Risk Assessment of Phytoestrogen Containing Diet in Cyclic Female Rats: Immunological, Biochemical and Oxidative Stress Evaluation

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ABSTRACT

Phytoestrogens are compounds that occur naturally in plants that contain a phenolic group positioned similarly to that of estrogenic steroids. Phytoestrogens components divided according to their chemical structure into isolavones, lignans, coumestans and stilbens soybean considered the richest sources of isolavones in normal food. Phytoestrogens has been used to treat numerous chronic diseases, including hormone related cancers, and coronary heart disease. The aim of the current study was to evaluate the effect of soy based diet containing phytoestrogens focused particularly on dietary isolavones on immunological, biochemical and oxidative stress parameters in female albino rats. Rats divided into 3 groups (each of 12 rats) the first group received control diet the other two groups received soy based containing high phytoestrogens for 30 days Twelve females from Phytoestrogens group are transferred into control diet after 30 days of administration and continued for another 30 days. Results revealed a significant reduction in body weight gain, pro-inflammatory cytokines and MDA as a marker to lipid peroxidation. On the other hand there is an elevation of GSH level. Serum protein electrophoresis and liver enzymes showed no significant changes among different groups. While, lipid profile revealed significant improvement with Phytoestrogens administration. In contrary, withdrawal group returned to normal after the end of withdrawal time.

INTRODUCTION

Phytoestrogens are plant compounds with estrogen-like biological activity. The use of certain plants in traditional medicine and folklore may be ascribed to their estrogenic properties. For example, the pomegranate is associated with fertility [1].

Phytoestrogens components divided according to their chemical structure into 1) isolavones (genistein, daidzein, biochanin A, formononetin). 2) lignans (matairesinol, secoisolariciresinol) 3) coumestans (coumestrol, 4-methoxycoumestrol), and 4) stilbens (resveratrol) [2]. In nature, isolavones mostly found in red clover, germs of alfalfa as well as linseed. Specifically, soybean considered the richest sources of isolavones in normal food. Dry soybeans contain 1.2-4.2 mg/g Isolavones [3].

The consumption of Phytoestrogens has been linked to reduction of numerous chronic diseases, including hormone related cancers, coronary heart disease, osteoporosis, and hypercholesterolemia [4]. Estrogens have been reported to affect adiposity either directly, by modulating lipogenesis, lipolysis or adipogenesis, or indirectly, by modulating appetite or energy expenditure [5]. Furthermore, estrogens exert positive effects on hepatic functions [6] and have anti-oxidant properties [7]. Moreover, isolavones including aglycones was known to be able to scavenge free radicals and to modulate the expression of genes encoding antioxidant enzymes [8].

Many authors reported the reduced rate of lipid peroxidation by Phytoestrogens taking as [9] in ovariectomized rats and [10] revealed that soy product consumption for 8 weeks could reduce MDA levels in

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patients with the metabolic disorder. [11] was reported that, Yellow Soybean and Black Soybean significantly lowered hepatic thiobarbituric acid reactive substances (TBARS), hepatic total lipid and cholesterol levels. Superoxide dismutase (SOD) and catalase (CAT) activities were significantly higher. Soy protein feeding may affect intracellular pathways, concentrations of several growth factors and cytokines as insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [12].

Genistein has a wide range of biological actions that suggest it may be of use in cancer treatment. Its molecular actions include: an inhibitory effect on protein tyrosine kinases, DNA topoisomerase I and II, and ribosomal S6 kinase; anti-estrogenicity; antioxidant activity; anti-angiogenesis activity; suppression of cell proliferation; induction of differentiation and modulation of apoptosis [13]. Administration of genistein (1 mg kg\(^{-1}\), i.v., 5 min after coronary artery occlusion) lowered myocardial necrosis, decreased serum CPK activity, increased myocardial contractility, decreased the occurrence of ventricular arrhythmias, and reduced serum and macrophages levels of TNF-\(\alpha\) [14].

Phytoestrogens acting as natural estrogen receptor modulators may preserve anti-inflammatory property and may function as potential chondro-protective compounds by inhibit IL-1\(\beta\)production [15].

In an attempt to develop an available food additive therapy, the aim of the current study was to determine the isoflavones in soybean and to evaluate its immunomodulatory, antioxidant and hepato-protective impacts.

**MATERIALS AND METHODS**

**Animals model and experimental design:**

A total of 36 female albino rats, were purchased from Lab Animal House, National Research Center, Dokki, Cairo. Animals were kept in metallic cages. Animals were kept at room temperature and natural day light rhythm. Food and water were allowed ad libitum. Animals were kept for 2 weeks for acclimatization before starting the experiment.

