CLINICAL INVESTIGATION

Soy isoflavones (*Glycine max*) attenuates bilateral ovariectomy (experimental menopause) induced alteration in the hepatic and renal metabolic functions in female Wistar rats

Abstract

The alarming increase in the rate of premature menopause has been reported in the in India. The reason for this early menopause is still unclear, but it is a major public health problem because of its influence on the development of components of metabolic syndrome. Abnormalities in hepatic and renal metabolic functions after menopause considered as a major trigger for the postmenopausal metabolic consequences. In the present study, we investigated the effects of surgical induced menopause on hepatic and renal functions and the beneficial effects of soy isoflavones on the same. Thirty-two female Wistar rats were divided into four different groups. Bilateral ovariectomy was done to create a model of experimental menopause. Those groups of rats received soy isoflavones, the dose of 150 mg/kg BW/day was given via drinking water for the 12 weeks. Bilateral ovariectomy in rats caused impaired liver and kidney functions as indicated by increased levels of plasma functional enzymes; Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), AST/ALT ratio, total protein, total bilirubin, plasma urea, creatinine, uric acid, blood urea nitrogen (BUN, BUN/creatinine ratio. Treatment with soy isoflavones significantly inhibited the ovariectomy-induced changes in these liver and renal parameters, suggesting the use of this natural product as an alternative remedy in the management of liver and kidney abnormalities associated with the postmenopausal women.

Keywords: bilateral ovariectomy • liver function • kidney function • soy isoflavones

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Introduction

Menopause, an age-related loss of ovarian follicular activities and estrogen production. The natural menopause occurs between 45 and 55 years of age worldwide [1]. In India, it has been estimated that the mean age of menopause between 41.9 and 49.4 [2]. The public health impacts of postmenopausal complications are substantial since women spend virtually one-third of their lives in the postmenopausal state worldwide [3]. In the direction of reports of the third consensus meeting of the Indian Menopause Society, the number of postmenopausal women in India is approximately 43 million and it may reach 103 million by 2026 [4]. These observations highlight the importance of empathizing the molecular and cellular mechanisms that underlie the pathology of metabolic complications concomitant with the postmenopausal population.

Recently, concern has been expressed that rates of premature menopause (before age 40 years) are high in India and may be increasing in certain sections of the population [4]. It has been observed that nearly four percent of women of the age group of 29-34 years attain menopause and its incidence increases to eight percent in women of age group between 35 and 39 years [4,5]. The reason for this early menopause is still unclear, but it is a major public health problem due to its influence on the development of components of metabolic syndrome.

Postmenopausal state emerged as the major risk for several metabolic diseases and is associated with a wide spectrum of liver and kidney abnormalities, known as Nonalcoholic Fatty Liver Disease (NAFLD), characterized by an excessive accumulation of fat in the hepatocytes (i.e., hepatic steatosis) and Chronic Renal Disease (CKD) [6,7]. NAFLD and CKD becoming a major public health problem due to its high prevalence and increased risk of developing metabolic syndrome and its components: Obesity, insulin resistance, type 2 diabetes mellitus, hypertension, dyslipidemia and coronary heart diseases [7-9]. Furthermore, studies have documented that the postmenopausal women three times prone to develop NAFLD and CKD is twice common in the postmenopausal state than the premenopausal state, suggesting a protective role of estrogen against NAFLD, CKD and their metabolic consequences [8-10]. Though the pathophysiology of isolated NAFLD and CKD are well established in the literature; to the best of our knowledge, there is no clearly established evidence available regarding the molecular basis of development of liver and kidney metabolic abnormalities associated with the postmenopausal state.

Hormone Replacement Therapy (HRT), a present strategy widely used in clinical practice in the management of postmenopausal related symptoms. Though HRT improves menopausal symptoms, the prolonged use of HRT is not recommended due to its adverse effects in causing a certain type of cancers such as breast cancer and ovarian cancer in addition to its negative impact on cardiovascular risks [11,12]. Hence, there is no effective pharmacological strategy is available in order to manage postmenopausal complications by causing any unwanted deleterious effects. Thus, the search for effective therapeutic strategies to tackle this problem has seen a renewed interest. Various medicinal plants, containing phytoestrogens have been used to combat this problem. One such a medicinal natural phytoestrogen is soy isoflavones from *Glycine max*.

