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Soybean isoflavone affects in rabbits: Effects on metabolism, antioxidant capacity, hormonal balance and reproductive performance

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ABSTRACT

Though soybean isoflavones (SBI) have pharmaceutical properties, the compounds also have endocrine disrupting activities that may adversely affect fertility of mammals. The effects of SBI on metabolism, antioxidant capacity, hormonal balance and reproductive performance of male rabbits were investigated. Adult male rabbits (n = 21) fed an isoflavone-free diet were orally treated with 0 (control; CON), 5 (small; LSBI) or 20 (large; HSBI) mg of SBI/kg body weight/day for 12 weeks. Both SBI doses resulted in lesser blood plasma total protein concentrations, while there were no effects on glucose and cholesterol concentrations compared to CON. The HSBI-treated males had the greatest ($P <$ 0.05) blood plasma total antioxidant capacity and least malondialdehyde. Treatment with both SBI doses induced a 43% increase in triiodothyronine concentrations ($P < 0.05$) and 82% in reaction times ($P <$ 0.001), while decreased sperm concentrations ($P =$ 0.01) and blood plasma testosterone concentrations ($P =$ 0.017) 26% and 19%, respectively. The total functional sperm fraction was less ($P <$ 0.05) in the HSBI group; however, there was no effect of the LSBI treatment as compared to values for the CON group. The kindling rates of females mated to HSBI-treated males tended to be less ($P =$ 0.081) than those of does mated with LSBI or CON males. In conclusion, only the HSBI treatment improved antioxidant status; whereas, treatment with both LSBI and HSBI doses induced a hormonal imbalance which led to an impaired testis function indicating the sensitivity of the adult male reproductive system to SBI actions.

1. Introduction

Isoflavones are a group of plant secondary metabolites that belong to phyto-oestrogenic compounds naturally synthesised by many legumes used for human nutrition or included in the diets of animals. Chemically, isoflavones are non-steroidal phenolic compounds with a structure similar to mammalian oestrogens. This unique structure results in isoflavones having wide-ranging biological activities as a result of actions in both genomic and non-genomic pathways when consumed by mammalian species (Wocławek-Potocka et al., 2013). There has been a wealth of scientific data indicating the antioxidant, antitumor, anti-cancers, anti-diabetic activities of these phytogenic molecules. In addition, these molecules can function to decrease the risk of cardiovascular diseases, menopausal symptoms and osteoporosis (reviewed by Rietjens et al., 2017). Growing public awareness regarding the therapeutic properties of isoflavones has encouraged direct consumption of plant-rich sources or as dietary supplements.

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Additionally, inclusion of these plant materials has become common in the diets of farm animals for producing phyto-oestrogen metabolites in animal products (for example, equol a metabolite of daidzein), which in some cases have greater oestrogenic potency (Andersen et al., 2009). Isoflavones, however,havea hormone-like activity due to the capacity to bind and activate oestrogen receptors (ER), resulting in oestrogen agonistic or antagonistic effects. Thus, isoflavones have been recognised as one of the environmental disrupting chemicals that might alter mammalian endocrine and reproductive systems in both males (Hashem et al., 2018a) and females (Hashem et al., 2016, 2018b).

Interest in the effects of phyto-oestrogens on fertility of males has increased in recent years because oestrogens have been confirmed to have important roles in male reproduction (Akingbemi, 2005). Oestrogens are synthesised by Sertoli, Leydig and testicular germ cells (Cooke et al., 2017). Additionally, both subtypes of ER, ERα and ERβ, are expressed in several hypothalamic nuclei, such as the hypothalamic preoptic area (POA, center of sexual behavior), pituitary gonadotropes (Shughrue et al., 1998), along the male reproductive tract, secondary sexual glands (Yamashita, 2004), round spermatids, Sertoli cells (Martínez-Traverso and Pearl, 2015) and Leydig cells (Schulster et al., 2016). Thus, the male reproductive system may be susceptible to endocrine, morphological and functional alterations by consumption of these compounds; however, there is still inadequate knowledge and conflicting results about the effects of isoflavones on reproductive performance of adult males (Rietjens et al., 2017). Consumption of kiwi fruit extract, rich in isoflavones and flavonoids resulted in a decrease in circulating testosterone, oestradiol and sperm cell count in adult male rats (Panjeh-Shahin et al., 2005). The consumption of soybean-rich diets and soybean supplements containing daidzein and genistein isoflavones resulted in a decrease in concentrations of sperm and testosterone and advanced the timing of the sperm acrosome reaction in mice, rats and rabbits (Pan et al., 2008; Hashem et al., 2018a). Administration of daidzein chronically to adult male rabbits for 12 weeks caused erectile dysfunction by potentiating norepinephrine-induced anti-erectile contraction of the penile corpus cavernosum (Srilatha and Adaikan, 2004). Conversely, feeding soybean meal or SBI supplements had no adverse effects on rabbit genital morphology and mouse erectile function (Cederroth et al., 2010) and, in another, study improved semen quality (Yuosef et al., 2004). In many epidemiological studies, consumption of large amounts of soybean phyto-oestrogen by humans such as Asiatic populations (China and Japan) resulted in reductions in sperm concentration and sperm motility (Giwercman, 2011; Xia et al., 2013) and evoked erectile dysfunction (Bai et al., 2004). Accordingly, there is not resolution as to whether the potential health benefits of phyto-oestrogens (antioxidant property) can compensate for possible health risks on reproductive functions of adult males. Soybeans are an isoflavone-rich legume (genistein and daidzein)that is become the main source of protein in animal diets (especially, dairy cows, pigs, and poultry species) because of banned usage of animal protein sources such as bone meal in Europe since 1995 (Wocławek-Potocka et al., 2013; Attia et al., 2017). The present study, therefore,was conducted to investigate the effects of administration of small and large doses of SBI on physiological responses, antioxidant biomarkers, hormone profile and fertility of adult male rabbits.

