The Estrogenic Effects of the Soy Phytoestrogen Genistein on the Liver and Bone of Chickens

by

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Abstract

Commercial chickens are known to develop various estrogen dependent diseases, including liver steatosis and osteoporosis. Studies involving both humans and animals have indicated that consuming soy protein may reduce the incidence of both these diseases. Soy phytoestrogens have been shown to reduce lipid accumulation in the liver and increase bone mineral content and bone density in humans. No previous research has been performed on the effects of genistein in the liver or bone of chickens.

Abnormal liver lipid accumulation, known as fatty liver or hepatosteatosis, is associated with many factors including plasma estrogen levels, excessive alcohol consumption, metabolic derangements, and obesity. Research evidence suggests that soy phytoestrogens may have a protective effect against liver lipid accumulation induced by estrogens in mammals. A series of studies was performed to determine if genistein would have a protective effect against liver lipid accumulation induced by exogenous estrogen in the chicken. Three experiments were performed using different types and ages of chickens; aged hens, mature hens, and male broiler chicks. Genistein doses were given by a daily oral gavage for fourteen days. Estrogen doses were given by injection in the subcutaneous tissue in the back of the neck three times during each experiment. There were no significant differences in most of the items measured. Exogenous estrogen was used to induce heavy liver lipid accumulation, but failed to induce this accumulation in

all of the experiments. Because of this, it was difficult to determine if genistein had a protective effect on the liver.

Research has suggested that the primary soy phytoestrogen, genistein, may help alleviate osteoporosis in women by increasing bone density through its estrogenic action. It is unknown whether genistein may improve bone density in aging laying hens or actually contribute to the problem through negative interactions with the hen's endocrine system. The objective of this study was to characterize and quantify the effects of the genistein on bone physiology in aged hens. Sixty White Leghorn laying hens, which were 3 years old and had never been through an induced molting process, were randomly selected and divided into 4 treatments: Sham Control, Low Genistein, Medium Genistein, and High Genistien. Doses were given by subcutaneous injection in the back of the neck every other day for 8 weeks. There were significant differences between femurs belonging to birds in the High Genistein and Medium Genistein treatments for all of the bone parameters measured. Neither the Low Genistein treatment (10 mg/kg body weight) nor the Medium Genistein treatment (15 mg/kg body weight) showed a significant positive effect on the bone as compared to the Sham Control. The results of this study suggest that the High Genistein treatment (20 mg/kg body weight) has a beneficial effect on femur quality of aged laying hens.

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This manuscript is dedicated to the author's parents, John and Colleen Stevenson, and siblings, Nichole and Matthew, for always being there and believing anything is possible.

Who would have thought that pet chickens would lead to this?

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General Introduction

Isoflavones from soybeans and other plants have become an area of interest to researchers because of their many estrogenic, antifungal, and antibacterial activities. Soybeans and soy-based products are the most significant sources of isoflavones for humans and livestock (Kurzer et al., 1997). Soybeans contain three major isoflavones: genistein, daidzein, and glycitein. Processing of soybeans for livestock feed does little to alter the isoflavone content of the resulting meal. Because of this, defatted soybean meals will contain essentially all of the isoflavones present in the starting soybeans (Eldridge and Kwolek, 1983). Alcohol extraction can be used to extract isoflavones and produce low isoflavone soy protein products.

Genistein is the primary soy isoflavone and shares structural features with the endogenous animal estrogen, 17-β estradiol. This structural similarity allows genistein to bind to estrogen receptors and sex hormone binding proteins. Through binding to estrogen receptors, genistein can exert both estrogenic and anti-estrogenic activity (Dixon and Ferreira, 2002). Genistein can also displace bound estrogen and testosterone from human sex steroid binding protein. This affects the clearance rates of androgens and estrogens and the availability of the hormones to target cells (Dixon and Ferreira, 2002).

The estrogenic activity of genistein and other isoflavones, with the possibility of both positive and negative effects on human health, have made them subjects of considerable research interest. Diets containing high levels of isoflavones are known to

negatively affect the fertility of livestock and laboratory animals. As with livestock feeds, most laboratory animal feeds contain soybeans or alfalfa, which are rich in isoflavones (Boettger-Tong et al., 1998; Degen et al., 2002).

The development of a fatty liver occurs widely in humans and animal species. It is often a response to various forms of acquired or inherited metabolic disorders (Hermier et al., 1988). Excessive lipid accumulation in the liver occurs commonly in avian species, especially in the laying hen. Research with human subjects suggests that soy protein may have beneficial effects on insulin resistance and may slow or stop the pathologic progression of diseases caused by excessive accumulation of lipid in the liver (Yang et al., 2011). Several studies on both humans and animals have indicated that consuming soy protein can improve lipid profiles, may decrease hepatic fat, and decrease the incidence of fatty liver (Yang et al., 2011).

The selection of the modern hen to continuously produce eggs over a prolonged period makes it highly susceptible to osteoporosis (Whitehead, 2004). Soy isoflavones have been shown to increase bone mineral content and bone density in humans (Greendale et al., 2002; Fitzpatrick, 2003; Sahin et al., 2007). Research has also shown that soy isoflavones are able to improve the absorption of calcium from the intestines (Arjmandi et al., 2002; Sahin et al., 2007).