Soy isoflavones, a group of naturally occurring phytoestrogens shares the structural and functional homogeneity with estrogen. It has been believed that consumption of phytoestrogens rich diet could have protective effects against postmenopausal symptoms [13]. Numerous studies including epidemiological studies to human and animal experiments have also shown anti-oxidant, anti-inflammatory, anti-diabetic and hypolipidemic effects of soy isoflavones against metabolic and cardiovascular diseases [14-18]. Consumption of SIFs also improved dyslipidemia and hepatic lipid accumulation by modulating the expression of some of the proteins involved in lipid metabolism [19-22]. In addition, recently several studies have reported the beneficial effects of SIFs on the management of fatty liver diseases, obesity and its associated metabolic complications, such as oxidative stress, insulin resistance, inflammation, and lipid abnormalities. However, the molecular mechanisms by which SIFs exhibit its beneficial effects are not clear and the molecular effect of SIFs on the pathogenesis of these metabolic complications in the model of the postmenopausal state is unknown. The beneficial effects of soy isoflavones have been studied in an animal the model; however, its efficacy in ameliorating the liver and renal functions in the postmenopausal state is largely unclear. Hence, in the present study is aimed to study the beneficial effects of soy isoflavones on liver and kidney functions in experimental menopause.

Materials and Methods

Animals and ethical approval

Thirty-two (n=32) female Wistar albino rats

weighing between 100 to 120 g aged 3 months were supplied by the central animal house of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER, Puducherry, India 605006). Rats were housed in stainless steel cages in a well-ventilated room with a 12-hour light/12 hour dark cycle at ambient temperature. Experiments were carried out in accordance with the guidelines for the Care and Use of laboratory animals (National Research Council, 1985) and the study was approved by the Institutional Animal Ethical Committee with JIP/IAEC/SC/1/2010-11 reference number for notice of approval.

Experimental groups

Thirty-two female rats were divided into four groups based on their body weight. The details of treatment for the different experimental groups as follows:

- Control: Sham-operated rats fed with normal chow for 12 weeks

- Control+SIF: Ovariectomy rats fed with normal chow for 12 weeks

- Ovariectomy: Sham-operated rats fed with normal chow for 12 weeks along with soy isoflavones (150 mg/kg BW/day) via drinking water

- Ovariectomy+SIF: Ovariectomy rats fed with

normal chow for 12 weeks along with soy isoflavones (150 mg/kg BW/day) via drinking water
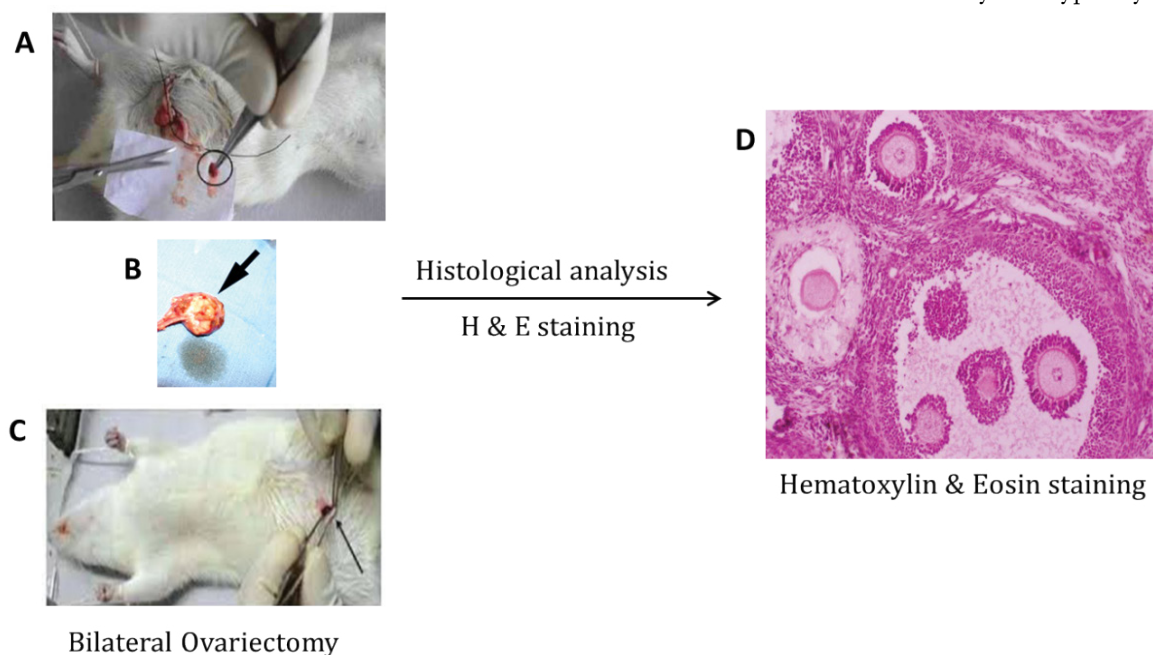
Surgical procedure for bilateral ovariectomy