2. Materials and methods

2.1. Animals and experimental design

The present study was conducted in the Laboratory of Rabbit Physiology Research, Agricultural Experimental station, Animal and Fish Production Department, Faculty of Agriculture, Alexandria University, Egypt (31° 20′N, 30 °E).The experimental design and animal handling were conducted in accordance with the guidelines of the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Rabbits used in this study were of V-line breeding; a maternal line selected based on litter size at weaning (Department of Animal Science, UPV, Valencia, Spain; Estany et al., 1989). Sexually mature male rabbits ($n = 21$; 8.5 months old and 3.00 ± 0.06 kg body weight, BW) were used. Rabbits were housed in a naturally ventilated lighted rabbitry using similar management and hygienic conditions, and were individually kept in galvanized wire cages (60 cm × 55 cm × 40 cm) equipped with feeders and automatic drinkers. The means of ambient temperature, relative humidity and daily photoperiod (daylight length) during the entire experimental period were 11.50 ± 0.21 °C, 76.30 ± 0.89% and 11.21 ± 0.79 h, respectively. Rabbits were fed a basal pelleted diet that covers daily maintenance requirements according to NRC (1977). Fresh water was available ad libitum. The basal diet was formulated to be completely phyto-oestrogen-free, mainly isoflavones. Lintels and chick peas were included in the basal diet as protein sources due to the minimal contents of desired isoflavones (Hashem et al., 2018a). The ingredient and chemical compositions of the basal diet are shown in Table 1. Before allocation of male rabbits into their respective groups, libido, semen volume, sperm concentration and sperm progressive motility were evaluated for each male. The mean (± pooled s.e.m.; $P > 0.05$) of libido (± 0.38), semen volume (± 0.01), sperm concentration (± 16.45) and sperm progressive motility (± 0.78) was:11.0 s, 0.51 ml, 307.5×10^6/ml and 77.23% for CON;11.75 s, 0.54 ml, 339.2×10^6/ml and 78.46% for LISO and 11.4 s, 0.56 ml, 315.0×10^6/ml and 79.0% for HISO. The male rabbits were assigned to one of three experimental groups ($n = 7$ rabbits/group). Rabbits in the first group served as a control (CON), while those in the second and third groups were orally administered either 5 (small dose, LSBI) or 20 (large dose, HSBI) mg soybean isoflavones (SBI) supplement (Pro-S, Mepaco Mediford, Egypt) per kg BW/day for 12 consecutive weeks, during which the first 2 weeks were for adaptation and the later 10 weeks for semen evaluation and data collection.Isoflavones (genistein and daidzein) were extracted from the basal diet and SBI supplementations occurred in the manner previously described (Zgórka, 2009) and the concentrations were determined using a HPLC coupled with a UV–vis diode-array detector using the separation conditions described by Hashem et al. (2018b). The analysis indicated the basal diet contained a negligible concentration of genistein (0.71 mg/100 g DM) and was daidzein-free (Table 1). The concentrations of isoflavones in SBI supplement was 166.7 mg/g that made up of 24.9 mg/g genistein and 141.8 mg/g daidzein with a ratio of 1 genistein: 5.7daidzein molecules.

Table 1
Ingredients, chemical composition and isoflavone (genistein and daidzein) concentrations of the basal diet.

Ingredients (%)	Basal diet
Wheat bran	40
Yellow corn	20
Lentils	17
Chick peas	10
Wheat straw	9.0
Molasses	2.5
Salts	1.0
Premix mixture	0.5
Chemical composition (%)	
Organic matter	89.9
Crude protein	13.3
Neutral detergent fibre	44.0
Acid detergent fibre	18.8
Ash	10.2
Metabolisable energy (MJ/kg DM)	10.15
Isoflavones content (mg/100 gDM)	
Genistein	0.71
Daidzein	not detectable

Each 3 kg of premix mixture contains: 13.340 IU vitamin A; 2680 IU vitamin D_3; 10 IU vitamin E; 2.68 mg K; 10.68 mg calcium pantothenate; 0.022 mg vitamin B_{12}; 0.668 mg folic acid; 400 mg choline chloride; 26.68 mg chlorotetracycline; 133.34 mg manganese; 66.68 mg iron; 53.34 mg zinc; 3.2 mg copper; 1.86 mg iodine; 0.268 mg cobalt; 0.108 mg selenium.

