



Dietary soy isoflavones during pregnancy suppressed the immune function in male offspring albino rats



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ABSTRACT

Phytoestrogens have an impact on both animals and humans due to use of legumes in animal diets as well as the increase of vegetarian diets in some human populations. Phytoestrogens thought to have varieties of adverse effects, among which immune system was involved. The present study aimed to investigate the effect of prenatal exposure to dietary soy isoflavones on some immunological parameters in male albino rat offspring. The pregnant rats were divided to three groups (12/group). Control group (free soy isoflavones), low soy isoflavones group (6.5%) and high soy isoflavones group (26%). The male offspring cell-mediated immune response was determined using phytohemagglutinin (PHA) injection and the intumescence index which was calculated on postnatal day 50 (PND 50). At PND 50, blood samples were collected for interleukin 12 (IL-12), interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) determination. Spleen, thymus, and PHA injected footpads were fixed for histopathology. Intumescence index, IL-12, IFN- γ , spleen and thymus relative weights were significantly ($P < 0.05$) decreased in offspring born to dams fed low and high dietary soy isoflavones. In contrary, TNF- α was significantly ($P < 0.05$) increased in offspring born to dams fed high dietary soy isoflavones. Spleen of rats born to dams fed high dose of dietary soy isoflavones showed coagulative necrosis in white pulp. In conclusion, male offspring born to dams fed different levels of soy isoflavones showed marked immunosuppression after PHA stimulation. This effect was mediated through the reduced IFN- γ that interacts with the IL-12 production pathway.

1. Introduction

Progressive accumulation of endocrine disruptors in the environment has deteriorated the ecological balances in natural populations and affected human health [1]. Although estrogen hormone and estrogen like substances can promote both humoral and cell-mediated immune responses, there are a considerable number of reports that show the suppressing effect of estrogens on some cell-mediated immune responses [2,3]. Phytoestrogens are natural polyphenolic non-steroidal plant compounds with estrogen-like biological activities [4,5] and structurally are similar to mammalian estrogen 17 β -estradiol (E2) [5]. The isoflavones genistein (GEN) and daidzein are among the most abundant phytoestrogens in human diets [6]. They can be classified as selective estrogen receptor modulators (SERMs) [7,8], where they have the ability to trigger estrogenic activity to act as agonist or antagonist [9,10] depending on the tissue, estrogen receptors (ERs) and

concentration of circulating endogenous estrogens [10]. The interaction between isoflavones and nuclear estrogen receptors that activates estrogen response elements is called genomic signaling pathway [11]. Another more faster and rapid action of isoflavones is mediated through binding of membrane ERs [12]. Binding membrane ERs promotes a cascade of intracellular events that comprises activation of G-proteins, protein kinase, phospholipase, or adenylate cyclase activities [13]. Isoflavones can act as tyrosine kinase inhibitors [14]. Moreover, these compounds possess antioxidant activity [15] due to its polyphenolic nature [16]. Isoflavones exert myriad effects on different body systems and organs. They can affect immune system [2,17], reproductive system [4,18], nervous system [19], liver [6], bone [20] as well as their potential antioxidant effect and antidiabetic effect [21].

Interleukin 12 (IL-12) is an important immunoregulatory cytokine that is produced mainly by antigen-presenting cells. The expression of IL-12 during infection regulates innate responses and outlines the type

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of the adaptive immune responses to be triggered. IL-12 can provoke the production of interferon- γ (IFN- γ) and triggers CD4⁺ T cells to differentiate into type 1 T helper (Th1) cells [22]. TNF- α has a crucial role in immune regulation by modulating lymphocyte proliferation and apoptosis, which is implicated in maintaining immune homeostasis and self-tolerance [23]. TNF- α activates cell inflammation, proliferation, survival and cell death depending on autocrine/paracrine signals, and on the cellular context [24,25].

In toxicity studies, endocrine-mediated effects have been reported in rat pups of dams treated with GEN during the gestational and/or lactation periods [26–28]. Moreover, most studies have focused on the effects of estrogenic pesticides and toxic substances on immune function [29,30]; less attention has been paid to the effects of naturally occurring phytoestrogens administered during pregnancy on the immune system of the male's first generation. Therefore, the aim of this study was to examine the effects of maternal exposure to soy isoflavones on cellular immune response of male offspring through determination of the immune response to intradermal PHA injection, IL-12, IFN- γ and TNF- α levels and to investigate the histopathological changes in foot pad, spleen and thymus.

2. Materials and methods

2.1. Rats

Forty five adult (36 females and 9 males), Wister albino rats, weighing from 180 to 250 g, were housed in a plastic cage (3/cage) at Laboratory Animal House, Faculty of Veterinary Medicine, Suez Canal University, Egypt. They were maintained under standard natural day light with a temperature of 25°C (\pm 1°C) and allowed to diet and water *ad libitum*. The animals were treated according to ethical guidelines described by Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

2.2. Monitoring estrous cycle and breeding

The estrous cycle of the rats was checked daily by cytological examination of vaginal smear to determine the females with regular cycles. Vaginal smears were obtained, processed and evaluated according to Ebeid et al. [31]. A mature male was presented with three proestrus females and mating was confirmed by the presence of spermatozoa in the vaginal smears or the occurrence of vaginal plug and this was considered the pregnancy Day zero [32].