Bilateral Ovariectomy (OVX), the removal of both the ovaries have been well recognized and widely used model of human menopause to study the reproductive changes as well as the biochemical basis for the development of metabolic and endocrine complications concomitant with the postmenopausal state [23-27]. Hence, we created an experimental model of menopause by subjecting the rats to bilateral ovariectomy.

The animal was anesthetized under ketamine anesthesia (100 mg/Kg body weight). The ventral aspect of the animal was shaven and cleaned with 70% alcohol and then with sterile saline. A single midline 2-3 cm long incision was made under sterile conditions. The ovaries were located and excised. The incision was then sutured using aseptic techniques and wiped clean with sterile saline. Antibiotic cream was applied locally over the wound. The success of the bilateral ovariectomy was confirmed histologically (Figure 1). The control rats were sham operated in order to neutralize the surgical induced biochemical changes.

Methanolic extraction of soy isoflavones

Isoflavones were extracted from soybean hypocotyls

Figure 1: (A and C) Surgical procedure for bilateral ovariectomy; (B) Removed ovary gland; (D) Histological confirmation (Hematoxylin and Eosin staining of ova) of successful of bilateral ovariectomy. Image was captured using Olympus BX43 Cell Culture inverted microscopy (Olympus, Japan) at 10X magnification.

according to the method described by Yoon-Bok Lee et al. [28]. The soybean hypocotyls were purchased from the local market (PAPSCO, Puducherry, India). The soybean hypocotyls were mixed with 10 volumes of 80% aqueous methanol and stirred for 4hrs at room temperature. The methanolic extract was then concentrated in a rotary evaporator at 50°C. The final step of the preparation involved freeze-drying the concentrated methanol extract.

In-vitro assessment of the anti-oxidant property of soy isoflavones extract

2,2'-dipicryl 2'-phenylhydrazine (DPPH) assay: The free radical scavenging capacity of soy isoflavones was assessed by 2,2'-dipicryl 2'-phenylhydrazine (DPPH) assay [29]. A standard graph was plotted using Gallic acid as a standard in the range of 0-100 μM/L concentration. It was found that the free radical scavenging capacity of soy isoflavones was 6.23 ± 1.04 μM/L equivalents of Gallic acid.

Route of soy isoflavones administration

In those groups of rats were isoflavones were administered, it was given mixed in drinking water at a dose of 150 mg/kg body weight/day for 12 weeks [28].

Blood sample collection and process

Fasting blood samples were collected under the deep diethyl ether anesthesia by retro-orbital vein at baseline (beginning of the study) and at the end of the study (twelve weeks). The blood samples were then centrifuged at 8,000 rpm for 10 minutes to separate the plasma. The plasma was stored at -80°C until assays were carried out. All tests were performed in accordance with the manufacturer's instructions.

Assessment of liver functional parameters

The bio-makers of liver injury such as: Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and Lactate Dehydrogenase (LDH) activities in the plasma were estimated enzymatically using commercial reagent kits adapted to Olympus AU400 fully automated Clinical Chemistry Analyzer (Bayer's Diagnostics, USA) as per the manufacturer's instructions. The plasma total protein was estimated using commercial reagent kits adapted to Olympus AU400 fully automated Clinical Chemistry Analyzer (Bayer's Diagnostics, USA) as per the manufacturer's instructions.