2.2. Physiological variables, antioxidant biomarkers and hormones

Body weight (BW), rectal temperature and feed intake were recorded weekly for each buck throughout the entire experimental period. Blood samples were collected from the ear vein of each buck into tubes coated with heparin (blood anticoagulant) at weeks 2, 4, 6, 8, 10 and 12 of the experimental period. Plasma was obtained by centrifugation at 700 $\times g$ for 20 min and stored at -80 °C. Plasma samples were colourimetrically assayed for total protein, albumin, globulin, glucose and cholesterol (Biosystem S.A., Barcelona, Spain). Concentrations of total antioxidant capacity (TAC) and malondialdehyde (MDA) were determined at weeks 4, 8 and 12 of the treatment period usinga colourimetric method of a commercial kit (Biodiagnostic, Giza, Egypt). The linearity of assay was up to 2 mM/l for TAC and up to 100 nmol/ml for MDA. Activities of reduced glutathione (GSH; Habig et al., 1974) and superoxide dismutase (SOD; Misra and Fridovich, 1972) antioxidant enzymes were also determined. Concentrations of triiodothyronine (T_3) and testosterone (T) in blood plasma were assayed at weeks 4, 8 and 12 using enzyme immunoassay commercial kits (Monobind Inc., Lake Forest, USA). The lower limit of assay detection was 0.15 ng/ml for T_3 and 0.05 ng/ml for T. The intra- and inter-assay coefficients of variation were 5.4% and 7.6%, respectively for T_3; and 6.73% and 8.90%, respectively for T.

2.3. Evaluation of libido, semen quality and fertility

2.3.1. Semen collection and libido

Semen samples were weekly collected between weeks 2 and 12 of the treatment period using an artificial vagina (maintained at 45 °C) fitted with a graduated collection tube and aid of "teaser" does. Three "teaser" does were used at each semen collection; each "teaser" doe was placed with seven males assigned randomly among the treatment groups. Libido (sexual activity indicator) was evaluated by recording reaction time in seconds using a stop watch. The reaction time was estimated as the time point at which the "teaser doe" was placed in the cage with males until the time point at which the ejaculate was obtained (El-Desoky et al., 2017).

2.3.2. Evaluation of semen physical properties

The volume of each semen ejaculate was recorded immediately after semen collection and removing the gel mass, if present, and the ejaculate contents was placed in a water bath (37 °C) for subsequent evaluations. A trained technician was devoted to assess different semen variables (sperm concentration, progressive motility, viability and morphology), following the guidelines of IRRG (2005). The percentage of sperm motility (% of progressive motility) was evaluated in several microscopic fields for each semen sample by visual examination at 40 × magnifications using a light microscope with a heated stage with classifications of subjective assessments ranging from 0% to 100%. Sperm concentration ($\times 10^6$/ml) was determined using the improved Neubauerhemocytometer slide (GmbH + Co., Brandstwiete 4, 2000 Hamburg 11, Germany) after dilution (1:100). A drop of each semen ejaculate was stained with an eosin–nigrosine blue staining mixture for determining percentage of sperm viability (live or dead) and sperm abnormality (normal or abnormal) by counting 200 sperm cells in several microscopic fields. The sperm cells were classified according to the staining pattern into: complete or partial purple-stained sperm cells, which were considered non-viable; and unstained sperm

cells, which were considered viable. The previously described semen variables were used to calculate the total sperm output (TSO, 10^6/ejaculate) as a yield of semen ejaculate volume (ml) by sperm concentration (10^6/ml); total motile sperm (TMS, 10^6/ejaculate) as a yield of TSO by percentage of progressive motility and total functional sperm fraction (TFSF, 10^6/ejaculate) as a yield of TMS by percentage of normal sperm morphology.

2.3.3. Evaluation of seminal plasma chemical properties

Seminal plasma was obtained by centrifugation of semen samples at 700 $\times g$ for 20 min. The samples were stored at -20 °C. Concentrations of total protein, albumin and cholesterol in the seminal plasma were measured colourimetrically (BioSystem S.A., Barcelona, Spain) at biweekly intervals (2, 4, 6, 8, 10 and 12 weeks). Seminal plasma globulin concentration was estimated by subtracting albumin value from the corresponding value of total protein. Concentration of seminal plasma fructose (initial fructose) was determined immediately after semen collection at the end of the treatment (week 12) using commercial kits (Biodiagnostics, Giza, Egypt).

2.3.4. Fertility assessments

To evaluate fertility and reproductive performance of male rabbits, at the end of week 12 (the end of semen collection period for evaluation), every male in each group was mated with three nulliparous receptive female rabbits every other day (IRRG, 2005). Values for reproductive variables including kindling rate and litter size, litter viability and litter weight at birth were recorded.