Review of Literature

Estrogens

Estrogens perform numerous physiological actions and are a member of the steroid hormone family (Norris, 1985; van Tienhoven, 1983; Korach et al., 1997; Bennink, 2003). This hormone family also contains androgens and progestagens (Saunders, 1998). The base core of all steroid hormones consists of seventeen carbon atoms formed into four fused rings. Three of the rings are cyclohexane rings. The fourth ring is a cyclopentane ring. The various steroid types differ based on the functional groups that are attached to the rings and by the oxidation state of the rings (Korach et al., 1997).

Steroid hormones are synthesized from the cholesterol molecule (Goldstein and Sites, 2002). As with all steroid hormones, the production of estrogens involve a very complex pathway (Figure 1). The first step in the pathway produces androstenedione from 17-hydroxyprogesterone. Androstenedione is an important intermediary in the pathway. A portion of the androstenedione produced is then converted into testosterone (Korach et al., 1997). The aromatization of the A-ring of testosterone by aromatase enzymes, found primarily in the gonads, converts testosterone into an estrogen (Bennink 2003). This conversion allows for the regulation of both hormones in the bodies of both male and female animals. Aromatase enzymes can also be found in the placenta, brain, adipose tissue, and many other tissues (Korach et al., 1997).

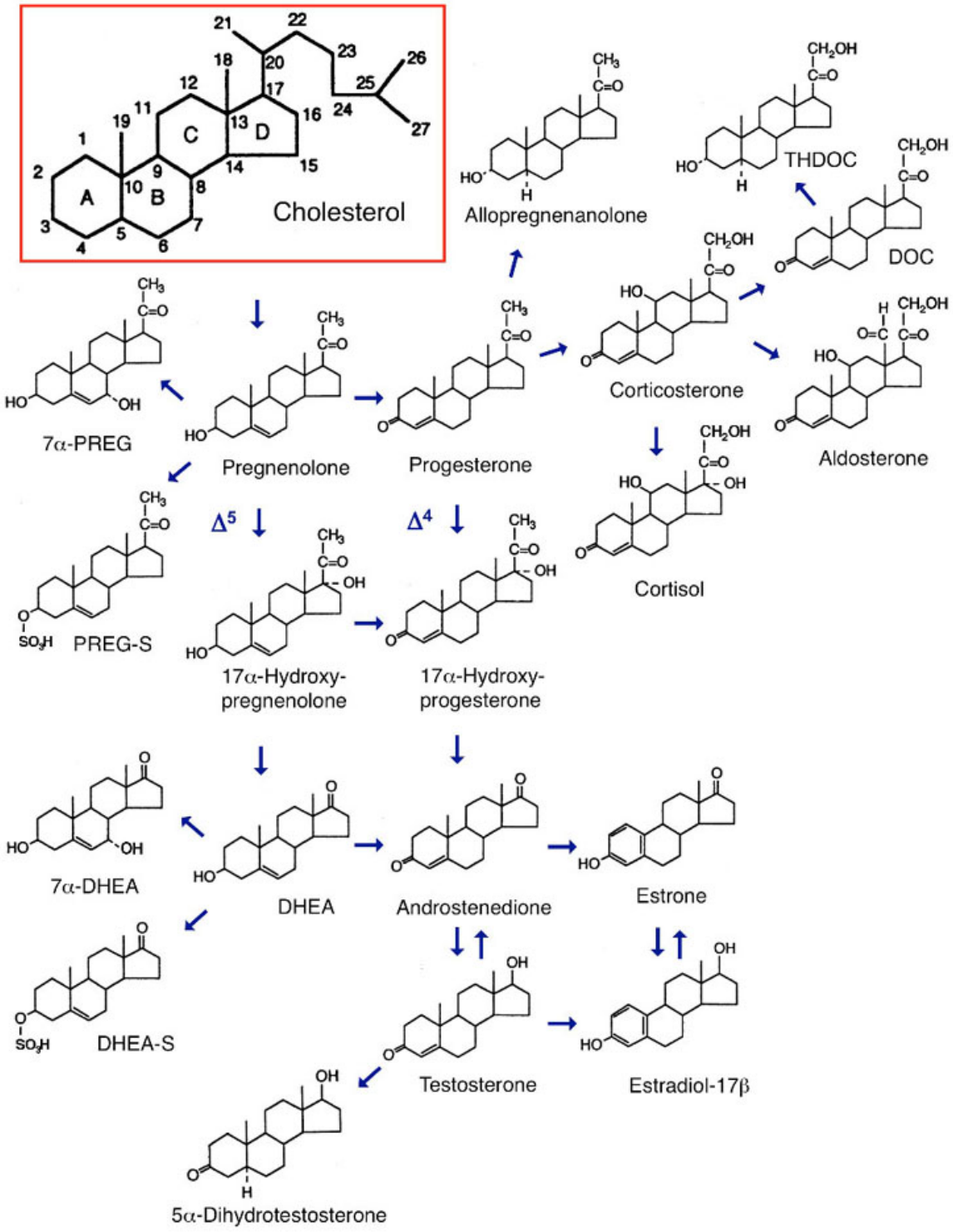


Figure 1: The steroid hormone synthesis pathway. (Sakamoto et al., 2012)

