Hepatoprotective and Metabolic Effects of Dietary Soy Phytoestrogens against Hyper Caloric Diet in Cyclic Female Wistar Rats is Mediated through Estradiol Receptors

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ABSTRACT

This study aimed the assessment of the hepatoprotective effects of dietary soy isoflavones (26%), subclass of phytoestrogens, against hepatic deteriorations induced by hypercaloric diet in cyclic female Wistar rats for 30 days. A total of 32 cyclic female Wistar rats were equally divided into control; (fed on casein based hyper caloric diet) and the other group received soy (26%) fed hyper caloric diet group for 30 days. Weekly food intake and body weight gain, serum lipid profile, leptin, hepatic GSH and MDA were determined. Liver histopathological examination and immunohistochemistry of hepatic ERβ receptors content were also determined. Weekly food intake and body weight gain were significantly (P<0.05) decreased in soy group than control. HDL was significantly (P<0.01) higher in treated group than control during all phases of estrous cycle. Total cholesterol (TC), Triglycerides (TG), and leptin were significantly (P<0.01) decreased in soy treated group than control. Hepatic GSH was significantly increased during proestrus (P<0.01), estrus and metestrus (P<0.05). MDA was significantly reduced in soy fed group during proestrus (P<0.01), estrus, metestrus and diestrus (P<0.05) than control. Histopathology of soy fed group revealed absence of steatosis and fatty infiltrations that were present in control. Hepatic ERβ increased significantly (P<0.01) in soy treated group than control in all phases of estrous cycle. It was concluded that, soy genistein and daidzein have a positive effect toward prevention of adiposity and hepatic oxidative stress caused by hypercaloric diet through ERβ signaling.

Keywords: isoflavones, leptin, ERβ, liver, GSH, MDA.

INTRODUCTION

Many foods and phytochemicals are considered to have therapeutic or health-promoting properties. Among these compounds; phytoestrogens have received much attention nowadays. They are ubiquitous within the plant kingdom and they have estrogen-like activity[1]. They have a phenolic group positioned similarly to that of estrogenic steroids[2]. They are categorized into 3 classes; lignans, coumestans and isoflavones[3]. Phytoestrogens have been widely consumed by human and animals. Their consumption is attributed to the reduction of numerous chronic diseases;

including hormone related cancers, coronary heart disease, osteoporosis, and hypercholesterolemia[4]. Phytoestrogens have numerous metabolites that vary greatly in structures but they resemble to the human estrogen; 17ß-estradiol[5]. They can bind both estrogen receptors (ERs) alpha (ERβ) and beta (ERβ)[6] with more preference to ER[2] than ERα[7]. Phytoestrogens are considered natural selective estrogen receptor modulators (SERMs) as they are associated with both estrogenic and anti-estrogenic activity in the body[8, 9]. Variation in physiological response to phytoestrogens is tissue specific and dependent on receptors number, extent of protein binding, distribution of receptor subtype and competing estrogen concentrations[5].

ERs have been demonstrated by numerous studies in humans and rodents to be essential mediators of the estrogen action on glucose and lipid metabolism[10,11]. Moreover, the direct and indirect effects of estrogen on adiposity has been extensively reported as it act by appetite or energy expenditure modulation via controlling release of some hormones such as leptin[11].

The oxidative damage is induced by free radicals, highly reactive molecules produced during cell metabolism, and exert their deleterious effects on proteins, lipids and DNA according to the age[12]. They cause numerous diseases, such as inflammation, cancer, arteriosclerosis, hypertension and diabetes mellitus[13].

Recently, studies have implicated for the importance of ERβ as an essential regulator of metabolic diseases. However, the potency of ERβ selective ligands to offset adiposity is not clear. Therefore the aim of this study is to clarify the hepato-protective effect of soy isoflavones (genistein and daidzein) in cyclic female Wistar rats with reference to hepatic ERβ expression. Furthermore, confirm their role on alleviation of hepatic oxidative stress beside the remote effect of soy isoflavones on appetite and leptin secretion.

MATERIAL AND METHODS

Animals

A total of 32 adult cyclic female Wistar rats weighing 190-200 g were purchased from Helwan Lab Animal House, Helwan, Egypt. They were kept in metallic cages 4 rats/ cage. They were kept at natural light rhythm of the day and at room temperature with *ad libitum* food and water access. Animals were kept for 2 weeks to claim before the start of experiment with casein based diet feeding.

