weight ratio revealed that the low-concentration phytoestrogens group exhibited a significantly higher value (0.257 ± 0.017 , 95% CI: 0.223–0.291, $p = 0.04$) in comparison to the high-concentration group (0.176 ± 0.010 , 95% CI: 0.156–0.196). Furthermore, the control group did not exhibit any statistically significant differences in comparison to the experimental groups (0.246 ± 0.021 , 95% CI: 0.204–0.288). Although there were fluctuations in the values of food intake and splenic weight ratio, the differences between the different groups were not statistically significant ($p > 0.05$). This suggested that phytoestrogens had a negligible effect on these variables.

Table 2: Influence of dietary soy phytoestrogens on feed intake, weight gain, thymus weight and spleen weight.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability (p)		
				Low dose vs control*	high dose vs control*	Low vs high dose#
Feed intake (g/day)	12.06 $\pm$ 0.38	12.27 $\pm$ 0.34	11.37 $\pm$ 0.37	0.99	0.96	0.06
Body weight gain (g/day)	1.93 $\pm$ 0.08	2.37 $\pm$ 0.12 [#]	1.21 $\pm$ 0.11 [#]	0.03	0.01	0.02
Splenic relative weight (%)	0.372 $\pm$ 0.011	0.348 $\pm$ 0.015	0.387 $\pm$ 0.014	0.07	0.11	0.06
Thymic relative weight (%)	0.246 $\pm$ 0.021	0.257 $\pm$ 0.017 [#]	0.176 $\pm$ 0.010 [#]	0.19	0.03	0.01

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons between groups. (*) indicate a statistically significant difference from the control group at $p < 0.05$. (#) denotes a statistically significant difference between the high and low dose of phytoestrogens at $p < 0.05$. Probability p -values are provided for pairwise comparisons between the groups.

Table 3: Influence of dietary soy isoflavones on TLC ($\times 10^3/\mu$ L) and DLC (%) in ovariectomized female albino rats.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability(p)		
				Low dose vs control*	high dose vs control*	Low vs high dose#
TLC($\times 10^3/\mu$ L)	12.6 $\pm$ 1.31	16.4 $\pm$ 0.66 [*]	19.1 $\pm$ 0.78 [*]	0.001	0.001	0.055
Neutrophils (%)	17.25 $\pm$ 1.65	17.13 $\pm$ 1.21	16.72 $\pm$ 1.74	0.13	0.59	0.28
Eosinophils (%)	2.64 $\pm$ 0.29	2.61 $\pm$ 0.32	2.08 $\pm$ 0.19	0.21	0.053	0.054
Basophils (%)	0.15 $\pm$ 0.15	0.23 $\pm$ 0.23	0.17 $\pm$ 0.17	0.08	0.11	0.29
Monocyte (%)	3.41 $\pm$ 0.26	3.62 $\pm$ 0.48	3.53 $\pm$ 0.00	0.17	0.06	0.057
Lymphocyte (%)	76.62 $\pm$ 1.39	76.42 $\pm$ 1.80	77.58 $\pm$ 1.93	0.38	0.26	0.13

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons between groups. (*) indicate a statistically significant difference from the control group at $p < 0.05$. (#) denotes a statistically significant difference between the high and low dose of phytoestrogens at $p < 0.05$. Probability p -values are provided for pairwise comparisons between the groups.

The influence of dietary soy isoflavones on TLC ($\times 10^3/\mu\text{L}$) and DLC (%) in ovariectomized female albino rats was summarized in Table 3. The results demonstrated that both low-dose and high-dose phytoestrogens groups exhibited statistically significant increases in TLC when compared to the control group, with the low-dose group reaching $16.4 \pm 0.66 \times 10^3/\mu\text{L}$ ($p = 0.001$) and the high-dose group reaching $19.1 \pm 0.78 \times 10^3/\mu\text{L}$ ($p = 0.001$). Although the differences between the low and high-dose groups approached significance ($p = 0.055$), they did not reach the conventional threshold for statistical significance. Neutrophils count, however, did not show statistically significant differences across groups ($p > 0.05$), with values remaining relatively stable between the control, low, and high-dose groups. Eosinophils demonstrated a borderline significant reduction in the high-dose group ($2.08 \pm 0.19\%$, $p = 0.053$) when compared to the control group ($2.64 \pm 0.29\%$), suggesting a potential trend toward decreased eosinophils percentages with higher phytoestrogen doses, though the difference did not reach statistical significance. Likewise, basophils exhibited tendencies for elevation in both the low and high-dose groups relative to the control; however, these variations were not statistically significant ($p > 0.05$). The impact of phytoestrogens on monocytes and lymphocytes was minimal, exhibiting small variances among groups without any statistically significant alterations. The high-dose group had a tendency for decreased monocyte percentages ($3.53 \pm 0.00\%$, $p = 0.06$) relative to the control group ($3.41 \pm 0.26\%$), however this difference did not achieve statistical significance. The findings demonstrated that phytoestrogens, especially at elevated doses, markedly affect TLC, although their effect on DLC, including neutrophils, eosinophils, and basophils, was more inconsistent and tended to approach, but did not

consistently achieve, significance. The absence of statistical significance in certain comparisons indicated that although phytoestrogens impact specific immunological parameters, their effect on leukocyte subtypes may be more nuanced or reliant on dosage. Table 4 illustrated the impact of dietary soy phytoestrogens on TNF- α , IL-6, IL-2, resisting hormone levels, NO levels, CRP, ANA, and COX-2 in ovariectomized female albino rats. The findings indicated that both low-dose and high-dose phytoestrogens interventions markedly decreased TNF- α levels in comparison to the control group. The low-dose group demonstrated a mean TNF- α concentration of 6.816 ± 0.166 pg/mL, but the high-dose group revealed a more significant decrease to 5.165 ± 0.042 pg/mL (both $p=0.00$ relative to control). The disparity between the low and high-dose cohorts was statistically significant ($p=0.03$). IL-6 levels were significantly reduced in both the low-dose group (13.182 ± 0.211 pg/mL, $p=0.00$) and the high-dose group (10.519 ± 0.135 pg/mL, $p=0.00$) relative to the control group (19.122 ± 0.314 pg/mL), with the high-dose group exhibiting a markedly greater decrease in IL-6 levels compared to the low-dose group ($p=0.01$). For IL-2, although both the low-dose (0.73 ± 0.04 pg/mL) and high-dose groups (0.71 ± 0.02 pg/mL) showed reductions in levels relative to the control group (0.86 ± 0.02 pg/mL), the differences were only statistically significant for the low-dose group ($p = 0.02$), while the high-dose group showed a weaker significance ($p = 0.04$). Resistin hormone levels exhibited a comparable trend, with notable reductions in both the low-dose (4.264 ± 0.085 pg/mL) and high-dose groups (3.345 ± 0.041 pg/mL) relative to the control group (5.729 ± 0.076 pg/mL), yielding $p=0.03$ for the low-dose versus control and $p=0.02$ for the high-dose versus control. The high-dose group demonstrated markedly reduced

