Original Research

Suppressive effects of genistein and daidzein on pituitary–thyroid axis in orchidectomized middle-aged rats

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Abstract

High intake of soybean phytoestrogens, isoflavones genistein (G) and daidzein (D), has been associated with health benefits. However, isoflavones were reported to affect adversely thyroid function in the presence of other goitrogenic factors. As the thyroid gland *becomes* functionally *impaired* with *age*, we examined whether supplementary doses of G or D would affect morphology and function of pituitary–thyroid axis in middle-aged male rats. Sixteen-month-old orchidectomized Wistar rats were treated with 10 mg/kg of either G or D, while the control sham-operated and orchidectomized group received just the vehicle for three weeks. The animals were fed soy-free diet with increased iodine content, and killed 24 h after the last treatment. Their pituitaries and thyroids were excised and prepared for further immunohistochemical and morphometric investigation. The concentrations of thyroid-stimulating hormone (TSH), total T_4 and T_3, in the serum were determined. In both isoflavone-treated groups, pituitary TSH-immunopositive cells had increased cellular volume and relative volume density ($P < 0.05$), as well as increased serum TSH levels ($P < 0.05$) in comparison to the controls; their thyroid tissue was characterized by increased volume of thyroglobulin-immunopositive epithelium ($P < 0.05$), epithelial height and index of activation rate ($P < 0.05$), while the volume of luminal colloid, and total serum T_4 and T_3 levels decreased ($P < 0.05$) in comparison to the controls. In conclusion, this study provides the first direct evidence that both G and D can induce microfollicular changes in the thyroid tissue and reduce the level of thyroid hormones in Orx middle-aged male rats, a model of andropause. This reduction consequently led to a feedback stimulation of pituitary TSH cells. The detected stimulatory effect was higher in the daidzein-treated rats.

Keywords: genistein, daidzein, TSH cells, pituitary, thyroid, rats

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Introduction

Soybean isoflavones, namely genistein (G) and daidzein (D), are increasingly used as dietary supplements; the rational being potential health benefits of soy isoflavones, including protection against breast or prostate cancer, cardiovascular disease and osteoporosis.[1–3] Structurally, G and D are similar to estradiol-17β and bind with a weaker potency to both types of estrogen receptors (ERs), with higher affinity for ERβ.[4,5] G has substantially greater ER-binding capacity and estrogenic activity than daidzein.[6] Besides their mild estrogenic/antiestrogenic activity, isoflavones also have anti-proliferative, antiangiogenic and antioxidative activities.[7,8]

Aging is associated with alterations in the function of the hypothalamic–pituitary–thyroid (HPT) axis. Thyrotropin-releasing hormone (TRH) level is found to be reduced in aged rat hypothalami,[9] while normal circulating levels of thyroid-stimulating hormone (TSH) have been reported, despite low serum thyroid hormone levels.[10,11] In humans, the frequency of hypothyroidism and autoimmune thyroid disorders increase in elderly populations.[12,13]

Many substances from the environment, including dietary components, may alter HPT axis function, thus expressing endocrine disruptive properties. Despite numerous beneficial effects of soy isoflavones,[1–3] epidemiological and experimental data have also shown adverse effects on human health.[14] Rats provide a useful risk assessment model for various thyroid toxicants,[15] although compared with the humans, the rodent thyroid gland is more sensitive to adverse chemicals.[16]

Isoflavones have been reported to be goitrogenic in studies both in humans and rats, but only in the presence of iodine deficiency or other goitrogenic factors.[17–19] In humans, goiter has been reported in infants fed non-iodized

soy-based formula.[20,21] Rats receiving low iodine diet that included 20% of defatted soybeans developed severe hypothyroidism, characterized by a reduction in serum thyroxin and an increase in serum TSH.[18] In addition, a diet containing higher percentage of soy (40% of defatted soybeans) in combination with iodine deficiency induced the development of thyroid carcinoma in rats.[17]

Doerge and associates[22–24] have demonstrated, both *in vitro* and *in vivo*, that G and D inhibit the activity of thyroid peroxidase (TPO), the key enzyme in the synthesis of thyroid hormones. Despite significant inactivation of this enzyme, serum thyroid hormone levels were unaffected by isoflavone treatments in young adult rats of both sexes. The authors hypothesize that soy could cause goiter, but only in animals or humans consuming diets that are only marginally adequate in iodine, or individuals who are predisposed to developing goiter.

The elderly tend to use more isoflavones as a nutritional supplement,[13] although the prevalence of thyroid dysfunction increase with age.[12] Data from the animal studies on the possible effects of soy isoflavones on the HPT system are still lacking. The aim of our study was to examine the effects of purified soy phytoestrogens G and D on structure and function of pituitary–thyroid axis in orchidectomized middle-aged rats.

Materials and methods

Animals and diets

All protocols were approved by the Animal Care and Use Committee of our Institute, following recommendations provided in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123, Appendix A).

The study was performed on male middle-aged (15–16 months old) Wistar rats, housed in the unit for experimental animals at the Institute for Biological Research 'Siniša Stanković' in Belgrade, Serbia. They were kept individually under constant laboratory conditions – room temperature ($22 \pm 2^{\circ}C$) and lighting (12L:12D). The animals were fed a soy-free diet,[25] prepared in cooperation with PKB INSHRA, and the Department of Food, School of Veterinary Medicine, Belgrade, Serbia, with corn oil as the main source of fat. The food contained (in grams per 100 g): casein, 20.3; cornstarch, 45; sucrose, 20; corn oil, 5.2; fiber (crystalline cellulose), 3.7; vitamin/mineral mix (Ca-P deficient), 1.5; calcium phosphate dibasic, 1.8; calcium carbonate, 1; DL-methionine, 1.5. Food contained 1.1 mg iodine/kg. Casein and crystalline cellulose were purchased from Alfa Aesar (Johnson Matthey GmbH & Co KG, Karlsruhe, Germany). DL-Methionine was obtained from the Sigma Chemical Company (St Louis, MO, USA). All other ingredients were from PKB INSHRA (Belgrade, Serbia). Food and water were provided *ad libitum*.

Experimental groups

Orchidectomy (Orx; $n = 24$) and sham-operations (SO; $n = 8$) were performed under ketamine anesthesia (15 mg/kg body weight; ketamine hydrochloride, Richter Pharma, Wels, Austria). Two weeks after the surgery, Orx animals were divided into two groups ($n = 8$), according to the treatments received: Orx + G and Orx + D groups received 10 mg/kg body weight of G or D, respectively (Nutraceutica, Monterenzio, Bologna, Italy). Both isoflavones showed high purities of over 98%, as estimated by high-performance liquid chromatography assay. Rats in the control SO and Orx groups were given the same volume of vehicle solution (minimal volume of absolute ethanol, approx. 0.17 mL; in sterile olive oil, approx. 0.33 mL). All substances were injected subcutaneously, every day for three weeks, except on Sundays. Subcutaneous injection was previously reported to be a suitable model for oral exposure to genistein.[26]

All animals were decapitated 24 h after the last treatment. Blood was collected from the trunk. The sera were stored at $-70^{\circ}C$. The pituitaries were excised, weighed, fixed in Bouin's solution for 48 h and then processed for embedding in paraffin wax according to routine protocols.