2.3. Isoflavones analysis and administration

Isoflavones were extracted, detected and quantified from the diet using high performance liquid chromatography (HPLC) according to Thiagarajan et al. [33]. Briefly, soy isoflavones were extracted from the experimental diet by mixing 1 g of diet with 20 mL of solution of 0.1 mol/L HCL and 80 mL of methanol, and then sonication of mixture was performed for 20 min and left at room temperature for 2 h. Filtration using Whatman filter paper (Clifton, New Jersey) was performed. The obtained filtrate was subjected to centrifugation at 10,000 rpm. The obtained supernatants were quantified for isoflavones contents. Isoflavones were quantified by comparison with genistein (Applichem GmbH Co., Germany) and daidzein, (Fullcco Co., Japan) with HPLC standards.

The female rats at Day zero of pregnancy were allocated to three groups. The first group (n = 12) were fed a control diet (soy isoflavones free). The second group (n = 12) were fed low dose of soy isoflavones (6.5%). The third group (n = 12) were fed high dose of soy isoflavones (26%). All diets were formulated to fulfill all the nutritional requirements of pregnant rats [34]. The percentages of soy isoflavones in both treated groups covered the level of 20–50 g of daily soy as a source of phytoestrogens that consumed by Asian population [35]. Experimental

diets were offered from Day zero of pregnancy to the Day of birth to the dams. After parturition, male offspring were selected and were given control diet up to Day 50 after birth which was defined as post natal day 50 (PND 50).

2.4. Cell mediated immune response in male offspring

Offspring's cell mediated immune response was carried out by injection of 0.1 mL of 10% PHA (Sigma L 9017, St. Louis, MO, USA) in left foot pad of each male in all experimental groups at PND 49. The right foot pad of the same rat was injected with 0.1 mL of PBS as a control. After 24 h (PND 50), thickness of dorso-ventral and lateral aspects of left footpad at point of injection was measured by using a manual micrometer [36]. The injections and measurements were made by the same person to reduce the error.

2.5. Determination of intumesce index

The ankle circumference was calculated according to $= 2\pi [\sqrt{a^2 + b^2/2}]$, where [a] is the dorso-lateral diameter and [b] is the dorso ventral diameter [37,38]. This was followed by calculation of intumesce index [39]. Intumesce Index = (measured ankle size – primary ankle size)/primary ankle size.

2.6. Determination of serum IL-12, IFN- γ and TNF- α

Blood samples were collected at the end of experimental period (PND 50). Serum was separated and kept at -20°C until analysis. Serum IL-12 was measured using rat IL-12/P70 sandwich ELISA kit (CUSABIO, China). Serum IFN- γ was measured using rat IFN- γ sandwich ELISA kit (R&D systems, China). Serum TNF- α was measured using rat TNF- α enzyme linked immunosorbent assay sandwich ELISA kit (IBL Co., Japan) according to manufacturer instructions.

2.7. Spleen and thymus relative weights

At PND 50, male offspring were scarified and the relative weight of spleen and thymus was calculated in relation to body weight [40,41].

2.8. Histopathology

PHA stimulated foot pads, spleen and thymus of males were fixed in 10% formalin buffer saline. They were gradually dehydrated then embedded in paraffin wax. Several 5- μm sections were cut then stained with hematoxylin and eosin (H&E) for histopathological examination [42].

2.9. Statistical analysis

The results were presented as the mean \pm standard error of mean (SEM). Statistically significant differences between groups were calculated using one way analysis of variance (ANOVA) followed by Duncan's post hoc multiple comparison test (SPSS software, version 16.0; SPSS Inc., Chicago, IL, USA). Dose-response to soy supplementation on the immune parameters was evaluated and soy-supplemented groups were compared with the control group for linear and quadratic contrasts using the generalized linear models procedure of SAS 9.3 (SAS Institute Inc., Cary, North Carolina, USA). The criterion for significance was set at $P < 0.05$.

3. Results

3.1. The number of male offspring

The number of male pups/dam was 3.0 ± 0.6 in control group, while, it was 3.2 ± 0.7 and 2.8 ± 0.4 in dams received low and high

Table 1
Effect of prenatal exposure to dietary soy isoflavones on intumesce index, IL-12, IFN- γ , TNF- α levels and relative organ weights of male offspring after 24 h of PHA injection.

Parameters	Control	Low soy isoflavones (6.5%)	High soy isoflavones (26%)	P value	
				Linear	Quadratic
Intumesce index	0.08 \pm 0.004 ^a	0.04 \pm 0.005 ^b	0.03 \pm 0.006 ^b	0.067	0.0001
IL-12 (pg/ml)	14.8 \pm 0.5 ^a	10.8 \pm 0.6 ^b	8.7 \pm 0.3 ^c	0.0001	0.0001
IFN- γ (pg/ml)	666.6 \pm 2.4 ^a	612.4 \pm 2.5 ^b	432.5 \pm 5.5 ^c	0.0001	0.0001
TNF- α (pg/ml)	19.1 \pm 0.5 ^a	22.3 \pm 0.4 ^a	32.6 \pm 2.0 ^c	0.0001	0.0001
Relative spleen weight (g/100 g)	0.5 \pm 0.04 ^a	0.3 \pm 0.01 ^b	0.3 \pm 0.01 ^b	0.0029	0.0008
Relative thymus weight (g/100 g)	0.3 \pm 0.02 ^a	0.2 \pm 0.01 ^b	0.2 \pm 0.02 ^b	0.0048	0.0007

Different superscripts within the same row indicated significant differences at $P < 0.05$.