**Ethical statement:**

All animals were treated and sampled in accordance with the guideline for care and use of animals which approved by the research ethics committee in the Faculty of Veterinary Medicine, Suez Canal University.

**Group I:**

Control group, include twelve females, they were fed on a casein based ration which was formulated to fulfill all the nutritional requirements of adult rat (basal diet) according to NRC, 1995 as shown in Table 1.

**Group II:**

Phytoestrogens group, Include twenty-four females received high Phytoestrogens diet.

**Dietary phytoestrogens composition [Table 1]:**

Phytochemical screening of the current dietary Phytoestrogens (Isoflavones) by [16] revealed that control diet contains 45 \(\mu\)g/g genistein and 28 \(\mu\)g/g daidzein respectively, while the high phytoestrogens diet contains 1320 \(\mu\)g/g genistein and 704.7 \(\mu\)g/g daidzein, respectively.

**Group III:**

Withdrawal of Phytoestrogens where twelve females from are transferred into control diet after 30 days of administration and continued for another 30 days.

**Performance parameter:**

Body weight of rats per group was recorded on individual basis at weekly intervals [17].

**Blood and organs sampling:**

At the end of the experimental period (30 days), all the animals were fasted for 24 hr and euthanized under general anesthesia with diethyl ether. Blood was collected directly from heart and serum was separated by centrifugation for enzyme analysis. Liver was excised, washed in ice cold saline, and removed of adhering fat and connective tissues. A 10% homogenate of the liver tissue was prepared in Tris–HCl buffer (0.1 M; pH 7.4), centrifuged (2500 rpm for 10 min at 4 °C) to pellet the cell debris and the supernatant was harvested for biochemical assays.

**Assessment of pro- inflammatory and immune parameters:**

Serum levels of IL-6, IL-1 and Tumor Necrosis Factor (TNF)-\(\alpha\) were purchased from BD Pharmingen (San Diego, CA, USA). They were estimated according to the manufacturer’s protocols (Becton Dickinson, San Diego, CA, USA).
**Determination of serum protein fractions:**  
Serum protein determined using polyacrylamide gel electrophoresis in the presence of SDS. The gel stained with Coomassie Brilliant Blue R250 followed with silver stain [18]. The dry gel was scanned with photographed. Interpretation was carried out using Syn Gene Gene Tools version, 4.01.02 software. The electrophoretic results recorded as percentage to the concentration of total protein.

**Table 1: Composition of experimental diets.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>High Phytoestrogens group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried skimmed milk powder</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Casein</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>Soybean seeds*</td>
<td>2.60</td>
<td>7.00</td>
</tr>
<tr>
<td>Corn gluten</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dried Alfa Alfa hay</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Yellow corn</td>
<td>59.00</td>
<td>71.38</td>
</tr>
<tr>
<td>Cellulose</td>
<td>-</td>
<td>2.00</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>Soybean oil</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Decalcium phosphate</td>
<td>1.65</td>
<td></td>
</tr>
<tr>
<td>Ground limestone</td>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Common salt</td>
<td>0.15</td>
<td>0.153</td>
</tr>
<tr>
<td>Premix**</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td>Lysine</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td>Total</td>
<td>100.00</td>
<td>100.00</td>
</tr>
</tbody>
</table>

* Soybean was autoclaved at 1100x for 30 minutes according to [50] to inactivate trypsin inhibitor, tannins, saponins, phytate, protease inhibitors, lectins and goitrogens.

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

Phytochemical screening of the current dietary Phytoestrogens (Isoflavones) by [16] revealed that control diet contains 45 μg/g genistein and 28 μg/g daidzein respectively, while the high phytoestrogens diet contains 1320 μg/g genistein and 704.7 μg/g daidzein, respectively.

**Evaluation of liver function tests:**
Alanine Aminotransferase (ALT, GPT) and Aspartate Aminotransferase (AST, GOT) were measured according to [19] using reagent kits purchased from Randox Company (United Kingdom)

**Determination of lipid profile:**
Serum levels of high-density lipoprotein cholesterol (HDL), total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL) were measured using enzymatic calorimetric kits (Biodiagnostic Co., Egypt) according to [20].

Assessment of some liver Oxidative stress parameters: Reduced glutathione (non-enzymatic antioxidants, GSH) was estimated by the method of [21] using reagent kits purchased from Bio diagnostic company (Egypt). MDA as a marker for lipid peroxidation was determined by the procedure of [22] using reagent kits purchased from Bio diagnostic company (Egypt).