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

Experiment design

Cyclic female Wistar rats were divided into two groups; Group I: control group, n=16 females, they were fed on a hypercaloric casein based ration. Group II: n=16 females received hypercaloric soy diet containing 26% soybeans. The calorie protein ratio for both control and soy isoflavones diet were the same. Both experimental diets were offered for 30 days.

Dietary isoflavones analysis

Dietary Isoflavones, genistein and Daidzein, were determined after their extraction from both experimental diets (control and soy ones) according to Thiagarajan *et al*[14].

Food intake and Body weight gain

Average weekly food intake (gram/ week) and body weight gain (gram/ week) were determined.

Blood and tissue sampling

After 30 day treatment, the experimental rats were overnight fasted and weighed. Four animals representing each stage of estrous cycle/ group were sacrificed under the effect of chloroform anaethesia to obtain blood. Separated sera were kept at -20ºC for estimation of lipid profile and leptin hormone.

Samples from liver were excised from 4 females/ group for each stage of estrous cycle and divided into two parts. First part was kept at -80ºC until preparation of liver homogenate for reduced glutathione (GSH) and malondialdehyde (MDA). The remaining part was immersed in 10% neutral buffered formalin for histopathological and immunohistochemical examination.

Lipid profile

The serum levels of total cholesterol (TC) (ELITech Diagnostic, France), triglycerides (TG) (ELITech Diagnostic, France) and high-density lipoprotein (HDL) cholesterol (Stanbio Laboratory, USA), were measured according to Tietz (15). Serum low density lipoprotein (LDL) was calculated by Friedwald formula described by Davidson and Rosenson (16).

LDL-C = Total Cholesterol- (Triglycerides/5 +HDL-Cholesterol)

Plasma leptin level

Plasma leptin concentrations were assayed using commercial enzyme linked immunoassay rat kit (Code No. 27295, IBL, Japan) according to manufacturer instruction.

Reduced glutathione activity

GSH activity in liver homogenate was assayed calorimetrically using BioVision, USA kit at 412 nm absorbance[17].

Estimation of malondialdehyde

The liver homogenate MDA content was calorimetrically assayed at 532 nm absorbance using BioVision, USA commercial kit [18]

Histopathology

Formalin fixed liver sections were processed using standard histopathological procedures according to Bancroft *et al.*[19].

Immunohistochemistry

The livers, were cut into 4 ¼m sections then mounted on positively charged slides. Primary ERβ antibody (Cat. No. RB- 10658-R7, Thermo Scientific Co., UK) was used for IHC according to Bancroft and Cook[20]. For quantitative analysis, the intensity of immunoreactive parts was performed using an image analyzer (Image J program). Seven random fields were selected from each slide of both experimental groups. Within each field, integrated density (IntDen) of eight random parts were analyzed and the mean for them was expressed as field IntDen[21].

Statistical analysis

All data in the current study were expressed as mean ± SE. they were subjected to student T test using GraphPad Prism software (Version 5.01, USA).

The criterion of probability for the significance was P> 0.05 and P<0.01 for the high significance.
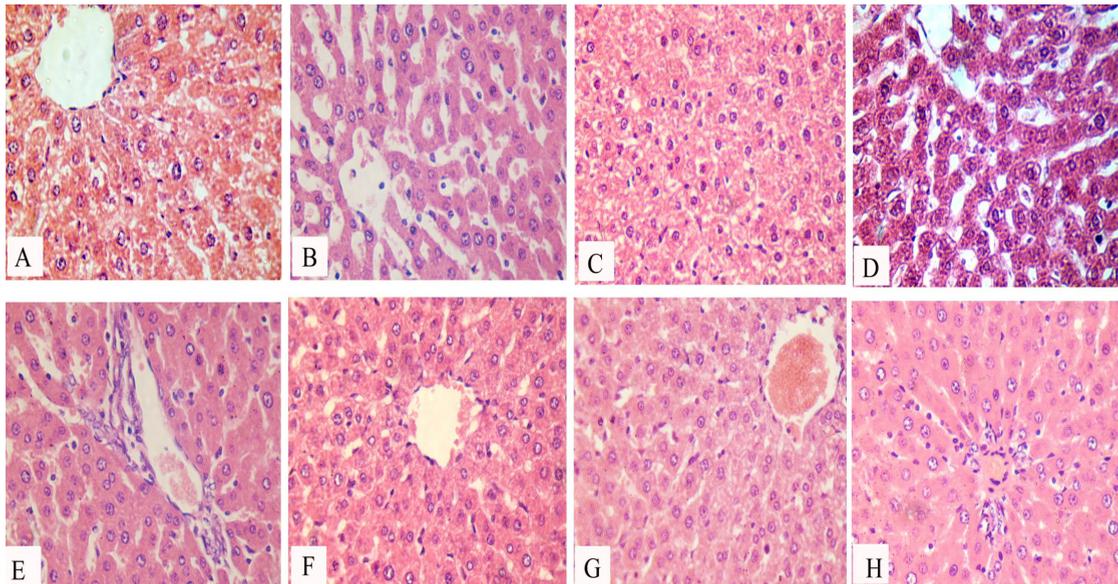
RESULTS