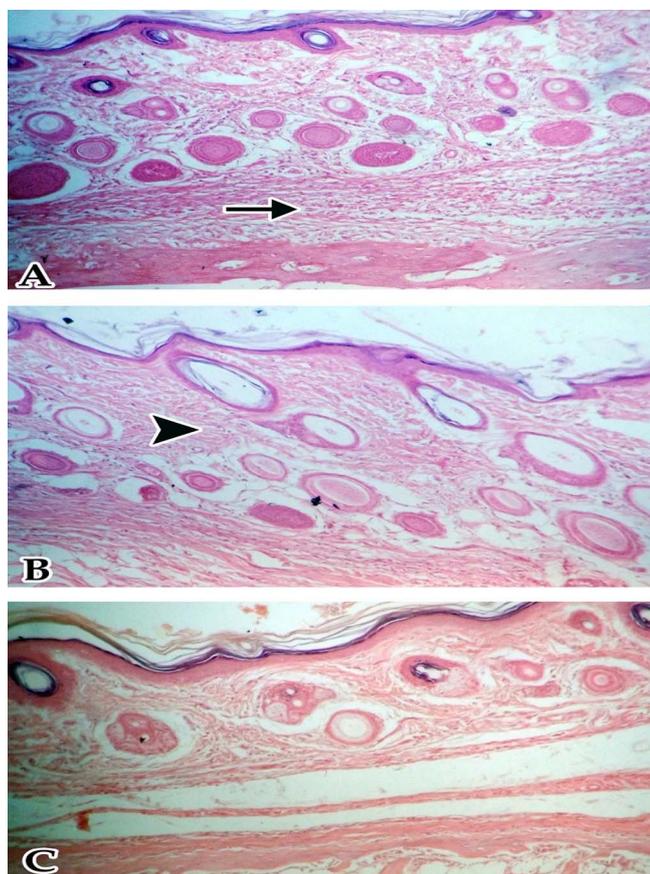


Fig. 1. Left foot pad of male offspring after 24 h of PHA injection. Male rats born to control dams (A) showing severe lymphocytic infiltration (arrow). Male rats born to dams exposed to low dietary soy isoflavones (B), mild lymphocytic infiltration was observed (head arrow). Male rats born to dams received high dietary soy isoflavones (C), no lymphocytic infiltration was detected (H&E; 200X).

soy isoflavones diet, respectively.

3.2. Soy isoflavones level in the diet

HPLC analysis for the experimental diet showed that control diet had zero level of genistein and daidzein, while low soy isoflavones diet contained 400 $\mu\text{g/g}$ genistein and 195 $\mu\text{g/g}$ daidzein. High soy isoflavones diet contained 1500 $\mu\text{g/g}$ genistein and 800 $\mu\text{g/g}$ daidzein.

3.3. Effect of soy isoflavones on intumesce index

Prenatal exposure to dietary soy isoflavones significantly ($P < 0.05$) reduced the intumesce index in male offspring born to dams received low and high soy isoflavones compared to those born to dams in control group. However, there was no significant differences in

intumesce index between male offspring born to dams exposed to low and high soy dietary soy isoflavones (Table 1).

3.4. Effect of soy isoflavones on IL-12, IFN- γ and TNF- α

IL-12 and IFN- γ were significantly ($P < 0.05$) decreased in offspring born to dams received high and low dietary soy isoflavones groups compared to that in control group. Moreover, offspring born to dams exposed prenatally to high dietary soy isoflavones group showed a significant ($P < 0.05$) decrease in IL-12 levels compared to that in low dietary soy isoflavones. In contrary, TNF- α was significantly ($P < 0.05$) increased in offspring born to dams received high dietary soy isoflavones group compared to that in low soy isoflavones and control groups (Table 1).

3.5. Effect of dietary soy isoflavones on relative weights of spleen and thymus

The relative weights of spleen and thymus were significantly ($P < 0.05$) decreased in rats born to dams exposed to high and low dietary soy isoflavones compared to that in control groups (Table 1).

3.6. Dose-response of soy supplementation on the immune parameters

In male offspring, IL-12, IFN- γ (linear, $P < 0.0001$) and relative spleen (linear, $P = 0.0008$) and thymus weights (linear, $P = 0.0007$) were decreased whereas intumesce index tended to decrease (linear, $P = 0.067$) with the increase in dietary soy isoflavones in the diets of pregnant rats. However, TNF- α concentration was increased (linear, $P < 0.0001$) with the increasing of dietary soy isoflavones in the diets of pregnant rats (Table 1).

3.7. Histopathology

Mild lymphocytic infiltration was observed in foot pads of offspring born to dams exposed to low dietary soy isoflavones supplemented diet. However, no lymphocytic infiltration was observed in foot pads of offspring born to dams received high dietary soy isoflavones (Fig. 1). The spleen of rats born to dams received low dietary soy isoflavones showed lymphocyte depletion in white pulp, while coagulative necrosis was seen in white pulp of rats born to dams received high dose of dietary soy isoflavones (Fig. 2). The thymus of rats born to dams received a low dietary soy isoflavones displayed tingible body macrophages with intracytoplasmic apoptotic bodies, while the thymus of rats born to dams administrated high dietary soy isoflavones showed advanced atrophy and severe lymphocyte depletion and vacuolation (Fig. 3).