2.4. Statistical analysis

All statistical procedures were conducted using Statistical Analysis Systems (SAS, 2001, version 8, Institute Inc., Cary, NC, USA). Data are expressed as least-squares mean ± pooled standard error of mean (± pooled s.e.m.). The square root transformation was used to normalize all data expressed as percentages. Biochemical variables of blood plasma and seminal plasma and semen characteristics were analysed using the two-way repeated-measures analysis of variance (ANOVA). The statistical model included the fixed effect of treatment (CON, LSBI and HSBI), time of sampling/data collection and the interactions, also the random effect of an individual buck was considered. A one-way ANOVA was used to determine the effect of treatment on the variables measured only once per buck including seminal plasma initial fructose, litter size, litter viability and litter weight at birth. Kindling rate (categorical data) was analysed using CATMOD procedure. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects on physiological variables

Effects of large and small doses of SBI on values for the physiological variables, antioxidant biomarkers and concentrations of hormones (T_3 and T) of adult male rabbits are presented in Table 2. The results indicate that neither of the LSBI nor HSBI doses affected body weight, feed intake and rectal temperature, while there were lesser concentrations of blood plasma total protein ($P < 0.001$), albumin ($P = 0.023$) and globulin ($P = 0.053$) as a result of these treatments. Treatment of adult male rabbits with LSBI and HSBI doses resulted in greater ($P = 0.022$) concentrations of blood plasma T_3, however, ($P = 0.017$) concentrations of blood plasma T were lesser compared to the CON. Male rabbits treated with the HSBI dose had greater ($P = 0.052$) concentrations of blood plasma TAC and lesser ($P = 0.044$) concentrations of blood plasma MDA than the CON, while those treated with the LSBI dose had intermediate values. Treatments at both doses of SBI did not affect ($P > 0.05$) activities of SOD and GSH antioxidant enzymes.

3.2. Effects on libido, semen quality and fertility

Effects of the large and small doses of SBI on libido and values for physiochemical variables of semen of adult male rabbits are shown in Table 3 and are depicted in Fig. 1. Treatment with LSBI or HSBI doses increased ($P < 0.001$) reaction times of male rabbits (lesser libido) compared to the CON (Table 3 and Fig. 1). Semen volume was not affected by treatment. Concentrations of sperm cells were less ($P = 0.01$) as a result of treatment with either dose of SBI (Table 3 and Fig. 1), however, these reductions were only observed near the end of the experimental period (weeks 11 and 12). The percentage of progressively motile sperm was greater ($P < 0.05$) for the LSBI- compared with HSBI-treated and CON male rabbits (Table 3 and Fig. 1). Neither the LSBI or HSBI doses affected ($P < 0.05$) percentages of either live sperm or abnormal sperm (Table 3 and Fig. 1). Male rabbits treated with the HSBI dose had less TSO ($P < 0.04$) and TMS ($P = 0.05$) than the CON male rabbits, while those treated with the LSBI dose had intermediate values (Table 3 and Fig. 1). Treatment with the HSBI dose resulted in a lesser ($P < 0.01$) TFSF compared with the other two groups (Table 3 and Fig. 1). Concentrations of seminal plasma total protein, albumin, globulin, cholesterol and initial fructose of ejaculates obtained at weeks 2, 4, 6, 8, 10 and 12 did not differ among the experimental groups (Table 3).The rabbit does mated with HSBI-treated males tended to have lesser ($P = 0.081$) kindling rates than those mated with CON males, while those mated with the LSBI-treated males had intermediate values. Litter size, litter weight and litter viability at birth did not differ among the experimental groups (Table 4).

4. Discussion

The main purpose of the present study was to assess whether supplementing SBI would positively or negatively affect health status

Table 2
Least-squares means (± pooled s.e.m.) for physiological responses, plasma metabolites, antioxidant biomarkers and hormone profile of adult male rabbits fed an isoflavone-free diet and administered a small (5 mg/kg BW/day; LSBI) or large (20 mg/kg BW/day; HSBI) dose of soybean isoflavones or a diet with no SBI supplementation (0 mg/kg BW/day; CON) for 12 consecutive weeks.