There are three endogenous or naturally occurring estrogens in vertebrate animals: estradiol, estrone, and estriol (Figure 2). Estradiol or 17_{β}-estradiol is the most potent naturally occurring estrogen. It is the predominant estrogen during the premenopausal or earlier period of life (Bennink, 2003). Estradiol is mainly secreted by the ovaries and has the greatest bioavailability of the three endogenous estrogens (Korach et al., 1997). It can be reversibly oxidized into esterone, but its bioavailability is reduced 10-fold by this oxidation (Korach et al., 1997; Birkhauser, 1996). Estrone is the main estrogen in female mammals after menopause (Bennink, 2003). Estriol is produced in large quantities by the placenta during pregnancy (Bennink, 2003). It is synthesized through a process independent of estradiol (Lieberman, 1996).

Figure 2: Structure of endogenous estrogens. (Desai, 2000)

Steroid hormones are lipid soluble and can pass easily through cell membranes. They are able to easily diffuse in and out of all cells and are retained with high affinity in specific target cells. These target cells contain intra-nuclear binding proteins called estrogen receptors (ER) (Kuiper et al., 1997). Most of estrogen's effects are mediated thorough its binding to estrogen receptors (Matthews et al., 2000). There are two receptors specific to estrogen, ERα, and ERβ. Estradiol is able to bind well with both.

Steroid hormones are carried through the blood bound to carrier proteins such as sex hormone binding globulin. These proteins allow estrogens to be easily transported to

the target cell without having effects on other cells (Korach et al., 1997). Estradiol is able to freely enter target cells and bind with the ERs. After binding, estradiol enters the nucleus of the cell and regulates gene transcription. This leads to the formation of messenger RNA. The messenger RNA then interacts to produce the specific proteins needed to express the effect of estrogens on the cell (Kuiper et al., 1997).

Throughout life, estrogens influence the growth, development, behavior and regulation of reproductive tissues in all vertebrates (Matthews et al., 2000; Zava et al., 1997; Peterson and Barnes, 1996; Femo et al., 2000). Estrogens are involved in the development and function of both the male and female genital tract and in the development of female secondary sexual characteristics (Norris, 1985; van Tienhoven, 1983; Sonnenschein and Soto, 1998). They are also involved in the sexual differentiation of the nervous system and in the development and function of bone (Sonnenschein and Soto, 1998; Azuma et al., 2004; Norris, 1985; van Tienhoven, 1983). Estrogens protect against loss of bone mineral density through the inhibition of bone resorption and can increase bone mineral density in a dose-dependent manner (Bennink, 2003). They can also induce the synthesis and secretion of cell specific proteins such as vitellogenin in avian species (Sonnenschein and Soto, 1998; Norris, 1985; van Tienhoven, 1983). Estrogens are involved in vascular dilation and can regulate circulating cholesterol levels, which provide protective effects on the vascular system (Jordan, 1996; Azuma et al., 2004).

Estrogen Receptors

The nuclear receptor superfamily is a large family of intracellular receptor proteins that bind to specific signal proteins. Members of this superfamily possess

modular structures composed of a cysteine-rich DNA-binding domain in the middle of the receptors. They also contain a carboxy-terminal hormone-binding domain. The amino-terminal ends of the various receptors in this family can vary extensively in both content and size (Cato and Ponta, 1989).

There are multiple classes of receptors in the nuclear receptor superfamily. One class of receptors is designated by having a high-affinity ligand-receptor interaction. Receptors in this class include estrogen, androgen, and vitamin D (Shay and Banz, 2005). Another class of receptors has a lower affinity for ligands. This class includes peroxisome proliferator-activated receptors. A third class of nuclear receptors is called the "orphan receptors" (Shay and Banz, 2005). These orphan receptors are the group of nuclear receptors that have not yet had a specific receptor ligand identified. It is possible that some orphan receptors may not actually have a ligand that will activate them (Shay and Banz, 2005).

There are two known types of estrogen receptors in mammals and birds. The two N-terminal domains of these types are similar. The DNA binding domains share 96% identity and the ligand binding domains are 50-60% identical (Harris et al., 2002). Although these types of estrogen receptors are so similar, the ability of a compound to selectively bind to a particular estrogen receptor subtype can be species dependent (Harris et al., 2002). Estrogen receptors in different animals have been shown to have different structures. The estrogen receptors from human, mouse, chicken, reptiles, pig, and fish exhibit differential preferences for ligands and relative binding affinities for many natural and synthetic compounds (Matthews et al., 2000). One example of these differential preferences is that the pig estrogen receptor exhibits a significantly greater

affinity for the estrogenic mycotoxin zearalenol than does the estrogen receptor from the chicken (Fitzpatrick et al., 1989).