Analysis of renal functional parameters

The biomarkers of renal injury such as: Fasting plasma urea, creatinine, uric acid were measured by Olympus AU400 fully automated Clinical Chemistry Analyzer (Bayer's Diagnostics, USA) using commercially available kits according to the manufacturer's instructions. Moreover, the Blood Urea Nitrogen (BUN) and BUN/creatinine ratio were calculated as follows [30]:

Blood Urea Nitrogen (BUN)=Blood urea (mg/dL)/2.142

Statistical Analysis

Results were displayed as means ± Standard Deviation (SD). The data were analyzed statistically using Statistical Package of Social Service Windows program version 19.0 (SPSS Institute, Inc., Chicago, IL, USA) statistical packages. The One-Way Analysis of Variance (ANOVA) followed by *Tukey post hoc* test was used to analyze the results. A p-value of less than 0.05 (P<0.05) was considered to be significant.

Results

In-vitro anti-oxidant assay for soy isoflavones

2,2'-dipicryl 2'-phenylhydrazine (DPPH) assay: The free radical scavenging capacity of soy isoflavones was assessed by 2,2'-dipicryl 2'-phenylhydrazine (DPPH) assay. A standard graph was plotted using Gallic acid as a standard in the range of 0-100 μM/L concentration. It was found that the free radical scavenging capacity of soy isoflavones was 6.23 ± 1.04 μM/L equivalents of Gallic acid.

Effect of Soy isoflavones extract on body weight and organ weights in ovariectomized Wistar rats

In Table 1, bilateral ovariectomy in rats caused increased body weight, liver weight, kidney weight, and reduced uterus weight when compared to the sham-operated rats (control group). Treatment with soy isoflavones markedly prevented the ovariectomy-induced increase in the body weight and organ weights; liver, kidney.

Effects of soy isoflavones extract on plasma bio-markers of liver injury in ovariectomized Wistar rats

The plasma biomarkers of liver injury: Aspartate Transaminases (AST), Alanine Transaminase (ALT), Lactate Dehydrogenase (LDH), alkaline phosphatase, total bilirubin was significantly increased in response to ovariectomy. Soy isoflavones treatment markedly inhibited the increment in these plasma liver function parameters in all the experimental rats (Figures 2 and 3). In light of the above findings in the plasma, the

Table 1. Soy isoflavones alleviates bilateral ovariectomy (experimental menopause) induced increment in the body weight and organ weights in female Wistar rats				
Parameters	Whole body weight (grams)	Relative LW (g/100 g BW)	Relative KW (g/100 g BW)	Relative UW (g/100 g BW)
Control	150.8 ± 7.36	2.24 ± 0.18	0.97 ± 0.08	0.51 ± 0.08
Ovariectomy	168.8 ± 6.13* (p 0.013)	3.16 ± 0.22* (p 0.000)	1.54 ± 0.10* (p 0.008)	0.41 ± 0.04
Control+SIF	143.0 ± 9.16	2.17 ± 0.11	1.01 ± 0.21	0.57 ± 0.10
Ovariectomy + SIF	149.3 ± 4.37¥ (p 0.000)	2.63 ± 0.31¥ (p 0.000)	1.03 ± 0.08¥ (p 0.000)	0.49 ± 0.03