The relative pituitary and thyroid weights are expressed as an absolute organ weight (mg) per final body weight (g) $\times$ 100.

Immunohistochemical analyses

Series of seven, 5-μm-thick, pituitary sections from the dorsal, middle and ventral pituitary and thyroid tissue levels were stained by the peroxidase–antiperoxidase immunohistochemical method.[27] For immunohistochemical studies of pituitary thyrotrophs, we applied the primary rabbit antisera directed against the rat TSH-β (generously received from Dr A F Parlow, NIH, Bethestda, MD, USA), while for thyroids we used the primary rabbit antisera directed against human thyroglobulin (Tg; Dakopatts, Glostrup, Denmark) that is not species specific.[28]

In brief, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 15 min. The reduction of non-specific background staining was achieved by incubation with normal porcine serum (Dakopatts, Glostrup, Denmark) diluted 1:10, for 45 min. The following sequence of antisera was then applied: first, for pituitaries the rabbit anti-rat TSH-β was applied at a dilution ratio of 1:1000 at room temperature for one hour, while for thyroids the rabbit antisera directed against human Tg were applied at a dilution of 1:500 for two hours at $37^{\circ}C$. Further, swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) was applied at a dilution ratio of 1:500 for 45 min; following was the rabbit peroxidase–anti-peroxidase complex (Dakopatts, Glostrup, Denmark) at a dilution ratio of 1:100, for 45 min. All washes and dilutions were performed using 0.1 mmol/L phosphate-buffered saline (PBS) (pH 7.4). Binding sites were then visualized using 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.03% hydrogen peroxide in 0.2 mmol/L TRIS-HCl buffer (pH 7.4; Serva, Heidelberg, Germany). The sections were counterstained with hematoxylin and mounted in Canada balsam (Molar Chemicals KFT, Budapest, Hungary). For the control pituitary sections, the primary antibody was substituted with PBS (pH 7.4).

Morphometric analyses

Morphometric analyses were carried out using a point-counting method.[29] Four to five transverse sections from the anterior, central and posterior parts of the pituitary and the thyroid were analyzed using the M_{42} multipurpose test grid inserted into the ocular of a Zeiss light microscope (Jena, Germany). Fifty test fields were counted per animal at total magnification of $\times 1000$ for pituitaries and $\times 400$ for thyroids.

For pituitary TSH-immunopositive cells, the relative volumes of the TSH-immunopositive cells (V_c; μm^3) and their nuclei (V_n; μm^3) were calculated.[30] The relative volume density of TSH-immunoreactive cells was expressed as a percentage of total pituitary cell volume within the pituitary unit volume (V_V; %).

In brief, the following parameters were counted: P_n is the number of points hitting on the nuclei of TSH-immunoreactive cells; P_{tc} is the number of points hitting on cellular cytoplasm and N_n the total number of TSH-immunoreactive cell nuclei inside the test field. Relative volume of the TSH cell nuclei was calculated as follows: $V_n = V_V n/N_V$, while the TSH cell volume was calculated as $V_c = 1/N_V$, considering $V_V n$ is the volume density of TSH cell nuclei and N_V is the numerical density of TSH cells. $V_V n$ is calculated using the formula: $V_V n = \Sigma P_n/\Sigma P_{tc}$. Since TSH cells are mononuclear, the N_V corresponded to the number of cells per cubic millimeter, according to the formula: $N_V = (\kappa/\beta) \times (N_A^{3/2}/V_V n^{1/2})$. On the basis of earlier karyometric studies, the shape coefficient β for pituitary cells was estimated to be 1.382, κ is the factor related to cell distribution according to their size (in case of TSH cells its value is 1) and N_A is the number of cells in the plane of pituitary tissue section. N_A is calculated using the formula: $N_A = \Sigma N_n/\Sigma P_{tc} \times a$, where a represents area that belongs to every point of test system ($a = d^2 \times 3^{1/2}/2$, considering d as the test line length of the applied test system). Considering that the applied M_{42} test system has 42 points, and given that all parameters were counted at 50 test fields, the relative volume density of TSH-immunoreactive cells was calculated as follows: $V_{Vc} = (P_n + P_{tc})/50 \times 42$.

For thyroids, the relative volume density (V_V; %) of the corresponding tissue components, defined as the ratio of this component in a volume unit – the thyroid follicles (epithelium plus colloid), follicular epithelium, interstitium and colloid, were calculated as follows:

$$V_v(\%) = P_p/P_t$$

where P_p is the counted points on the component and P_t is the total of points of the test system.[29] By assuming that the thyroid follicular epithelium can be regarded as forming an approximate hollow sphere, the average follicular epithelium cell height (τ) was calculated as follows:

$$\tau = 3P_e \times L_t/2P_t \times (I_e + I_i) + (I_e + I_i)^{1/2}$$

where P_e is the counted points on epithelium, L_t is the test line, P_t is the total of points of the test system and I_e and I_i are the external and internal intersections of the structures with the test line, respectively.[31] The follicular cell activation index (i_a) represents the epithelial volume/colloid volume ratio and indicates the reaction of the thyroid gland structure to the blood TSH concentration.[32]

Serum hormone assays

The sera were separated from the trunk blood after decapitation and stored at -70°C until assayed. Rat serum TSH was measured with a commercially available radioimmunoassay (rTSH [^{125}I] system, Amersham Biosciences, Piscataway, NJ, USA). The levels of total T_4 and T_3 were measured using commercially available electrochemiluminescence immunoassays (Roche Diagnostics GmbH, Mannheim, Germany). All samples were measured in duplicate within a single assay, with intra-assay coefficient of variations for TSH of 3.7% (4 ng/mL), for T_4 of 4.7% (33.4 nmol/L) and for T_3 of 3.6% (1.22 nmol/L).

Statistical analyses

Statistical analyses were performed using Statistica 6.0 software (Statsoft, Tulsa, OK, USA). The data for the experimental groups were tested for normality of distribution with the Kolmogorov–Smirnov test and then subjected to one-way analyses of variance. Duncan's multiple range test was used for *post hoc* comparison of means between the groups. A confidence level of $P < 0.05$ was considered statistically significant. The data are presented as means $\pm$ standard deviation.

Results

Body and organ weights

Five weeks after Orx, a 10% decrease ($P < 0.05$) in mean body weight was observed in comparison with the SO animals. Subsequent treatments with either G or D did not change body weight significantly in comparison to the Orx animals (Table 1).

The absolute pituitary weight of the animals was not affected by Orx or following treatment with soy isoflavones (Table 1). The pituitary weight/body weight ratio for the Orx group increased by 16% ($P < 0.05$), in comparison to the SO group (Table 1). Treatments of Orx rats with G and D did not change the pituitary weight in comparison with the Orx control group (Table 1).