Table 4: Influence of dietary soy phytoestrogens on TNF- α , IL-6, IL-2 (Pg/ mL), resistin hormone levels (Pg/mL), NO levels ($\mu\text{mol/L}$), CRP (mg/dL), ANA and COX-2 (ng/mL) levels in ovariectomized female albino rats.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability (p)		
				Low dose vs control*	high dose vs control*	Low vs high dose#
TNF- α (Pg/mL)	9.893 $\pm$ 0.127	6.816 $\pm$ 0.166*#	5.165 $\pm$ 0.042*#	0.00	0.00	0.03
IL-6 (Pg/mL)	19.122 $\pm$ 0.314	13.182 $\pm$ 0.211*#	10.519 $\pm$ 0.135*#	0.00	0.00	0.01
IL-2 (Pg/mL)	0.86 $\pm$ 0.02	0.73 $\pm$ 0.04*	0.71 $\pm$ 0.02*	0.02	0.04	0.21
Resisting hormone (Pg/mL)	5.729 $\pm$ 0.076	4.264 $\pm$ 0.085*#	3.345 $\pm$ 0.041*#	0.03	0.02	0.01
NO ($\mu\text{mol/L}$)	26.76 $\pm$ 1.21	22.20 $\pm$ 0.63*	19.98 $\pm$ 0.42*	0.002	0.003	0.06
CRP (mg/dL)	10.222 $\pm$ 0.075	8.316 $\pm$ 0.121*#	7.390 $\pm$ 0.100*#	0.01	0.005	0.03
ANA (ng/mL)	0.207 $\pm$ 0.008	0.136 $\pm$ 0.004*#	0.083 $\pm$ 0.004*#	0.02	0.013	0.006
COX-2 (ng/mL)	1.98 $\pm$ 0.08	1.54 $\pm$ 0.14*#	0.90 $\pm$ 0.01*#	0.02	0.004	0.001

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons between groups. (*) indicate a statistically significant difference from the control group at $p < 0.05$. (#) denotes a statistically significant difference between the high and low dose of phytoestrogens at $p < 0.05$. Probability p -values are provided for pairwise comparisons between the groups.

resistin levels in comparison to the low-dose group ($p=0.01$). Significant reductions in NO levels were noted in both the low-dose ($22.20 \pm 0.63 \mu\text{mol/L}$, $p=0.002$) and high-dose groups ($19.98 \pm 0.42 \mu\text{mol/L}$, $p=0.003$) when compared to the control group ($26.76 \pm 1.21 \mu\text{mol/L}$). The disparity between the low and high-dose groups was marginally insignificant ($p=0.06$). CRP levels were considerably diminished in both the low-dose ($8.316 \pm 0.121 \text{ mg/dL}$, $p=0.01$) and high-dose groups ($7.390 \pm 0.100 \text{ mg/dL}$, $p=0.005$) in comparison to the control group ($10.222 \pm 0.075 \text{ mg/dL}$). The difference between the low and high-dose groups was statistically significant ($p=0.03$). ANA levels were markedly diminished in the low-dose ($0.136 \pm 0.004 \text{ ng/mL}$, $p=0.02$) and high-dose groups ($0.083 \pm 0.004 \text{ ng/mL}$, $p=0.013$) relative to the control group ($0.207 \pm 0.008 \text{ ng/mL}$), exhibiting a statistically significant difference between the low and high-dose groups ($p=0.006$). Finally, COX-2 levels showed a substantial reduction in both the low-dose ($1.54 \pm 0.14 \text{ ng/mL}$, $p=0.02$) and high-dose groups ($0.90 \pm 0.01 \text{ ng/mL}$, $p=0.004$) compared to the control ($1.98 \pm 0.08 \text{ ng/mL}$), with a significant difference between the two treatment groups as well ($p=0.001$).

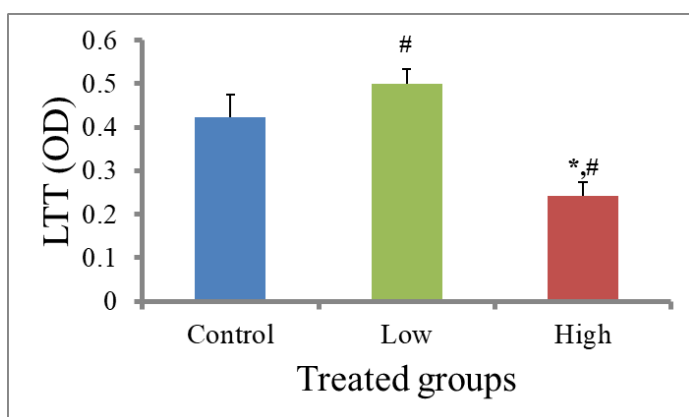


Figure 1: Effect of dietary soy phytoestrogens on LTT (OD) in ovariectomized female albino rats. Data is expressed as mean $\pm$ SE. (*) denotes a statistical ($p < 0.05$) difference between the treated groups and the control. (#) denotes a statistical ($p < 0.05$) difference between high and low dietary soy phytoestrogens concentrations. the p -value for the difference between the “High” group and the control group is $p = 0.04$. p value for the difference between the “High” and “Low” dietary soy phytoestrogen concentrations is $p = 0.02$.

As shown in Figure 2A, the spleen tissue from the control group displayed a normal architecture of red pulp, white pulp with lots of lymphocytes, and a well-defined marginal zone that encircles the periarteriolar lymphoid sheath (PALS) around the central artery. The splenic tissues treated with low-dose-dietary phytoestrogens (6.6%) exhibited a decrease in lymphocytes in the follicular sections of the white pulp (WP) and the periarteriolar lymphoid sheath (PALS) (Figure 2B). Additionally, small and localized

regions of lipidosis were detected. Severe depletion of lymphocytes was frequently detected in both the white pulp and red pulps of the spleen treated with high-dose-dietary isoflavones (Figure 2C).

