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Soybean Phytoestrogens – Friends or Foes?

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1. Introduction

Proper and balanced nutrition is very important in prevention and treatment of chronic diseases. Many individuals modify their diet and/or take different nutraceuticals expecting to attain optimum health, extend their lifespan and prevent diseases such as cardiovascular, cancer, osteoporosis, obesity, or diabetes type II.

Based on „Japanese phenomenon“ (Adlercreutz, 1998), numerous advertisements suggest that soy-based diet, and its phytoestrogens (PE) in particular, provide protection against many chronic diseases and contribute to the long lifespan often observed in Asia. That is why soy and other phytoestrogen - rich plants became increasingly popular in the U.S. and western countries in the past 30 years. Furthermore, in these countries, PEs are often consumed in its purified form, as nutritional supplements, “designed” for special medical purposes. These supplements are freely available in pharmacies, health food shops, grocery shops and are usually consumed without medical control. There is a lack of awareness that uncontrolled consumption of natural PEs may be potentially harmful to human health. Even more concerning is that some people consume supplements in excess of suggested daily dosage (Wuttke et al., 2007).

The soybean (*Glycine max*), compared to other legumes, is richer in protein levels and quality, based on its digestibility and concentration of essential amino acids (Rand et al., 2003). It is also good source of fiber, certain vitamins and minerals, such as folate and potassium (Rochfort and Panozzo, 2007). It has very high antioxidant content, similar to fruits famous for their antioxidant activity (Galleano et al., 2010). Also, despite their high carbohydrate content, the glycemic load of soybeans is relatively low due to their low glycemic index. In addition, soy-food has high levels of iron in the form of ferritin (Lönnerdal et al., 2006). The concentration of calcium in soymilk is much lower than in cow milk, however, its absorption from soy milk is similar to that from cow milk (Reinwald and Weaver, 2010).

Besides the favorable nutritional attributes, soybean contains a number of biologically active components (saponins and lunasin, phytic acids, phytosterols, trypsin inhibitors, and peptides) including isoflavones genistein (G), daidzein (D) and glycitein (Gy). As soybean phytoestrogens, isoflavones are considered the most important in prevention and treatment of hormone-dependent cancers, cardiovascular diseases, osteoporosis, menopausal symptoms and other age-related diseases. In addition, some studies suggest that soy and its isoflavones affect body weight homeostasis.

Modern world is a controversy with ever-increasing obesity on one side, and a high percent of starving people around the globe, on the other side. Having that in mind, combined with

observed beneficial health and weight-lowering effects, high nutritional value makes soy probably one of the most strategically important plants.

However, aside from potential beneficial effects (still under intensive investigation and not fully proven), soybean phytoestrogens may also act as endocrine disruptors, by interfering with the function of reproductive system, as well as with other endocrine systems, namely thyroid and adrenal, and may, under some circumstances, increase cancer risk. This is why scientists are intensively trying to precisely evaluate potential benefits versus adverse effects of soy. Due to the importance, the researches are done both *in vitro* and *in vivo*, using different experimental approaches, animal models and various human studies. Results obtained so far are highly inconsistent and depend on experimental conditions, applied doses, animals and humans' age and sex, type of diet, presence of other PE sources in the diet, or other factors. Moreover, it remains unclear whether soy extracts, soy concentrate and purified isoflavones have identical effects. This is why the role of soy food in diet became a somewhat confusing topic in recent years. With approximately 2000 soy-related papers published annually, and half of it related to isoflavones (Messina, 2010), it is becoming extremely difficult to compare all of the available data.

Due to the many differences in the chemical composition of soy products, and the fact that two thirds of human population cannot produce equol (Setchell et al., 2002), the authors decided to primarily focus their attention on effects of purified genistein and daidzein. We will evaluate the latest findings, using clear statements from the literature, as well as our own results, focusing on major potential healthful effects while also considering adverse effects of purified soybean phytoestrogens. More important, the authors will try to analyze the data in order to evaluate whether the net beneficial /adverse effect for each targeted organ system depends on sex and age.

2. Structure, absorption and bioavailability of soybean phytoestrogens

Soybeans and its products are the most abundant source of isoflavones in the human diet. Isoflavones are normally taken up with food, absorbed in the gastrointestinal tract, and eliminated via urine. The absorption and bioavailability of isoflavones has been the subject of frequent debates among scientists. One of the main factor influencing the absorption and bioavailability is the chemical structure of the compound (D'Archivio et al., 2010). Structurally, soy isoflavones G, D and Gy are diphenolic compounds, which are present in soy and non-fermented soyfood isoflavones in its glycosylated forms, as glycones genistin, daidzein and glycitin (Setchell, 1999; Fig. 1).

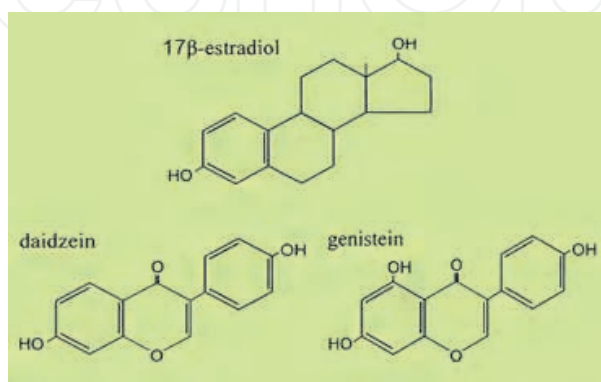


Fig. 1. Structure of soybean phytoestrogens and mammalian 17β - estradiol.

As a prerequisite for absorption, the sugar must be removed from the compound at some point during ingestion (Setchell et al., 2002). Soy isoflavone glycosides are hydrolyzed to their aglycones by lactase phloridizin hydrolase in the apical membrane of the lumen of the small intestine, as well as by bacterial intestinal glucosidases (Wilkinson et al., 2003). Aglycones undergo passive diffusion across the small and large intestinal brush border (Larkin et al., 2008). However, some authors claim that glycosides may be absorbed also through the active sodium-dependent glucose transporter (Gee et al., 2000). Results obtained when we examined effects of soy extract on fluidity of erythrocyte membrane, showed that genistein and isoflavone glucosides intercalate and increase the order and rigidity of the outer layer of cellular membrane. Therefore, isoflavone glucosides may be also transported across the cell membrane directly, via entropy-driven flip-flop (Ajdžanović et al., 2010, 2011). Biological significance of this mechanism is unclear.

The absorption and bioavailability of isoflavones depends to some extent on interaction with other food components (Birt et al., 2001). The assumption that isoflavones are absorbed more efficiently from fermented than from non-fermented soy foods was re-examined and then rejected (Maskarinec et al., 2008). Since intestinal microflora is capable of hydrolyzing the isoflavone glycosides from nonfermented soyfood, recommendations favoring fermented soyfood cannot be justified.

Genistein is stronger than daidzein in its agonistic activity for the ERs, as well as in its antioxidative potential. On the other hand, daidzein can be further metabolized into its bacterial metabolite equol, which has stronger estrogenic and antioxidative properties than both genistein and daidzein, or some other isoflavone metabolites (Mitchell et al., 1998). Although it appears that all animals produce equol following soy ingestion, in humans this is the case in approximately 30% of population (Lampe et al., 1998; Setchell et al., 2002). This is thought to be dependent on inter-individual variability in the presence of specific intestinal bacteria (Rowland et al., 2000). Besides the microflora composition, individual differences in gut transit time and redox potential of colon and genetic polymorphisms are likely to contribute to this great variability (Duffy et al., 2007). When evaluating the effects of age on equol production, it was demonstrated that during the first months of life, equol levels in plasma and urine were significantly lower than in adults, which may be due to the immature intestinal flora (Setchell et al. 2002). Lampe et al. (1998) detected no significant differences in the prevalence of equol production between genders.

3. Biological basis of soybean phytoestrogen actions

The estrogenic effect of the isoflavones was first recognized when examining impaired fertility in grazing animals (Bennetts et al., 1946). Three decades later, Setchell et al. (1987) established that isoflavone-rich soy was a factor in reduced fertility of cheetahs in North American zoos. Isoflavones were classified as phytoestrogens following *in vivo* and *in vitro* demonstration of their binding potency of isoflavones for estrogen receptors (ER), as well as for sex-hormone binding globuline (Kuiper et al., 1998).

Testosterone actions in numerous male tissues are mediated through its conversion to estrogen catalyzed by aromatase enzymes. Specific α and β ER are detected in different male and female tissues (Korach, 1994), but the ratio between ER α and ER β is different (Rosen, 2005). This finding has finally changed the classical view of the estrogens as exclusively female hormones. ER β is known to modulate ER α transcriptional activity acting as an activator at low concentrations of mammalian estrogen - estradiol 17 β (E2) and as an

inhibitor at high concentrations of E2. E2 has equal binding affinities for ER α and ER β , while isoflavones have a higher potency for ER β .

The molecular structures of genistein, daidzein and E2 are similar in many aspects. The intra-molecular distance between the hydroxyl groups at each end of the molecules is almost identical for both isoflavones and E2. These distances determine hydrogen bond interaction with amino acids of the ligand-binding site of the ER (Vaya and Tamir, 2004). Though molecular binding for ER between isoflavones and E2 are similar, both G and D binding potency for ERs is significantly lesser in comparison to E2. In addition, they bind with higher potency to estrogen receptor (ER) β in comparison to ER α (Kuiper et al., 1998). These features classify them as potential natural selective estrogen receptor modulators (Phyto SERMs). Thus, soybean isoflavones may exert estrogenic, antiestrogenic, or estrogen non-reactive biological actions, depending on their concentration and concentration of endogenous estrogen, tissue, and amount and type of estrogen receptors present in the tissue (Wuttke et al. 2007). Therefore, it is of importance to determine the estrogenic action of isoflavones compared with the effects of E2 (in females) and both testosterone and estradiol (in males) in each individual organ.

Phyto SERMs represent a new and very promising class of potential hormonal therapy agents. Major potential advantage of SERMs over estrogen analogue therapy is that it may demonstrate all of the favorable effects of estrogens. However, in order to declare isoflavones as safe, it needs to be demonstrated that they do not share the risks associated with estrogens used in hormone replacement therapy, osteoporosis treatment or in treatment of prostate carcinoma (Wuttke et al., 2007).