The absolute thyroid weight of Orx rats was not significantly different, while its relative weight was 14% higher than the value obtained for the SO controls. Subsequent treatments of Orx rats with G and D did not affect these parameters significantly in comparison with the Orx control group (Table 1).

Pituitary thyrotroph cells

In SO middle-aged males, TSH-immunopostive cells were often present in the central part of the pituitary pars distalis, as small groups or single cells in close proximity to numerous capillaries. TSH immunopositivity was seen as a diffuse granular brown cytoplasmic stains. They varied in size and appearance (polygonal, elongated or ovoid; Figure 1a).

Table 1 Body and organ weights after orchidectomy and subsequent subcutaneous administration of genistein (Orx + G) and daidzein (Orx + D) in male orchidectomized middle-aged rats

Group	Body weight (g)	Absolute pituitary weight (mg)	Relative pituitary weight (pituitary weight in mg × 100/body weight in g)	Absolute thyroid weight (mg)	Relative thyroid weight (thyroid weight in mg × 100/body weight in g)
SO	650 ± 29	17.00 ± 1.87	2.20 ± 0.13	32.00 ± 2.90	5.00 ± 0.36
Orx	586 ± 35*	16.60 ± 1.41	2.53 ± 0.2*	34.00 ± 3.40	5.70 ± 0.43*
Orx + G	625 ± 35	18.25 ± 2.87	2.94 ± 0.34	37.50 ± 2.60	6.02 ± 0.48
Orx + D	612 ± 52	18.00 ± 1.15	2.83 ± 0.23	36.00 ± 2.20	5.80 ± 0.40

The results are mean ± standard deviation ($n = 8$)
*$P < 0.05$; orchidectomized (Orx) versus sham-operated control (SO) group

After Orx, pituitary TSH cells were somewhat less numerous and darker, though their location and shape remained the same as in the SO controls. A small number of TSH-immunopositive cells strongly resembled morphology of hypertrophic gonadotroph cells after gonadectomy (Figure 1b).

After genistein treatment of Orx rats, TSH-immunopositive cells appeared larger and more numerous in comparison to the Orx and SO controls (Figure 1c). In comparison to the control groups, TSH-immunopositive cells of Orx + D rats were more numerous, lighter and hypertrophed (Figure 1d).

Morphometric analyses demonstrated no significant changes of TSH cells after Orx (Figure 2a–c). After genistein treatment, both volume and the relative volume density of TSH cells were significantly increased by 11% and 12%, respectively ($P < 0.05$; Figure 2a and c) compared with the Orx group. After daidzein treatment, the changes of examined morphometric parameters for pituitary TSH cells were more prominent than after genistein treatment. In comparison with the values calculated for Orx controls, V_c increased by 19% ($P < 0.05$; Figure 2a), V_n increased by 12% ($P < 0.05$; Figure 2b) and V_V increased by 23% ($P < 0.05$; Figure 2c).

Thyroid gland tissue

The thyroid tissue in middle-aged male rats consisted of follicles of different size and morphology. Most central follicles were of medium size, lined by a *cuboid* or low columnar Tg-immunopositive follicular epithelium (Figure 3a and b). Tg immunopositivity was granular and diffusely

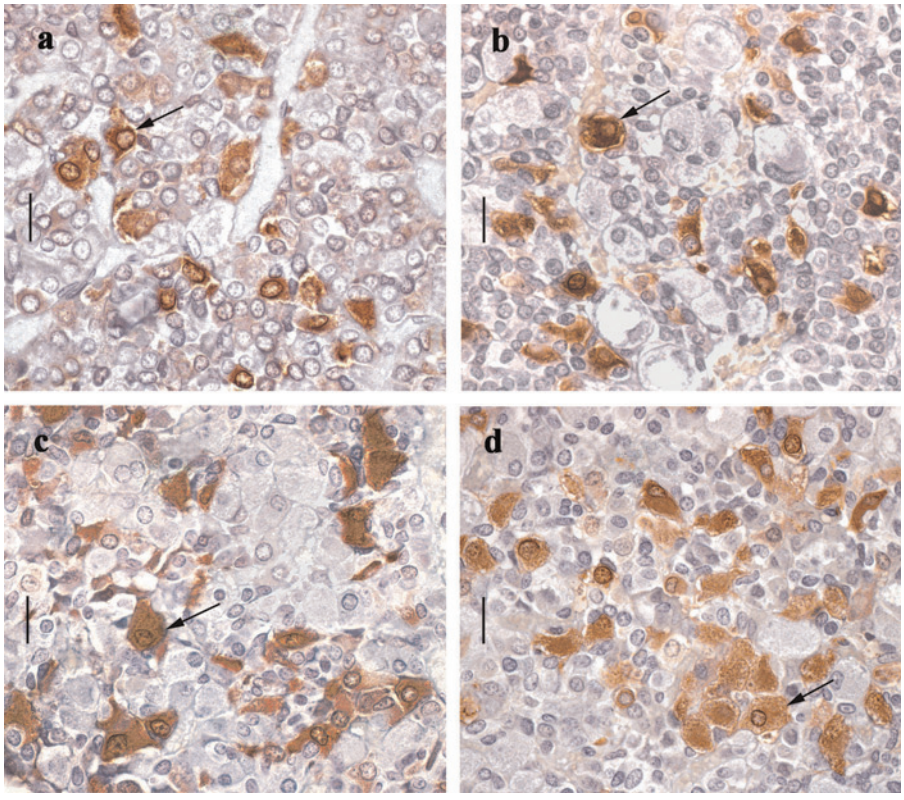

Figure 1 Immunoreactive thyroid-stimulating hormone (TSH) cells in the pars distalis of the pituitary gland from a sham-operated middle-aged rat were often present as small groups or single cells in close proximity to blood capillaries (a; arrow); TSH cells from an orchidectomized rat are somewhat less numerous and darker, though some strongly resembled the morphology of hypertrophic gonadotroph cells after gonadectomy (b; arrow); TSH cells from orchidectomized genistein-treated rat appeared more numerous and larger than in the control groups (c; arrow); TSH cells of orchidectomized daidzein-treated rat are more numerous, lighter and larger than TSH cells of all other experimental groups (d; arrow). Bar = 20 μm (A color version of this figure is available in the online journal)