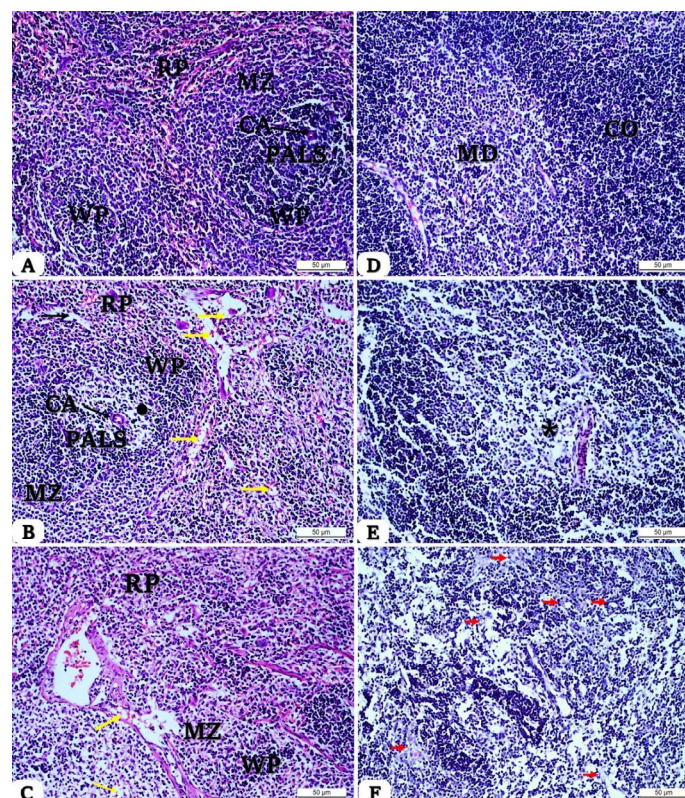


Figure 2: Histomorphology of control spleen (A) showed a normal appearance of red pulp (RP), white pulp (WP) with a well-differentiated marginal zone (MZ), central artery (CA), and periarteriolar lymphoid sheath (PALS). (B) The section of the spleen from the low-dose-dietary soy (6.6%) phytoestrogens group showed a mild depletion of T cells in PALS. Signs of lipidosis were also detected (yellow arrows). (C) Severe lymphocytes depletion in the WP compartments and RP was observed with high-dose dietary soy (26.41%) phytoestrogens in the spleen section (*). (D) The thymus (control group) displayed normal architecture of the cortex (CO) and medulla (MD). (E) Thymus section of a low-dose-dietary soy (6.6%) phytoestrogens group showed a cellularity decrease in the medullary area (*). (F) Thymus section of a high-dose-dietary soy (26.41%) phytoestrogens group exhibited severe depletion of lymphocytes in the cortex, in addition to the presence of apoptotic lymphocytes (red arrows), [H&E stain; original magnification: $\times 20$].

The thymus typically consists of a cortex and medulla, as observed in the control group (Figure 2D). As indicated in Figure 2E, the medullary area decreased in the group treated with low-dose dietary phytoestrogens (6.6% soy). While the thymic tissue of the group treated with high-dose dietary phytoestrogens (26.41% soy) exhibited severe depletion in cortical lymphocytes and frequent presence of apoptotic lymphocytes (Figure 2F) indicating a marked

disruption in thymic immune cell maintenance.

EXPERIMENT II

In Experiment II, the influence of dietary soy phytoestrogens withdrawal on feed intake, body weight gain, and the relative weights of the thymus and spleen in ovariectomized female albino rats was represented in Table 5. No significant differences were observed in the daily feed intake of the groups with respect to the influence of dietary soy phytoestrogens withdrawal. Intakes of 17.95 ± 0.58 g/day and 16.83 ± 0.73 g/day were observed in the low and high dose phytoestrogens withdrawal groups, respectively, while the control group reported an intake of 17.99 ± 0.71 g/day. The probability values for comparisons between the low dose and control ($p = 0.22$), high dose and control ($p = 0.16$), and low vs high dose ($p = 0.15$) did not reveal statistically significant changes. In terms of body weight gain, there was a trend toward variation among the groups. The control group gained weight at a rate of 1.85 ± 0.39 g/day, the low dose withdrawal group showed a slightly higher gain of 2.29 ± 0.14 g/day, while the high dose group

exhibited a low weight gain of 1.69 ± 0.35 g/day. Statistical analysis indicated marginal significance in body weight gain between the high dose and control groups ($p = 0.06$), with other pairwise comparisons (low dose vs control, $p = 0.09$; low vs high dose, $p = 0.09$) also approaching significance. In comparison to the control group, the phytoestrogens-treated groups exhibited a lower relative spleen weight. The control group had a spleen weight of $0.52 \pm 0.13\%$, while the low and high dose groups exhibited $0.36 \pm 0.03\%$ and $0.39 \pm 0.02\%$, respectively. The comparison between the low dose and control groups approached statistical significance ($p = 0.056$), suggesting a possible effect of the phytoestrogens withdrawal, while the high dose vs control ($p = 0.09$) and low vs high dose ($p = 0.15$) comparisons did not reach significance. There were no significant differences in thymus relative weight between the groups. The control group had a thymus weight of $0.21 \pm 0.02\%$, the low dose group had $0.22 \pm 0.02\%$, and the high dose group exhibited $0.21 \pm 0.01\%$. Probability values for all comparisons (low dose vs control, $p = 0.33$; high dose vs control, $p = 0.24$; low vs high dose, $p = 0.33$) indicated no significant changes.

Table 5: Influence of dietary soy phytoestrogens withdrawal on feed intake, body weight gain, thymus and spleen relative weights in ovariectomized female albino rats.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability (p)		
				Low dose vs control	high dose vs control	Low vs high dose
Feed intake (g/day)	17.99 ± 0.71	17.95 ± 0.58	16.83 ± 0.73	0.22	0.16	0.15
Body weight gain (g/day)	1.85 ± 0.39	2.29 ± 0.14	1.69 ± 0.35	0.09	0.06	0.09
Spleen relative weight (%)	0.52 ± 0.13	0.36 ± 0.03	0.39 ± 0.02	0.056	0.09	0.15
Thymus relative weight (%)	0.21 ± 0.02	0.22 ± 0.02	0.21 ± 0.01	0.33	0.24	0.33

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons between groups. Probability p -values are provided for pairwise comparisons between the groups.

Table 6: Influence of dietary soy phytoestrogens withdrawal on TLC ($\times 10^3/\mu\text{L}$) and DLC (%) in ovariectomized female albino rats.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability (p)		
				Low dose vs control*	high dose vs control*	Low vs high dose#
TLC($\times 10^3/\mu\text{L}$)	9.83 ± 1.27	12.55 ± 1.79	9.63 ± 0.63	0.21	0.18	0.16
Neutrophils (%)	13.52 ± 3.39	12.36 ± 3.28	15.02 ± 0.63	0.11	0.07	0.08
Eosinophils (%)	2.72 ± 0.71	2.34 ± 0.76	3.00 ± 0.19	0.23	0.19	0.15
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.04	0.33	0.24	0.32
Monocyte (%)	3.48 ± 0.89	3.24 ± 0.90	3.80 ± 0.52	0.22	0.16	0.25
Lymphocyte (%)	60.28 ± 15.07	61.78 ± 15.49	78.14 ± 0.56	0.18	0.16	0.19

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD) test for pairwise comparisons between groups. Probability p -values are provided for pairwise comparisons between the groups.