4. Discussion

Although knowledge on the effects of soy isoflavones has increased recently, due to the consumption of soy isoflavones via soy supplements

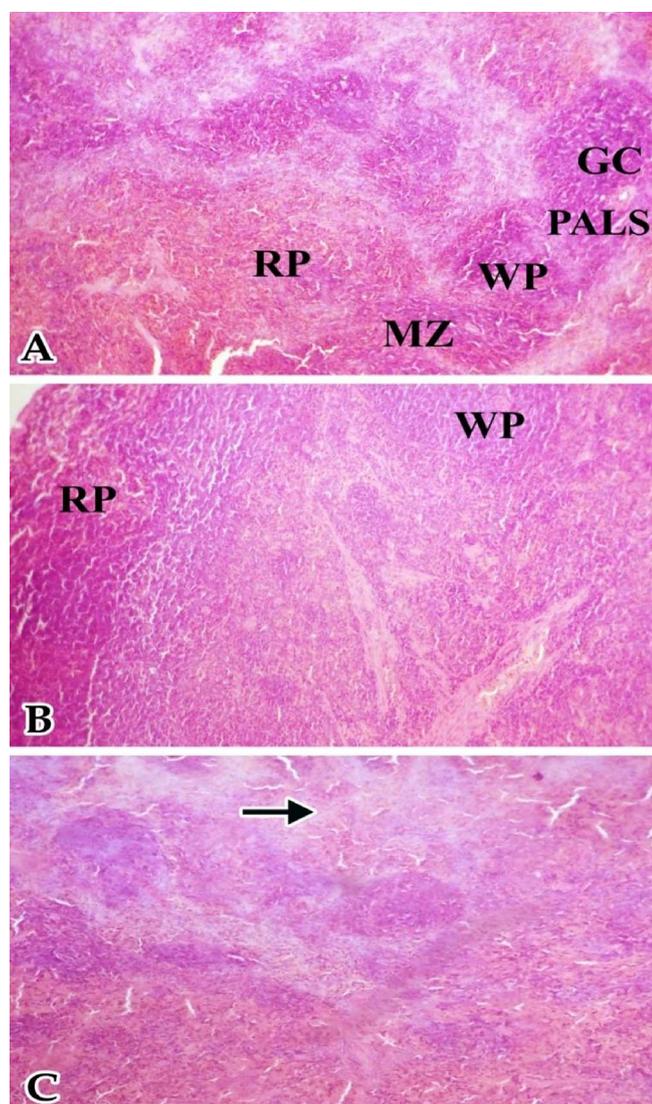


Fig. 2. Spleen of male offspring after 24 h of PHA injection. Normal spleen of a control group (A) showing white pulp (WP) area, red pulp (RP) area, marginal zone (MZ), germinal center (GC) and periarteriolar lymphoid sheath (PALS). Male rats born to dams exposed to low dietary soy isoflavones (B), spleen showed mild depletion in WP cellularity. Male rats born to dams received high dietary soy isoflavones (C), showed coagulative necrosis in WP (arrow) (H&E; 200X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and food products in adult and infant diets, there have been only scarce studies concerning the prenatal exposure to soy isoflavones and its effect on cellular immune response in offspring of male rats.

Male offspring born to dams received low and high dietary soy isoflavones showed reduction in intumescence index after 24 h of PHA injection. This might be attributed to the effect of prenatal exposure to estrogenic chemicals that could produce weaker inflammatory responses to PHA as compared with control individuals [43], suggesting that prenatal exposure to dietary soy isoflavones had an immunosuppressive effect. Furthermore, *in vivo* immunosuppressive effect of high soy diet or genistein was also reported by O'Connor et al. [44] who indicated that they could delay the rejection of rat cardiac allografts.

The present study demonstrated that prenatal exposure to high and low dietary soy isoflavones resulted in a significant reduction in IL-12 and IFN- γ levels in male offspring. The remarkable decrease of serum IFN- γ concentrations might delay the IL-12 production due to the immunosuppressive effects of soy isoflavones. These cytokines are