Estrogen receptor alpha (ERα) was the first estrogen receptor to be identified and is found mainly in the uterus and mammary gland (Gustafsson, 2000). It consists of 595 amino acids with a molecular weight of 66kDa (Kuiper et al., 1996; Furlow et al., 1990; Grohe et al., 1998). After binding to the ligand ERα dimerizes and becomes a homodimer. The homodimer then binds to estrogen response elements (EREs) in the transcriptional control regions of the target genes (Mosselman et al., 1996).

Estrogen receptor beta (ERβ) was discovered nearly 10 years after ERα. It is a protein that consists of 485 amino acids with a molecular weight of 54.2 kDa (Kuiper et al., 1996; Furlow et al., 1990; Grohe et al., 1998). It can bind estradiol and transactivate estrogen regulated reporter genes. Estrogen receptor beta is a less efficient activator of estrogen dependent genes than ERα and anti-estrogens can inhibit its effect (Mosselman et al., 1996; Kuiper et al., 1996).

Estrogen receptors are modular in structure and consist of six distinct domains (Evans, 1988). A typical estrogen receptor contains a hypervariable N-terminal region that is called the activation function 1 (AF-1) that contributes to the transactivation function. They have a DNA binding domain, a hinge region, and a C-terminal ligand-binding domain called activation function 2 (AF-2) (Azuma et al., 2004). The dimerization function of AF-2 is very important since dimerization is a prerequisite for functional activity of the estrogen receptors (Cato and Ponta, 1989). Both of the AF-1 and AF-2 domains of estrogen receptor alpha have been shown to have a transcriptional activation function (Tora et al., 1989). They both also interact with transcriptional

mediators and co-factors (Endoh et al., 1999; Rachez et al., 1999; Belandia et al., 2002; Belandia and Parker, 2003; Fernandes et al., 2003).

Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that are involved in cellular lipid homeostasis and insulin action (Chinetti et al., 2000; Neve et al., 2000; de Villiers and Smart, 1999; Napoli et al., 2001; Nagy et al., 1998). PPARs have been called promiscuous receptors because they can be activated by many different ligands (Mezei et al., 2003). They can regulate the transcription of genes involved in lipid and glucose homeostasis. PPARS can also regulate lipid metabolism within the cell (Mezei et al., 2003). There are two distinct types of PPARs, alpha (PPARα) and gamma (PPARγ). PPARα generally controls the transcription of many of the genes involved in lipid catabolism. PPARγ generally controls the expression of genes involved in the differentiation of adipocytes and in insulin sensitization (Neve et al., 2000). The activation of both types of PPARs leads to increases in beta-oxidation and insulin sensitization. It also leads to reductions in blood and liver lipid concentrations (Picard and Auwerx, 2002).

When a ligand binds to the peroxisome proliferator-activated receptor, the receptor becomes activated. This causes it to bind to peroxisome proliferator response element (PPRE) sequences that are located within the promoter regions of PPAR-regulated genes (Neve et al., 2000; Qi et al., 2000). PPARs have been shown to have multiple ligands. These include some unsaturated fatty acids and their derivatives and drugs used to treat diabetes such as glitazones (Neve et al., 2000; Ricote and Glass, 2001). Phytoestrogens have also been shown to activate PPAR-mediated gene expression

(Mezei et al., 2003). The primary soy isoflavones, genistein and daidzein, have been shown to activate both PPARα and PPARγ. This activation may explain the lipid-lowering effect seen with soy consumption (Mezei et al., 2003; Shay and Banz, 2005).

Soy isoflavones have been investigated for their estrogenic actions for many years. Research into their actions on other nuclear receptors has recently begun (Shay and Banz, 2005). Studies have shown that an anti-estrogenic compound does not block the adipogenic activity of genistein. This suggests that the adipogenic activity of genistein is due to a mechanism other than activation of the estrogen receptors (Dang et al., 2003). The adipogenic activity of genistein could be due to activation of PPARγ. Genistein has been shown to dose-dependently stimulate PPAR-directed gene expression in micromolar ranges (Dang et al., 2003). Based on the various in vitro results of Dang et al., it is thought that the effects of genistein at low concentrations are similar to estrogen-mediated effects and the effects of genistein at higher concentrations are similar to PPAR-mediated effects (Shay and Banz, 2005).

Phytoestrogens

Phytoestrogens are chemicals produced by plants that resemble endogenous animal estrogens in either their structure or function (Whitten and Naftolin, 1998; Bradbury and White, 1954). The term phytoestrogen first appeared in the literature in the late 1980's (Dixon, 2004). The classical definition of phytoestrogens refers to compounds that can exert estrogenic effects on the central nervous system, induce estrus, and stimulate growth of the genital tract of female animals (Lieberman, 1996).

There are three main classes of phytoestrogens. All three classes include diphenolic compounds with structural similarities to natural and synthetic estrogens and

anti-estrogens (Kurzer, 1997). The three classes are isoflavones, coumestans, and lignans (Figure 3). Of the three, isoflavones are the most prevalent and have the largest known effects on human and animal health.