Regarding the effects of dietary soy phytoestrogens withdrawal on TLC, DLC, and LTT were declared in Table 6. The results showed that there were no statistical significant differences between the studied groups. In the low dosage withdrawal group, the TLC was $12.55 \pm 1.79 \times 10^3/\mu\text{L}$, while in the high dose group, it was $9.63 \pm 0.63 \times 10^3/\mu\text{L}$. The TLC in the control group was $9.83 \pm 1.27 \times 10^3/\mu\text{L}$. Pairwise comparisons' probability values (low dosage compared. control, $p = 0.21$; high dose vs. control, $p = 0.18$; low vs. high dose, $p = 0.16$) did not demonstrate statistical significance. In terms of neutrophils percentages, the low and high dose groups showed $15.02 \pm 0.63\%$ and $12.36 \pm 3.28\%$, respectively, while the control group showed $13.52 \pm 3.39\%$. No significant differences were found, despite a tendency towards a difference in neutrophils count across the groups (low dosage versus control, $p = 0.11$; high dose vs control, $p = 0.07$; low vs high dose, $p = 0.08$). P-values showed no significant differences between low dosage and control ($p = 0.23$), high dose and control ($p = 0.19$), and low dose and high dose ($p = 0.15$). Eosinophils percentages were also similar among the groups (control: $2.72 \pm 0.71\%$, low dose: $2.34 \pm 0.76\%$, high dose: $3.00 \pm 0.19\%$). The high dose group had $0.04 \pm 0.04\%$ basophils, whereas the control and low dose groups had none at all ($0.00 \pm 0.00\%$). However, the difference between the two groups was not statistically significant (low dose vs control, $p = 0.33$; high dose vs control, $p = 0.24$; low versus high dose, $p = 0.32$). The percentages of monocytes in the control ($3.48 \pm 0.89\%$), low dosage ($3.24 \pm 0.90\%$), and high dose ($3.80 \pm 0.52\%$) groups did not differ significantly from one another (low dose vs control, $p = 0.22$; high dose vs control, $p = 0.16$; low versus high dose, $p = 0.25$). Finally, the lymphocytes percentages did not reach statistical significance (low dose vs control, $p = 0.18$; high dose vs control, $p = 0.16$; low vs high dose, $p = 0.19$). However, the lymphocytes percentages were higher in the high dose group ($78.14 \pm 0.56\%$) than in the control ($60.28 \pm 15.07\%$) and low dose groups ($61.78 \pm 15.49\%$). These findings implied that the immunological parameters in ovariectomized rats were not significantly affected by the removal of soy phytoestrogens from their diet.

A statistically significant difference was noted between the control and treated groups when it came to the effect of dietary soy phytoestrogen withdrawal on lymphocyte transformation test (LTT) optical density (OD) in female albino rats who had their ovaries removed. Figure 3 showed that when comparing the three groups, the control (WC), low dosage withdrawal (WL), and high dose withdrawal (WH) all had lower LTT values. Statistically significant reductions in LTT following phytoestrogens withdrawal were observed in the WL and WH groups, as compared to the control group, which had a significantly higher LTT value ($p=0.03$, $p=0.02$, respectively). In comparison to the control group, these results indicate that lymphocytes

transformation was considerably affected by both low and high dosages of phytoestrogens removal.

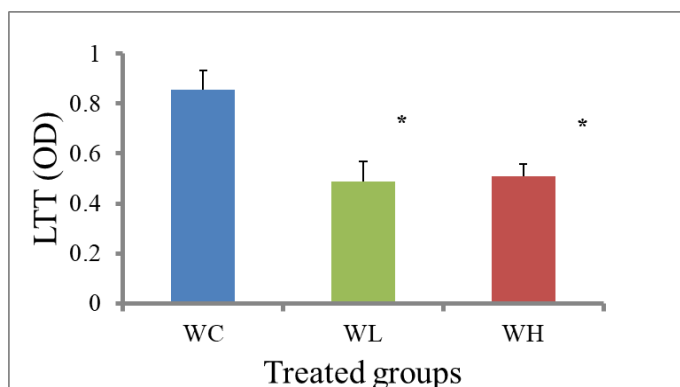


Figure 3: Effect of dietary soy phytoestrogens withdrawal on LTT (OD) in ovariectomized female albino rats. Data is expressed as mean $\pm$ SE. (*) denotes a statistical ($p < 0.05$) difference between the treated groups and the control. p -value for the WL group compared to the control is $p=0.03$, while the estimated p -value for the WH group compared to the control is $p=0.02$.

The elimination of dietary soy isoflavones had a substantial effect on the inflammatory markers that were examined in ovariectomized female albino rats, as shown in Table 7. The group that served as the control displayed significantly higher levels of TNF- α (8.165 ± 0.149 Pg/mL) as compared to the groups that received a low dose (5.811 ± 0.040 Pg/mL) and the group that received a high dose (5.156 ± 0.026 Pg/mL). It was shown that there were highly significant variances for both doses ($p = 0.00$), as well as a significant difference between the low dose group and the high dose group ($p = 0.03$). In a manner that is comparable, the groups that were treated demonstrated a significant reduction in the levels of IL-6, while the group that served as the control recorded 15.095 ± 0.149 Pg/mL. In contrast, the low and high dose groups presented levels of 12.417 ± 0.272 Pg/mL and 10.244 ± 0.042 Pg/mL, respectively ($p = 0.00$ for both compared to control, $p = 0.02$ between doses). A notable decline was recorded for IL-2, with the control group with value equal to 0.86 ± 0.02 Pg/mL compared to the treated groups (low dose: 0.77 ± 0.02 Pg/mL, high dose: 0.72 ± 0.02 Pg/mL). The p -values were 0.01 and 0.03, indicating significance, while no difference was found between the low and high dose groups ($p = 0.92$).

In relation to the resistin hormone, both treated groups exhibited notable decreases when compared to the control (4.522 ± 0.149 Pg/mL). The low dose group recorded a level of 3.687 ± 0.077 Pg/mL, while the high dose group measured at 3.251 ± 0.037 Pg/mL ($p = 0.023$ for low dose, $p = 0.011$ for high dose, and $p = 0.032$ between doses). The levels of NO showed no significant differences among the groups, with the control group measuring 23.94 ± 0.76 $\mu\text{mol/L}$

Table 7: Effect of dietary soy isoflavones withdrawal on TNF- α , IL-6 and IL-2 levels (Pg/ml) in ovariectomized female albino rats.