Besides their estrogenic activities, isoflavones also exhibit non-hormonal actions such as antioxidant effects. Antioxidant properties are one of the most important claims for food ingredients, dietary supplements and anticancer products. In addition, the free radical theory of aging continues to be among the most popular theories. Therefore, the antioxidant property of isoflavones offers an additional important mechanism through which they protect against age-related diseases. All soy isoflavones act as antioxidants, playing role in scavenging free radicals that can cause DNA damage and lipid peroxidation (Kruk et al., 2005) and activate antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and glutathione reductase (Mitchell et al. 1998). The determining factors for isoflavone antioxidant activities are the absence of the 2, 3-double bond and the 4-oxo-group on the isoflavone nucleus and the position of the hydroxyl groups, with hydroxyl substitution being of utmost importance at the 4' position, of moderate importance at the 5 position, and of little significance at the 7 position. That is why G has higher antioxidant capacity than daidzein and the reason why both have stronger antioxidant activity than their glycosides (Cherdshewasart and Sutjit, 2008).

Genistein in high concentration is a potent inhibitor of Tyr kinases (Akiyama et al., 1987), DNA topoisomerases I and II, and ribosomal S6 kinase, resulting in inhibition of cell growth. Tyrosine kinases are responsible for <1% of protein phosphorylation within cells, but they appear to phosphorylate many proteins required for regulation of cell functions. Genistein has been shown to induce cell cycle arrest and apoptosis in numerous cell lines, including ER (+) and ER (-). Many of the reported beneficial effects of isoflavones and particularly those on tumor growth may be attributed to this mechanism. However, some authors criticize this by underlying that the concentrations necessary for such inhibition in the tested cell systems or organs by far exceed the serum concentrations achieved by isoflavone ingestion alone (Jiménez and Montiel 2005; Wuttke et al., 2007).

Growing evidence shows that isoflavones may also modulate the activity/expression of steroidogenic enzymes. These enzymes are present in the adrenal glands and gonads but also in many tissues that have the ability to convert circulating precursors into active hormones (i.e. brain, liver, reproductive tracts, adipose tissue, skin and breast tissue). Genistein and daidzein were reported to inhibit the activity of 3β -hydroxysteroid dehydrogenases (HSD) purified from bovine adrenal microsomes (Wong & Keung, 1999). The same isoflavones were also shown to inhibit 3β -HSD type II in mitochondrial and microsomal preparations of the human adrenocortical H295R cell line, and subsequently a similar inhibition of the conversion of dehydroepiandrosterone (DHEA) to androstenedione by these isoflavones was observed in total membrane fractions of Sf9 insect cells in which human 3β -HSD had been over-expressed (Ohno et al., 2002). However, Mesiano et al. (1999) showed that genistein and daidzein specifically inhibited the activity of 21-hydroxylase (P450c21/CYP 21) in H295 cells but had no effect on other steroidogenic enzymes, including 3β -HSD.

4. Soybean phytoestrogens in prevention and therapy of cancer

The incidence of hormon-dependent cancers, namely breast and prostate, is lower in Asia than in western countries (Messina et al., 2006; Parkin, 2005). Migrants from Asia, who maintained their traditional diet, even when living in the West, had a lower risk of these diseases. However, shifting towards a more of a western diet increased the risk (Ziegler et al., 1993). Once SERM properties of soybean isoflavones were discovered, it was hypothesized that high soy dietary intake might be associated with low incidence of hormone-dependent cancers in Asian population, as well as with other putative health benefits (Setchell, 1999). That is why soyfood and its isoflavones in a form of dietary supplements or concentrated extracts have been increasingly used in the western populations in the recent years.

However, when Patisaul and Jefferson (2010) discussed potential safety of infant soy formula, they stressed the essential difference between Asians (on a traditional „soy-reach“ diet) and Caucasians (on a traditional „Western“ diet) in exposure to soy over the lifespan. In Asia, soy consumption is high during entire lifespan, except for a brief breast-feeding period in early infancy. People in the West feed their babies soy infant formula, so the pattern is just the opposite - the highest intake of isoflavones occurs in the first year of life and then drop to near zero, with eventual increase later in advanced adult age. In relation to this, some authors support the opinion that lower incidence of breast cancer in Asian women is due to their continuous exposure to soy from early life throughout their whole lifespan (Warri et al., 2008). Maskarinec et al. (2004) concluded that Caucasian women who ate more soy during their lifespan had denser breast tissue (a risk factor for breast cancer) than those who did not.

4.1 Effects on breast cancer

Overexposure to estrogen (early menarche, short duration of breastfeeding and low parity) is a major contributing factor in the development of breast cancer. As soybean isoflavones have a relatively high binding potency for ERs, a concern has been raised that high phytoestrogen intake may promote growth of estrogen-sensitive tumors or put breast cancer survivors at risk of reoccurrence (Helferich et al., 2008; Messina & Loprinzi, 2001).

The data about the role of isoflavones in prevention and therapy of breast cancer are controversial. Some authors proposed that genistein at low, physiologically relevant level, may stimulate ER-positive tumors due to their estrogenic properties, while at higher level, anti-cancer actions of isoflavones may be predominant (Duffy et al., 2007). Shu et al. (2009) also suggested dose-dependent effects of ingested soybean isoflavones: intake of low doses was associated with increased mortality rate and breast cancer recurrence, while intake of more than 40 mg per day appeared to have antiproliferative effects. These results were evident in women with both ER-positive and ER-negative breast cancer. The authors suggested that soy isoflavones protect against breast cancer by competing with estrogens in binding to the estrogen receptor. At the same time soy isoflavones increase the synthesis of sex hormone-binding globulin, lowering the biological availability of sex hormone, inhibit 17β -hydroxysteroid dehydrogenases (thus reducing estrogen synthesis), and increase clearance of steroids from the circulation (Taylor et al., 2009). However, Harris et al. (2004) showed that isoflavones inhibit sulfotransferase (enzymes that catalize estrogen inactivation in mammary gland) ten times more than sulfatase enzymes, which catalize local estrogen production. This may lead to increase in free estrogen levels in the tumor tissue, which in turn may stimulate tumor growth.

Recent epidemiological and clinical data were summarized in review article of Messina & Wood (2008) and the authors concluded that isoflavone intake have either a modest protective role or no effect on breast tissue density in pre and postmenopausal women and on breast proliferation in postmenopausal women with or without a history of breast cancer. The results of animal studies are also controversial. Experiments on monkeys that examined effects of soy on mammary gland indicated to the possibility that proliferating effect of estradiol may be antagonized by isoflavone-rich soy protein diet (Jones et al., 2002; Wood et al., 2004).

A number of studies conducted in immunodeficient nu/nu or SCID mice strains demonstrated enhanced proliferation, or no effect of isoflavones on tumor development and progression (Allred et al. 2004; Hsieh et al., 1998). In addition, Heferich and co-workers (2008) implanted estrogen-dependent tumors into ovariectomized mice and found that dietary genistein was able to reduce the inhibitory effect of tamoxifen on tumor growth. However, prevention and inhibition of the progression of experimentally induced mammary tumors by isoflavones was also detected, as well as that post pubertal soy treatment before the induction of tumor had a slightly preventive effect (Pei et al., 2003; Sarkar et al., 2002). Results on Sprag-Dawley rats also proposed that pre-pubertal exposure to soybean isoflavones have highly significant tumor preventive effects (Gallo et al., 2001; Lamartiniere et al., 2002).

More recent review of the animal models used to investigate the health benefits of soy isoflavones concluded that results obtained in different animal models demonstrate minimal effects of isoflavones in breast and prostate cancer prevention (Cooke, 2006).

In vitro genistein inhibited proliferation of ER-positive and ER-negative breast cancer cells at high doses ($>10\text{M}$), but promote tumor growth at lower, more nutritionally relevant doses (Wang et al., 1996). Tamoxifen is the oldest and most-prescribed SERM for breast cancer treatment and it also have mixed effects depending on dose. The SERM-like activities of soy isoflavones makes dietary guidelines particularly difficult to be issued with confidence. Carcinogen-induced mammary cancers predominantly express $\text{ER}\alpha$, and there are some indications that substances that activate mainly $\text{ER}\beta$ have an antiproliferative effect. In addition, it was reported that genistein may interact with tamoxifen, both synergistically

and antagonistically (Shu et al., 2009; Taylor et al., 2009). The inhibition of proliferation in human breast cancer cell line with tamoxifen could be overridden by physiological concentration of genistein (Jones et al., 2002), which indicate that genistein may negate healing effect of tamoxifen on breast cancer patients.

Besides the ER-dependent mechanisms, high doses of genistein may inhibit tumor development and growth by other molecular mechanisms: by antiproliferative actions through inhibition of tyrosine kinase and DNA topoisomerase activities (Akiyama et al., 1987; Markovits et al., 1989), by induction of cell cycle arrest and apoptosis (Bektic et al., 2005), as well as by exerting anti-angiogenic actions (Fotsis et al., 1993).

4.2 Effects on prostate cancer

Lifelong exposure to isoflavones plays a role in the low incidence of prostate cancer observed in Asian males. However, the effects of soy consumption on existing prostate cancer may differ in relation to disease stage. Kurahashi et al. (2007) reported that soy isoflavones in the diet decreased the risk of localized prostate cancer, while soy-containing miso soup increased the risk of advanced prostate cancer. The obtained results may be due to loss of estrogen receptors in advanced tumors, or due to possible errors in food measurement and small sample of men with advanced prostate cancer.

Hamilton-Reeves et al. (2007) reported that soy protein isolate with or without isoflavones affected hormone receptor expression patterns in men at high risk for developing advanced prostate cancer. Intake of soy protein isolate with isoflavones significantly suppressed androgen receptor expression but did not alter estrogen receptor beta expression in prostate, while intake of soy protein isolate without isoflavones tended to suppress AR expression ($P = 0.09$). The authors concluded that soy protein isolate consumption may be beneficial in preventing prostate cancer, and hypothesized that soy isoflavones may attenuate but not prevent progression of latent prostate cancer.

Hussain et al. (2003) found that patients with prostate carcinoma consuming a soy-enriched diet had a statistically significant drop in prostate-specific antigen (PSA) levels, compared to the control group. However, more recent study of deVere White et al. (2010) demonstrated that higher amounts of aglycone isoflavones genistein and daidzein did not lower PSA levels in men with low-volume prostate cancer.

Osterweil (2007) observed a dose-dependent decrease in the risk of localized prostate cancer with isoflavone consumption. Men with higher intake of isoflavones had a decreased risk of prostate cancer compared to those with lower intake of isoflavones.