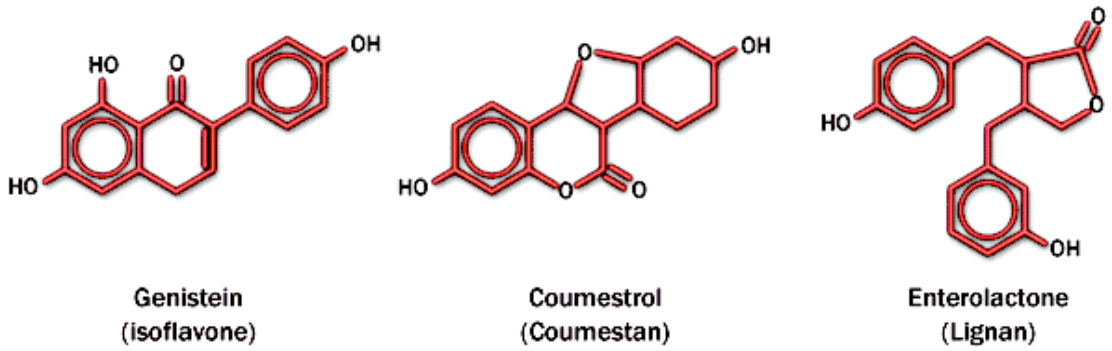


Figure 3: Structure of the three types of phytoestrogens. (Riuz-Larrea and Ruiz-Sanz, 2008)

Phytoestrogens may exert their biological activity in many different ways. One way is by mimicking the action of endogenous estrogens produced in animals. They can act as estrogen agonists or antagonists. They can also act by altering the pattern of synthesis and metabolism of endogenous hormones or by modifying hormone receptor values in the animals (Sonnenschein and Soto, 1998; Whitten et al., 1995; Dibb, 1995).

Phytoestrogens are able to exert estrogenic effects through binding to estrogen receptor (ER) alpha or beta (Figure 4). Unlike endogenous estrogens, many phytoestrogens preferentially bind to ERβ. In order to bind with estrogen receptors, the phytoestrogen must have a specific chemical structure (Adams, 1995a). A phenolic ring structure is necessary for binding and any estrogen-like activity (Korach et al., 1997). This ring allows binding to the ER by mimicking the ring of the endogenous estrogen that binds with ER binding site. The effects phytoestrogens have are dose-dependent and vary based on the specific tissue.

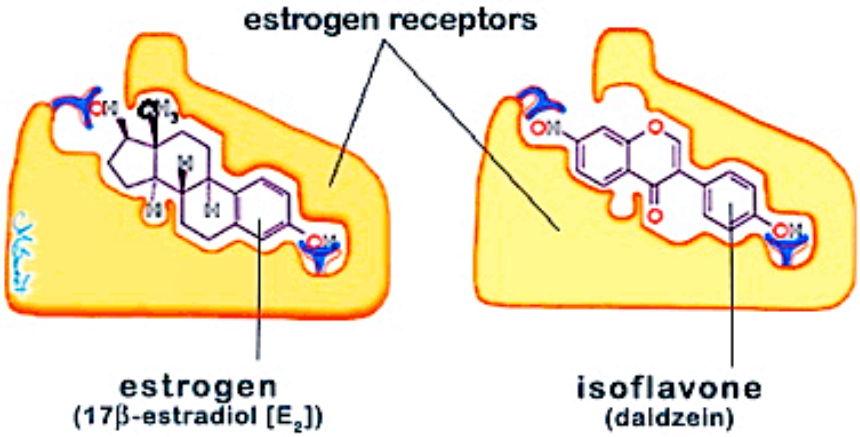


Figure 4: Estrogen and daidzein bound to estrogen receptors. (Women to Women, 2008)

Phytoestrogens can also act by altering the pattern of synthesis and metabolism of endogenous hormones (Sonnenschein and Soto, 1998; Whitten et al., 1995; Dibb, 1995). Phytoestrogens often mimic the actions of endogenous estrogen, but their effects are not necessarily identical (Adams, 1995a). In addition to binding to ERs, phytoestrogens can exert effects by changing the concentration of circulating endogenous estrogens. They can inactivate various enzymes or change estrogens' bioavailability by stimulating or suppressing the carrier protein, sex hormone binding globulin, which transports estrogens throughout the body (Korach et al., 1997).

Phytoestrogens do not bioaccumulate in the body and remain unbound or free while in the blood stream. They are generally easily metabolized and excreted quickly (Thomas 1997). Although they spend relatively little time in the body, they can have significant effects on body systems (Barrett, 1996). Phytoestrogens may act as either estrogen agonists or antagonists depending on the dose, duration of use, individual metabolism, intrinsic estrogenic state, age of the animal, and number of repeated exposures (Barrett 1996; Cassidy, 2003; Zhao et al, 2005; Kaladas and Hughes, 1989;

Whitten and Patisaul, 2001). The ability for the effects of phytoestrogens to be reversed also depends on these same factors (Kaldas and Hughes, 1989; Dibb, 1995).