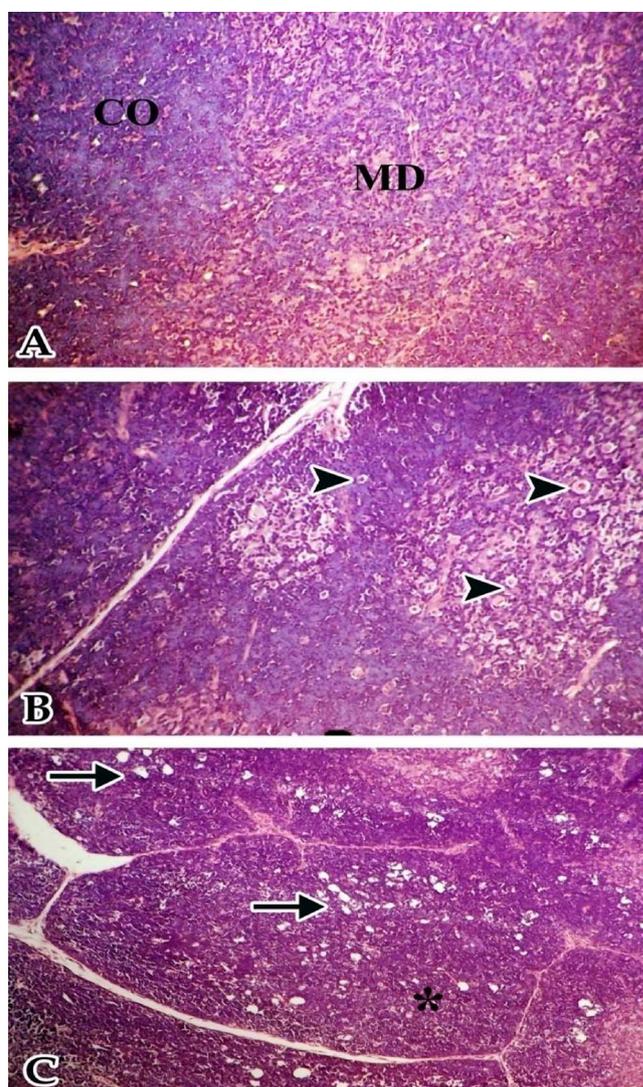


Fig. 3. Thymus in male offspring after 24 h of PHA injection. Normal thymus of control group (A) with cortex (CO) and medulla (MD), cortex has uniformly dense population of lymphocyte. Thymus of male offspring born to dams exposed to low dietary soy isoflavones (B), showing tangible body macrophages with intracytoplasmic apoptotic bodies (head arrows). Thymus of offspring born to dams exposed to high dietary soy isoflavones (C), showing severe lymphocyte depletion and vacuolation (arrows) and absence of distinct cortico-medullary junction (*). (H&E; 200X).

responsible for chemotaxis of leukocytes [45,46] to the site of inflammation induced by PHA injection. The decrease in IFN- γ concentrations and IL-12 production was consistent with marked depletion in cell populations of thymus and spleen. The decrease in these cell populations may imply for the lower concentrations of both IFN- γ and IL-12 [47,48]. IL-12 is a critical cytokine that drives the differentiation of IFN- γ producing Th1 cells [49]; the decrement in IL-12 might be due to decrease of IFN- γ . NF- κ B has been shown to interact with ERs and also can regulate IFN- γ transcription [50]. In addition, the direct role of ER α in regulation of IFN- γ production during inflammation was previously proven by Curran et al. [51]. Furthermore, these results could be attributed to inhibitory effect of dietary soy genistein on NF- κ B production or prevention of NF- κ B binding to DNA [52], leading to decrease IFN- γ levels in male offspring.

Interestingly, TNF- α was markedly elevated in males born to dams received high soy isoflavones, that contradicted the lower levels of IFN- γ and IL-12 after PHA injection. This result was in agreement with Vasiadi et al. [53]. In attempt to explain these phenomena, Castro et al. [54] declared that there were no relation between increment of IL-12

and TNF- α when exposed to lipophilic genestein derivatives *in vitro* which enable to induce IL-12 inhibition but fail to inhibit TNF- α . A similar effect on IL-12 and TNF- α was reported in a study using lipopolysaccharides-stimulated dendritic cells (DCs) treated with aspirin as an anti-inflammatory drug [55]. However, in an *in vitro* study, bone marrow-derived DCs treated with the catechin polyphenol epigallocatechin gallate produced less IL-12, in contrast with increased TNF- α [56].

The isoflavones especially genestein could be able to bind to estrogen receptor (ER α) that induces numerous cellular alterations [57,58]. Among these changes, the reduced T-cell abundance and reduced cell-mediated immune function [2,59] which manifested in the current experiment by lower relative weights of spleen and thymus beside the depletion in the lymphoid cells. The decrease in relative spleen weight associated with the down regulating effect of genestein on relative percentages of spleen T cells [60]. 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 thymus atrophy and immune suppression in developed rodents [61,62]. Other mechanism could involve the 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 genestein concentrations *in vitro* [63,64].

Few lymphocytes infiltration was detected in foot pad of male offspring born to dams fed low soy isoflavones, however, no infiltration was seen in footpad of offspring born to dams fed high soy isoflavones, when compared with perivascular accumulation of lymphocytes migration in the dermis layer in offspring born to dams of control group. In attempt to explain these phenomena, Halloran et al. [37] and Kennedy and Nager [65] reported a rapid recruitment / proliferation of WBCs at the PHA injection site. Neutrophils, eosinophils and macrophages were rapidly increased at the PHA site of injection, peaking within 12 h. Eosinophil and neutrophil numbers subsequently declined but macrophage continued to increase for a further 12 h. On the other hand, lymphocytes did not present in large numbers at the injection site until 24 h post-injection. This pattern suggested an immediate innate response by neutrophils, eosinophils and macrophages, followed by a secondary response consisting largely of lymphocytes which is slower in progression. Although lymphocytes are expected to be produced in large number by the specific mitogenic effect of PHA on T cells, lymphocytes appearing at the injection site after 24 h [66].

5. Conclusion

Dietary soy isoflavones at prenatal period might have immunosuppressive effect on cell mediated immunity of male offspring after PHA stimulation. This effect might possibly mediated by reduction of IL-12 and IFN- γ production, depletion in thymus and spleen lymphoid tissue and poor inflammatory and immune response to PHA stimulation in foot pad. A dose-response in the immune system of the offspring due to increasing soy concentrations in the diet of their dams was confirmed.