Few animal studies have been conducted to investigate the role of soy isoflavones on prostate cancer development and progression. Genistein markedly inhibited prostate tumor metastasis in mice (Lakshman et al., 2008). Isoflavone-containing diets retarded the development of prostate cancer in rats (Pollard & Suckow, 2006). In contrast to this, Naik et al. (1994) showed that genistein added to the drinking water or intraperitoneally injected have no effect on the growth of the subcutaneously implanted MAT-LyLu prostate carcinoma in rats.

Zhou et al. (1999) in their in vitro studies found that dietary soy products may inhibit experimental prostate tumor growth through a combination of direct effect on tumor cells and indirect effects on tumor neovasculature. In addition, dietary phytoestrogens down-regulated androgen and estrogen receptor expression in adult male rats prostate (Lund et al., 2004). More recent in vitro studies demonstrated that phytoestrogens at high concentrations exert an anti-androgen effect through the interaction with AR (Mentor-

Marcelet et al., 2001). In vitro tests also showed that soy isoflavone genistein induced apoptosis and inhibited growth of both androgen-sensitive and androgen-independent prostate cancer cells (Hussain et al., 2003).

Wuttke et al. (2010) in a recent review provided detailed analysis of both in vitro and animal experimental data and concluded that isoflavones may protect the prostate to make it less prone to develop cancer.

In conclusion, based on inconsistent evidence, it is apparent that the use of phytoestrogens as chemopreventive agents is still in its infancy, justifying a need for further research. Experimental studies based on nutritionally relevant doses are needed to clarify potential health benefits, as well as estrogenic, antiandrogenic and/or nonestrogenic isoflavone activities in the breast and prostate tumors.

5. Soybean phytoestrogens in prevention and therapy of cardiovascular diseases

Soy protein and isoflavones received great attention and provoked heated discussions due to their potential role in reducing risks of cardiovascular diseases. Following is a historical overview of the most relevant results and announcements related to clinical trials, as well as of animal and in vitro research, providing insight into potential mechanisms of isoflavone action.

5.1 Effects on serum lipid levels

Obesity is associated with disruption in lipid and sugar metabolism, and is a principal cause of chronic diseases, namely cardiovascular diseases, hypertension, atherosclerosis and type II diabetes mellitus. This makes obesity a major health problem, which has reached pandemic proportions. The treatment for obesity is lifestyle change, including diet restriction and exercise. However, pharmacological treatment is often necessary. Isoflavones are of particular interest as an alternative to statins or fibrates in potential lowering of serum lipid levels.

Epidemiologic studies demonstrated a reduced rate of mortality due to coronary heart disease in Japanese postmenopausal women populations consuming a traditional Japanese diet. On the other side expatriate Japanese living in the US had higher blood pressure and cholesterol levels than the Japanese still living in Japan. Some authors proposed that detected differences are not of genetic origin but are due to diet rich in soy products, fish and fiber (Adlercreutz et al., 1998).

Anderson et al. (1995) published a meta-analysis that attracted widespread attention, demonstrating that intake of at least 25g of soy protein per day lowered total and low density lipoprotein (LDL) cholesterol. Lipid lowering potential of soy protein was also demonstrated in various animal studies (Greaves, et al., 1999; Potter, et al., 1995). This led to U.S. Food and Drug Administration (FDA) issuing a health claim for soy protein and coronary heart disease (1999). FDA also claimed that the evidence did not support significant role of isoflavones in lipid-lowering effects of soy protein. Some more recent reports also demonstrated a significant reduction in plasma concentrations of total and LDL cholesterol in humans exposed to soy proteins (Greany et al., 2004; Teixeira et al., 2000).

Due to their estrogenic activity, isoflavones may be the bioactive component attributed to soy protein. This possibility was examined using different experimental approaches and

animal models. Some research studies highlighted a favorable hypolipidemic effect related to isoflavones, at least when consumed in combination with soy proteins. Removal of the isoflavone-containing fraction from soy protein resulted in a loss of its beneficial effect on the serum lipid profile and atherosclerosis progression in mice (Kirk et al., 1998), in golden Syrian hamsters (Lucas, et al., 2001), and in rhesus monkeys (Anthony et al. 1996). High isoflavone, combined with high soy protein intake leads to significantly decreased serum total and LDL cholesterol compared to low isoflavone intake. Some authors reported that ingested purified isoflavones exert lipid-lowering effects (Ae Park et al. 2006; Kojima et al. 2002; Sosić-Jurjević et al., 2007). However, others showed minimal or no effects of isolated isoflavones on blood lipid levels (Greaves et al., 1999; Molsiri et al., 2004).

Clinical trials also show diverse beneficial effects of isoflavone supplements on cardiovascular system. These discrepancies may be a result of different intestinal bacterial flora and hence bioavailability of soy isoflavone metabolites. Other reasons might be differences in dose-response effects (Hooper et al., 2008), sex and length of isoflavone supplementation (Zhan & Ho, 2005), limited number of subjects, or pre-existing metabolic status of subjects included in supplement trials (Villa et al., 2009).

In contrast to previously mentioned data, in 22 random trials, isolated soy protein combined with isoflavones, compared with milk or other proteins, decreased LDL cholesterol by approximately 3%. This reduction was small in comparison to amount of soy protein (average 50g per day) intake (Sacks et al., 2006). There was no detected benefit on level of HDL cholesterol, triglycerides or blood pressure. These authors concluded that soy food may be beneficial to cardiovascular health because of their high content of fiber, vitamins, high content of polyunsaturated fat, rather than and its isoflavone content. Recent review of the animal models used to investigate the health benefits of soy isoflavones also concluded that the efficiency of isoflavones in improving lipid profile is less than earlier research suggested (Cooke, 2006).

For this reason, American Heart Association issued a discouraging statement, and warned that earlier research indicating clinically important favorable effects of soy products on low density lipoprotein (LDL) is not confirmed by most studies during the past 10 years. U.S. FDA announced its intent to reevaluate the data related to cardio protective effects of soy (2007).

More recent research demonstrated that the combined intervention of genistein and l-carnitine act synergistically in reducing serum lipid and LDL levels, as well as reducing body weight in mice and rats (Che et al., 2011; Yang et al., 2006). In addition, synergy portfolio diet, containing plant sterols, viscous fibers and soy protein reduced serum LDL cholesterol similar to traditional statin drugs (Jenkins et al., 2003). Therefore, soybean isoflavones, either as natural components of food or as nutritional supplements, in combination with other functional food may favorably alter indicators of cardiovascular disease risk.

Though positive effects on metabolism in humans have been widely debated, studies in rodents should help in identifying and evaluating the biologically relevant mechanisms involved in isoflavone actions.

ERs are important mediators of the action of estrogen on lipid metabolism both in males and females. Men with mutations in the aromatase gene (enzyme that converts androgens to estrogens) display truncal obesity, insulin resistance and hyperlipidemia (Carani et al., 1997). Due to structural similarities of isoflavones and E2, G and D might also directly influence the regulation of adipogenesis. However, it must be noticed that genistein

preferably binds to ER β , while ER α is predominantly found in liver. In ovariectomized mice, estradiol and genistein did not increase estrogen-responsive genes in the liver, and the authors suggested that the cholesterol-lowering ability of estrogen requires estrogen receptors (they postulated crosstalk between ERs and NF κ B) but not estrogen receptor-dependent gene transcription (Evans et al., 2001).

Isoflavones may have distinct influences on metabolism in males and females. Males have a different number and distribution of ERs compared to females. It is important to realize the impact of other hormones such as androgens and thyroid hormones on liver and other metabolic tissues. Using ovariectomized Wistar rats Molsiri et al. (2004) obtained no significant difference in serum lipid levels after s.c. genistein injections, while we detected lipid lowering effect of both G and D (similar to this obtained for testosterone-treated groups) in orchidectomized young and middle-aged adults, as well as in testis-intact middle-aged male rats (Sosić-Jurjević et al., 2007 and our unpublished data).

Aside from having estrogenic activity (Potter et al., 1995), both G and D exert „phytofibrate“ and or „phytoglitzone“ activity, and activate peroxisome proliferator-activated receptors (PPAR) α and γ (Mezei et al., 2006). PPARs bind a wide number of ligands and directly affect lipid metabolism by enhancing transcription of PPAR-regulated genes (Shen et al., 2006). Generally, PPAR α controls the transcription of many genes involved in lipid catabolism, whereas PPAR γ controls the expression of genes involved in adipocyte differentiation and insulin sensation. PPAR α is important for β -oxidation and is mainly expressed in liver, kidney, heart, and muscle, where lipoprotein metabolism is important. PPAR γ is mainly expressed in adipose tissues and is considered the master regulator of adipogenesis (Rosen, 2005; Ørgaard & Jensen, 2008).

Isoflavones may also affect lipid metabolism indirectly, via effect on thyroid function and/or thyroid hormone action in liver. T3 and its receptor (TR) play important role in regulation of energy homeostasis, metabolic processes and body weight. Hypothyroidism causes hypercholesterolaemia characterized by increased levels of LDL (Sasaki, et al., 2006). TR β 1 is the major TR in the liver while T3 action is mediated via TR α 1 in the heart. TR β 1 agonist KB-141 lower cholesterol, increases metabolic rate and decreases body weight (Grover et al., 2005). Xiao et al. (2007) described that expression of the rat hepatic thyroid hormone receptor β 1 is upregulated by isoflavones. In addition, E interplay with TH in regulation of different physiological functions including effects on growth, bone mass, and triglycerides. E can be viewed as a modulator whose response relies on interplay with T3 signaling mechanisms (DiPippo et al., 1995).

Many researchers have tried to link effects of soy intake on lipid metabolism with modulation of thyroid hormone levels. However, it is still difficult to demonstrate clear-cut effects on thyroid (this topic would be analyzed in more details in a subchapter related to endocrine disruptive potential of isoflavones). On the other hand, most researchers who examined lipid-lowering potential of isoflavones did not include in their research examining of the thyroid status, or deiodinase I enzyme activity in liver. When examining the effects of G and D that should mimic exposure to supplements (10mg/kg) in orchidectomized middle-aged male rats our research team obtained that both G and D decreased the serum total cholesterol and LDL levels similar to control testosterone treatment, and brought about an increase in serum triglycerides similar to that observed after control estradiol treatment (Sosić-Jurjević et al., 2007). Within the same animal model we detected significant decrease of serum thyroid hormones (Sosić-Jurjević et al., 2010). However, when we examined deiodinase I enzyme activity in liver of G and D treated rats, it was significantly increased

(our unpublished data) in comparison to the control values. Therefore, the local production of T3 in liver was increased and the local increase of T3 might contribute to the detected decrease in total cholesterol and LDL levels.