Variables	Treatment (Trt)			Pooled s.e.m.	*P*-value		
	CON	LSBI	HSBI		Trt	Time	Trt × Time
Physiological responses[1]							
Body weight (kg)	3.07	3.02	3.13	0.10	0.720	< 0.001	0.952
Feed intake (g/day)	192	190	187	12.65	0.845	0.327	0.743
Rectal temperature (°C)	38.40	38.38	38.42	0.15	0.921	< 0.001	0.750
Plasma metabolites[2]							
Total protein (g/dl)	7.73 [a]	7.02 [b]	7.16 [b]	0.11	< 0.001	< 0.001	0.003
Albumin (g/dl)	3.22 [a]	2.87 [b]	2.99 [b]	0.08	0.023	< 0.001	0.214
Globulin (g/dl)	4.56 [a]	4.11 [b]	4.17 [b]	0.14	0.053	< 0.001	0.023
Glucose (mg/dl)	118.78	117.94	123.13	5.29	0.756	< 0.001	0.206
Cholesterol (mg/dl)	68.39	79.76	74.38	7.38	0.214	0.014	0.205
Antioxidant biomarkers[3]							
Total antioxidant capacity (mM/l)	0.98[b]	1.43[ab]	1.71[a]	0.28	0.052	0.229	0.474
Malondialdehyde (nmol/ml)	2.76[a]	2.27[ab]	1.87[b]	0.34	0.044	0.997	0.782
Superoxide dismutase (U/ml)	239.8	265.5	240.1	9.84	0.124	< 0.001	0.122
Glutathione-S-transferase (U/ml)	1.32	1.32	1.43	0.06	0.477	0.145	0.230
Hormone profile[3]							
Triiodothyronine (ng/ ml)	1.22[b]	1.81[a]	1.68[a]	0.14	0.022	0.450	0.217
Testosterone (ng/ml)	5.17[a]	3.95[b]	4.37[b]	0.58	0.017	0.010	0.146

Within row, [a,b]means with uncommon superscripts differ ($P < 0.05$).

[1] Means of body weight, feed intake and rectal temperature are for values recorded throughout 12 weeks of the experimental period (no. of observations = 84/treatment).

[2] Means of blood plasma metabolites are for values recorded atweeks 2, 4, 6, 8, 10 and 12 of the experimental period (no. of observations = 42/ treatment).

[3] Means of antioxidant biomarkers and hormone profile are for values recorded at weeks 4, 8 and 12 of the experimental period (no. of observations = 21/treatment).

Table 3
Least-squares means (± pooled s.e.m.) for libido and physical and chemical variables of semen of adult male rabbits fed an isoflavone-free diet and administered a small (5 mg/kg BW/day; LSBI) or large (20 mg/kg BW/day; HSBI) dose of soybean isoflavones or a diet with no SBI supplementation (0 mg/kg BW/day; CON) for 12 consecutive weeks.

Variables[1]	Treatment (Trt)			Pooleds.e.m.	*P*-value		
	CON	LSBI	HSBI		Trt	Time	Trt × Timee
Reaction time (s)[2]	10.84[b]	15.42[a]	16.77[a]	1.23	0.004	< 0.001	< 0.001
Physical [2]							
Volume (ml)	0.60	0.68	0.55	0.05	0.252	0.192	0.122
Sperm con ($\times 10^6$ / ml)	327.1[a]	260.6[b]	249.2[b]	26.71	0.01	0.012	0.045
Progressive motility (%)	71.07[b]	78.66[a]	71.06[b]	1.83	< 0.001	0.087	0.140
Live sperm (%)	75.17	79.18	75.66	1.47	0.103	< 0.001	0.118
Abnormal sperm (%)	12.05	13.22	13.42	0.654	0.282	< 0.001	0.393
TSO(10^6 /ejaculate)	196.3[a]	177.2[ab]	137.1[b]	21.90	0.033	0.045	0.257
TMS (10^6 /ejaculate)	139.4[a]	141.8[ab]	97.3[b]	14.25	0.031	0.100	0.431
TFSF (10^6 /ejaculate)	122.3[a]	119.3[a]	84.4[b]	12.51	0.010	0.120	0.524
Chemical[3]							
Total protein (g/dl)	3.21	3.18	3.27	0.15	0.911	0.117	0.697
Globulin (g/dl)	1.31	1.32	1.36	0.11	0.934	0.006	0.514
Albumin (g/dl)	1.89	1.81	1.89	0.09	0.780	0.043	0.897
Initial fructose (mg/dl)[4]	282.0	254.3	260.5	8.42	0.287	–	–

Within row, [a,b]means with uncommon superscripts differ ($P < 0.05$).

[1] Sperm con = sperm concentration; total sperm output (TSO) = semen ejaculate volume × sperm con; total motile sperm (TMS) = percentage of motile sperm × TSO; total function sperm fraction (TFSF) = TMS × percentage of normal sperm morphology.

[2] Means of reaction time and semen physical variables are for values recorded from week 2 to week 12 ofthe experimental period (no. of observations = 77/treatment).

[3] Means of seminal plasma chemical variables are for values recorded at weeks 2, 4, 6, 8, 10 and 12 of the experimental period (no. of observations = 42/treatment).

[4] Means of initial fructose are for values recorded once at week 12.