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References

- [1] D. Balabanic, M. Rupnik, A.K. Klemencic, Negative impact of endocrine disrupting compounds on human reproductive health, *J. Reprod. Fertil. Dev.* 23 (2011) 403–416.
- [2] S. Yellayi, M.A. Zakroczymski, V. Selvaraj, V.E. Valli, V. Ghanta, W.G. Helferlich, P.S. Cooke, The phytoestrogen genestein suppresses cell-mediated immunity in mice, *J. Endocrinol.* 176 (2003) 267–274.
- [3] P.S. Cooke, V. Selvaraj, S. Yellayi, Genistein, estrogen receptors, and the acquired immune response, *J. Nutr.* 136 (2006) 704–708.
- [4] I. Wocławek-Potocka, C. Mannelli, D. Boruszewska, I. Kowalczyk-Zieba, T. Waśniewski, D.J. Skarżyński, Diverse effects of phytoestrogens on the reproductive performance: cow as a model, *Int. J. Endocrinol.* 2013 (2013) 1–15.
- [5] G.G.C. Kuhnle, A. Vogiatzoglou, H.A. Ward, K.T. Khaw, Dietary phytoestrogens and health – a population study, *Proc. Nutr. Soc. 70 (OCE4)* (2011) (E254:1).
- [6] O.E. Kelany, H.E. Khaled, M. Amal, H.M. Abdelrazek, M.M. Abdel-Daim, Hepatoprotective and metabolic effects of dietary soy phytoestrogens against hypercaloric diet in cyclic female albino rats is mediated through estradiol receptors beta, *Biomed. Pharmacol. J.* 10 (2017) 1061–1069.
- [7] A. Brzezinski, A. Debi, Phytoestrogens: the ‘natural’ selective estrogen receptor modulators? *Eur. J. Obstet. Gynecol. Reprod. Biol.* 85 (1999) 47–51.
- [8] T. Oseni, R. Patel, J. Pyle, V.C. Jordan, Selective estrogen receptor modulators and phytoestrogens, *Planta. Med.* 74 (2008) 1656–1665.
- [9] E.K. Shanley, W. Xu, Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action, *Chem. Res. Toxicol.* 24 (2011) 6–19.
- [10] C.J. Gruber, W. Tschugguel, C. Schneeberger, J.C. Huber, Production and actions of estrogens, *N. Engl. J. Med.* 346 (2002) 340–352.
- [11] S.M. Belcher, A. Zsarnovszky, Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms, *J. Pharmacol. Exp. Ther.* 299 (2001) 408–414.
- [12] C. Watson, R. Alyea, Y.-J. Jeng, M. Kochukov, Nongenomic actions of low concentration estrogens and xenoestrogens on multiple tissues, *Mol. Cell. Endocrinol.* 274 (2007) 1–7.
- [13] T. Simoncini, E. Rabkin, J.K. Liao, Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells, *Arterioscler. Thromb. Vasc. Biol.* 23 (2003) 198–203.
- [14] S. Barnes, The biochemistry, chemistry and physiology of the isoflavones in soybeans and their food products, *Lymphat. Res. Biol.* 8 (2010) 89–98.
- [15] G.-A. Yoon, S. Park, Antioxidant action of soy isoflavones on oxidative stress and antioxidant enzyme activities in exercised rats, *Nutr. Res. Pract.* 8 (2014) 618–624.
- [16] R.P. Patel, B.J. Boersma, J.H. Crawford, N. Hogg, M. Kirk, B. Kalyanaraman, D.A. Parks, S. Barnes, V. Darley-Usmar, Antioxidant mechanisms of isoflavones in lipid systems: paradoxical effects of peroxyl radical scavenging, *Free Radic. Biol. Med.* 31 (2001) 1570–1581.
- [17] J. Yu, X. Bi, B. Yu, D. Chen, Isoflavones: anti-inflammatory benefit and possible caveats, *Nutrients* 8 (2016) 361.
- [18] K.A. Solak, F.M.J. Wijnolts, S.M. Nijmeijer, B.J. Blaauw, M. van den Berg, M.B.M. van Duursen, Excessive levels of diverse phytoestrogens can modulate steroidogenesis and cell migration of KGN human granulosa-derived tumor cells, *Toxicol. Rep.* 1 (2014) 360–372.
- [19] M. Tanida, K. Imanishi, M. Komatsu, J. Satomi, N. Yamamoto, M. Wang, Y. Kurata, T. Shibamoto, Soy isoflavone affects the autonomic nervous system in a tissue-specific manner in anesthetized rats, *Exp. Biol. Med.* (Maywood, NJ) 239 (2014) 477–483.
- [20] D.J. Cai, Y. Zhao, J. Glasier, D. Cullen, S. Barnes, C.H. Turner, M. Wastney, C.M. Weaver, Comparative effect of soy protein, soy isoflavones, and 17 β -estradiol on bone metabolism in adult ovariectomized rats, *J. Bone Miner. Res.* 20 (2005) 828–839.
- [21] D. Johar, A. Maher, O. Aboelmagd, A. Hammad, M. Morsi, H. Farag, H.I. Awad, T.A. Ibrahim, S. Zaky, Whole-food phytochemicals antioxidative potential in alloxan-diabetic rats, *Toxicol. Rep.* (2018) (in press, Accepted Manuscript).
- [22] T. Hamza, J.B. Barnett, B. Li, Interleukin 12 a key immunoregulatory cytokine in infection applications, *Int. J. Mol. Sci.* 11 (2010) 789–806.
- [23] M.P. Cancro, S.H. Smith, Peripheral B cell selection and homeostasis, *Immunol. Rev.* 27 (2003) 141–148.
- [24] D. Wallach, E.E. Varfolomeev, N.L. Malinin, Y.V. Goltsev, A.V. Kovalenko, M.P. Boldin, Tumor necrosis factor receptor and Fas signaling mechanisms, *Annu. Rev. Immunol.* 17 (1999) 331–367.
- [25] P. Rangamani, L. Sirovich, Survival and apoptotic pathways initiated by TNF- α : modeling and predictions, *Biotechnol. Bioeng.* 97 (2007) 216–229.
- [26] K.B. Delclos, C.C. Weis, T.J. Bucci, G. Olson, P. Mellick, N. Sadovova, J.R. Latendresse, B. Thorn, R.R. Newbold, Overlapping but distinct effects of genistein and ethinyl estradiol (EE(2)) in female Sprague-Dawley rats in multi-generational reproductive and chronic studies, *Reprod. Toxicol.* 27 (2009) 117–132.
- [27] W. Kouidhi, C. Desmetz, A. Nahdi, R. Bergès, J.P. Cravedi, J. Auger, M. El-May, M.C. Canivenc-Lavier, In utero and lactational exposure to low-dose genistein-vinclozolin mixture affects the development and growth factor mRNA expression of the submandibular salivary gland in immature female rats, *Toxicol. Pathol.* 40 (2012) 593–604.
- [28] R.S. Strakovsky, S. Lezmi, J.A. Flaws, S.L. Schantz, Y.X. Pan, W.G. Helferlich, Genistein exposure during the early postnatal period favors the development of obesity in female, but not male rats, *Toxicol. Sci.* 138 (2014) 161–174.
- [29] J.J. Segura, A. Jimenez-Rubio, R. Pulgar, N. Olea, J.M. Guerrero, J.R. Calvo, In vitro effect of the resin component bisphenol A on substrate adherence capacity of macrophages, *J. Endod.* 25 (1999) 341–344.
- [30] M.S. Christin, A.D. Gendron, P. Brousseau, L. Ménard, D.J. Marcogliese, D. Cyr, S. Ruby, M. Fournier, Effects of agricultural pesticides on the immune system of Ranapipeins and on its resistance to parasitic infection, *Environ. Toxicol. Chem.* 22 (2003) 1127–1133.
- [31] H.M. Ebaid, R.A. Elgawish, H.M.A. Abdelrazek, G. Gaffer, H.M. Tag, Prenatal exposure to soy isoflavones altered the immunological parameters in female rats, *Int. J. Toxicol.* 35 (2016) 274–283.