*,¥ $P<0.05$ considered as statistically significant
Data were expressed as mean ± SD (n=8)
*: Comparison with control, ¥: Comparison with Ovariectomy
SIF: Soy Isoflavones; BW: Body Weight; LW: Liver Weight; KW: Kidney Weight; UW: Uterus Weight
One way analysis of variance (ANOVA) followed by *Tukey post-hoc test*, SPSS Version 21.0
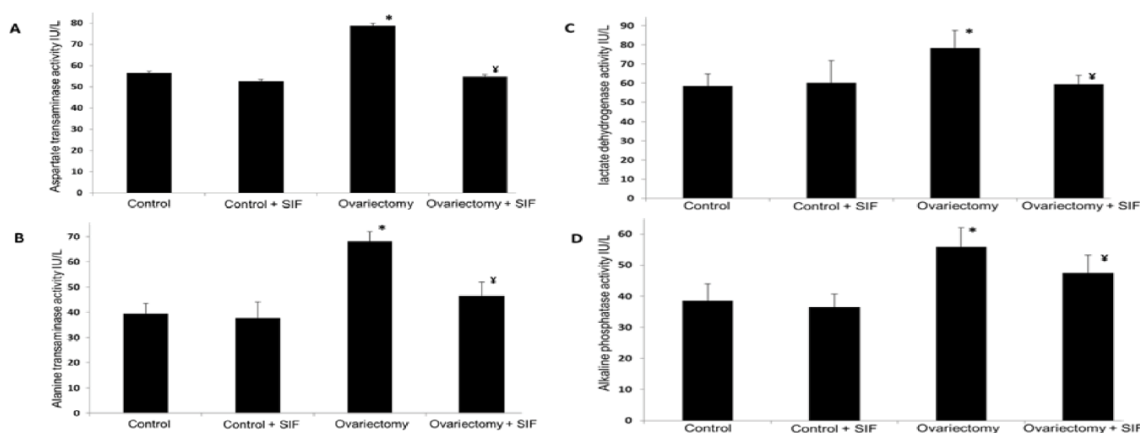
Figure 2: Soy isoflavones significantly improves the ovariectomy induced changes in the plasma liver functional enzymes activities in Wistar rats (A) Plasma aspartate transaminase; (B) Plasma alanine transaminase; (C) Plasma lactate dehydrogenase; (D) Plasma alkaline phosphatase. Bilateral ovariectomy in rats caused elevation the plasma functional enzymes activities. Treatment with soy isoflavones significantly inhibited the ovariectomy induced elevation in these parameters.
*,¥ $P<0.05$ considered as statistically significant. Data were expressed as mean ± SD (n=8)
*: Comparison with control, ¥: Comparison with Ovariectomy
SIF: Soy Isoflavones
One Way Analysis of Variance (ANOVA) followed by *Tukey post-hoc test*, SPSS Version 21.0

liver tissue also displayed with the elevated levels of AST, ALT, LDH and total bilirubin in response to ovariectomy (Table 2). Soy isoflavones treatment successfully inhibited this rise (Table 2).

Effects of soy isoflavones extract on plasma biomarkers of renal injury in ovariectomized Wistar rats

The biomarkers of renal damage: Plasma urea, creatinine, and uric acid were found to be elevated in the plasma of ovariectomized rats when compared to the plasma of sham-operated control rats. Supplementation of soy isoflavones significantly inhibited the ovariectomy-induced increased in these renal parameters (Table 3). In addition, the Blood Urea Nitrogen (BUN) was found to be increased in response to ovariectomy and this increment in the calculated BUN was inhibited by soy isoflavones supplementation (Table 3).

Discussion

Postmenopausal women are a higher risk to develop metabolic syndrome components; oxidative stress, inflammation, insulin resistance, dyslipidemia, atherosclerosis, and cardiovascular complications when compared to the premenopausal women [31]. However, the ground for this is poorly understood. This finding forms the necessity for further research to understand the molecular basis of postmenopausal obesity complications. Through estrogen deficiency after menopause reported to accelerate metabolic syndrome complications, the derangements in the metabolic functions of liver and kidney plays a major role in the development of postmenopausal metabolic complications. Soy isoflavones are known phytoestrogens believed to possess beneficial effects against postmenopausal complications [30,31]. In the present study, we investigated the preventive