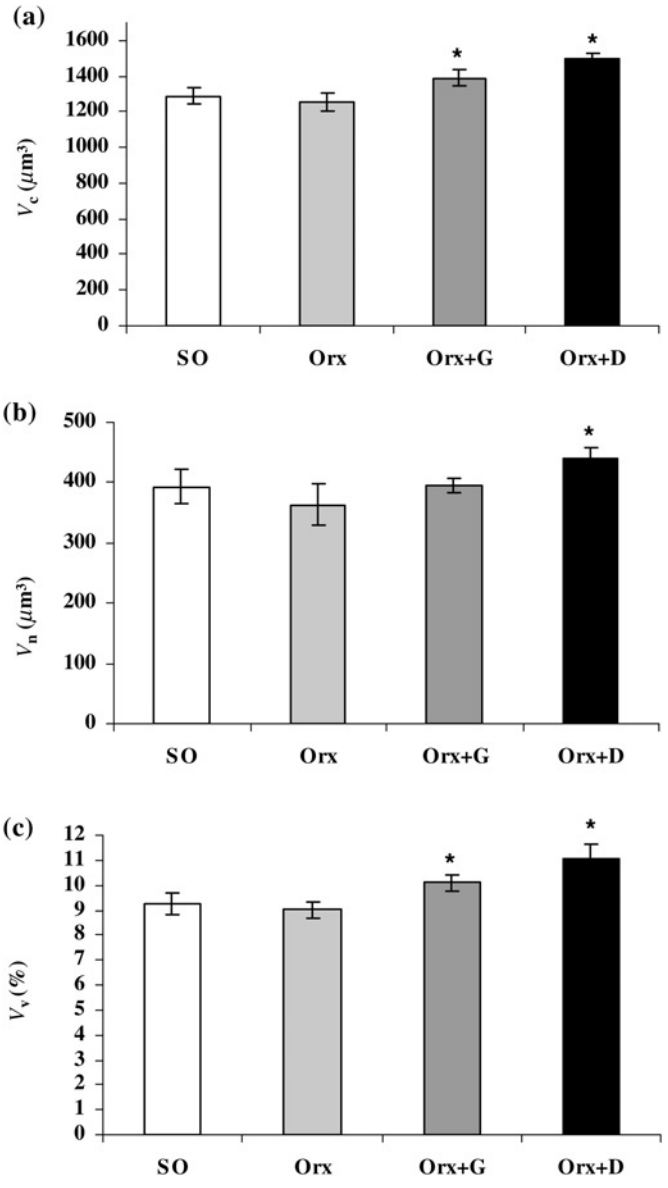

Figure 2 The cellular volume (V_c; a), nuclear volume (V_n; b), as well as the relative volume density (V_V; c) of immunoreactive thyroid-stimulating hormone cells in the pituitaries of sham-operated (SO), orchidectomized (Orx), orchidectomized genistein-treated (Orx + G) and daidzein-treated (Orx + D) middle-aged rats. The values are mean ± standard deviation ($n = 8$); $^*P < 0.05$ versus orchidectomized control (Orx) group

distributed throughout the thyrocytes, or more accumulated at the apical pole of cells (Figure 3a and b). The intensity of Tg immunopositivity varied between the follicles (Figure 3a). In some follicles of smaller size the luminal colloid was also Tg immunopositive (Figure 3a).

Five weeks after Orx, the thyroid structure of middle-aged male rats was not significantly changed in comparison to the SO controls (Figure 3c and d).

The thyroid tissue of G- and D-treated Orx rats was characterized by a microfollicular structure and the depletion of luminal colloid was clearly evident. The follicles were lined by cuboid or highly prismatic Tg-immunopositive follicular epithelium that was visibly enlarged in comparison to the controls (Figure 3e–h). The increased number of interstitial, Tg-negative cells was evident only after daidzein treatment (Figure 3g and h).

Morphometric analyses demonstrated that G administration brought about an increase in the volume densities of the follicular epithelium by 9% ($P < 0.05$; Figure 4a), while the volume density of the colloid was 20% less than in Orx rats ($P < 0.05$; Figure 4a). The epithelial height and index of activation rate increased by 8% and 41% ($P < 0.05$; Figure 4b and c), respectively, in comparison with the Orx rats.

Subsequent subcutaneous administration of daidzein increased V_Ve by 8% ($P < 0.05$; Figure 4a), V_Vi by 9% ($P < 0.05$; Figure 4a), τ by 11% ($P < 0.05$; Figure 4b), i_a by 47% ($P < 0.05$; Figure 4c) and decreased V_{Vc} by 26% ($P < 0.05$; Figure 4a) in comparison to the values obtained for Orx rats, respectively.

Serum TSH, total T_4 and T_3 levels

The mean serum levels of TSH, total T_4 and T_3, are summarized in Figure 5a–c. In comparison with the values obtained for SO controls, Orx did not change significantly the serum levels of examined hormones. However, subsequent treatments with soy isoflavones increased serum levels of TSH – this parameter was 18% and 48% ($P < 0.05$; Figure 5a) higher in the Orx + G and Orx + D groups, respectively, than in the Orx group.

In comparison to the Orx rats, in the Orx + G group the levels of total T_4 and T_3 were 19% and 11% lower, respectively ($P < 0.05$; Figure 5b and c).

Similar to G-treated animals, the level of total T_4 decreased by 16% and T_3 by 11% ($P < 0.05$; Figure 5b and c) in the Orx + D group when compared with Orx control values.

Discussion

This study clearly demonstrates that purified G or D can modulate pituitary–thyroid axis structure and induce primary hypothyroidism in Orx middle-aged rats.

In this experiment, Orx was performed to minimize effects of endogenous sex steroids on pituitary–thyroid axis,[16,33] although this effect was reported to become attenuated with increasing age.[34–36] Isoflavone dosage was selected to mimic human exposure to elevated isoflavone concentrations, during consumption as nutritional supplements.[37] To avoid the additional goitrogenic stimulus, rats were fed a semi-purified, soy-free diet with increased iodine content (concentration was five times higher than the required 150–200 μg of iodine per food kg[38,39]).

Under our experimental conditions, G or D did not affect the body weight. Reports on the effects of isoflavones on body weight of males are contradictory. Decrease,[40,41] increase[42] or, as in this study, no effect[19,43] has been reported, probably due to different treatment protocols and experimental conditions, including variations in diet (soy-free versus soy-containing diet), applied dose and duration of the treatment.

Pituitary TSH-immunoreactive cells of isoflavone-treated rats were larger and more numerous in comparison to the