**Statistical analysis:**
All data in the present study were expressed as mean ± SE, they were subjected to student F test using SPSS® software (Statistical Package for Social science, version 17.01, Illinois, USA). The probability criterion for significance was P>0.05 and P<0.01 for the high significance according to [23].

**Results:**

**Effect of dietary Phytoestrogens on body weight gain:**
Body weight gain that shown in table (2) revealed a significant decrease in body weight gain g / week in Phytoestrogens-fed group. There was no significant difference between control and withdrawal group.

**Effect of dietary Phytoestrogens on GSH as liver oxidative enzyme and MDA as a marker for lipid peroxidation:**
As illustrated in table (2) we noticed that GSH was significantly increased in Phytoestrogens- treated group in compare with control group mean while withdrawal group did not show significant change than control group. Concerning lipid peroxidation, there is a marked decrease in MDA level in Phytoestrogens- treated group comparing with control group. However, withdrawal group did not show significant change than control group.
Effect of dietary Phytoestrogens on pro-inflammatory and immune parameters:
The values of cytokines that shown in table (3) revealed that IL1ß, IL6 and TNF α were significantly decreased in Phytoestrogens treated group in compare with control group while; withdrawal group reached the normal control value.

Effect of dietary Phytoestrogens on serum protein electrophoresis:
Serum protein fraction (table 4), electrophoretogram (Fig 1) and subsequent scanning software analysis (Fig 2, Fig 3, Fig 4) revealed no significant difference among different groups.

Effect of dietary Phytoestrogens on liver function enzymes and lipid profile parameters:
The values of lipid profile that shown in table (5) revealed that elevated HDL-cholesterol was observed in Phytoestrogens treated group in compare with control group and the withdrawal group returned back to normal as control group. In contrary, LDL- cholesterol, TG and TC were significantly decreased in Phytoestrogens treated group in compare with control group while withdrawal group returned to control value. Phytoestrogens caused no changes in liver enzymes in all groups.

Fig 1: Separation of serum protein fraction in gel electrophoresis, Lane 1: control, Lane 2: phytoestrogen-treated group, Lane 3: withdrawal group.

Fig 2: Analysis of serum protein electrophoresis pattern in control group.

Fig. 3: Analysis of serum protein electrophoresis pattern in phytoestrogen- treated group.
Phytoestrogens may also function as antioxidants by enhancing antioxidant enzyme activity [24]. The current work revealed that high phytoestrogens diet containing 1320 μg/g genistein and 704.7 μg/g daidzein, respectively exerts significant increase in hepatic thiobarbituric acid reactive substances (TBARS), hepatic total lipid and cholesterol levels. Superoxide dismutase (SOD) and catalase (CAT) activities were significantly higher [11]. Major soy isoflavonegenistein and the daidzein metabolite equol are potent antioxidants increases in endogenous antioxidant mechanisms and reduced oxidative stress where decrease in GSSG/ GSH ratio.

Discussion:
Phytoestrogens may also function as antioxidants by enhancing antioxidant enzyme activity [24]. The current work revealed that high phytoestrogens diet containing 1320 μg/g genistein and 704.7 μg/g daidzein, respectively exerts significant increase in hepatic thiobarbituric acid reactive substances (TBARS), hepatic total lipid and cholesterol levels. Superoxide dismutase (SOD) and catalase (CAT) activities were significantly higher [11]. Major soy isoflavonegenistein and the daidzein metabolite equol are potent antioxidants increases in endogenous antioxidant mechanisms and reduced oxidative stress where decrease in NAD (P)H oxidase activity and superoxide levels in the brain. In addition, equol reduced plasma thiobarbituric acid reactive substances [26].
A possible contributory mechanism to the antioxidant activity of isoflavonoids related to its similarity to cholesterol and α-tocopherol, partition into the hydrophobic core of the membrane, cause a dramatic decrease in lipid fluidity in this region of the membrane hindering diffusion of free radicals, and thereby decrease the kinetics of free radical reactions [27]. Chronic dietary administration of genistein significantly elevated the activities of catalase, SOD, glutathione peroxidase, and glutathione reductase in murine skin and small intestine [28].

The exact underlying mechanism by which isoflavonoids increase GSH levels has not yet been elucidated. Possibly isoflavonoids affect various enzymes involved in GSH homeostasis such as glutamyl-S-transferase and gamma glutamyl cysteiny1 synthetase, thereby increasing intracellular GSH level [29]. Interestingly, Phytoestrogens play a role in the protection against oxidatively-induced DNA damage [30]. Consequently, and taking into account that Phytoestrogens are being increasingly proposed as an alternative with low risks to fight the menopause-related pro-oxidation status and associated lipid damage.