5.2 Effects on atherosclerosis progression

Atherosclerosis is part of the normal aging process but its progression depends on a wide range of environmental and genetic factors (Davies et al., 2004). Generally, atherosclerosis refers to the formation and hardening of fatty plaques (atheromas) on the inner surface of the arteries. The arteries not only harden, they become narrow. Such narrowed vessels can be easily blocked by constriction or objects in the bloodstream. Atherosclerosis begins with injury to endothelial cells, exposing portions of the artery surface below the endothelium. Free radicals or other irritants could start the process, as well as high blood pressure. Platelets cluster around the injured endothelial cells and release prostaglandins, which cause the endothelial cells to proliferate. LDL-cholesterol particles release their fat into the areas made porous by prostaglandins. Macrophages swell themselves on oxidized LDL-cholesterol until they become "foam cells" that invade atheromas. The atheromas are hardened by fibrin, which forms scar tissue, and finally calcium patches.

The atheroprotective effects of soy-based diets have been partly attributed to the associated reduction in cholesterol levels in human studies (Jenkins et al., 2002). Similar findings have also been reported in nonhuman primates fed soy-based diets (Anthony et al., 1997; Register et al., 2005). Animal studies with rabbits and hamsters, which are considered a good non-primate model for studies of atherosclerosis, demonstrated that soy isoflavones reduce atherosclerotic lesion areas in the aortic arch by means of LDL reduction (Alexandersen et al., 2001; Lucas et al., 2003). In addition, atherosclerotic changes induced by a cholesterol rich diet were prevented by isoflavones in rabbits, hamsters and premenopausal monkeys (Adams, et al. 2005; Lucas et al., 2001). The intake of genistein and daidzein decreases LDL oxidation (Tikkanen, et al., 1998). Both genistein and daidzein have also been shown to protect human umbilical cord endothelial cells and bovine aortic endothelial cells from the atherogenic effect of oxidized LDL (Kapiotis et al., 1997).

However, numerous animal studies suggest that dietary soy inhibits atherosclerotic lesion development by mechanisms other than lowering serum cholesterol.

Isoflavones are reported to prevent lipid peroxidation by scavenging lipid-derived peroxy radicals (Patel et al., 2001) and inhibit copper-dependent LDL oxidation (Kerry & Abbey, 1998). It is well known that oxidized LDL is more prone to induce atherosclerosis than unoxidized form. In addition, proteome analyses revealed protein targets that in response to soy isoflavones increase the anti-inflammatory response in blood mononuclear cells thereby contributing to the atherosclerosis-preventive activities of a soy-rich diet (Wenzel et al., 2008). Studies in apolipoprotein E knock-out mice showed that atherosclerotic lesions are reduced when fed a soy-containing diet despite unchanged serum lipid levels (Adams et al., 2002). Findings from a recent study in aged lipoprotein receptor knock-out mice has underscored the importance of oxidative stress coupled with a failure to up-regulate There is now compelling evidence that isoflavone supplementation have anti-inflammatory functions and hence can represent an effective therapeutic strategy to enhance Nrf2 activity to protect the aging vasculature (Adams et al., 2002; Mulvihill & Huff, 2010).

Vasodilatory effects of isoflavones may be also related to their estrogenic actions. Both estrogen receptors α and β are expressed in the arteries (Christian et al., 2006). Estrogens have been shown to stimulate inducible NO synthase in endothelial cells and the increased

NO production causes relaxation of arterial myocytes (Mahn et al., 2005). Research on the effect of genistein on plasma nitric oxide concentrations, endothelin-1 levels and endothelium-dependent vasodilatation in postmenopausal women revealed that genistein therapy improved flow-mediated endothelium-dependent vasodilatation in healthy postmenopausal women. This improvement is probably mediated by a direct effect of genistein on vascular function and could be the result of an increased ratio of nitric oxide to endothelin (Squadrito et al., 2002).

In conclusion, despite the fact that dietary soy products and isoflavones are heavily advertised for their hypolipidemic effect, their therapeutic potential is lesser than was previously hoped and depend on many factors related to inter-individual differences.

6. Soybean phytoestrogens in bone protection

Bone remodeling is a continuous process of bone resorption and bone formation for the purpose of maintaining normal bone mass. As a skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissues, osteoporosis is usually caused by a chronic imbalance in the bone remodeling cycle. This skeletal disorder occurs as part of the natural aging process and is associated with the rapid decline in ovarian function and subsequent reduction of circulating estrogen in women after the menopause and declining testosterone in middle-aged and older men. However, in contrast to postmenopausal osteoporosis in women, the age related bone loss in men is less well-defined. Observational studies have indicated that estrogen administration is important in bone remodeling. Thus, hormone replacement therapy (HRT) administered in a dose-dependent manner, not only significantly reduces bone loss, but also lowers the incidence of hip and vertebral fractures (Lindsay et al., 1976, 1984; Michaelsson et al., 1998). In the other hand, although HRT has a protective effect on bone tissue, it can increase the risk of breast, endometrial ovarian or prostate cancer developing (Davison & Davis, 2003; Loughlin & Richie, 1997; Nelson et al., 2002). For this reason, much attention has been paid to the examination of alternative therapeutic compounds that may have protective effects on bone, without adverse effects on other tissues. Epidemiological studies have demonstrated a low incidence of postmenopausal fractures and high bone mineral density (BMD) in Asian populations with a particularly soy-rich diet (Cooper et al., 1992; Lauderdale et al., 1997; Somekawa et al., 2002). Thus, phytoestrogens have been proposed as an alternative to conventional hormone therapy for preventing osteoporosis and have shown beneficial effects on bone health (Barnes, 2003; Morin, 2004).

Bone remodeling is regulated by the activity of two different cell lines. Osteoblasts stimulate bone formation and calcification, while osteoclasts promote bone resorption. It has been shown that isoflavones affect osteoblastic bone formation and osteoclastic bone resorption in vitro. The anabolic effects of genistein and daidzein on bone metabolism have been investigated in culture using femoral trabecular and cortical bone tissues obtained from elderly female rats (Gao & Yamaguchi, 1999; Yamaguchi & Gao, 1997, 1998). Genistein induced a significant increase in calcium content, alkaline phosphatase activity as a marker of osteoblasts, as well as DNA content, which is an index of bone cell numbers in bone tissues (Yamaguchi & Gao, 1997). In bone tissue culture medium daidzein significantly elevated bone components (Gao & Yamaguchi, 1999). Both genistein and daidzein increased newly synthesized protein content, alkaline phosphatase activity and DNA content in cultures of osteoblastic MC3T3-E1 cells (Sugumoto & Yamaguchi, 2000, 2000a; Yamaguchi & Sugumoto, 2000).

In addition to effects on osteoblasts, many authors have reported that isoflavones are efficacious in suppressing osteoclast activity *in vitro*. Genistein completely inhibited bone resorption and osteoclast-like multinucleated cells in culture with bone-resorbing factors (Gao & Yamaguchi, 1999a; Yamaguchi & Gao, 1998a). Also, daidzein inhibited the development of osteoclasts from cultures of porcine bone marrow and reduced bone resorption (Rassi et al., 2002).

While *in vitro* studies reveal possible actions of isoflavones on individual bone cells, *in vivo* studies provide insight into the effects of isoflavones on the intact system and coupling effects between osteoblasts and osteoclasts. Most of the animal bone studies investigating isoflavone action have been performed in rodents. Aged ovariectomized female and orchidectomized male rats represent a suitable model for simulating osteoporosis due to estrogen or androgen deficiency (Comelekoglu et al., 2007; Filipović et al., 2007; Pantelić et al., 2010; Turner, 2001; Vanderschueren et al., 1992). Using this animal model, supplementation with isoflavones has been shown to prevent bone loss (Fig. 2) induced by gonadal hormone deficiency (Filipović et al., 2010; Khalil et al., 2005; Lee et al., 2004; Om & Shim, 2007; Ren et al., 2007; Soung et al., 2006). In a randomized placebo controlled trial with estrogen and phytoestrogen on ovariectomized nonhuman primates, Ham et al. (2004) failed to show any efficacy of soy phytoestrogens in decreasing all indices of bone turnover as estrogen does, but soy phytoestrogens were able to increase bone volume, trabecular number and decrease trabecular separation, stressing the importance of phytoestrogens in postmenopausal osteoporosis prevention.

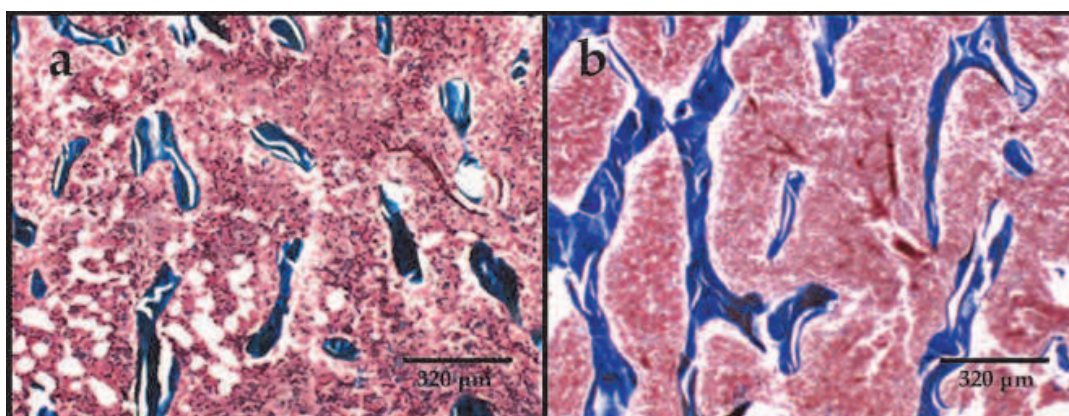


Fig. 2. Trabecular bone microarchitecture of the proximal tibia in control orchidectomized (a) and daidzein-treated orchidectomized (b) rat; azan staining method; unpublished image of Filipovic et al.