HPLC analysis of both experimental diets revealed that; control diet had no detectable level of genistein or daidzein, while the soy diet contained 1500µg/g genistein and 600 µg/g daidzein, respectively.

There was no significant difference between control and soy fed groups after the first week in body weight or food intake. Starting from the second week till 4th week, body weight gain and food intake (g /week) showed significant (P<0.05) decrease in group II than group I (Table 1).

The values of HDL were significantly (P<0.01) higher in soy treated group than control during all phases of estrous cycle. On the other hand, TG and TC were significantly (P<0.01) decreased in group II than group I during all phases of the estrous cycle. LDL revealed non-significant changes between group II and I among all stages of the estrous cycle. Serum leptin levels demonstrated highly significant (P<0.01) decrease in group II during proestrus, estrus, metestrus, and diestrus than group I. The level of hepatic GSH was increased with higher significance (P<0.01) in soy fed group during proestrus phase of the cycle. During estrus and metestrus hepatic GSH showed significant (P<0.05) increase in soy fed group than control meanwhile, during diestrus a non significant increment was observed in soy fed group than control. MDA, denoting Lipid peroxidation, was reduced with higher significance (P<0.01) in hepatic tissue of soy treated group than control during proestrus phase. During estrus, metestrus, and diestrus phases MDA showed a significant (P<0.05) reduction in soy treated livers than control ones (Table 2).

Liver histopathology demonstrated steatosis and fatty infiltrations in group I while livers of soy fed rats' showed normal architecture without fat infiltration during all phases of the cycle. ERβ immunostaining showed marked increase in soy fed group during proestrus, estrus, metestrus, and diestrus than control (Figure 1). Moreover, ERβ Integden domonstrated highly significant (P<0.01)

Table 1: The effect of dietary soy isoflavones on body weight gain (g/ week) and food intake (g/ week) in cyclic female Wistar rats during 4 weeks

Parameter	One week		Two weeks		Three weeks		Four weeks	
	Group I	Group II	Group I	Group II	Group I	Group II	Group I	Group II
Body weight gain	10.24±1.09	8.11±0.76	10.5±1.50	3.79±0.89*	3.93±1.50	1.81±0.49*	2.40±1.15	1.29±0.23*
Food intake	84.81±6.55	77.43±4.10	72.27±3.41	61.52±2.05*	68.40±2.04	57.54±1.72*	71.10±0.39	58.46±1.63*

*Means significant at (P<0.05) for each phase of the cycle in both groups.

Table 2: Effect of dietary soy isoflavones on serum leptin (ng/ml), HDL (mg / dl), TG (mg / dl), TC (mg / dl), LDL (mg / dl), hepatic GSH (mg/g) and MDA (nmol/g) in cyclic female Wistar rats after 30 days

Para-meters	Group I				Group II			
	Proestrus	Estrus	Metestrus	Diestrus	Proestrus	Estrus	Metestrus	Diestrus
HDL	13.87±1.88	12.23±0.69	14.28±2.56	15.36±1.52	20.29±2.13**	21.21±1.02**	21.58±3.61**	24.35±1.06**
TC	74.44±2.86	78.92±0.81	75.23±3.26	72.51±1.87	52.95±1.39**	51.76±1.98**	51.54±1.67**	53.59±2.04**
TG	113.00±4.72	116.00±3.46	119.00±4.16	114.01±3.21	88.93±1.89**	87.03±2.39**	81.97±2.16**	82.07±3.29**
LDL	72.23±2.53	70.85±2.95	71.50±1.10	71.74±2.46	72.65±1.53	72.89±2.18	73.26±1.65	73.63±2.02
Leptin	196.00±1.53	193.70±2.60	202.31±3.84	201.71±4.41	170.13±2.60**	171.30±2.84**	173.3±3.38**	175.7±2.84**
GSH	58.06±1.19	61.24±0.79	59.57±2.34	61.42±2.57	70.03±2.09**	74.38±2.98*	73.89±2.40*	68.43±5.80
MDA	3.387±0.09	2.55±0.25	2.49±0.30	2.917±0.26	1.72±0.12**	1.493±0.13*	1.56±0.07	1.95±0.12*

*Means significant at (P<0.05) for each phase of the cycle of control and soy groups.
**Means highly significant at (P<0.01) for each phase of the cycle of control and soy groups.