- [32] A. Piesta, T. Maj, A. Chelmońska-Soyta, The influence of mating on estrogen receptor alpha protein level in spleen and uterine macrophages in female mice, *J. Reprod. Biol.* 9 (2009) 225–240.
- [33] D.G. Thiagarajan, M.R. Bennink, L.D. Bourquin, F.A. Kavaam, Prevention of pre-cancerous colonic lesions in rats by soy flakes soy flour, genistein, and calcium, *J. Am. J. Clin. Nutr.* 68 (1998) 1394S–1399S.
- [34] NRC, Nutrient Requirements of Laboratory Animals, 4th revised ed., National Acad., Paris. Washington. D.C, 1995.
- [35] H. Adlercreutz, H. Honjo, A. Higashi, T. Fotsis, E. Hämäläinen, T. Hasegawa, H. Okada, Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet, *Am. J. Clin. Nutr.* 54 (1991) 1093–1100.
- [36] L.B. Martin, P. Han, J. Lewittes, J.R. Kuhlman, K.C. Klasing, M. Wikelski, Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoeological technique, *Funct. Ecol.* 20 (2006) 290–299.
- [37] M.M. Halloran, Z. Szekanez, N. Barquin, G.K. Haines, A.E. Koch, Cellular adhesion molecules in rat adjuvant arthritis, *Arthritis Rheum.* 39 (1996) 810–819.
- [38] C.B. Langman, K.K. Ford, L.M. Pachman, F. Glorieux, Vitamin D metabolism in rats with adjuvant-induced arthritis, *J. Bone Miner. Res.* 5 (1990) 905–913.
- [39] Y. Chen, L.P. Wu, Y.J. Liu, P.C. Deng, H.L. Yin, S.-L. Wen, C. Chen, L.-M. Ye, Urinary study on the biochemical effect of acupuncture on monosodium urate crystals-induced acute gouty arthritis in rats using 600MHz 1H NMR, *J. Bioequiv. Availab.* 4 (2012) 106–111.
- [40] S.O. Aniagu, F.C. Nwinyi, D.D. Akumka, G.A. Ajoku, S. Dzarma, K.S. Izebe, M. Ditse, P.E.C. Nwaneri, C. Wambebe, K. Gamaniel, Toxicity studies in rats fed nature cure bitters, *Afr. J. Biotechnol.* 4 (2005) 72–78.
- [41] A.V. Karaulov, I.V. Mikhaylova, A.I. Smolyagin, V.M. Boev, A. Kalogeraki, A.M. Tsatsakis, A.B. Engin, The immunotoxicological pattern of subchronic and chronic benzene exposure in rats, *Toxicol. Lett.* 275 (2017) 1–5.
- [42] A.A. Drury, E.A. Wallington, Carleton's Histological Technique, 5th ed., Oxford University press, New York, Toronto, 1980.
- [43] D.R. Ardia, E.D. Clotfelter, The novel application of an immunological technique reveals the immunosuppressive effect of phytoestrogens in bettasplendens, *J. Fish Biol.* 68 (2006) 144–149.
- [44] T.P. O'Connor, D.A. Liesen, P.C. Mann, L. Rolando, W.J. Banz, A high isoflavone soy protein diet and intravenous genistein delay rejection of rat cardiac allografts, *J. Nutr.* 13 (2002) 2283–2287.
- [45] A. Iellem, L. Colantonio, S. Bhakta, S. Sozzani, A. Mantovani, F. Sinigaglia, D. D'Ambrosio, Inhibition by IL-12 and IFN-alpha of I-309 and macrophage-derived chemokine production upon TCR triggering of human Th1 cells, *Eur. J. Immunol.* 30 (2000) 1030–1039.
- [46] D.L. Hodge, W.B. Schill, J.M. Wang, I. Blanca, D.A. Reynolds, J.R. Ortaldo, H.A. Young, IL-2 and IL-12 alter NK cell responsiveness to IFN-gamma-inducible protein 10 by down-regulating CXCR3 expression, *J. Immunol.* 168 (2002) 6090–6098.
- [47] A.O. Chua, R. Chizzonite, B.B. Desai, T.P. Truitt, P. Nunes, L.J. Minetti, R.R. Warrior, D.H. Presky, J.F. Levine, M.K. Gately, Expression cloning of a human IL-12 receptor component: a new member of the cytokine receptor superfamily with strong homology to gp130, *J. Immunol.* 153 (1994) 128–136.
- [48] J.R. Schoenborn, C.B. Wilson, Regulation of interferon-gamma during innate and adaptive immune responses, *Adv. Immunol.* 96 (2007) 41–101.