Phytoestrogens may elicit a bone sparing effect by both genomic and nongenomic mechanisms. They are able to interact with enzymes and receptors and, their stable structure and low molecular weight enables them to pass through cell membranes (Adlercreutz et al., 1998). The structural similarity of phytoestrogens to mammalian estrogens and their ability to bind to estrogen receptors (Setchell et al., 1999) suggests that the actions of phytoestrogens are mediated via estrogen receptors. ER α and ER β have been detected in bone (Arts et al., 1997; Onoe et al., 1997). The relative binding affinity of phytoestrogens for ER β is greater than that for ER α , and the protective effect of phytoestrogens on bone is probably produced through binding to estrogen receptors, particularly ER β (Kuiper et al., 1998). In addition, phytoestrogens such as coumestrol, genistein and daidzein increase alkaline phosphatase activity in osteoblast-like cells (Kanno et al., 2004). Daidzein stimulates

osteoblast differentiation, induces changes in the action of the cytoskeleton responsible for cell adhesion and motility and activates transcription factors associated with cell proliferation and differentiation (de Wilde et al., 2004; Ge et al., 2006; Jia et al., 2003). Also, isoflavones promote insulin-like growth factor-I (IGF-I) production which enhances osteoblastic activity (Arjmandi et al., 2000).

Isoflavones inhibit bone resorption, via direct targeting of osteoclasts. They can decrease differentiation and increase apoptosis of osteoclasts or interfere with signaling pathways such as intracellular calcium, cAMP or protein kinase and protein tyrosine phosphatase (Gao & Yamaguchi, 2000; Sliwinski et al., 2005). Furthermore, osteoblasts are essential for *in vitro* osteoclastogenesis through cell-to-cell interactions of cytokines. Isoflavones regulate the expression and osteoblastic production of osteoclastogenesis-regulatory cytokines, such as interleukin-6 (IL-6), which stimulates osteoclast formation, and osteoprotegerin (OPG), which is identical to osteoclastogenesis inhibitory factor, and the receptor activator of NF- κ B ligand (Chen et al., 2002).

In addition to ERs, it has been shown that PPAR are new targets of phytoestrogens. PPAR directly influences osteogenesis and adipogenesis in a divergent way (Dang & Lowik, 2005). These authors suggested that biphasic dose-dependent effects of phytoestrogens are the result of concurrent activation of ERs and PPARs. Dominant ER-mediated effects that increase osteogenesis and decrease adipogenesis can only be seen at low concentrations of phytoestrogens, whereas dominant PPAR-mediated effects that decrease osteogenesis and increase adipogenesis are only evident at high concentrations.

Calcitonin (CT), a hormone secreted from thyroid C cells is known to inhibit osteoclast activity directly through its receptors (Nicholson et al., 1986). It was shown that synthesis and release of CT from thyroid C cells decreased after ovariectomy in rats, due to lack of estrogens (Filipović et al., 2002; Sakai et al., 2000). On the other hand, estrogen treatment had a stimulatory effect on CT secretion in ovariectomized rats (Filipović et al., 2003; Grauer et al., 1993). However, chronic Ca treatment of ovariectomized rats positively affected CT release without any significant changes in morphometric parameters of the C cells, suggesting an important role for estrogen in the regulation of CT synthesis (Filipović et al., 2005). Exogenous CT administration was reported to inhibit CT secretion in rats and therefore CT treatment probably suppresses C cell function due to a negative feedback (Sekulić et al., 2005). Recently, daidzein was found to stimulate CT secreting thyroid C cell activity in addition to increasing trabecular bone mass and decreasing bone turnover (Filipović et al., 2010). These results suggest that, besides direct action, daidzein may affect bone structure indirectly through enhancement of thyroid C cell activity.

Although animal studies demonstrate a clear skeletal benefit of phytoestrogens, clinical trials have given different results. Soy isoflavones were observed to retard bone loss in some (Huang et al., 2006; Newton et al., 2006), but not in other studies (Arjmandi et al., 2005; Brink et al., 2008). To date, only one study indicated that supplementation of intact soy protein providing 83 mg isoflavones daily might increase both hip and spine BMD in men (Newton et al., 2006). Also, the results of meta-analyses of soy foods and isoflavones extracted from soy protein have given conflicting results concerning the prevention of bone loss (Liu et al., 2009; Ma et al., 2008). The large heterogeneity in these conclusions might have arisen because many results were pooled from different individual studies, involving different treatment durations, different doses of soy isoflavone and study quality (Liu et al., 2009). These authors suggested that, because changes in bone mineral density (BMD) occur

slowly over time, in short-term intervention studies this change may represent a transient remodeling rather than a long-term steady-state. In addition, a favorable effect on the spine BMD was achieved with large doses of isoflavones (≥ 80 mg/day, median 99 mg/day), but not with lower doses (< 80 mg/day, median 60 mg/day). Thus, the potential of soy isoflavones to prevent bone loss can be achieved by a dosage of 80 mg/day (Huang et al., 2006).

Finally, a wealth of supporting data from many in vitro mechanistic studies on bone cell lines and in vivo investigations using models of osteoporosis shows bone-sparing effects from phytoestrogens. These studies indicate that positive effects of phytoestrogens, as a SERM, may be achieved through estrogen receptors or other mechanisms. However, the results of clinical studies are more inconsistent. The different efficacy of phytoestrogen treatments, in studies involving either animal or human subjects, depended on dose, route and duration of administration. The data are, however, rather tantalizing because it is possible that soy isoflavones may offer the maximum benefit for prevention of osteoporosis. Therefore, it is necessary to perform large-scale clinical dietary intervention studies with phytoestrogens to determine their effects on bone tissue in humans.

7. Soybean phytoestrogens as potential endocrine disruptors

Endocrine systems of vertebrates have essential role in regulation of growth (including bone growth/remodeling), reproduction, stress, lactation, metabolism, energy balance, osmoregulation, and all other processes involved in maintaining homeostasis. Disruption in function of any endocrine system, involving either increased or decreased hormone secretion, result inevitably in disease, the effects of which may extend to many different organs and functions, and may even be life-threatening.

An endocrine-disrupting compound (EDC) is defined by the U.S. Environmental Protection Agency (EPA) as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process." All hormone-sensitive physiological systems are vulnerable to EDCs, including brain and hypothalamic neuroendocrine systems; pituitary; thyroid; adrenal gland; cardiovascular system; mammary gland; adipose tissue; pancreas; ovary and uterus in females; and testes and prostate in males.

The exposure to such chemicals does not necessarily mean that disturbance of the relevant endocrine system will occur, as much depends on the level, duration and timing of exposure. However, even subtle changes, however small, in combination and/or under different conditions and/or in later generations might reduce the ability of humans (animals) to adapt. It may also happen that the magnitude of the disruption becomes evident only in presence of an additional stress factor.

It is beyond the scope of this chapter to discuss the potential interference of soy isoflavones with all endocrine organs; instead, the focus will be on three major endocrine axes that are affected by soybean phytoestrogens: pituitary – gonadal, -thyroid and - adrenocortical systems.

7.1 Effects on female reproductive system

Soybean isoflavones are ligands for both ER α and ER β , despite the fact that their estrogenic potency is much lower than that of E2. Therefore, they can mimic and/or antagonize the

mechanisms of E2 action and thus interfere with both endocrine and reproductive functions of the pituitary-gonadal axis. The rat uterotrophic assay is a widely used screening test for the detection of estrogenic, endocrine-disrupting chemicals. Genistein administration to ovariectomized rats induced a dose-dependent uterine growth and altered expression of estrogen-regulated genes (Diel et al., 2004). As E2 does not stimulate these uterine parameters in ER α KO mice (Couse & Korach, 2001), this test is considered as the proof for estrogenic action of phytoestrogens via ER α .

The first recognized health benefit of isoflavones was their potential to alleviate climacteric complaints, namely hot flushes and night sweats in perimenopausal women (Adlercreutz, 1998). Within the short period of time, numerous isoflavone and soy products became available in a form of food supplements and remedies. They were advertised as natural alternative to hormone replacement therapy, useful in prevention of climacteric symptoms. However, majority of recent placebo-controlled clinical trials support the opinion that isoflavone preparations are not superior to placebo, as placebo effect is 30% to 50% when dealing with psychosomatic climacteric complaints (Patisaul & Jefferson, 2010). Animal studies also demonstrated that only high doses of isoflavones were able to suppress overactivation of hypothalamic gonadotropin-releasing hormone pulse generator induced by estrogen deprivation (the major cause of hot flushes and other climacteric symptoms (Wuttke et al., 2007). It is important to stress that exposure to high doses of soy isoflavones (150mg/kg) is similar in biological effects to classical hormone replacement therapy. Therefore, their consumption bears a risk of increased proliferation of endometrial and mammary gland tissue with so far unpredictable risk of cancer development.

Multiple human studies demonstrated that exposure of premenopausal women to soybean isoflavones have a suppressive effect on pituitary-gonadal axis; consumption of isoflavone-rich soy food suppresses serum estrogen and progesterone levels and attenuate the preovulatory surge of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Hooper et al., 2009; Nagata et al., 1998; Schmidt et al., 2006;). However, some researchers found no impact of isoflavones on female hormone levels (Maskarinec et al., 2002). Soybean phytoestrogens may also affect the women menstrual cycle, but findings are inconsistent. It was shown that a diet with soy protein delays menstruation and prolongs the follicular phase of the menstrual cycle (Cassidy et al., 1994). Other studies demonstrated increased or unchanged follicular phase length, decreased or unchanged midcycle LH and FSH, increased, decreased or unchanged estradiol, decreased dehydroepiandrosterone sulfate, and decreased or unchanged luteal phase progesterone in relation to isoflavone ingestion (Cassidy et al., 1994; Duncan et al., 1999). Therefore, women who try to become pregnant or have menstrual cycle irregularities should be cautious with consumption of isoflavone-enriched soy products or supplements.

Animal studies in rodents produced clear evidence of adverse effects of G on the female reproductive system following treatment during development (Chen et al., 2007; Kouki et al., 2003; National Toxicology Program, 2008). Studies that demonstrated clear evidence of developmental toxicity for G involved treatment during the period of lactation in rodents, as well as multigenerational studies that included exposure during gestation, lactation, and post-weaning. In adulthood, the effects of neonatal exposure to 50 mg G/kg bw/day were manifested as a lower number of live pups per litter (Padilla-Banks et al., 2006), a lower number of implantation sites and corpora lutea (Jefferson et al., 2005), and a higher incidence of histomorphological changes of the reproductive tract (i.e., cystic ovaries,

progressive proliferative lesions of the oviduct, cystic endometrial hyperplasia, and uterine carcinoma) relative to control females (Newbold et al., 2001).