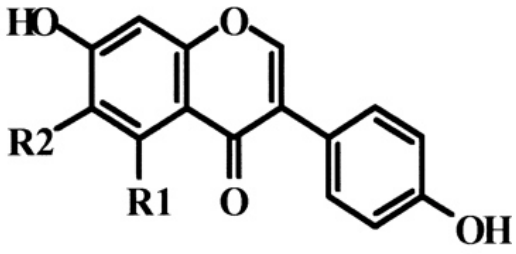
A low dose phytoestrogen diet can induce developmental and maturational abnormalities in many different types of animals (Burton and Wells, 2002). The apparent relative estrogenic potency of phytoestrogens is dependent upon the type of assay (*in vitro or in vivo*) used to measure hormonal activity. The effects of phytoestrogens on livestock and laboratory species have been studied most extensively. Field biology studies in many wild herbivore species are lacking (Hughes, 1988). Decreased fertility, due to dietary intake of phytoestrogens, has been reported in several different species (cattle, cheetahs, guinea pigs, mice, quail, rabbits, and sheep) (Chapin et al., 1996).

There are many different theories on why plants produce phytoestrogens. One theory is that they produce them to mimic the action of endogenous animal estrogens. The phytoestrogen acts as a defensive substance allowing plants to diminish the fertility of herbivores that feed on them (Hughes, 1988). Scientists disagree on this theory though, because plants generally use non-nutrient compounds for this type of chemical defense. Examples of these non-nutrient compounds include fiber, lignin, cellulose, toxicants, essential oils, and other volatile substances (Radwan, 1974). Other theories on the production of phytoestrogens include them acting to regulate plant hormones, protect plants against ultraviolet radiation, or act as fungicides (Barrett 1996).

Soybean Isoflavones

Isoflavones are polyphenolic compounds that are mainly produced by beans and other members of the legume family. The four most common isoflavones are genistein, daidzein, formononetin, and biochanin A (Figure 5). They are closely related to the

antioxidant flavonoids found in many plants, vegetables, and flowers and are produced through the same branch of the phenylpropanoid pathway (Thomas, 1997). This pathway begins with the amino acid phenylalanine and produces the intermediary compound naringenin. In legumes, naringenin is then converted to genistein by two legume-specific enzymes, isoflavone synthase and adehydratase. Another intermediate in the pathway, naringenin chalcone, is converted into daidzein. Isoflavones such as genistein and daidzein are only found in some plant families, because most plants do not contain the enzyme that converts the flavone precursor into an isoflavone (Dixon, 2004).



Compounds	R1	R2
genistein	OH	H
daidzein	H	H
glycitein	H	OCH_3

Figure 5: Structure of Common Isoflavones. (Pan et al., 2001)

Soybeans are the major source of isoflavones for animal and human nutrition. Soy isoflavones are each found in four chemical forms. The unconjugated forms or aglycones are daidzein, genistein and glycitein. The glucosides are daidzin, genistin, and glycitin. There are also acetylglucosides and malonylglucoside (Kudou et al., 1991). Soy isoflavones exist naturally in the glycoside form (Eldridge and Kwolek, 1983). The malonylglucosides are the predominant isoflavones in soybeans. Concentrations of the glucoside, acetylglucoside, and aglycone forms tend to increase during extraction, processing and cooking (Wang, 1994a; Coward, 1998).

Isoflavones exist in plant tissues as sugar derivatives called glycosides. Once plant isoflavones are consumed, these compounds are hydrolyzed into aglycones in the

digestive tract. From there, they can be excreted, absorbed into the gut, or metabolized further (Barrett, 1996). Aglycones are more bioavailable to animals than their glycoside precursors (Kurzer, 1997). The isoflavone aglycones and their metabolites are absorbed in the intestine, where they are mostly conjugated into glucuronides and/or sulphates prior to their release into the blood and transport to the liver (Wu et al., 2004).

Plants also use isoflavones and their derivative compounds to ward off disease-carrying fungi and microbes. Concentrations of the various isoflavones in plants vary depending on stress from prior viral, bacterial, fungal, or herbivore attacks. It is thought that soybean isoflavones induce the *nod* genes in *Bradyrizobium japonicum* to form nitrogen-fixing root nodules (Cho and Harper, 1991; Kape et al., 1991; Smit et al., 1992). Soybean isoflavones are also associated with the response of soybeans to infection by *Phytophthora megasperma* (Graham et al., 1990).

The various methods used in the production of foods (heat processing, enzymatic hydrolysis, and fermentation) can significantly alter the isomeric distribution of the isoflavones in soy foods. The amount of the malonyl forms, which are heat sensitive, will be different for raw and heat-treated soybeans (Wang and Murphy, 1996; Kurzer, 1997). The heat processing involved in producing dry soymilk reduces the concentration of malonylglucosides and increases the concentrations of glucosides and acetylglucosides (Song et al., 1998). Isoflavones are not removed in the process used to extract oil from soybeans. The resulting defatted soybean meals will contain essentially all of the isoflavones of isoflavone glucosides present in the starting soybeans (Eldridge, 1983).