Parameters	Control	Phytoestrogens				
		Low dose	High dose	Probability (p)		
				Low dose vs control*	high dose vs control*	Low vs high dose#
TNF- α (Pg/mL)	8.165 $\pm$ 0.149	5.811 $\pm$ 0.040 [#]	5.156 $\pm$ 0.026 [#]	0.00	0.00	0.03
IL-6 (Pg/mL)	15.095 $\pm$ 0.149	12.417 $\pm$ 0.272 [#]	10.244 $\pm$ 0.042 [#]	0.00	0.00	0.02
IL-2 (Pg/mL)	0.86 $\pm$ 0.02	0.77 $\pm$ 0.02 [*]	0.72 $\pm$ 0.02 [*]	0.01	0.03	0.92
Resisting hormone (Pg/mL)	4.522 $\pm$ 0.149	3.687 $\pm$ 0.077 [#]	3.251 $\pm$ 0.037 [#]	0.023	0.011	0.032
NO (μ mol/L)	23.94 $\pm$ 0.76	22.86 $\pm$ 0.82	22.94 $\pm$ 0.69	0.11	0.23	0.14
CRP (mg/dL)	9.479 $\pm$ 0.155	7.943 $\pm$ 0.036 [#]	7.358 $\pm$ 0.057 [#]	0.003	0.001	0.02
ANA (ng/mL)	0.171 $\pm$ 0.008	0.103 $\pm$ 0.004 [#]	0.082 $\pm$ 0.004 [#]	0.00	0.019	0.00
COX-2 (ng/mL)	1.47 $\pm$ 0.04	1.11 $\pm$ 0.01	1.52 $\pm$ 0.30	0.12	0.16	0.07

Data is expressed as mean $\pm$ SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA), followed by post-hoc comparisons using Tukey's Honestly Significant Difference (HSD)_{ss} test for pairwise comparisons between groups. (*) indicate a statistically significant difference from the control group at $p < 0.05$. (#) denotes a statistically significant difference between the high and low dose of phytoestrogens at $p < 0.05$. Probability p -values are provided for pairwise comparisons between the groups.

and no notable changes observed in the treated groups ($p > 0.1$ for all comparisons). In this study, CRP levels were notably reduced in both the low dose (7.943 $\pm$ 0.036 mg/dL) and high dose groups (7.358 $\pm$ 0.057 mg/dL) when compared to the control group (9.479 $\pm$ 0.155 mg/dL) with statistical significance ($p = 0.003$ and $p = 0.001$, respectively). Additionally, a significant difference was observed between the treated groups ($p = 0.02$).

The control group for ANA exhibited a notably elevated level (0.171 $\pm$ 0.008 ng/mL) compared to the low dose (0.103 $\pm$ 0.004 ng/mL) and high dose (0.082 $\pm$ 0.004 ng/mL) groups, with all comparisons demonstrating statistical significance ($p < 0.05$). Ultimately, there were no notable differences in COX-2 levels among the groups, with the control group (1.47 $\pm$ 0.04 ng/mL) exhibiting levels comparable to those of the treated groups ($p > 0.1$ for all comparisons). The findings revealed that the removal of dietary soy isoflavones significantly influenced various immune and inflammatory markers, especially TNF- α , IL-6, IL-2, resistin, CRP, and ANA, while showing no substantial effect on NO or COX-2 levels.

Splenic tissue from low (WL) 6.60 % and high dose (WH) 26.41 % of dietary phytoestrogens withdrawal group displayed normal architecture. Thymic tissue of low (WL) 6.60 % and high dose (WH) 26.41 % dietary phytoestrogens withdrawal groups revealed apoptotic lymphocytes (Figure 4).

Substantial amounts of soy and its derivatives are utilized as a feed component for laboratory and pet food animals. In addition, humans are consuming soy and its derivatives, such as soy isoflavones and soy protein supplements, in

large amounts (Kim, 2021). Although there have been several research conducted, the understanding of the biological efficacy of soy phytoestrogens in diet and their effects on various body systems is still far from complete. This study aimed to evaluate the probable estrogenic influences of dietary soy phytoestrogens on immunological response. Two doses of soy isoflavones (low 6.6% and high 26.41%) were administered to account for the variability in their levels in human and animal diets. Furthermore, it is necessary to evaluate whether there is variability in the immunological response to these substances based on the dosage, and determine whether their effects may be reversed upon discontinuation for one month.

The administration of low (6.60 %) and high levels of dietary soy phytoestrogens (26.41%) to ovariectomized female rats were within the human exposure limit that varies according to traditional feeding habits between nations. Asians consumed 20 to 50 g of soy daily, which constitutes their chief source of phytoestrogens, matched to a daily intake of 20 to 80 mg of phytoestrogens daily however, European and United States people may consume about 0.49 to 0.66 and 0.15-3 mg daily (Adlercreutz *et al.*, 1991; Sirtori *et al.*, 2005; Desmawati and Sulastri, 2019). This study found that the daily feed consumption of ovariectomized female albino rats was not influenced by exposure to soy phytoestrogens in diet. The findings of Weber and Lephart (2014) and Weber *et al.* (2001) are consistent with these results. However, Kelany *et al.* (2017) demonstrated that soy reduced feed intake in female rats. The rats that were nourished high soy phytoestrogens experienced a substantial decrease in their daily gain of the body weight in comparison to the control group. The findings align with the results published in the research

conducted by Tolba (2013), Helmy *et al.* (2014), Ma *et al.* (2014), and Abdelrazek *et al.* (2019). The decrease mentioned indicates that the activity of isoflavones, which have estrogenic hormone properties, is advantageous for regulating body fat (Szkudelska *et al.*, 2000; Tolba, 2013). On the other hand, Akhlaghi *et al.* (2017) demonstrated non-statistical variation in human body weights fed on soy phytoestrogens.