In addition, the reproductive performance of the neonatally-treated mice was tested during adulthood and there was a significant negative trend for the number of dams with litters. Because the effects were more pronounced in animals at 6 months of age than at 2 or 4 months of age, the authors suggested that reproductive senescence may occur earlier in these animals as a result of the neonatal G treatments (Jefferson et al., 2005). These authors explained that, although G-treated mice ovulate under exogenous hormonal influence, the ovulation rate was changed. The lower doses of G treatment enhanced ovulation rate, while the higher doses decreased this parameter. Ovulation of too many oocytes early in life may reduce the number of oocytes available for fertilization and lead to lower fertility rates later in life (McLachlan et al., 1982). The development of the ovary and ovarian follicles was altered following neonatal G treatment (Jefferson et al., 2002). Ovaries of G-treated mice contained multioocyte follicles (MOFs) at 19th postnatal day. This phenotype is a marker for altered development of the ovary, which lead to oocytes of poor quality (Jefferson et al., 2005). These oocytes are less potent, since the oocytes derived from single oocyte follicles were far more likely to be fertilized in vitro than oocytes derived from MOFs (Iguchi et al., 1990). In our laboratory, results obtained on the ovaries of immature rats treated with 50mg G/kg for three days (from 19th till 21th postnatal day) showed that G disturbed the follicular parenchyma-ovarian stroma ratio (Fig. 3), induced increase of total ovary volume (Medigović et al., 2009).

Data from experiments using DNA microarray analysis for examining the effects of genistein in the developing rat uterus indicate that genistein alters the expression of 6-8 times as many genes as does E₂, most of which were down-regulated (Barnes, 2004).

Data are not consistent about onset of puberty and sexual maturation in rats and mice following exposure during gestation and lactation or continuous exposure to soy diet or supplements. An earlier onset of vaginal opening was observed in mice exposed directly to G during the period of lactation (Nikaido et al., 2004.) and in rats treated by sc injection as neonates with 10 mg G/kg bw/day (Bateman & Patisaul, 2008). However, other authors reported delay in vaginal opening (Anzalone et al., 1998).

Only a very small number of studies have been published on D and its estrogenic metabolite equol, and no studies have evaluated the effects of developmental exposure to glycitein. Detection of typical estrogenic effects in these studies are controversial. Kouki et al. (2003) reported no effect on estrous cyclicity in rats treated by sc injection with ~19 mg D/kg bw/day on PND1-5. In contrast, treatment with the same dose levels of G caused the predicted estrogenic effect in all of these studies. Similar to these authors, in our laboratory (unpublished data) no uterotrophic response was detected after subcutaneous injection of immature female rats with 50mg D/kg/day (treatment lasted from 19th postnatal till 21th postnatal day), though the same treatment with G caused predicted estrogenic response.

Isoflavones can pass from mother to fetus through placenta. However, this exposure is considerably lower than in infants fed with soy formula. Initially developed as an alternative to bovine milk formulas for babies with a milk allergy, use of soy infant formula became more popular among environmentally oriented population with vegetarian life style. A recent prospective study in human infants observed that female infants fed soy-based formulas exhibit estrogenized vaginal epithelium at times when their breast fed or cow milk- based formula fed peers did not (Bernbaum et al., 2008). Patisaul and Jefferson

(2010) concluded that further determination if soy infant formula have long-term reproductive health effects should be a public health imperative.

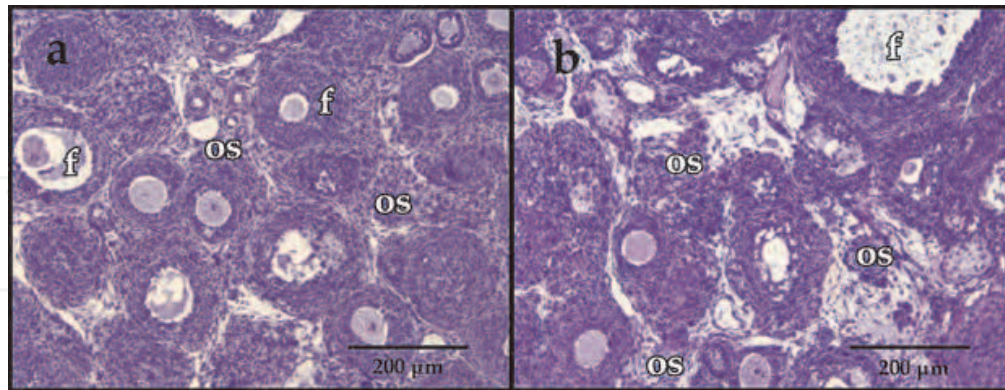


Fig. 3. Ovaries of 21 day old control (a) and genistein – treated rat; hematoxylin - eosin staining method; OS, ovarian stroma; f, follicular parenchyma; hematoxylin – eosin staining method; unpublished image of Medigović et al.

7.2 Effects on male reproductive system

Soy phytoestrogens, alone or in combination with some other EDC, may adversely affect androgen hormone production, spermatogenesis, sperm capacitation and fertility. Results of recent meta-analysis suggest that neither soy foods nor isoflavone supplements alter bioavailable T concentration in adult men (Hamilton-Reeves et al., 2010). However, Tanaka et al. (2009) reported that short-term administration of soy isoflavones decreased testosterone and dihydrotestosterone (DHT) and increased sex hormone-binding globulin levels.

Male reproductive system is particularly sensitive in prenatal stage and during early infancy, when disruption of the hormonal balance in favor of estrogens can lead to irreversible abnormalities in sex specific physiology and behavior in the adulthood (Patisaul & Jafferson, 2010).

Only few animal studies reported results on the developmental effects of exposure to soy infant formula. Their study designs were based on the same group of male marmosets treated during infancy, and assessed either as juveniles (Sharpe et al., 2002) or adults (Tan et al., 2006). The soy infant formula-fed male marmosets had significantly lower plasma testosterone levels than their cow milk formula-fed co-twins. Histopathological analysis on the testes of a subset of the co-twins revealed an increase in Leydig cell abundance per testes in the soy infant formula-fed marmosets compared to their cow milk formula-fed co-twin, in the absence of a significant change in testicular weight. A follow up study was conducted on the remaining animals when they were sexually mature (80 weeks of age or older). The males fed with soy infant formula as infants had significantly heavier testes and increased number of both Leydig and Sertoli cells per testicle compared to cow milk formula-fed controls. In addition, there was no significant onset of puberty, level of adult plasma testosterone, or fertility. The authors suggest that the increase in testes weight was likely due to an increase in testicular cell populations. Therefore, these results demonstrated permanent effects on testicular cell populations, but no obvious effects on reproductive function, namely fertility or permanent changes in testosterone levels of experimental animals.

Some studies on rats and mice demonstrated increased testicular weight when animals were treated with soy diet or isoflavone supplements during gestation and lactation or continuous exposure, similar to the effect described above in marmosets treated with soy infant formula during infancy (Akingbemi et al., 2007; McVey et al., 2004; Piotrowska et al., 2011; Ruhlen et al., 2008; Wisniewski et al., 2005). Other authors reported a decrease (Atanassova et al., 1999; Wisniewski et al., 2003) or no effect on testicular weight (Fielden et al., 2003; Kang et al., 2002).

Controversial results are found as to the effects of lifelong exposure of rodents to phytoestrogens on reproductive function, namely fertility or changes in testosterone levels. The litter size was not affected when male rats were exposed to dietary soy throughout life (Atanassova et al., 1999). Also, chronic dietary exposure to G did not adversely affect spermatogenesis or seminal vesicle weight in rats (Delclos et al., 2001; Roberts et al., 2000). On the other hand, a few studies indicate negative effects of phytoestrogens on male reproductive success. Thus, a continuous exposure to low combined doses of G and vinclozolin affects male rats' reproductive health by inducing reproductive developmental anomalies, alterations in sperm production and quality, and fertility disorders (Eustache, 2009).

Exposure to G was found to induce hyperplasia of Leydig cells in mice (Lee et al., 2004b). The exposure to isoflavones during 5 weeks decreased the level of circulating testosterone, depending on the dose used (Weber et al., 2001). No significant differences in serum testosterone concentration was detected in rats receiving high doses of G and D from intrauterine life through sexual maturity (Piotrowska et al., 2011). In vitro investigation showed that G can promote the testosterone production of rat Leydig cells at a low concentration, but both D and G can inhibit it at a higher concentration (Zhu et al., 2009).

Effect of phytoestrogens on male reproduction system is a complex process that depends on developmental stage and time of exposure, applied dosage, and other factors. Together, these factors determine the potential risk for adverse consequences with long-lasting effects on male reproductive function. At present, the evidence is insufficient to determine whether soy products cause or do not cause adverse developmental effect on male reproductive system, due to the small number of studies, limitations in their experimental designs, and failure to detect adverse functional effects.

7.3 Effects on pituitary-thyroid axis

Goitrogenic effects of a soybean diet in animals were reported in 1933 (McCarrison, 1933). Similar to animals, goiter and hypothyroidism were reported in infants fed with adapted soy formula without adequate iodine supply (Van Wik et al., 1959). This effect was eliminated by supplementing commercial soy infant formulas with iodine, or by switching to cow milk (Chorazy et al., 1995). However, infants with congenital hypothyroidism that were fed with iodine supplemented diet still needed higher doses of L-thyroxine (Jabbar et al., 1997). In addition, the incidence rate of autoimmune thyroid disease was doubled in teenage children who consumed soy formula as infants (Fort et al., 1990). However, results of clinical studies with adults are not consistent: some authors suggest that isoflavones have a mild or no effect on thyroid function (Dillingham et al., 2007; Duncan et al., 1999), while others indicate that isoflavones suppress the thyroid function (Haselkorn et al., 2003; Ralli, 2003; Sathyapalan et al., 2011).

Rats provide a useful risk assessment model for various thyroid toxins (Choksi et al., 2003). However, compared to the human, rodent thyroid gland is more sensitive to adverse chemicals (Capen, 1997). Several investigators have reported induction of goiter in iodine-deficient rats maintained on a soybean diet (Ikeda et al., 2000; Kajiya et al., 2005; Kimura et al., 1976), although only in cases of iodine deficiency or presence of some other goitrogenic factor. 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 (Ikeda et al., 2000). 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 (Kimura et al., 1976).