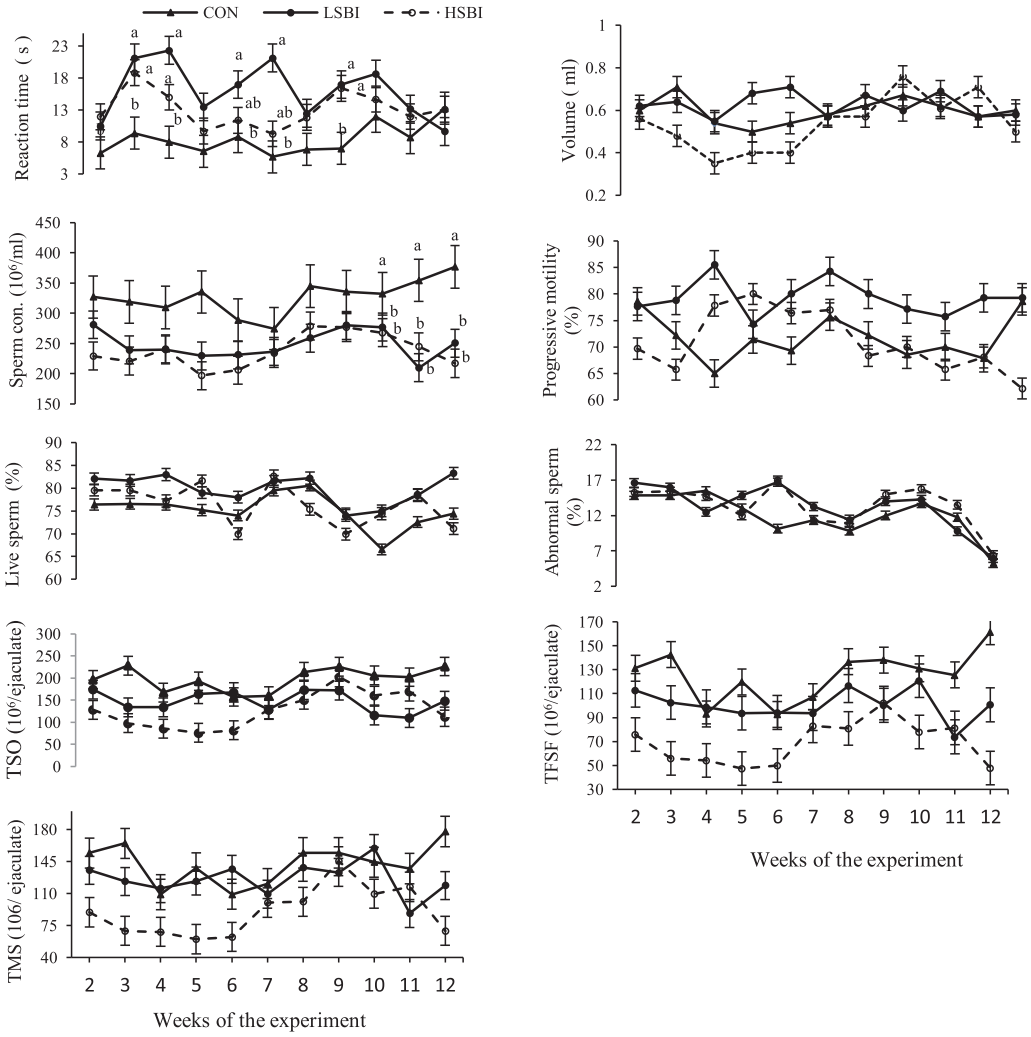

Fig. 1. Least square means (± SEM; $n = 7$/group) for weekly changes in reaction time(s) when placed in presence of females, semen volume (ml), sperm concentration (sperm con., 10^6/ml) and percentages of motile, live and abnormal sperm and total sperm output (TSO, 10^6/ejaculate), total motile sperm (TMS, 10^6/ejaculate), and total functional sperm fraction (TFSF, 10^6/ejaculate) of adult male rabbits fed an isoflavone-free diet and administered a small (5 mg/kg BW/day; LSBI) or large (20 mg/kg BW/day; HSBI) dose of soybean isoflavones or a diet with no SBI supplementation (0 mg/kg BW/day; CON) for 12 consecutive weeks; [a,b]Means within the same time point with different superscript letters differ ($P < 0.05$).

Table 4
Number of does, kindling rate, and least squares means (± pooled s.e.m.) for litter size, number of live litters, number of dead litters and litter weight for does mated with adult male rabbits fed an isoflavone-free diet supplemented with a small (5 mg/kg BW/day; LSBI) or large (20 mg/kg BW/day; HSBI) dose of soybean isoflavones or a diet with no SBI supplementation (0 mg/kg BW/day; CON) for 12 consecutive weeks.

Variables	Treatment (Trt)			Pooled s.e.m.	*P*-value
	CON	LSBI	HSBI		
No. of does	21	20	20	–	–
Kindling rate (%)[1]	72.7	60.0	50.0	–	0.081
Litter size at birth	7.1	7.4	7.9	0.424	0.784
No. of live litters	6.8	6.9	7.3	1.13	0.903
No. of dead litters	0.60	0.57	0.56	0.29	0.839
Litter weight at birth (g)	400.5	420	380	11.76	0.621

[1] Kindling rate (%) = no. of delivered does/no. of inseminated does × 100.

and reproductive performance of adult male rabbits. Soybeans are one of the main protein sources substantially included in rabbit diets at a proportion from 8% to 18% of the diet ingredients (Cardoso and B´ao, 2007; Hashem et al., 2018a). The range of isoflavone concentration, mainly genistein and daidzein, in soybean meal is between 98.4 mg/100 g DM (Sudar et al., 2012) to 209.6 mg/100 g DM (He and Chen, 2013). Thus, the calculated average of daily SBI intake of an adult rabbit consuming about 200 g soybean-based diet might range from 14.4 to 75.6 mg and about 4.8 to 25.2 mg/kg BW(calculated based on average BW about 3.0 kg). The doses of 5 and 20 mg/kg BW used in the present study, therefore, are consistent with amounts of SBI consumed by adult rabbits in field conditions.