Our data explored anti-inflammatory activity of dietary Phytoestrogens as confirmed by decreased IL-1β, IL-6 and TNF-α. Soy isoflavones, such as genistein, daidzein, and biochanin A, suppress nuclear factor-κB (NF-κB)-driven IL-6 expression, independently of their estrogenic activity. This occurs via attenuation of mitogen-activated protein kinase (MEK) and extracellular signal-regulated kinase (ERK) activities, which further down-regulates NF-κB p65 and histone H3 phosphorylation [31]. Moreover, isoflavone structure resembles that of 17β-estradiol. Thus, genistein is able to up-regulate the expression of longevity-related genes involving interactions with oestrogen receptors, activation of MAPK, ERK1/2 and NFκb [3]. In another study, treatment with soybean isoflavones (10(-5) M), in the presence of TNF-α for 48 hr inhibited production of IL-6 and PGE2. Genistein reduced IL-6 production not by tyrosine kinase inhibition but rather by interference with NF-jB subcellular localization and subsequent DNA binding [32]. Genistein Suppresses Japanese encephalitis virus induced TNF-α and IL1 production [33].

Phytoestrogens have a potential role in the treatment of post-menopausal and inflammatory bone loss directly inhibiting TNF-α-induced resorptions [34]. It was demonstrated that equol inhibits LPS-induced TNF-α gene expression, at least in part, by blocking NF-kB DNA binding and transcriptional activity [35]. Because equol is known to have strong free radical scavenging and antioxidant activities [36]. It is assumed that the inhibition of NF-kB by equol may be mediated by the regulation of ROS pathway [13]. Moreover, Equol inhibits TNF-α expression at least in part, by blocking NF-kB activation reflecting its osteoprotective effect [37].

We consistently observed that consumption of dietary Phytoestrogens reduced body weight that is coincide with those recorded by [38] The decrease of feed efficiency and increase of FCR as in this study is consequent due to loss of large amount of ingested food as energy lost during the increased locomotor activity. Exercise is known to activate the fuel-sensing enzyme 5' adenosine monophosphate-activated protein kinase (AMPK) in both adipose tissue and skeletal muscles which in turn improves glucose uptake and fatty acid oxidation in peripheral tissue [39] thus decreasing feed efficiency and increasing feed conversion ratio and reducing body weight in this study. This implies that the estrogenic hormone action of Phytoestrogens is beneficial to body fat regulation and the decreased level of leptin that is produced in adipose tissue, which is a sensing enzyme 5' adenosine monophosphate activated protein kinase (AMPK) in both adipose tissue and skeletal muscles which in turn improves glucose uptake and fatty acid oxidation in peripheral tissue [39].

It is important to highlights that, dietary Phytoestrogens evoked hypolipidemic impact in this experiment which are consistent with previous record of [42]. These finding may contributed to that, Phytoestrogens affected lipid metabolism in liver and adipose tissue, and decreasing triglycerides while increasing free fatty acids in serum [43]. In addition, it lower cholesterol levels by increasing LDL receptor activity, and the reduction in cholesterol may offer some protection against atherosclerosis [44]. Another explanation is that soy Phytoestrogens decrease intestinal cholesterol absorption increase in bile acid excretion that mediate the lipid-lowering effect of soy protein [45].

Furthermore, some beneficial effects of soy nut on inflammatory markers, which contain both ω -3 and ω -6 fatty acids, are likely to be associated with the lowest levels of inflammation [46]. Isoflavone genistein has recently been shown to cause decreases in lipoprotein lipase mRNA in adipose tissue with concomitant decreases in lipid filling of adipocytes [47]. These results suggest that the hypolipidemic effect of genistein could ascribed in part to the upregulation of genes involved in fatty acid catabolism, which is consistent with results from in vitro studies [48,49].

The primary outcomes of the present study showed an improvement in lipid profiles. Finally, Phytoestrogens treatment was well tolerated and caused no changes in liver enzymes as [50] who performed an experiment on women were randomized to use either Phytoestrogens (tablets containing 114 mg of isoflavonoids) for 3 months and they recorded no change in liver enzyme level. Notably, intake of soybean containing isoflavones resulted in the greatest prevention of obesity, hyperlipidemia that may further negatively liver activity.
Conclusion:
The present investigation highlights particularly on dietary Phytoestrogen containing isoflavones and summarized recent knowledge on their efficacious impacts on adiposity, immunity and liver antioxidant improvement in female rats and improved that withdrawal of Phytoestrogens returned rats to its normal control state.

REFERENCES