Doerge and his associates demonstrated that genistein and daidzein inhibit the activity of thyroid peroxidase (TPO), the key enzyme in the synthesis of thyroid hormones (TH), both in vitro and in vivo (Divi et al., 1997; Chang & Doerge, 2000; Doerge et al., 2002). However, despite significant inactivation of this enzyme, serum thyroid hormone levels were unaffected by isoflavone treatments in young adult rats of both sexes. Most other authors, who performed their studies on young adult animals of both sexes, also 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 (Chang & Doerge, 2000; Schmutzler et al., 2004). The authors suggested that soy could cause goiter, but only in animals or humans consuming diets marginally adequate in iodine, or who were predisposed to develop goiter, or exposed to additional goitrogenic compounds such as perchlorate, a potent inhibitor of the sodium-iodide-symporter (NIS) of thyrocytes.

Increasing evidence is available that set points of the HPT axis change during various life phases and tend to be less sensitive to negative feedback by thyroid hormones in aging individuals. However, the results on isoflavone effects in aged humans and rodents are scarce. In rodent models, we are the first who demonstrated that both genistein and daidzein induce micro-follicular changes in the thyroid tissue, including hypertrophy of Tg-immunopositive follicular epithelium and colloid depletion (Fig.4), and reduce the level of serum thyroid hormones in orchidectomized (Orx) middle-aged male rats, a model of andropause (ŠošiĆ-Jurjević et al., 2010). The concentration of total T4 in serum decreased more prominently than concentration of total T3 in serum in comparison to the corresponding control values. This reduction consequently led to a feedback stimulation of pituitary TSH cells, detected by the increase in cell volume and relative volume density of TSH β -immunopositive cells per pituitary unit volume, as well as by the increased concentration of TSH in serum. Besides the TPO, there might be other molecular targets for isoflavone interference with the pituitary-thyroid axis.

Soy isoflavones may interfere with thyroid hormones at binding sites of serum distribution proteins such as transthyretin (TTR). In vitro analysis demonstrated that soy isoflavones are potent competitors for T4 binding to TTR in serum and cerebrospinal fluid (Radović et al., 2006). As an outcome of this interference, isoflavones may alter free thyroid hormone concentrations, resulting in altered availability and metabolism of thyroid hormones in target tissues (Köhrle, 2008; Radović et al., 2006). The role of serum binding proteins for thyroid hormone in thyroid homeostasis is not well understood. No single serum T4 - binding protein is essential for good health or for the maintenance of euthyroid state in humans (Robbins, 2000). There are a number of clinical situations in which serum binding proteins are elevated or reduced (even completely absent) and the thyroid state remain

normal (Refetoff, 1989). In contrast, there is evidence that the role of serum binding proteins is to allow the equal distribution of hormone delivery to tissues (Mendel et al., 1987). In rats, TTR is a major serum transport protein of thyroid hormones. In humans TTR is produced in the choroid plexus and appears to be important for thyroid hormone action in the brain (Richardson et al., 2007). Thus, TTR may mediate transport of environmental chemicals into various compartments such as placenta (Meerts et al., 2002). Chemical binding to the TTR may not only decrease the availability of thyroid hormone to various tissues, it may also selectively target these chemicals for transport and uptake.

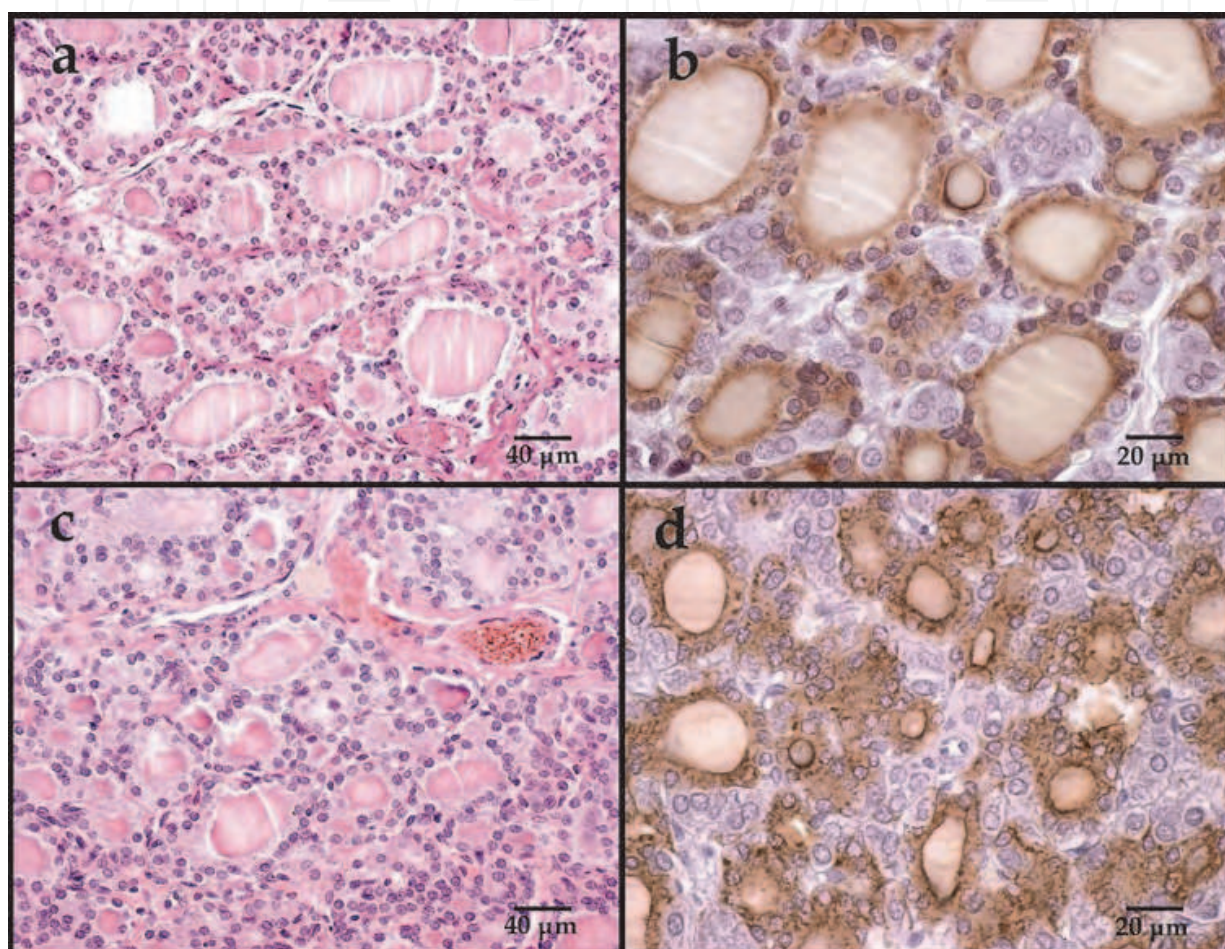


Fig. 4. Thyroid gland tissue of control orchidectomized (a and b) and daidzein-treated orchidectomized (c and d) rat; hematoxylin - eosin and immuno-staining for thyroglobulin; unpublished image of Šošić-Jurjević et al.

In order to accurately assess thyroid function it must be understood that deiodinase enzymes are essential control points of cellular thyroid activity that determine intracellular activation and deactivation of thyroid hormones. Apart from the hormone synthesis by the thyroid gland, deiodination pathways in liver and kidney are the main contributors to thyroid hormone metabolism, turnover and homeostasis. Enzyme 5'-deiodinase type I (5'DI) is the key enzyme in thyroid hormone activation and inactivation in extra thyroidal tissues. This enzyme catalyzes deiodination of the thyroid hormone precursor thyroxine (T4) to the biologically active triiodo-thyronine (T3), as well as the inactivation of T4 and T3 to „reverse” T3 and T2. It is expressed in different tissues, with

highest expression rate found in rat liver, kidney, thyroid gland and pituitary (Bianco et al., 2002). It is regulated in a TH-dependent manner (Köhrle, 2002). In response to iodine deficiency or hypothyroidism, plasma TH values are reduced, TSH is increased and the organism tries to restore normal T3 levels by down-regulation of 5'DI in brain and liver, respectively. In addition, activity of 5'DI in different tissues seems to be sex- (Köhrle et al., 1995; Lisboa et al., 2001) and age- dependent (Corrêa da Costa et al., 2001). According to Corrêa da Costa et al. (2001) decreased serum T3 was detected only in old males, which was explained by a two-times-higher hepatic deiodination of T4 to T3, detected in aged females in comparison to males. Genistein was shown to increase hepatic 5'DI activity of about 33% in young adult female rats, but the detected increase was not statistically significant (Schmutzler et al., 2004). In our model - system (orchidectomized middle-aged rats) G significantly increased ($p < 0.05$) 5'DI activity by 33% (unpublished data). However, neither 5'DI in thyroid nor pituitary 5'DII activity were affected by G or D treatment (unpublished data). These data indicate that although pituitary-thyroid axis in male rats is more vulnerable compared to the one in young adults, it still has great ability to compensate the adverse effects of isoflavones.

Isoflavones may also affect the thyroid function indirectly, via its estrogenic action. Estrogen receptors were located both in pituitary thyrotrophs and in thyroid follicular cells (González et al., 2008; Hampl et al., 1985). Donda et al. (1990) found that pituitary TSH cells in adult female rats have a higher density of T₃ and TRH receptors than in male rats, probably due to a modulatory effect of estradiol. Males are more prone to develop goitrogenesis in response to goitrogenic stimuli, probably due to higher TSH levels in comparison to females (Capen, 1997). It seems that estradiol make the TSH cells more sensitive to the negative feedback regulation with thyroid hormones (Ahlquist et al., 1987). In orchidectomized middle-aged rats we demonstrated that pharmacological doses of testosterone and estradiol disturbed the endocrine homeostasis of pituitary-thyroid axis, but in different directions. Testosterone acted stimulatory, probably through central stimulation of pituitary TSH cells, since both serum TSH and T4 levels were increased. Estradiol acted inhibitory and, though detected structural changes corresponded to centrally induced hypothyroidism, the level of TSH in serum was not significantly altered, suggesting that estradiol may interfere with TSH action within the thyrocytes (Sekulic et al., 2010).