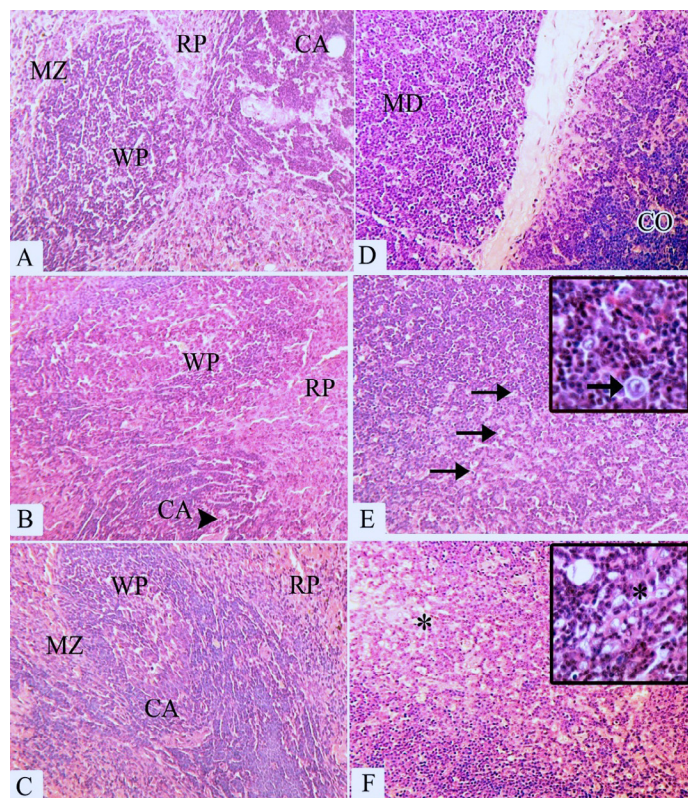


Figure 4: Histomorphology of (A) spleen (control group, WC) showed RP: Red pulp, WP: White pulp CA: Central artery, MZ: Marginal zone, (B) low dose of dietary soy (6.6%) phytoestrogens withdrawal group (WL) showing mild depletion of T cells in the PALS. The lymphocytes in the PALS have been replaced by small numbers of macrophages, granulocytes, and plasma cells, Brown fat was present (arrows). (C) high dose of dietary soy (26.41%) phytoestrogens withdrawal (WH) group. (D) thymus (control group) showing CO: Cortex, MD: Medulla. (E) thymus of low dose of dietary soy (6.6%) phytoestrogens withdrawal group (WL) showed apoptotic bodies (arrow). (F) thymus of high dose of dietary soy phytoestrogens withdrawal group (WH) showed striking tinctorial change is obvious in the treated thymus, especially in the cortical regions due to marked apoptosis of the lymphocytes in this region of the treated rat (astriks). [H&E stain; original magnification: $\times 20$].

This study found that the intake of dietary soy isoflavones did not have an impact on the relative weights of the spleen. This is consistent with the findings reported by Kakehashi *et al.* (2012), Nishide *et al.* (2013), and

Abdelrazek *et al.* (2019) while Gaffer *et al.* (2018) found significant reduction in splenic weight of male rats exposed to soy phytoestrogens during pregnancy. The source of contradictory may be attributed to difference in exposure route and animal model. The thymic relative weight was significantly reduced in the OVX rats that consumed a high intake of soy phytoestrogens matched to the group supplemented with low soy phytoestrogens and control one. These findings are consistent with the previous ones conducted by Yellayi *et al.* (2002), Zhao *et al.* (2006), and Abdelrazek *et al.* (2019). The decrease in thymus weight can be ascribed to the impact of genistein on the thymus over two potential machineries: Estrogen receptors and mechanisms that do not include estrogen receptors (non-ER-mediated mechanisms) (Yellayi *et al.*, 2002). On the other hand, Zhang *et al.* (1997) demonstrated an increment in mice splenic weight after exposure to soy daidzein (20 and 40 mg/kg) for 7 days.

The thymus possesses both estrogen receptors, ER- β and ER- α . It is important to mention that isoflavones, particularly genistein, have a strong attraction to both receptor subtypes because of their similar chemical properties to estradiol (Sakai and Kogiso, 2008). Estrogen treatment has been shown to cause thymic atrophy and immune suppression in mature rodents (Kohen *et al.*, 1998; Taves and Ashwell, 2022). In addition, genistein, an isoflavone of phytoestrogens, has been found to affect the thymus through a mechanism that does not depend on estrogen receptors (Yellayi *et al.*, 2003). This mechanism involves the influences of isoflavones on topoisomerase II and/or protein tyrosine kinases, which have been demonstrated to be repressed in thymocytes and other cell types when exposed to high levels of genistein in laboratory studies (Mustelin *et al.*, 1990; Essex, 1996). Topoisomerase II is a nuclear protein catalyzing the reaction of DNA relinking and breakage. Hence, soy phytoestrogens inhibit this enzyme it could impede its interplay in keeping DNA topology needed for DNA transcription, replication, and recombination of cellular genes (Zhou *et al.*, 2009), therefore causing apoptosis in lymphoid organs and depletion as noted in the present study. Tyrosine kinases are important signals transduction enzymes tangled in regulation of differentiation and growth of thymocytes and splenocytes (Rinke de Wit *et al.*, 1996), therefore their inhibition via soy phytoestrogens could cause the observed lymphoid depletion. The suppression of both topoisomerase II or protein tyrosine kinases may have an impact on other factors, particularly IL-2, which is essential for the growth and specialization of thymocytes and T-lymphocytes (Ross and Cantrell, 2018). Consequently, there was a notable decrease in the proliferation of T-lymphocytes in the group that had a high amount of dietary soy (26.41%) phytoestrogens compared to the groups that consumed a

low amount (6.6%) of dietary soy phytoestrogens and the control group. Therefore, the usage of soy phytoestrogens in diet may be applied as a therapeutic immunosuppressive agent.

Leukocytes are potential part of immune system that contribute in humoral and innate immune responses. They are found in the blood circulation and tissues where they can take part in cellular and inflammatory responses to pathogens or injury (Sollome and Fry, 2015). The loss of endogenous estrogen has been widely linked to reduction in TLC in women (Chen *et al.*, 2016; Eledo *et al.*, 2018) that needs a compensatory supplement. The consumption of soy isoflavones had a significant impact on TLC in both the high and low soy fed groups, compared to the control one. However, the dietary soy phytoestrogens therapy did not change the DLC. These findings align with that mentioned by Zhao *et al.* (2005). The rise in TLC seen in this study may be linked to the reduction in interleukin levels, which hinders the chemotactic response of leukocytes (Trifilieff *et al.*, 2000; Pfeilschifter *et al.*, 2001). This result takes us to the possible compensatory effect of soy phytoestrogens on TLC depletion after menopause that possibly improves immune response.