Figure 3: Soy isoflavones significantly improves the ovariectomy induced changes in the plasma liver functional parameters in Wistar rats (A) Plasma total protein; (B) Plasma total bilirubin. Bilateral ovariectomy in rats caused increase in plasma total protein and bilirubin levels. Soy isoflavones treatment significantly restored these changes.
*,¥ P<0.05 considered as statistically significant. Data were expressed as mean ± SD (n=8)
*: Comparison with control; ¥: Comparison with Ovariectomy
SIF: Soy Isoflavones
One way Analysis of Variance (ANOVA) followed by *Tukey post-hoc test*, SPSS Version 21.0
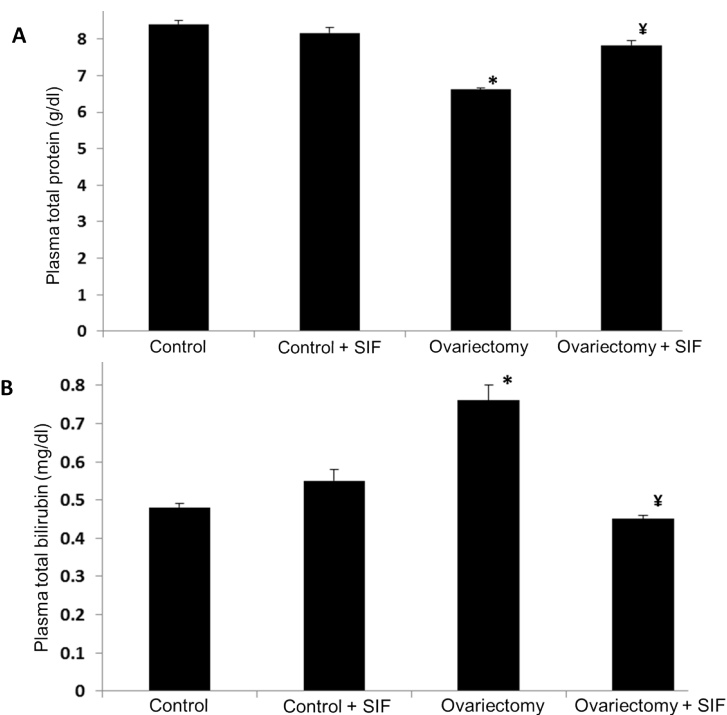
Table 2. Soy isoflavones ameliorates bilateral ovariectomy (experimental menopause) induced derangements in the hepatic functional parameters in the liver of female Wistar rats				
Parameters	Control	Ovariectomy	Control+SIF	Ovariectomy+SIF
hepatic AST, IU/mg protein	6.21 ± 0.18	7.89 ± 0.08* (p 0.000)	6.14 ± 0.07	6.11 ± 0.24¥ (p 0.000)
hepatic ALT, IU/mg protein	1.22 ± 0.03	2.11 ± 0.09* (p 0.031)	1.22 ± 0.05	1.27 ± 0.31¥ (p 0.028)
hepatic LDH, IU/mg protein	7.41 ± 1.13	11.14 ± 1.03* (p 0.006)	8.01 ± 0.14	6.21 ± 1.07¥ (p 0.000)
hepatic ALP, IU/mg protein	1.31 ± 0.02	2.88 ± 0.13* (p 0.000)	1.18 ± 0.06	1.76 ± 0.07¥ (p 0.000)
*,¥ P<0.05 considered as statistically significant
Data were expressed as mean ± SD (n=8)
*: Comparison with control; ¥:Comparison with Ovariectomy
SIF: Soy Isoflavones; AST: Aspartate Transaminase; ALT: Alanine Transaminase; LDH: Lactate Dehydrogenase, ALP: Alkaline Phosphatase
One way analysis of variance (ANOVA) followed by Tukey post-hoc test, SPSS Version 21.0				

Table 3. Soy isoflavones ameliorates bilateral ovariectomy (experimental menopause) induced derangements in the plasma renal functional parameters in female Wistar rats				
Parameters	Control	Ovariectomy	Control+SIF	Ovariectomy+SIF
Blood urea mmol/L	5.93 ± 0.24	13.38 ± 0.29* (p 0.000)	6.09 ± 0.35	9.53 ± 0.98¥ (p 0.000)
Blood Creatinine μmol/L	26.43 ± 3.91	50.77 ± 4.77* (p 0.000)	30.75 ± 1.75	36.50 ± 2.21¥ (p 0.002)
Blood Uric acid μmol/L	185.93 ± 17.05	296.75 ± 23.24* (p 0.000)	149.49 ± 58.51	198.58 ± 15.56¥ (p 0.000)
BUN mg/dL	16.62 ± 0.67	37.46 ± 0.82* (p 0.000)	17.07 ± 0.99	26.71 ± 2.76¥ (p 0.010)
BUN/Creatinine mg/dL	56.80 ± 9.27	65.86 ± 6.91	49.19 ± 2.54	65.09 ± 9.05
*,¥ P<0.05 considered as statistically significant.
Data were expressed as mean ± SD (n=8).
*: Comparison with control; ¥: Comparison with Ovariectomy
SIF: Soy Isoflavones; BUN: Blood Urea Nitrogen
One way analysis of variance (ANOVA) followed by Tukey post-hoc test, SPSS Version 21.0				

effects of soy isoflavones on derangements in the metabolic activities of liver and kidney in the setting of experimental postmenopausal state.