Isoflavones are phenylpropaniod-derived compounds that constitute from 0.3% to more than 0.8% of the soybean seed on a dry weight basis or as much as 3 mg/g dry

weight (Lee et al., 2003). Nearly 90% of the total isoflavones are located in the cotyledon with the rest located in the hypocotyls (Tsukamoto et al., 1995). It is estimated that the isoflavone content in soybean ranges from 0.14-1.53 mg/g and from 1.3-1.98 mg/g in soy flour. (Safford et al., 2003)

The content of phytoestrogens within plants varies from place to place depending upon several factors. These factors include the climate, humidity, age of the bean, temperature during germination, general location, and amount of fertilizer used (Thomas, 1997; Chapin et al., 1996). This means non-controllable conditions such as temperature, rainfall, soil fertility, pest and disease damage could modify the levels of these compounds. The isoflavone content of soybeans can vary by year and variety by a factor of 3–5 (Franke et al., 1994). The variation in the isoflavone content of soybeans is reflected in soy products that are made from different crop years and different varieties of soybeans (Song et al., 1998).

The yearly difference in isoflavone content can also be associated with the temperature during seed development. Research has shown that seeds maturing at low temperatures had greater isoflavone concentrations than seeds maturing at high temperatures (Lee et al., 2003). Isoflavone concentrations were also found to increase in soybeans that were produced on low- to medium- potassium soils (Wu et al., 2004).

Soybean isoflavones, mainly genistein and daidzein, possess numerous biological activities that seem to support the health of normal cells. Isoflavones also encourage apoptosis in diseased cells (McCue and Shetty, 2004) and can affect the development of reproductive and non-reproductive tissues (Mardon et al, 2008). In the absence of estrogens, isoflavones have been shown to have a weakly estrogenic or agonistic effect.

They may exhibit anti-estrogenic, or antagonistic, effects when estrogens are present (Cline and Hughes, 1998). Reproductive problems, infertility, thyroid disease and liver disease has been attributed to dietary intake of isoflavones in several species of animals including mice, cheetah, quail, pigs, rats, sturgeon, and sheep (Fallon and Enig, 2000).

Isoflavones may be used as a feed supplement to decrease fat deposition in animals due to their ability to act as an estrogen antagonist (Payne et al., 2001). At very high concentrations, soy isoflavones depress the growth rate and feed conversion ratio in commercial broiler type chickens (Payne et al., 2001). Supplementing soy isoflavones to diets was shown to increase growth rate and carcass muscling in pigs (Sahin et al., 2007). Research has also shown that laboratory animals consuming soy, in place of other proteins, generally developed 25-50% fewer tumors than control animals, although not all studies show these protective effects (Kurzer, 2003).

Genistein

Genistein (4', 5, 7-trihydroxyisoflavone) is biosynthetically the simplest of the isoflavone compounds of the soybean (Barnes and Peterson, 1995; Dixon and Ferreira, 2002). It was first isolated in 1899 from the woody plant known as Dyer's Bloom or *Genista tinctoria*. Genistein received its chemical name from this plant and was first synthesized in the laboratory in 1928 (Walter, 1941). It is a central intermediate in the biosynthesis of more complex isoflavones (Dixon and Ferreira, 2002). Genistein is a diphenolic planar molecule with aromatic A-ring and a molecular weight similar to steroidal estrogens (Lamartiniere et al., 1995). Genistein contains a phenolic ring structure (Figure 6), which is necessary to bind to the estrogen receptors in animals (Setchell, 1998).

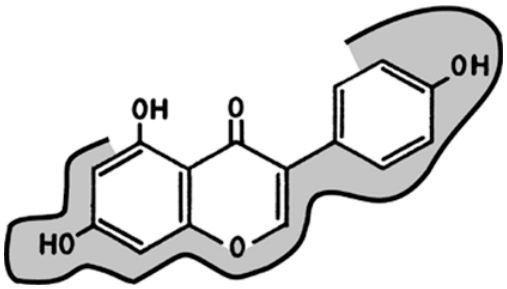


Figure 6: Structure of Genistein. (Demmig-Adams and McCauley, 2005)

Once consumed, genistein is naturally deglycosylated and is absorbed into the blood stream where it is converted into the inactive β-glucuronide form (Bennetau-Pelissero et al., 2001;Yuan et al., 2003). Genistein's principal metabolite, 7-O-β-glucuronide, is easily absorbed from the intestines, extracted from the portal blood, and excreted into bile (Sfakianos et al., 1997). It is taken up by the liver and undergoes efficient enterohepatic circulation. This allows genistein to be accumulated in the enterohepatic circuit or be excreted with a long half-life. In the first twenty-four hours genistein is quickly excreted. It then shows a slow rate of excretion (Sfakianos et al., 1997). The bioavailability of genistein has been determined based on renal excretion times (Xu et al., 1994).

Genistein undergoes rapid clearance from blood serum following injections in mice, but with a high dose, it often collects under the skin at the injection site (Supko and Malspeis, 1995). When injected in high doses, genistein treatment results in alterations to ovarian steroid hormone production. This is likely to occur through changes in signaling through the hypothalamic-pituitary-ovarian axis (Cotroneo et al., 2001). Genistein has been shown to bind estrogen receptors in the uterus, to be uterotrophic, and act on sexual differentiation in mammals (Levy et al., 1995; Hopper et al., 1998; Milligan et al., 1998).