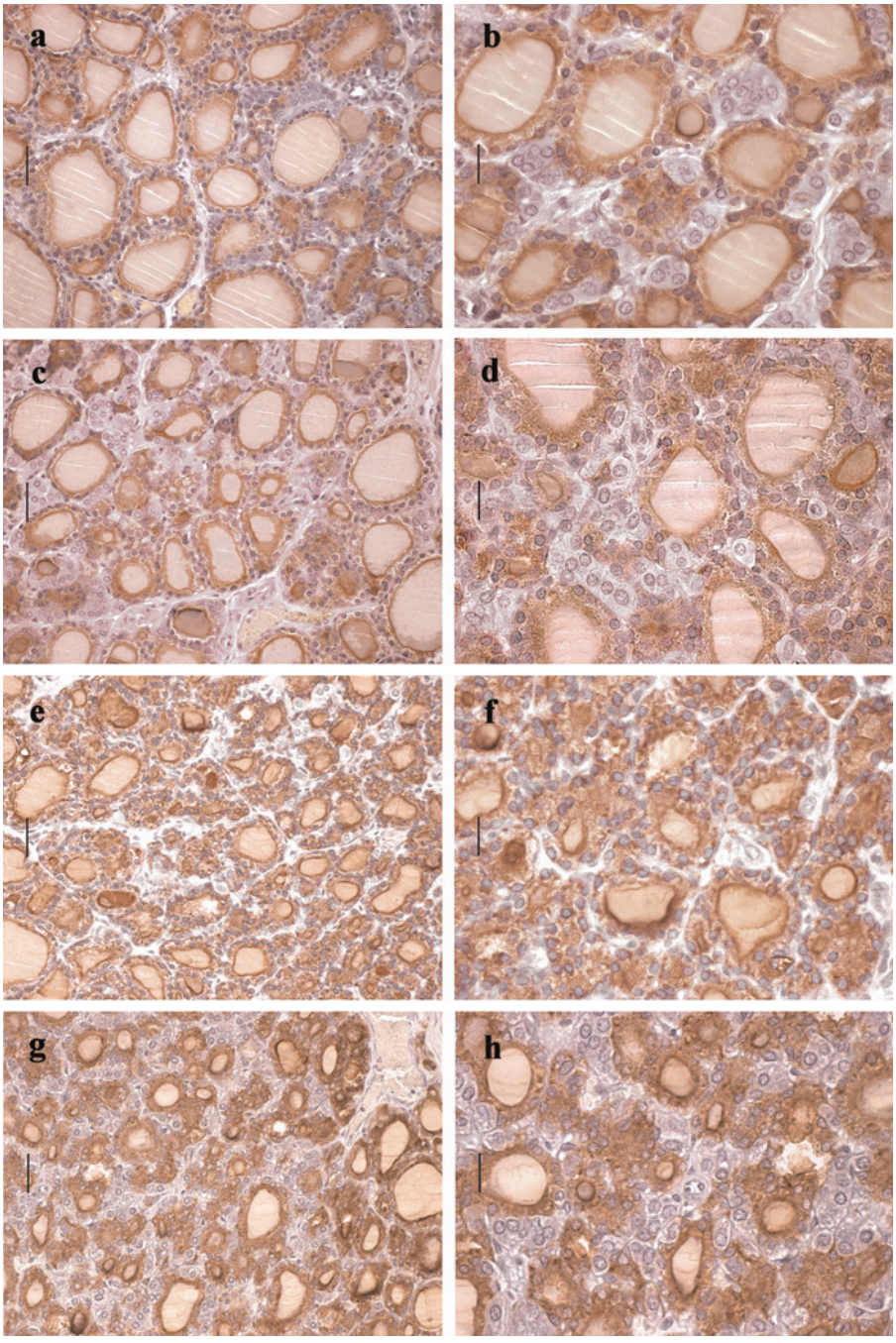

Figure 3 Thyroid gland tissue from the sham-operated (a, bar = 40 μm; b, bar = 20 μm) and orchidectomized (c, bar = 40 μm; d, bar = 20 μm) animals is mainly composed of colloid-rich, uniform follicles, lined by a cuboid or low prismatic, strongly thyroglobulin (Tg)-immunopositive epithelium. Thyroid tissue of the orchidectomized genistein-treated (e, bar = 40 μm; f, bar = 20 μm) and orchidectomized daidzein-treated (g, bar = 40 μm; h, bar = 20 μm) rats is characterized by a small size follicles *poor* in colloid that were lined by cuboid or highly prismatic Tg-immunopositive epithelium (A color version of this figure is available in the online journal)

controls. Their relative cellular volumes, as well as the relative volume density per pituitary unit volume, significantly increased in comparison to the corresponding control values. These changes are probably due to the release from the negative feedback effect of thyroid hormones detected under our experimental conditions. Similar structural changes of pituitary thyrotrophs were described in protracted primary hypothyroidism.[44] In addition, increased numbers of pituitary thyrotrophs were detected in young rats fed soy-rich plus iodine-deficient diet.[19]

G and D treatments did not affect the thyroid weight in comparison to the Orx controls. Further histomorphometric examinations of rat thyroids showed microfollicular structure, characterized by hypertrophied Tg-immunopositive follicular epithelium and colloid-depleted follicles. Experimental stimulation of the thyroid gland by TSH induces similar changes in thyroid structure as detected under our experimental conditions.[45] A direct negative effect of isoflavones on thyroid hormone synthesis, by significant blocking of TPO activity (more than 60%), has been well described.

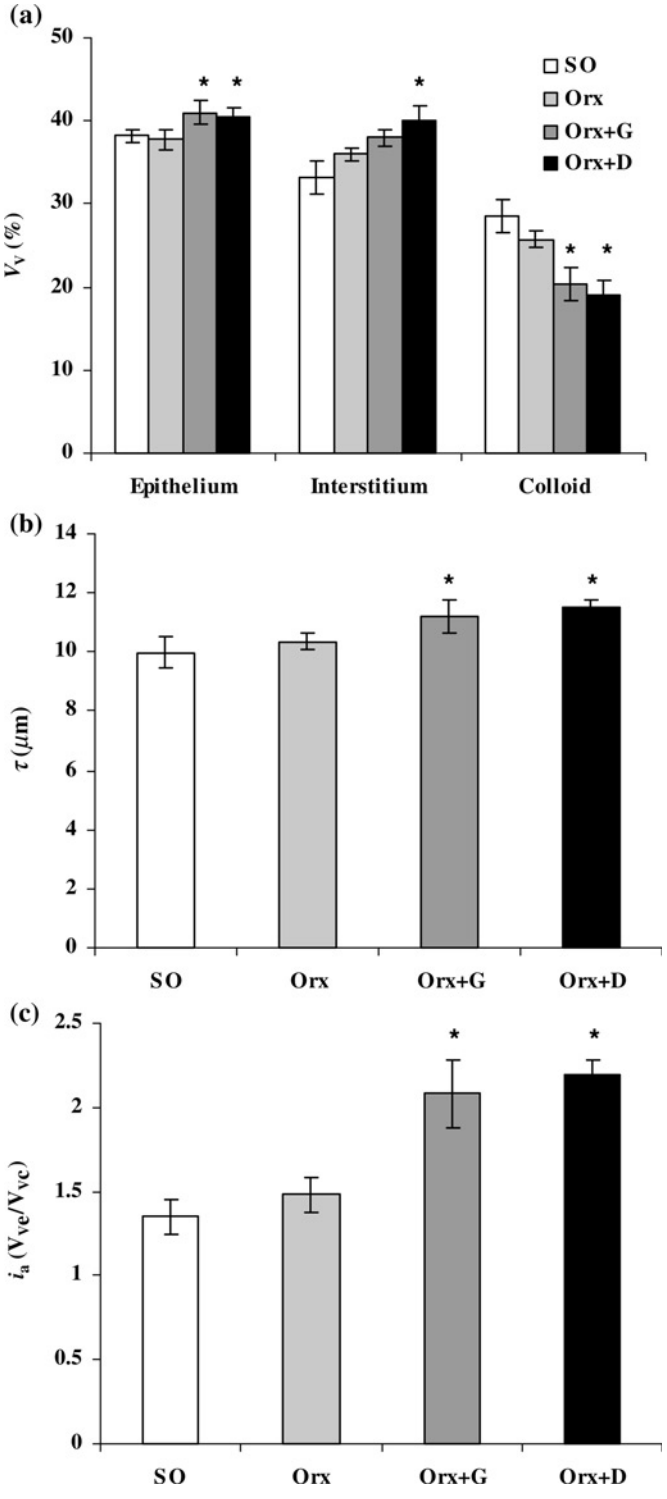

Figure 4 The relative volume densities (V_V; a) of thyroglobulin-immunopositive epithelium, interstitium and colloid, height of thyroid follicular cells (τ; b) and index of activation rate (i_a; c) in the thyroids of sham-operated (SO), orchidectomized (Orx), orchidectomized genistein-treated (Orx + G) and daidzein-treated (Orx + D) middle-aged rats. The values are mean ± standard deviation ($n = 8$); $^*P < 0.05$ versus orchidectomized control (Orx) group