Previous studies have reported that the postmenopausal women are more prone to liver dysfunction [32], conditions underlined by leakage

of cellular enzymes: ALT, AST and LDH [33]. In the present study, the activities of these enzymes: AST, ALT, and LDH were elevated in the plasma of ovariectomized rats. This finding indicating the occurrence of liver dysfunction. This increase in the activities of these enzymes was normalized by soy isoflavones, demonstrating an attenuation effect on liver dysfunction, which is linked with the maintenance of hepatocytes membrane integrity [34]. Previously we reported the abnormal liver histology and liver inflammatory cells in response to ovariectomy and the hepatoprotective effect of soy isoflavones on the same [35-37]. From the proteomics analysis, the ovariectomy caused over-expression of pro-oxidants and pro-inflammatory proteins; NOX, MPO, HO-1, p38 MAPK, ERK 1/2, COX2, THFα, IL6 in association with suppressed expression of anti-oxidant and anti-inflammatory proteins; SOD1, Catalase, GPx, GSH in the liver [37,38]. The changes in these proteins might have caused hepatic oxidative stress, inflammatory response with the resultant liver dysfunction. Treatment with soy isoflavones significantly improves inhibited the ovariectomy-induced changes in the expression levels of these proteins and improved liver function. In the present study, we found that the soy isoflavones extract exhibited potent antioxidant property as indicated by DPPH free radical scavenging assay. The anti-oxidant property of soy isoflavones extract might have attributed to the hepatoprotective effects against the postmenopausal state.

Previously, we have reported that the ovariectomy in rats per se leads to the lipid metabolic derangements which ultimately leads to hypertriglyceridemia and hepatic steatosis [39]. This effect was attributed by the overexpression of lipogenic proteins, such as LXR, SREBP1, FAS, ACC, and PPARγ, in association with suppressed expression of lipolytic proteins, such as FXR, SHP, insig2 and PPARα [39]. The ovariectomy-induced hypertriglyceridemia may have caused fat accumulation in the liver leading to fatty liver. This might be reflected in the elevated plasma levels of liver function enzymes. Treatment with SIF significantly restored the lipid metabolism and improved hypertriglyceridemia and hepatic steatosis, suggesting the use of this natural phytoestrogen as an alternative strategy/remedy for relieving the lipid abnormalities concomitant with the postmenopausal population.

In addition, the surgical ovariectomy in rats caused kidney dysfunctions as evidenced by increased levels of blood urea, creatinine, uric acid, and BUN. We have previously reported the ovariectomy-induced up-regulation of renal NOX4, COX2, p38MAPD in association with down-regulation of SOD and GPx [36,38]. The overexpression of pro-oxidants proteins might have caused oxidative stress and inflammatory response leading to kidney damage. This may be reflected at plasma levels of elevated urea, creatinine, uric acid, and BUN. Treatment with soy isoflavones significantly improves these derangements and improved renal metabolic functions.

Conclusion

In the present study, we can conclude that bilateral ovariectomy (experimental menopause) in rats per se caused liver dysfunction as well as renal dysfunction. Supplementation of soy isoflavones significantly improved/reverted these dysfunctions, suggesting the use of this natural product as an alternative remedy in the management of liver and kidney metabolic dysfunctions associated with the postmenopausal state.

Conflict of Interest

We declare that we have no conflicts of interest.

Acknowledgment

Executive summary

The alarming increase in the rate of premature menopause has been reported in the in India. The reason for this early menopause is still unclear, but it is a major public health problem because of its influence on the development of components of metabolic syndrome. Abnormalities in hepatic and renal metabolic functions after menopause considered as a major trigger for the postmenopausal metabolic consequences. In the present study, we investigated the effects of surgical induced menopause on hepatic and renal functions and the beneficial effects of soy isoflavones on the same. Thirty-two female Wistar rats were divided into four different groups. Bilateral ovariectomy was done to create a model of experimental menopause. Those groups of rats received soy isoflavones, the dose of 150 mg/kg BW/day was given via drinking water for the 12 weeks. Bilateral ovariectomy in rats caused impaired liver and kidney functions as indicated by increased levels of plasma functional enzymes; Aspartate Transaminase (AST), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP), Lactate Dehydrogenase (LDH), AST/ALT ratio, total protein, total bilirubin, plasma urea, creatinine, uric acid, blood urea nitrogen (BUN, BUN/creatinine ratio. Treatment with soy isoflavones significantly inhibited the ovariectomy-induced changes in these liver and renal parameters, suggesting the use of this natural product as an alternative remedy in the management of liver and kidney abnormalities associated with the postmenopausal women.

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