The IL-6 and TNF- α are considered inflammatory mediators that play a substantial part in aging associated low grade of inflammation, inflammatory diseases and autoimmunity through NF- κ B and STAT3 pathways (Kim *et al.*, 2012; Hirano, 2021). Gonadectomy in females or reaching menopause age in women makes them susceptible to inflammatory diseases and elevation of cytokines (Kim *et al.*, 2012). The serum concentrations of IL-6 and TNF- α were statistically lower in both the high (26.41%) and low (6.6%) dietary soy phytoestrogens groups compared to the control OVX rats. These findings align with the results mentioned by Abdelkarem *et al.* (2011) and Abdelrazek *et al.* (2019). This data was in partial agreement with Jenkins *et al.* (2002) who found significant reduction of IL-6 in middle aged women after high soy isoflavones intake however, TNF- α did not affected. They suggested the anti-inflammatory potential of genistein and daidzein. Previous investigations have demonstrated that genistein can hinder NF- κ B/TNF- α signaling pathways through both ER-dependent and ER-independent mechanisms (Seibel *et al.*, 2009; Messina *et al.*, 2011). The suppressing effect of phytoestrogens could be due their selective binding to estradiol receptors 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). NF- κ B/IKK-dependent signaling pathway is also prevented via phytoestrogens where NF- κ B gene expression is impeded via blockage of the key intermediate activating unit I κ B kinase (IKK), which phosphorylates the inhibitor subunit (I κ B) of this

transcription factor (Zhou *et al.*, 2010; Pavlova *et al.*, 2016). Moreover, phytoestrogens reduce protein tyrosine kinase activity via non ER- non-dependent machinery (Gredel *et al.*, 2008) that is associated with the stimulation of TNF- α and IL-6 production in response to introduction of LPS in murine macrophages (Geng *et al.*, 1993; Beaty *et al.*, 1994). Another possible explanation is that the consumption of soy phytoestrogens in the diet caused a reduction in body weight increase and fat accumulation, as seen in this study. This decrease in weight gain was allied with a decrease in IL-6 levels, which is believed to be formed by adipocytes and is considered one of the adipokines, as proposed by Mohamed-Ali *et al.* (1997) and Bastard *et al.* (2000).

This study found a substantial reduction in resistin hormone levels in both the high and low dietary soy phytoestrogens groups, as matched to the control group. The findings align with that achieved by Liu *et al.* (2006), Fazliana *et al.* (2009), and Abdelrazek *et al.* (2019) in animals and as reviewed by Domínguez-López *et al.* (2020) in human. Resistin is synthesized by adipose tissue and stimulates an inflammatory response by triggering the generation of TNF- α by macrophages via the NF- κ B pathway (Silswal *et al.*, 2005). The current investigation revealed that dietary soy isoflavones have a lowering effect on serum resistin levels, indicating potential anti-inflammatory activity of genistein and daidzein (Steppan and Lazar, 2004; Reilly *et al.*, 2005). Soy isoflavones decreased the concentrations of cytokines such as IL-6, TNF- α , and CRP, along with a decrease in resisting levels. This suggests that resistin levels are regulated and associated with the levels of these cytokines (Lehrke *et al.*, 2004) and the inflammatory protein (Shetty *et al.*, 2004), where IL-6 stimulates the liver to produce CRP (Heilbronn *et al.*, 2001). There is statistical significant connotation between CRP level and severity of various inflammatory diseases, including autoimmunity in human (Morrow and Ridker, 2000). CRP has the ability to bind antigens or autoantigens in autoimmune diseases via deposition of complement cleavage fragments on both ligand and on the CRP (Volanakis and Narkates, 1983). CRP can intercede complement-dependant chromatin solubilization (Robey *et al.*, 1985) therefore, help in the nuclear material clearance from the blood circulation by engaging Fc γ R and complement in human (Szalai, 2004). Reports cleared that administration of human CRP to mice influence clearance of plasma nucleosome core-particles and chromatin (Burlingame *et al.*, 1996). The more accumulation of apoptotic cell debris and the formation of ANA are pathognomonic for autoimmune dysfunction in human (Russell *et al.*, 2004) and animal models (Theofilopoulos and Dixon, 1987). Therefore, the abridging of CRP and ANA by soy phytoestrogens denoted their beneficial affects against autoimmunity or inflammation induced by estrogen depletion. Vafeiadou *et al.* (2006) confirmed this result in their study on

postmenopausal women given isoflavones. They pointed out that isoflavones potentially lowered the CRP levels without altering other inflammatory cytokines.

The levels of serum NO exhibited a noteworthy decrease in both the high (26.41%) and low dietary (6.6%) soy phytoestrogens groups compared to the control one. These findings align with the results attained by [Hooshmand et al. \(2007\)](#) and [Jantaratnotai et al. \(2013\)](#). The observed outcomes may be ascribed to the suppressive impact of phytoestrogens on iNOS expression, as reported by [Jantaratnotai et al. \(2013\)](#). NO is a redox molecule that has a role in the development and regulation of infectious diseases, malignancies, autoimmune processes, and chronic degenerative disorders ([Wink et al., 2011](#)). NO is considered a toxic or signalling agent. It is produced from macrophages toward infectious agent liberating free radicals that kills the causative agent ([Coleman, 2001](#)). Also, NO is preferentially formed by the Th1 lymphocytes ([Taylor-Robinson et al., 1994](#)) and selectively inhibits Th1 responses ([Wei et al., 1995](#)) suggesting an autocrine down-regulation of Th1 by NO. At the same time NO provokes Th2 responses, that enhances the manufacture of IgE and promotion of IgE-mediated diseases such as asthma ([Barnes and Liew, 1995](#)) or auto immune diseases ([Olewicz-Gawlik and Kowala-Piaskowska, 2023](#)). The decrease in the amount of NO in the treated groups is expected, given these chemicals are known for their antioxidant impact ([Röhrdanz et al., 2002](#); [Rimbach et al., 2008](#); [Park et al., 2010](#)). The decrease in NO will impact cellular signaling in all physiological pathways associated with this compound. For instance, it will affect the leukocytes chemotactic response, as evidenced by an increase in the TLC in the circulation of rats treated with isoflavones ([Triflieff et al., 2000](#); [Pfeilschifter et al., 2001](#)). In addition, the herein study found that the role of NO as an intracellular messenger in chemokine signaling pathways ([Cherla and Ganju, 2001](#)) was reduced. This was demonstrated by the drop in levels of IL-6, IL-2, and TNF- α in the groups treated with soy phytoestrogens. All of these factors enhance the anti-proliferative and anti-inflammatory belongings of soy genistein ([Jeong et al., 2014](#)) and daidzein ([Peng et al., 2017](#)) on the immune system.

This study demonstrated a notable reduction in ANA levels in both the high and low dietary soy isoflavones groups as matched to the control one. The decrease in circulating levels of ANA could be attributed to the impact of isoflavones, particularly genistein, on splenic CD4⁺CD25⁺ cells. This effect was not estimated in this study. The influence on ANA levels is likely due to the impact on the deficiency of the complement component 4 (C4) gene, as demonstrated by [Leaungwutiwong et al. \(2011\)](#). All of these factors reduce the removal of dying cells, which could potentially create autoantigens. These autoantigens can then activate

T and B cells, leading to the production of autoantibodies. This process also decreases the levels of ANA ([Jørgensen et al., 2003, 2004](#); [Shim et al., 2004](#); [Munoz et al., 2005](#); [Leaungwutiwong et al., 2011](#)).