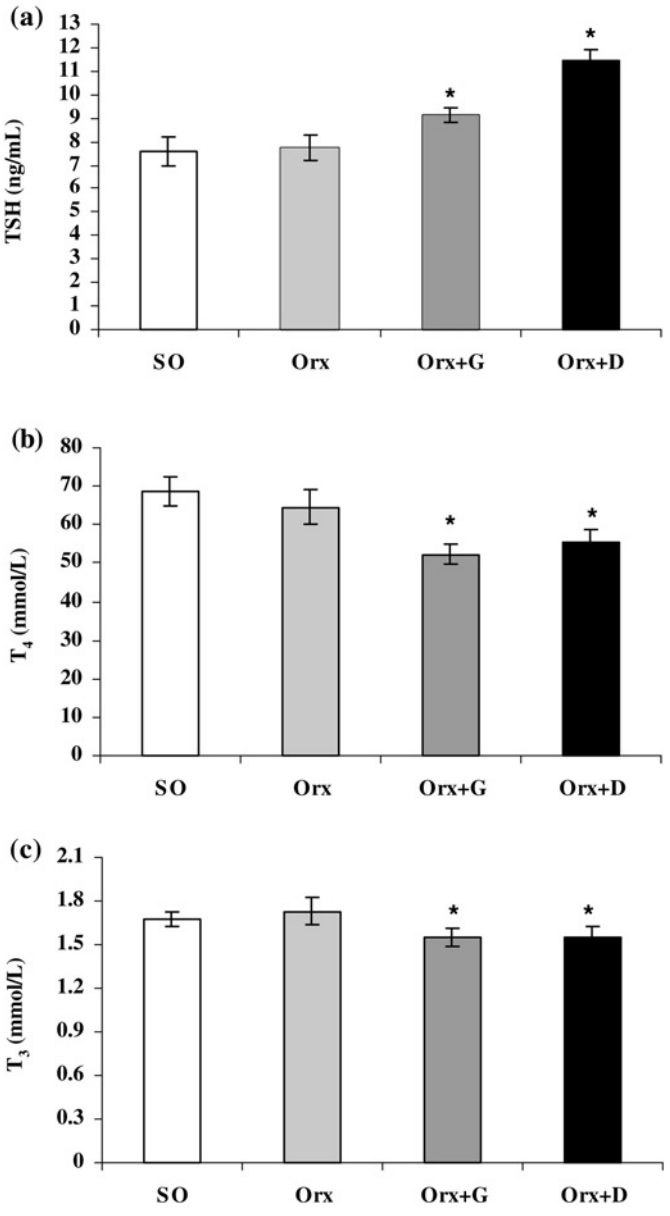

Figure 5 Serum levels of thyroid-stimulating hormone (TSH) (a), total T_4 (b) and T_3 (c) in sham-operated (SO), orchidectomized (Orx), orchidectomized genistein-treated (Orx + G) and daidzein-treated (Orx + D) middle-aged rats. The values are mean ± standard deviation ($n = 8$); $^*P < 0.05$ versus orchidectomized control (Orx) group

G and D were demonstrated to block both TPO-catalyzed reactions: iodination of thyrosine residues of Tg, and T_4 formation by coupling reactions (the half maximum inhibitor concentration (IC_{50}) values were calculated as 1–10 μmol), but this effect was eliminated by iodine.[22,23]

Serum levels of TSH increased, while T_4 and T_3 levels decreased similarly in Orx + G and Orx + D experimental groups in comparison to the controls. It is well accepted that the receptor-mediated TSH stimulation of the thyroid gland related to goitrogenic chemicals leads to morphological changes of follicular cells that include hypertrophy, hyperplasia and eventually, in the later phases, neoplasia.[16] Altogether, our results indicate that purified G and D acted as goitrogenic factors and induced thyroid follicular cells' hypertrophy, stimulated by increasing serum TSH, following a reduction in thyroid hormone synthesis.

Consistent with our findings, Mitsuma *et al.*[46] reported reduced levels of thyroid hormones in aged rats fed 40 volume percent soy protein diets. However, most authors,

who performed their examinations on young adult animals of both sexes, reported that soy or isoflavones alone, in the absence of other goitrogenic stimulus, did not affect thyroid weights, histopathology and the serum levels of TSH and thyroid hormones.[19,23,47] After exposing young adult rats to 20% defatted soybean diet, only a mild irregularity of thyroid follicular structure, accompanied by increased serum levels of TSH and unaffected levels of thyroid hormones were detected.[18]

This study design minimizes the presence of food goitrogen or iodine deficiency contributing to the detected changes. Therefore, our results highlight the increased vulnerability of pituitary–thyroid axis for adverse effects of soy isoflavones with advancing age.

An important finding of this study is that subcutaneous administration of the same dose of daidzein stimulated TSH cells more effectively than G, although both G and D similarly affected thyroid follicular tissue and decreased the levels of thyroid hormones to a similar extent.

The only significant histomorphometric difference between thyroids of G- and D-treated rats is the increase in the relative volume density of the interstitium, detected after daidzein treatment. Under our experimental conditions, increased volume of the calcitonin-immunoreactive thyroid C cells was detected after daidzein,[48] but not after G treatment (unpublished data). In addition, ipriflavone, a synthetic isoflavone that is metabolized into daidzein, was reported to stimulate calcitonin synthesis and secretion from the thyroid glands, as detected by immunohistochemistry and Northern blot analysis.[49]

On the other hand, TSH cells are estrogen responsive – ERα has been detected by *in vitro* autoradiography[50] as well as by immunohistochemistry.[51] The latter study also identified no immunoreactivity for ERβ in the TSH cells. Therefore, the difference in response of TSH cells to G and D could be due to the higher estrogenic potency of G in comparison to daidzein (half maximal effective concentration (EC_{50}) for ERα was 15 μmol for G and >300 μmol for daidzein[6]). We have previously reported that pharmacological doses of estradiol decreased the number of TSH-immunopositive cells in female rats.[52,53] Other researchers have reported that higher doses of E2 inhibited TSHβ mRNA levels.[54,55]