- [49] J. Magram, J. Sfarra, S. Connaughton, D. Faherty, R. Warrior, D. Carvajal, C.Y. Wu, C. Stewart, U. Sarmiento, M.K. Gately, IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses, *Immunity* 4 (1996) 471–481.
- [50] L.I. McKay, J.A. Cidlowski, Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways, *Endocr. Rev.* 20 (1999) 435–459.
- [51] E.M. Curran, B.M. Judy, L.G. Newton, D.B. Lubahn, G.E. Rottinghaus, R.S. Macdonald, C. Franklin, D.M. Estes, Dietary soy phytoestrogens and ER alpha signalling modulate interferon gamma production in response to bacterial infection, *Clin. Exp. Immunol.* 135 (2004) 219–225.
- [52] W.L. Smith, D.L. Dewitt, R.M. Garavito, Cyclooxygenases: structural, cellular, and molecular biology, *Annu. Rev. Biochem.* 69 (2000) 145–182.
- [53] M. Vasiadi, J. Newman, T.C. Theoharides, Isoflavones inhibit poly (I:C)-induced serum, brain and skin inflammatory mediators – relevance to chronic fatigue syndrome, *J. Neuroinflammation* 11 (2014) 1–12.
- [54] S.B. Castro, C.O. Junior, C.C. Alves, A.T. Dias, L.L. Alves, L. Mazzocoli, M.T. Zoet, S.A. Fernandes, H.C. Teixeira, M.V. Almeida, A.P. Ferreira, Synthesis of lipophilic genistein derivatives and their regulation of IL-12 and TNF- α in activated J774A.1 cells, *Chem. Biol. Drug Des.* 79 (2012) 347–352.
- [55] L. Ho, D.M. Chang, H.Y. Shiau, C.H. Chen, T.Y. Hsieh, Y.L. Hsu, C.S. Wong, J.H. Lai, Aspirin differentially regulates endotoxin-induced IL-12 and TNF- α production in human dendritic cells, *Scand. J. Rheumatol.* 30 (2001) 346–352.
- [56] J. Rogers, I. Perkins, A.V. Olphen, N. Burdash, T.W. Klein, H. Friedman, Epigallocatechin Gallate modulates cytokine production by bone marrow-derived dendritic cells stimulated with lipopolysaccharide or muramyl dipeptide, or infected with Legionella pneumophila, *Exp. Biol. Med.* 230 (2005) 645–651.
- [57] P.L. Whitten, H.B. Patisaul, Cross-species and interassay comparisons of phytoestrogen action, *Environ. Health Perspect.* 109 (2001) 5–20.
- [58] D. Nikitovic, A.M. Tsatsakis, N.K. Karamanos, G.N. Tzanakakis, The effects of genistein on the synthesis and distribution of glycosaminoglycans/ proteoglycans by two osteosarcoma cell lines depends on tyrosine kinase and the estrogen receptor density, *Anticancer Res.* 23 (2003) 459–464.
- [59] S. Yellayi, A. Naaz, M.A. Szweczykowski, T. Sato, J.A. Woods, J. Chang, M. Segre, C.D. Allred, W.G. Helferich, P.S. Cooke, The phytoestrogen genistein induces thymic and immune changes: a human health concern? *Proc. Natl. Acad. Sci. U. S. A.* 99 (2002) 7616–7621.
- [60] T. Sakai, M. Kogiso, Soy isoflavones and immunity, *J. Med. Invest.* 55 (2002) 167–173.
- [61] N.J. Olsen, W.J. Kovacs, Gonadal steroids and immunity, *Endocr. Rev.* 17 (1996) 369–384.
- [62] F. Kohen, L. Abel, A. Sharp, Y. Amir-Zaltsman, D. Sömjen, S. Luria, G. Mor, A. Knyszynski, H. Thole, A. Globerson, Estrogen-receptor expression and function in thymocytes in relation to gender and age, *Dev. Immunol.* 5 (1998) 277–285.
- [63] T. Mustelin, K.M. Coggeshall, N. Isakov, A. Altman, T cell antigen receptor-mediated activation of phospholipase C requires tyrosine phosphorylation, *Science* 247 (1990) 1584–1587.
- [64] C. Essex, Phytoestrogens and soy based infant formula, *Br. Med. J.* 313 (1996) 507–508.
- [65] M.W. Kennedy, R.G. Nager, The perils and prospects of using phytohaemagglutinin in evolutionary ecology, *Trends Ecol. Evol.* 21 (2006) 653–655.
- [66] G.P. Brown, C.M. Shilton, R. Shine, Measuring amphibian immunocompetence: validation of the phytohemagglutinin skin-swelling assay in the cane toad *Rhinella marina*, *Methods Ecol. Evol.* 2 (2011) 341–348.