Fig. 1: Photomicrographs showing liver histopathology of control group (A,B, C,D) and soy isoflavones treated group (E,F,G, H) cyclic female Wistar rats during proestrus, estrus, metestrus and diestrus, respectively. Steatosis and fat infiltration were noted in control group during all phases of the estrous cycle. The soy group revealed no fatty infilterations with normal appearance of hepatic architecture in all phases of the estrous cycle. [hematoxylin and eosin (H&E) stain; original magnification: ×20]
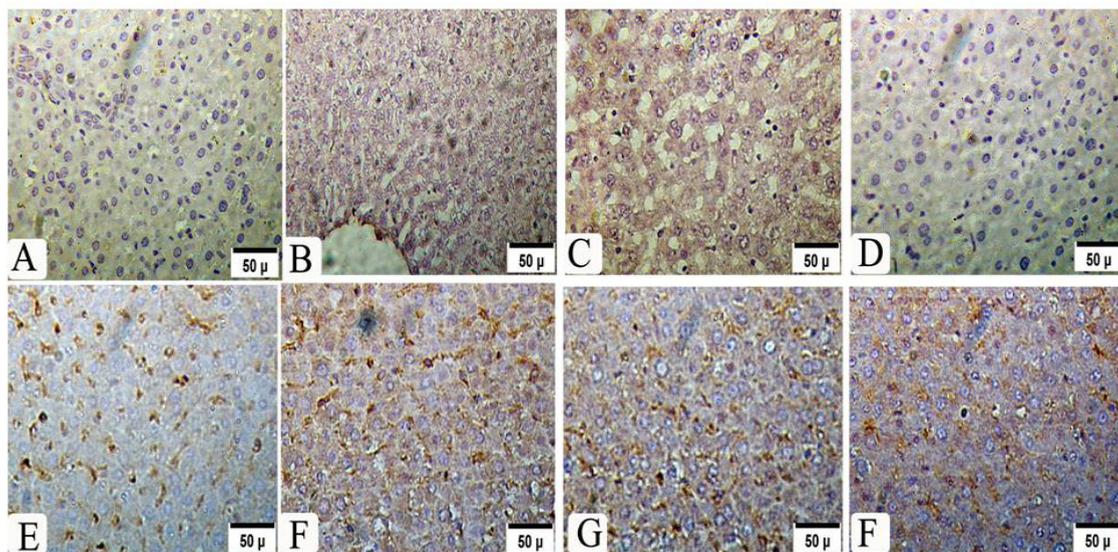
Fig. 2: Immunostaining of cyclic female Wistar rats' livers A: Control proestrus, B: control estrus, C: control metestrus, D: control diestrus, E: soy isoflavones during proestrus, F: soy isoflavones during estrus, G: soy isoflavones during metestrus, F: soy isoflavones during diestrus. The Figure demonstrated that ERβ were predominantly localized within the cytoplasm. Soy treatments produced up regulation of ERβ expression in hepatocytes relative to control during all phases of the cycle.X20
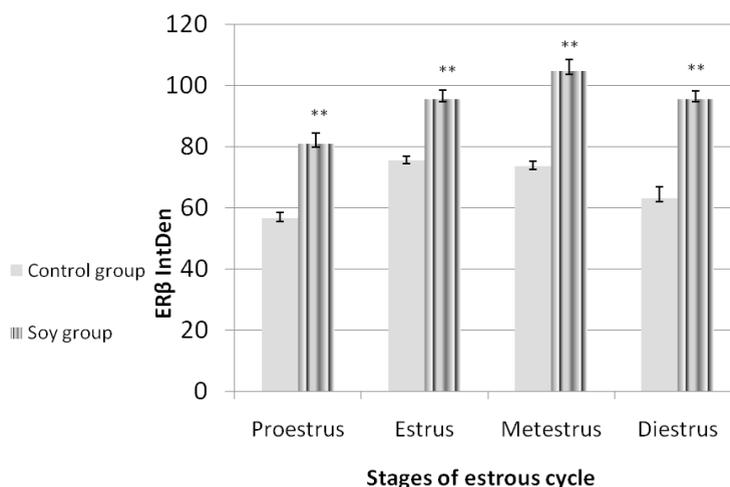
Fig. 3: Effect of dietary soy isoflavones on estrogen receptor beta (ERβ) expression integrated density (IntDen) in cyclic female Wistar rats

increase in soy group than control among all phases of estrus cycle (Figure 2&3).