An additional mechanism that might contribute to the detected difference in the effect of G and D at the level of pituitary thyrotrophs is the difference in their affinity for binding to transthyretin, a major blood transport protein of thyroid hormones in rats. Radović *et al.*[56] demonstrated G to be a much more potent competitor of the *in vitro* binding of T_4 to transthyretin. The biological significance of this mechanism is still unclear, though it might affect the free thyroid hormone concentrations, resulting in altered tissue availability and disturbed feedback to the pituitary.[57]

In conclusion, this is the first report demonstrating that soy isoflavones disrupt pituitary–thyroid axis function in aging male rats. Both G and D similarly affected thyroid follicular structure and were equipotent in reducing the level of total thyroid hormones. This reduction consequently led to a feedback stimulation of pituitary TSH cells. However, daidzein was found to be more potent in this stimulatory effect, supporting the opinion that another mechanism(s), besides the direct inhibition of thyroid follicles, may contribute to this action.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. BŠ-J, BF and VA conducted the experiments; BŠ-J contributed to study design, data collection, statistical analyses, data interpretation, manuscript preparation and literature search; BF contributed to study design, data collection and literature search; VA contributed to study design, data collection and literature search; SS contributed to blood hormonal data collection and literature search; VM contributed to study design, literature search and fund collection; and MS contributed to study design, data interpretation, manuscript preparation, literature search and fund collection.

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REFERENCES

1 Setchell KD. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am J Clin Nutr* 1998;**68**(Suppl.):1333S–46S

2 Tham DM, Gardner CD, Haskell WL. Potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab* 1998;**83**:2223–35

3 Šošić-Jurjević B, Filipović B, Ajdzanović V, Brkić D, Ristić N, Stojanoski MM, Nestorović N, Trifunović S, Sekulić M. Subcutaneously administrated genistein and daidzein decrease serum cholesterol and increase triglyceride levels in male middle-aged rats. *Exp Biol Med (Maywood)* 2007;**232**:1222–7

4 Miksicek RJ. Estrogenic flavonoids:structural requirements for biologic activity. *Proc Soc Exp Biol Med* 1995;**208**:44–50

5 Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* 1998;**139**:4252–63

6 Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors α and β to the estrogen response element. *J Agric Food Chem* 2003;**51**:7632–5

7 Ramos S. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *J Nutr Biochem* 2007;**18**:427–42

8 Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. *Arch Biochem Biophys* 1998;**360**:142–8

9 Cizza G, Brady LS, Calogero AE, Bagdy G, Lynn AB, Kling MA, Blackman MR, Chrousos GP, Gold PW. Central hypothyroidism is associated with advanced age in male Fischer 344/N rats: *in vivo* and *in vitro* studies. *Endocrinology* 1992;**131**:2672–80

10 Donda A, Lemarchand-Béraud T. Aging alters the activity of 5′-deiodinase in the adenohypophysis, thyroid gland, and liver of the male rat. *Endocrinology* 1989;**124**:1305–9

11 Reymond F, Dénéréaz N, Lemarchand-Béraud T. Thyrotropin action is impaired in the thyroid gland of old rats. *Acta Endocrinol (Copenh)* 1992;**126**:55–63

12 Robuschi G, Safran M, Braverman LE, Gnudi A, Roti E. Hypothyroidism in the elderly. *Endocr Rev* 1987;**8**:142–53

13 Eisenbrand G, Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG). Isoflavones as phytoestrogens in food supplements and dietary foods for special medical purposes. Opinion of the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG)-(shortened version). *Mol Nutr Food Res* 2007;**51**:1305–12

14 National Toxicology Program. NTP toxicology and carcinogenesis studies of genistein (CAS No. 446-72-0) in Sprague–Dawley rats (feed study). *Natl Toxicol Program Tech Rep Ser* 2008;**545**:1–240

15 Choksi NY, Jahnke GD, St Hilaire C, Shelby M. Role of thyroid hormones in human and laboratory animal reproductive health. *Birth Defects Res B* 2003;**68**:479–91

16 Capen C. Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicol Pathol* 1997;**25**:39–48

17 Kimura S, Suwa J, Ito M, Sato H. Development of malignant goiter by defatted soybean with iodine-free diet in rats. *Gann* 1976;**67**:763–5

18 Ikeda T, Nishikawa A, Imazawa T, Kimura S, Hirose M. Dramatic synergism between excess soybean intake and iodine deficiency on the development of rat thyroid hyperplasia. *Carcinogenesis* 2000;**21**:707–13

19 Kajiya H, Takekoshi S, Miyai S, Ikeda T, Kimura S, Osamura RY. Dietary soybean enhances Pit-1 dependent pituitary hormone production in iodine deficient rats. *J Mol Histol* 2005;**36**:265–74

20 Van Wyk JJ, Arnold MB, Wynn J, Pepper F. The effects of a soybean product on thyroid function in humans. *Pediatrics* 1959;**24**:752–60

21 Chorazy PA, Himelhoch S, Hopwood NJ, Greger NG, Postellon DC. Persistent hypothyroidism in an infant receiving a soy formula: case report and review of the literature. *Pediatrics* 1995;**96(Pt 1)**:148–50

22 Divi RL, Chang HC, Doerge DR. Anti-thyroid isoflavones from soybean: isolation, characterization, and mechanisms of action. *Biochem Pharmacol* 1997;**54**:1087–96

23 Chang HC, Doerge DR. Dietary genistein inactivates rat thyroid peroxidase *in vivo* without an apparent hypothyroid effect. *Toxicol Appl Pharmacol* 2000;**168**:244–52

24 Doerge DR, Chang HC. Inactivation of thyroid peroxidase by soy isoflavones, *in vitro* and *in vivo*. *J Chromatogr B* 2002a;**777**:269–79

25 Picherit C, Coxam V, Bennetau-Pelissero C, Kati-Coulibaly S, Davicco MJ, Lebecque P, Barlet JP. Daidzein is more efficient than genistein in preventing ovariectomy-induced bone loss in rats. *J Nutr* 2000;**130**:1675–81

26 Jefferson WN, Padilla-Banks E, Newbold RR. Disruption of the female reproductive system by the phytoestrogen genistein. *Reprod Toxicol* 2006;**23**:308–16

27 Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG. The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen–antibody complex (horseradish peroxidase–antihorseradish peroxidase) and its use in identification of spirochetes. *J Histochem Cytochem* 1970;**18**:315–33

28 Gérard AC, Denef JF, Colin IM, van den Hove MF. Evidence for processing of compact insoluble thyroglobulin globules in relation with follicular cell functional activity in the human and the mouse thyroid. *Eur J Endocrinol* 2004;**150**:73–80

29 Weibel ER. *Stereological Methods, Vol. 1, Practical Methods for Biological Morphometry*. London: Academic Press, 1979

30 Weibel ER, Gomez DM. A principle for counting tissue structures on random sections. *J Appl Physiol* 1962;**17**:343–8

31 Bogataj M, Kališnik M, Poredoš P. A simplified indirect method of the average shell thickness of the hollow spheres from samples insections. *Folia Anat Yugosl* 1977;**6**:45–9