The consumption of soy phytoestrogens led to a substantial decrease in blood COX-2 levels in both the high soy (26.41%) and low soy (6.6%) dose groups, in a way that was dependent on the dosage, when matched to the control OVX rats. The results align with the studies conducted by [Hooshmand et al. \(2007\)](#) in human chondrocytes, [Valles et al. \(2010\)](#) in cultured astrocytes, [Khan et al. \(2012\)](#) in mice, [Takaoka et al. \(2018\)](#) in human endometrial cells and [Abdelrazek et al. \(2019\)](#) in rat. In fact, the estrogen depletion is associated with promotion of inflammation and proinflammation in women ([McCarthy and Raval, 2020](#)). The later response is characterized by accumulation of leukocytes, variations in vascular permeability, and inflammatory mediators production, such as TNF- α , IL-6, and other chemokines ([Feghali and Wright, 1997](#)). Some of these cytokines can stimulate NF- κ B expression, a that regulates the transcription of various acute phase proteins and numerous stress response genes, such as inducible nitric oxide synthase and COX-2 ([Pahl, 1999](#)) that is a crucial pro-inflammatory enzyme responsible for converting arachidonic acid into prostaglandins that are associated with pain and inflammation ([Smith et al., 2000](#)). The reduction in COX-2, may account for the anti-inflammatory impact of soy genistein and daidzein, which imitate estrogen as selective estrogen receptor modulators. The study by [Hermenegildo et al. \(2005\)](#) investigated the influences of isoflavones on estrogen receptors, specifically whether they operate as agonists or antagonists. These receptors interplay in regulating the amalgamation and bioactivity of COX-2. The results can be claimed to the repressive consequence of isoflavones, particularly genistein, on NF- κ B, which subsequently suppresses the formation of COX-2 ([Largo et al., 2003](#); [Hooshmand et al., 2007](#)). In addition, soy isoflavones, genistein ([Park et al., 2010](#)) and daidzein ([Röhrdanz et al., 2002](#)), have been found to possess antioxidant properties, as reported by [Morvaridzadeh et al. \(2020\)](#). Antioxidants are known to exert inhibitory effects on the activation of protein kinase C mediated by phorbol esters, as demonstrated by [Lee and Lin \(1997\)](#), as well as on activator protein-1, as shown by [Huang et al. \(1991\)](#), both of which are involved in COX-2 promoter activity.

Withdrawing dietary soy phytoestrogens did not result in significant changes in daily food consumption, body weight growth, splenic relative weight, thymic relative weight, TLC, DLC, NO levels, and COX-2 levels among the three groups. This phenomenon can be attributed to the fact that the elimination half-lives of daidzein and genistein, although not determined in this work, are brief

due to their quick metabolism (Shelnutt *et al.*, 2000). Previous explanation of Setchell and Adlercreutz (1988) who stated that isoflavones have well-known dilapidation pathways that hinge on intestinal flora, dietary habits, redox activity, and intestinal motility. Once the phytoestrogens are ingested, they are in glycoside form in plant which is transformed to aglycon form by acidic gastric juice. Once isoflavones reach intestine, it is processed by gut flora. Genistein is transferred into inert hormonal form called *p*-ethylphenol and daidzein is transformed into equol (about 70%), isoflavan (about 10–25%) and desmethylangolensin (about 5–20%). The composition of intestinal bacteria and their number influence the later transformation rates that decline in case of antibiotic therapy, ileostomy and feeding soy milk in early life that dramatically down regulate intestinal flora (Setchell *et al.*, 2001; Lampe, 2003). Diet nature influence transit time in intestine and redox state that influence intestinal bacterial composition and count (Setchell and Adlercreutz, 1988). Bioavailability of isoflavones varies according feeding habits where it is equal 30%–40% in vegetarians and Asians and reaches 13% to 35% on non-vegetarians (Xu *et al.*, 1995; Glazier and Bowman, 2001). Therefore, these factors that influence the blood concentrations of daidzein and genistein, as well as their plasma half-lives (which were not evaluated in this study), can result in changes in the time it takes for these substances to be eliminated from the body. The elimination of dietary soy isoflavones in this study led to a substantial decrease in LTT, TNF- α , IL-2, IL-6, resistin hormone, ANA, and CRP in rats that were previously treated with phytoestrogens, in comparison to the control group. The decline was unexpected, as the impact of isoflavones on these parameters persisted long after their discontinuation. Therefore, the influence of these chemicals on immunological processes, particularly cell-mediated immunity, could not be reversed within one month after their absence. The current study elucidated a close relationship between withdrawal of soy phytoestrogens and the persistent reduction in LTT, TNF- α , IL-2, IL-6, resistin hormone, ANA, and CRP that ensure continuity of anti-inflammatory, anti-lymphopoietic and anti-autoimmune effects of soy phytoestrogens. These results didn't support our hypothesis that soy phytoestrogens withdrawal could reverse their influence on ovariectomy induced cellular immune system perturbations. Therefore, further studies are required to clarify the existing mechanism beyond the persistence of soy phytoestrogens influence on ovariectomy induced immune perturbations.

CONCLUSIONS AND RECOMMENDATIONS

The utilization of dietary soy phytoestrogens in a state of estrogen deficiency balanced the cellular immunological

parameters to counteract the negative effects of estrogen depletion, aging, and autoimmune reactions. This impact remained partially irreversible even one month after they stopped following the diet. The observed effects suggest that soy phytoestrogens could be a beneficial alternative to estrogen replacement therapy in older individuals, menopausal women, and those with estrogen shortage. This could help mitigate the negative impact of these circumstances on the immune system. A limitation of this was to measure the half-life of genistein and daidzein in plasma for accurate correlation of their withdrawal time from blood. Another limitation was the absence of mechanistic approach toward studying the influence of phytoestrogens withdrawal, therefore, further studies are required to perfectly elucidate the actual mechanisms and pathways that make some parameters resistant to change upon withdrawal while others are not.

ACKNOWLEDGEMENT

The authors would like to acknowledge Dr. Mona Osama, Lab animal assisting staff, Faculty of Veterinary Medicine, Suez Canal University, for her help in ovariectomy procedures.

NOVELTY STATEMENT

This research gave a new focus about the effect phytoestrogens withdrawal on cell mediated immune parameters.

AUTHOR'S CONTRIBUTION

Conceptualization, H.M.T, H.M.A.A, S.N.A; Methodology, H.M.A.A, H.M.T, H.M.T, R.M.G; Formal analysis, R.M.G, H.M.A.A, H.M.T; Investigation, H.M.A.A, R.M.G, H.M.T, S.N.A; Resources, H.M.A.A, R.M.G, H.M.T; Writing- original draft preparation, R.M.G, S.N.A; Writing-review and Editing, H.M.A.A., H.M.T, H. M. T, S.N.A all authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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