DISCUSSION

Current study demonstrated the possible protective effects of soy isoflavones against the risks of adiposity and hepatic oxidative stress in cyclic female Wistar rats. Exposure of mature cyclic female rats to soy isoflavones significantly (P<0.05) reduced food consumption and body weight gain than control ones. Given the physiochemical similarity between soy phytoestrogens and estradiol, it is not surprising that exposure to dietary soy phytoestrogens mimics estrogen's hormone action as it acts as estradiol. These results are generally consistent with those reported by studies of Tolba[22] and Ebaid *et al.* [23]. Reduction in food intake may be due to the appetite repressing action of estrogen[24] as dietary phytostrogens decrease food intake and hence decrease body weight. The decrease implies that the estrogenic hormone action of soy isoflavones which is beneficial to body fat regulation. Consequently, leptin hormone level that is produced from fat tissue was decreased. Leptin hormone influences hypothalamic neuropeptide Y levels which regulates feeding behaviour[25].

Dietary soy isoflavones was shown to have direct effects on lipid metabolism as it decreased TC & TG and increased HDL significantly (P<0.01) in all phases of estrus cycle. These results are consistent with previous records of Uesugi *et al.*[26] and Tolba,[22]. This could be attributed to the effect of soy isoflavones on hepatic lipid metabolism and adipose tissue that led to decrease in triglycerides[27]. This was reflected by decrease in hepatic fatty changes in treated group among all phases of the estrous cycle. These results suggests the hypolipidemic effect of soy isoflavones that are ascribed to their structural similarities to estradiol which acts predominantly via two distinct nuclear ERs, ERα and ERβ, that are defined as ligand-inducible transcription factors[28]. Another explanation for the lipid-lowering effect of soy isoflavones; is it might lower cholesterol levels through reduction of intestinal cholesterol absorption with increase in bile acid excretion[29].

Soy isoflavones significantly (P<0.01) decreased serum leptin level during proestrus, estrus, metestrus, and diestrus than control. Leptin is a mediator of long-term regulation of energy balance[30]. Taking together, the depression in TG and TC as well as the reduced hepatic fatty changes this allowed us to believe the direct influence of soy isoflavones on adipocytes which are the main source of leptin[31]. Moreover, soy isoflavones especially genistein inhibit some enzymes in adipocytes that abates their leptin production in spite of unchanged gene expression[32].

Soy phytoestrogens increased GSH significantly (P<0.01) during proestrus phase of the cycle and at P<0.05 during estrus and metestrus. On the other hand hepatic MDA was reduced significantly at P<0.01 during proestrus and at P<0.05 during estrus, metestrus, and diestrus phases in soy isoflavones treated livers than control ones. These results were coincide with those of Baeza *et al.*[33]. The lipid profile improvements (TC and TG) in soy isoflavones group could be attributed to such effect. Lipid profile alterations is a causal factor for reduction in lipid peroxidation[34] and oxidative stress[35] that resulted from increase in reactive oxygen species production and reduction in antioxidant enzymes. Hepatic steatosis that shown in group I leaded to an increase in lipid peroxidation in hepatocytes, which, in turn, activates hepatic stellate cells (HSCs). The HSCs are thought to be the primary target cells for inflammatory and oxidative stimuli, and to produce the components

of extracellular matrix[36]. Moreover, soy isoflavones as SERMs could exert positive effects on liver functions[37] as they possess anti-oxidant properties[38] thus increasing the hepatic expression of ERβ. Finally, soy isoflavones significantly (P<0.05) increased hepatic ERβ that could modulate energy homeostasis, oxidative stress and lipid peroxidation. However this point needs further investigations and confirmation.

CONCLUSION

Soy isoflavones improved serum lipid profile, inhibited leptin, modulate hepatic oxidative stress and lipid peroxidation, reduced accumulation of fat in the liver and increased hepatic ERβ expression. Collectively, these results suggest that soy isoflavones can represent a new class of ERβ ligand to prevent/treat adiposity and hepatic oxidative stress.

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