Estrogen was also demonstrated to inhibit activity of thyroid follicular cells in the absence of TSH both in vitro and in vivo (Furlanetto et al., 2001; Vidal et al., 2001). Our previous research of a young adult and middle-aged rat menopause models indicated that chronic estradiol treatment modulated pituitary TSH cells and thyroid structure and decreased serum levels of thyroid hormones, with no significant changes in serum TSH level (Šošić-Jurjević et al., 2005, 2006). Genistein acted as estrogen agonist in an estrogen-responsive pituitary cell line (Stahl et al., 1998).

In conclusion, though there are multiple molecular targets for interference of isoflavones with pituitary-thyroid-peripheral network, this system has considerable capacity to compensate disturbances of its feedback mechanism. If thyroid function is impaired, the risk of developing hypothyroidism increases. Elderly population and individuals with thyroid dysfunction should be aware of potential risk when use isoflavone supplements.

7.4 Effects on pituitary-adrenocortical axis

Results concerning the potential effects of the soy phytoestrogens on pituitary-adrenocortical axis in humans are very limited. The animal studies and in vitro experiments

demonstrated remarkable influence of isoflavones on morphology and function of adrenal cortex. The continuous administration of genistein (40mg/kg) to weanling rats resulted in greater total protein content in zona fasciculata (ZF) and zona reticularis (ZR) of adrenal cortex, and low serum corticosterone concentration (corticosterone is a major glucocorticoid hormone in rats; Ohno et al., 2003). Genistein administration to orchidectomized middle-aged rats, as a model of andropause, increased zona glomerulosa (ZG), ZF (Fig. 5) and ZR cell volumes, and decreased serum aldosterone and corticosterone concentrations ($p < 0.05$), whereas serum DHEA concentration significantly increased (Ajdžanović et al., 2009a). Genistein and daidzein increased androgen and decreased glucocorticoid production (Mesiano et al., 1999) in human adrenocortical cells in a culture. Recent study on human adrenocortical H295R cell line demonstrated that daidzein and genistein strongly inhibited secretion of cortisol with IC50 values below 1 μ M (Ohlsson et al., 2010).

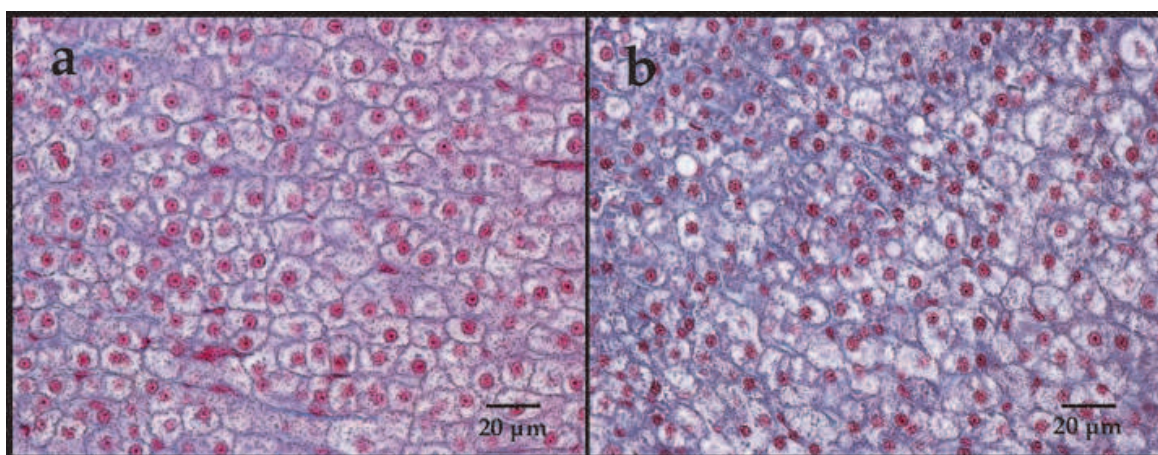


Fig. 5. Zona fasciculata of adrenal cortex in control orchidectomized (a) and genistein-treated orchidectomized (b) rat; azan staining method; unpublished image of Ajdžanović et al.

The isoflavones possess structural features similar to estradiol, which enables them to act via ERs (Lephart et al., 2004). Production of steroids in human fetal adrenocortical cells is modulated by estrogens (Fujieda et al., 1982; Mesiano & Jaffe, 1993; Voutilainen et al., 1979). It was shown that 17β -estradiol in high concentrations increased ACTH-stimulated androgen production and inhibited glucocorticoid synthesis in cultured human fetal adrenal cortical cells (Mesiano & Jaffe, 1993). Although these results indicate the influence of estrogens on the adrenocortical cells in vitro, their physiological significance is still unclear. Under physiological conditions the endogenous estrogen concentration does not reach 1 μ M/L in nonpregnant adults. However, it is possible that dietary phytoestrogens, as estrogen-related compounds, could reach circulating levels high enough to exert estrogenic actions. Consuming the large amounts of soy-derived foods, for example in Japanese diet, circulating concentrations of phytoestrogens can reach higher levels (1-5 micromole/L) (Adlercreutz & Mazur, 1997).

Isoflavones may also affect activity or expression of steroidogenic enzymes, which seems to be the case for its action on rat adrenal cortex (Malendowicz et al., 2006; Mesiano et al., 1999; Ohno et al., 2003). Within the adrenals, steroids are produced through the action of five forms of cytochrome P450 and 3β -hydroxysteroid dehydrogenase (3β HSD) (Simpson & Waterman, 1992). Differential expression of these enzymes in the three adrenocortical zones

leads to the production of specific steroids within each zone (Suzuki et al., 2000). As a precursor of steroidogenesis, the glomerulosa cells use pregnenolone which can be metabolized by either 3β HSD or 17α -hydroxylase, $17, 20$ -lyase. The relative expression of these enzymes influences the synthesis of aldosterone and cortisol/corticosterone in ZG and ZF, as well as adrenal androgens in ZR (Conley & Bird, 1997). The major physiological regulators of adrenal aldosterone production are angiotensin II (Ang II) and potassium. Ang II stimulates aldosterone production through the activation of multiple intracellular signaling pathways including a number of tyrosine kinases (Berk & Corson, 1997; Ishida et al., 1995). It was showed that genistein, as a potent inhibitor of various tyrosine kinases may inhibit aldosterone production (Akiyama et al., 1987; Dhar et al., 1990). Genistein and daidzein are also potent competitive inhibitors of human adrenocortical 3β HSD and cytochrome P450 21 -hydroxylase, suppressing cortisol and stimulating DHEA production in vitro (Mesiano et al., 1999). Part of the inhibition of aldosterone production may result from an increase in 17α -hydroxylase, $17, 20$ -lyase activity, which removes the substrate from the pathway leading to aldosterone and directs it towards the synthesis of adrenal androgens (Sirianni et al., 2001). Isoflavones could also affect adrenal function indirectly, by affecting pituitary ACTH cells. It was previously reported that estrogen replacement lowered the *proopiomelanocortin* (POMC) gene mRNA level and the ACTH response to repeated stressful stimuli in ovariectomized rats (Redei et al., 1994). A certain synergism between CRH (*corticotrophin releasing hormone*) and the various cytokines, namely IL-1, IL-2 and IL-6, has been shown to exist in stimulation of the pituitary ACTH secretion (Bateman et al., 1989; Besedowsky & del Ray, 1996). Genistein may interrupt the stimulatory effects of CRH and cytokines on POMC gene transcription and reduce the level of ACTH, through inhibition of tyrosine kinase phosphorylation cascades (Katahira et al., 1998), but the biological significance of this mechanism is still unclear. We treated orchidectomized middle-aged rats with different doses of genistein or daidzein (10 and 30mg/kg body weight); (Ajdžanović et al., 2009; Ajdžanović et al., 2010; Milošević et al., 2009), and detected similar decrease in pituitary ACTH cellular volume and plasma ACTH levels. Corticosterone levels were also decreased, supporting that some other mechanism, aside from feedback regulation, is involved in effect of isoflavones on pituitary ACTH cell regulation. Keeping in mind that aging is associated with augmented activity of the pituitary-adrenal axis and higher incidence of stress-related psychiatric disorders (Hatzinger et al. 2000), this decline might be considered beneficial at some point.

On the other hand, chronic treatment of weanling rats with genistein (40mg/kg body weight) elevated ACTH level, most probably due to decreased serum corticosterone level and thus release from a negative feedback regulation (Ohno et al., 2003). This finding is of importance since glucocorticoids have important "programming" effects during development. This means that alternations in the circulating levels of glucocorticoid hormones may affect the timing and set points of other endocrine axes (Manojlovic-Stojanoski et al., 2010), as well as brain development, memory and learning capabilities in adults (de Kloet et al., 1988).

Based on animal studies and in vitro research it may be concluded that soy isoflavones interfere with the function of pituitary-adrenocortical axis. This hormonal axis plays a major role in control of stress response and regulation of numerous body processes (digestion, metabolism of carbohydrates, protein and fat, attenuation of the inflammatory response, mood, emotions and sexuality). Therefore, the biological impact of this interference is high. Potential health risks for various age groups should be further assessed.

8. Conclusion

So, are soy isoflavones friends or foes? The answer is complex and may ultimately depend on age, sex, health status, quantity of intake, and even the composition of an individual's intestinal micro flora. In vitro and animal research, as well as human research including both clinical and epidemiologic data, suggests that isoflavone-containing products pose a risk to estrogen-sensitive breast cancer patients and in women at high risk of developing this disease. Results of animal and human studies suggest a modest benefit in prevention of prostate cancer. Exposure to isoflavones by feeding soy infant formula bears a risk of adverse effects on the long-term development of infants. Women who tend to get pregnant or have irregularities in menstrual cycle, as well as persons who are at risk of thyroid dysfunction, should avoid soy isoflavone supplements. The usage of soy protein (with or without isoflavones) seems to have a modest beneficial effect on cardiovascular system and protective role in prevention and treatment of osteoporosis. Research of potential synergy of isoflavones and drugs, and/or other functional food could be a new promising strategy in reducing risk of age-related diseases, improving life quality and expanding life span.

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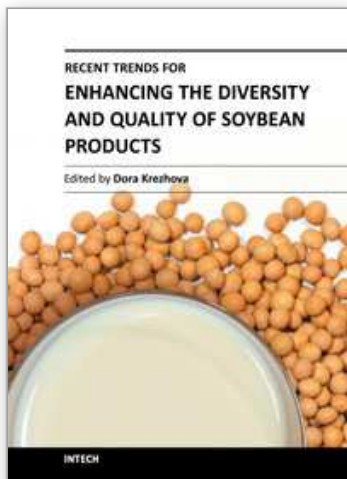
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