32 Kališnik M, Vraspir-Porenta O, Kham-Lindtner T, Logonder-Mlinsek M, Pajer Z, Stiblar-Martincic D, Zorc-Pleskovic R, Trobina M. The interdependence of the follicular, parafollicular, and mast cells in the mammalian thyroid gland: a review and a synthesis. *Am J Anat* 1988;**183**:148–57

33 Gray LE Jr, Wilson V, Noriega N, Lambright C, Furr J, Stoker TE, Laws SC, Goldman J, Cooper RL, Foster PM. Use of the laboratory rat as a model in endocrine disruptor screening and testing. *ILAR J* 2004;**45**:425–37

34 Chen HJ. Age and sex difference in serum and pituitary thyrotropin concentrations in the rat: influence by pituitary adenoma. *Exp Gerontol* 1984;**19**:1–6

35 Bagchi N, Shivers B, Brown TR. Effects of castration and sex steroids on the thyroid response to thyrotropin. *Endocrinology* 1984;**114**:1652–6

36 Borges PP, Curty FH, Pazos-Moura CC, Moura EG. Effect of testosterone propionate treatment on thyrotropin secretion of young and old rats *in vitro*. *Life Sci* 1998;**62**:2035–43

37 Doerge DR, Sheehan D. Goitrogenic and estrogenic activity of soy isoflavones. *Environ Health Perspect* 2002b;**110**:349–53

38 Parker HE, Andrews FN, Hauge SM, Quackenbush FW. Studies on the iodine requirements of white rats during growth, pregnancy and lactation. *J Nutr* 1951;**44**:501–11

39 Penel C, Rognoni JB, Bastiani P. Thyroid autoregulation: impact on thyroid structure and function in rats. *Am J Physiol* 1987;**253(Pt 1)**:E165–172

40 Schleicher RL, Lamartiniere CA, Zheng M, Zhang M. The inhibitory effect of genistein on the growth and metastasis of a transplantable rat accessory sex gland carcinoma. *Cancer Lett* 1999;**136**:195–201

41 Michael McClain R, Wolz E, Davidovich A, Pfannkuch F, Edwards JA, Bausch J. Acute, subchronic and chronic safety studies with genistein in rats. *Food Chem Toxicol J* 2006;**44**:56–80

42 Penza M, Montani C, Romani A, Vignolini P, Pampaloni B, Tanini A, Brandi ML, Alonso-Magdalena P, Nadal A, Ottobrini L, Parolini O, Bignotti E, Calza S, Maggi A, Grigolato PG, Di Lorenzo D. Genistein affects adipose tissue deposition in a dose-dependent and gender-specific manner. *Endocrinology* 2006;**147**:5740–51

43 Faqi AS, Johnson WD, Morrissey RL, McCormick DL. Reproductive toxicity assessment of chronic dietary exposure to soy isoflavones in male rats. *Reprod Toxicol* 2004;**18**:605–11

44 Alkhani AM, Cusimano M, Kovacs K, Bilbao JM, Horvath E, Singer W. Cytology of pituitary thyrotroph hyperplasia in protracted primary hypothyroidism. *Pituitary* 1999;**1**:291–5

45 Gerber H, Studer H, Conti A, Engler H, Kohler H, Haeberli A. Reaccumulation of thyroglobulin and colloid in rat and mouse thyroid follicles during intense thyrotropin stimulation. A clue to the pathogenesis of colloid goiters. *J Clin Invest* 1981;**68**:1338–47

46 Mitsuma T, Ito Y, Hirooka Y, Kayama M, Iyumi M, Hasegawa M, Shin K, Mori Y, Adachi K, Sato T, Tauchi H. The effects of soybean diet on thyroid hormone and thyrotropin levels in aging rats. *Endocr Regul* 1998;**32**:183–6

47 Schmutzler C, Hamann I, Hofmann PJ, Kovacs G, Stemmler L, Mentrup B, Schomburg L, Ambrugger P, Grüters A, Seidlova-Wuttke D, Jarry H, Wuttke W, Köhrle J. Endocrine active compounds affect thyrotropin and thyroid hormone levels in serum as well as endpoints of thyroid hormone action in liver, heart and kidney. *Toxicology* 2004;**205**:95–102

48 Filipović B, Šošić-Jurjević B, Ajdžanović V, Trifunović S, Brkić D, Sekulić M. Influence of chronic daidzein treatment on thyroid C cells and trabecular bone structure in orhidectomized male rats. *50th Symposium of the Society for Histochemistry*, Interlaken, Switzerland, –4 October 2008, Poster Session abstracts, 18pp

49 Watanabe K, Takekoshi S, Kakudo K. Effects of ipriflavone on calcitonin synthesis in C cells of the rat thyroid. *Calcif Tissue Int* 1992;**51**(Suppl. 1):S27–9

50 Keefer DA, Stumpf WE, Petrusz P. Quantitative autoradiographic assessment of ^{3}H-estradiol uptake in immunocytochemically characterized pituitary cells. *Cell Tissue Res* 1976;**166**:25–35

51 González M, Reyes R, Damas C, Alonso R, Bello AR. Oestrogen receptor alpha and beta in female rat pituitary cells: an immunochemical study. *Gen Comp Endocrinol* 2008;**155**:857–68

52 Sekulić M, Lovren M, Milosević V. Immunoreactive TSH cells in the pituitary of female middle-aged rats after treatment with estradiol or calcium. *Acta Histochem* 1998;**100**:185–91

53 Šošić-Jurjević B, Filipović B, Milosević V, Nestorović N, Negić N, Sekulić M. Effects of ovariectomy and chronic estradiol administration on pituitary–thyroid axis in adult rats. *Life Sci* 2006;**79**:890–7

54 Franklyn JA, Wood DF, Balfour NJ, Ramsden DB, Docherty K, Sheppard MC. Modulation by oestrogen of thyroid hormone effects on thyrotrophin gene expression. *J Endocrinol* 1987;**115**:53–9

55 Böttner M, Christoffel J, Rimoldi G, Wuttke W. Effects of long-term treatment with resveratrol and subcutaneous and oral estradiol administration on the pituitary–thyroid-axis. *Exp Clin Endocrinol Diabetes* 2006;**114**:82–90

56 Radović B, Mentrup B, Köhrle J. Genistein and other soya isoflavones are potent ligands for transthyretin in serum and cerebrospinal fluid. *Br J Nutr* 2006;**95**:1171–6

57 Schmutzler C, Gotthardt I, Hofmann PJ, Radovic B, Kovacs G, Stemmler L, Nobis I, Bacinski A, Mentrup B, Ambrugger P, Grüters A, Malendowicz LK, Christoffel J, Jarry H, Seidlovà-Wuttke D, Wuttke W, Köhrle J. Endocrine disruptors and the thyroid gland – a combined *in vitro* and *in vivo* analysis of potential new biomarkers. *Environ Health Perspect* 2007;**115**(Suppl. 1):77–83

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