It is widely believed that many of the therapeutic actions of SBI are related to the antioxidant activity. Isoflavones can directly scavenge lipid peroxyl radicals and contribute in hydrogen-atom-donation reactions due to the presence of phenolic rings in the structure of these molecules (Fran et al., 2000). Furthermore, phyto-oestrogens can stimulate cellular antioxidant functions through various genomic pathways such as evoking activation of the antioxidant/electrophile response element) ARE/EpRE)-mediated gene, improving cellular defense against the toxicity of electrophiles and free radicals (Jungbauer and Medjakovic, 2014). In the present study, the antioxidant activity of SBI has been confirmed; however, this effect was only evident with the administration of the larger dose (20 mg/kg BW/day) of SBI. Yousef et al. (2004) found that oral administration of 5 mg/kg BW SBI (made up of 1genistein: 1daidzein) every other day for 13 weeks was sufficient to reduce lipid peroxidation in blood plasma, testis, liver and brain. The major isoflavone detected in the SBI supplement used in the present study was daidzein which was about a 6-fold greater amount than genistein. Daidzein has lesser antioxidant activity than genistein (Hu et al., 2007) which might explain the lesser antioxidant activity of SBI supplement observed in the present study with administration of LSBI (5 mg/kg BW/day) dose. Administration of either the small or large doses of SBI did not induce a change in the activities of antioxidant enzymes (GSH and SOD). Similarly, Hashem et al. (2018a) reported that feeding a soybean-based diet providing 27.4 mg/kg BW/day isoflavones (genistein and daidzein) or a linseed-based diet providing 28.1 mg/kg BW/day (made up of secoisolariciresinol and daidzein) did not result in a change in activities of SOD and GSH antioxidant enzymes. Liu et al. (2005), however, reported that rats fed diets containing large amounts of SBI (150 and 200 ppm) had marked increases in SOD and catalase activities in various organs. Accordingly, it could be suggested that the mechanism by which SBI supplements exert antioxidant activities depends on the isoflavone type and/or dose. Large doses of SBI might be required to induce enhancements in the activity of antioxidant enzyme actions, while small or moderate doses might be sufficient only to scavenge free radicals.

Another therapeutic action of SBI that has been proposed relates to the association of SBI dietary inclusions with a reduction in risks of cardiovascular diseases and diabetes (reviewed by Rietjens et al., 2017). In the present study, the cholesterol and glucose-lowering activities of SBI were not substantiated by the results obtained. Consistent with this finding in the present study, are results of previous studies where SBI supplements were ineffective in lowering circulating cholesterol concentration (Hodgson et al., 1998; Nestel et al., 1999). Setchell et al. (2001) suggested that the presence of the protein matrix of soybeans may be necessary for the effectiveness of isoflavones in lowering blood cholesterol. With regard to blood plasma proteins, reductions in the concentrations of blood plasma total proteins, albumin and globulin were observed with the LSBI- and HSBI-treatments of male rabbits in the present study. This effect seems to be related to the reductions in the concentrations of blood plasma T (male anabolic hormone) in the LSBI and HSBI-treated males.

The endocrine disrupting role of SBI at the thyroid gland was evident based on results from the present study. Treatment with both the LSBI (5 mg/kg BW/day) and HSBI (20 mg/kg BW/day) doses increased concentrations of blood plasma T_3. Similarly, Gunnarsson et al. (2009) reported that phyto-oestrogens (24.5 mg biochanin A, 8 mg formononetin, 4 mg genistein and 3.5 mg daidzein) stimulated the secretion of T_3 in peri-pubertal male goats. Also, Hashem et al. (2018a) reported that inclusion of soybean meal (isoflavone phyto-oestrogens) or linseed meal (lignan phyto-oestrogens) in the diets of adult male rabbits increased concentrations of blood plasma T_3 when fed for 12 weeks. The increases in T_3 concentrations following SBI treatments may lead to the effects of SBI in increasing a hyperthyroidism risk. This assumption is consistent with the finding that oestrogens can stimulate proliferation of thyroid cells (Santin and Furlanetto, 2011) and that the oestrogenic activity of isoflavones may become manifested when endogenous oestrogens are at relatively lesser concentrations such as in males (Shanle and Xu, 2011). There, therefore could be actions of SBI, as an oestrogen agonist in the thyroid gland tissues of males, stimulating cellular proliferation and thus leading to an increase in T_3concentrations.

Treatment with LSBI or HSBI doses in the present study resulted in a decrease in libido of the experimental rabbits. This finding is in consistent with results reported by Retana-Márquez et al. (2016) in adult rats. Interestingly, isoflavones as well as oestrogens can pass through the blood-brain barrier and bind with both types of oestrogen receptors, ER-α and ER-β, down-regulating hypothalamic ER gene expression, and interfering binding capacity of oestradiol to its receptors, evoking changes in sexual behavior (Loutchanwoot et al., 2014).Furthermore, in the present study, the behavioural alterations caused by both doses of SBI might be explained by the decrease in the concentration of blood plasma T observed for the LSBI- and HSBI-treated-males. Testosterone is an important androgen for the expression of male sexual behaviour through its aromatization to oestradiol in the POA hypothalamic nucleus (Hull et al., 2006). Isoflavones can inhibit the aromatization of T to oestradiol as a result of both ligands competing for binding sites of aromatase (Aldercreutz et al., 1993), disturbing the androgen-dependent sexual behavioral axis (Zingue et al., 2015).

In the present study, it was observed that the accessory glands and testis had different sensitivity to SBI actions as indicated by the relative functionality of these two tissues. Both values for semen ejaculate volume and seminal plasma constituents are mainly dependent on the functions of the accessory glands (Flint et al., 2015) which, in the present study, were not affected by either dose of SBI. Treatment with either LSBI or HSBI, however, impaired functions of the testis by reducing T concentrations and sperm cell counts. Retana-Márquez et al. (2016) reported that treatment of adult male rats with 5 mg/kg BWgenistein or daidzein was enough to impair testis functions by decreasing spermatogenesis and steroidogenesis processes. Consistent with this finding, Meena et al. (2017)

reported that in adult male rats, treatment with 20 or 100 mg/kg BW/day genistein decreased T and sperm concentrations, while a decrease in prostate weight was only evoked by treatment with 100 mg/kg BW/day genistein. These findings support results of the present study that the testis appears to be more sensitive to the endocrine disrupting action of SBI compared with the accessory glands.

The decrease in T concentration during the period of isoflavones administration could be attributed to the capacity of these compounds to: 1) diminish Leydig cell sensitivity to LH stimulation (Akingbemi et al., 2007); 2) reduce the gene expression and activity of steroidogenic enzymes such as cytochrome P450, 3β-hydroxysteroid dehydrogenase, cytochrome P450 17α-hydroxylase/17–20 lyase, and 17β-hydroxysteroid dehydrogenase (Opalka et al., 2012) and 3) disrupt the positive action of oestrogens on testicular germ cells by acting as an anti-oestrogenic agent, evoking germ cell apoptosis (Assinder et al., 2007). Furthermore, the decrease in the sperm concentrations could have resulted from the reduction in the concentrations of blood plasma T of SBI-treated males because T is an essential hormone for the completion of the spermatogenesis process (Retana-Márquez et al., 2016). Also, there are many types of phyto-oestrogens that evoke germ cell apoptosis, specifically spermatocytes and round spermatids (Retana-Márquez et al., 2012) by attenuating the positive action of oestrogens on testicular germ cells (Assinder et al., 2007).

Evaluation of sperm quality variables in the present study indicated sperm viability and morphology were not affected by the LSBI and HSBI doses; however, sperm motility of the LSBI-treated male rabbits was 12% greater than those of HSBI and CON male rabbits. Mitochondrial adenosine triphosphate (ATP) is one of the energy sources that allows for sperm motility and mitochondrial dysfunction can contribute to a reduction in sperm motility in some men (Folgerø et al., 1993). Interestingly, isoflavones, such as oestrogens, are suspected to affect the function of the mitochondria in the gametes. Kotwicka et al. (2016) reported that17β-oestradiol affected the human sperm mitochondrial function with relatively lesser concentrations of 17β-oestradiol leading to improvements in mitochondrial membrane potential, while relatively greater concentrations led to a decrease in mitochondrial membrane potential. This might, in part, explain why the treatment with the HSBI dose did not improve sperm motility in the present study.

With regard to fertility, the reductions in sperm concentrations due to treatment with large and small doses of SBI were associated with lesser kindling rates in the present study. This reduction, however, was more evident in the HSBI-treated male rabbits. This could be attributed to the enhanced sperm motility of the LSBI-treated male rabbits which compensated for the lesser sperm concentration, and thus TFSF was not different than that for controls, and was greater than that for the HSBI-treated male rabbits.

5. Conclusion

The results of the present study indicate that the smaller dose (5 mg/kg BW/day) of SBI supplement induced a hormonal imbalance and impaired libido, spermatogenesis and fertility of adult male rabbits. The positive effects on antioxidant activity when there was treatment with the large dose (20 mg/kg BW/day) of SBI supplement is considered to have not been beneficial for adult male fertility during the sexually activity period. These results indicate the importance of the re-evaluation of the reproductive performance of adult male rabbits fed soybean based-diets.

Conflict of interest

The authors declare no conflicts of interest

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