Overall, the effects of genistein can be simply summarized by stating that it delays reproduction in females and accelerates it in males (Bennetau-Pelissero et al., 2001).

Unlike other isoflavones, genistein exerts toxicity only at concentrations greatly in excess of those at which it first exerts its biological and pharmacological effects (Dixon and Ferreira, 2002). This makes it a potentially important molecule for research in dietary cancer chemoprevention. Research has shown that genistein and other related isoflavones can have protective effects on breast cancer, inhibit tumor cell growth, and induce apoptosis in cancer cells (Spinozzi et al., 1994; Constantinou et al., 1998; Katdare et al., 2002). They can also inhibit the development of chemically induced cancers, such as cancers of the stomach, bladder, lung, prostate, and blood (Dixon and Ferreira, 2002). The inhibition of the growth of human stomach cancer cell lines *in vitro* by genistein and biochanin A involves stimulation of a signal transduction pathway that leads to apoptosis (Yanagihara et al., 1993). Genistein demonstrates a broad range of antioxidant and free radical-scavenging activity (Yen and Lai, 2003; Rimbach et al., 2003) and can suppress oncogene expression, and induce cell differentiation of malignant cells (Okura et al., 1988; Zwiller et al., 1991; Yamashita et al., 1990; Linassier et al., 1990).

Genistein has been shown to inhibit protein tyrosine kinases, DNA topoisomerases, and angiogenesis in *in vitro* experiments (Blair et al, 1996; Barrett, 1996). Genistein is a well-known inhibitor of tyrosine kinases that are central components in signal transduction processes (Chapin et al., 1996). These tyrosine kinases include protein kinase C (PKC) and a protein tyrosine kinase (PTK) (Osada et al., 1988; Akiyama et al., 1987). Genistein can also influence various cell functions through protein kinase A (PKA) and estrogen receptor activation (Makarevich et al., 1997). In addition,

genistein has been shown to inhibit DNA topoisomerase II, cell cycle progression, angiogenesis, and various oxidation reactions (Barnes and Peterson, 1995; Peterson, 1995; Barnes et al., 1995). Genistein also modulates tissue differentiation, cell proliferation, and the growth and development of neoplasia in several organs (Peterson and Barnes, 1993; Rokhlin and Cohen, 1995; Peterson and Barnes, 1996).

Foods that are rich in genistein, such as soybeans, may have a beneficial effect on osteoporosis in postmenopausal women without the negative side effects associated with hormone substitution (Wisemann, 2000). Genistein has been shown to bind preferentially to ER-β. It is able to stimulate the activity of osteoblasts, which is thought to be related to ER-β, without inducing cellular proliferation in breast or uterine tissue (Windahl et al., 2000). This osteoblastic activity suggests that genistein may be useful as an inhibitor of osteoclasts at pharmacologically attainable levels (Blair et al., 1996). Genistein is able to inhibit osteoclastic activity directly by a mechanism independent of cellular attachment. It does this at levels similar to those necessary to inhibit tyrosine kinase auto-phosphorylation *in vitro* (Blair et al., 1996).

Hepatic Steatosis

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disorder in affluent societies and is the most common cause of abnormal liver tests (Ayala et al., 2009). NAFDL is a complex disorder involving environmental factors and genetic predisposition (Martin-Castillo et al., 2010). It represents the metabolic consequences of over-nutrition and altered dietary intake on the liver coupled with low physical activity and sedentary lifestyles (Ayala et al., 2009). Non-alcoholic fatty liver disease describes a group of liver diseases that vary in severity. These liver diseases include simple steatosis

(non-alcoholic fatty liver) and the progressive, inflammatory form of non-alcoholic steatohepatitis (NASH).

The term non-alcoholic steatohepatitis (NASH) was first introduced in 1980. Liver biopsies from patients with NASH show steatosis with changes similar to biopsies from patients with alcoholic hepatitis (Yang et al., 2011). NASH ultimately leads to cirrhosis of the liver and the development of hepatocellular carcinoma without excessive alcohol intake (Martin-Castillo et al., 2010).

The clinical characteristics of NASH include obesity, hyperlipidemia, diabetes mellitus, and hypertension. These characteristics have also been associated with insulin resistance (Yang et al., 2011). Non-alcoholic fatty liver disease is characterized by the accumulation of more than 5-10% intracellular lipids in the liver. It also involves hepatomegaly, increases in serum aminotransferases, and low-grade systemic inflammation (Salih et al., 2009). Hepatic steatosis is the initial stage of chronic non-alcoholic fatty liver disease. It is characterized by a single large fat droplet that displaces the nucleus or by multiple smaller intra-cytoplasmic droplets (Kim et al., 2010). Steatohepatitis can be distinguished from simple steatosis with the presence of hepatocyte ballooning (Ayala et al., 2009).

Although the etiological mechanisms of NASH have not been fully understood, the “two hit theory” provides the most widely accepted explanation describing the progression of NASH. The first hit is fat accumulation in liver hepatocytes, which marks the first stage of NASH development. The second hit is aggravating factors such as inflammatory cytokines, oxidative stress of endotoxins, which contribute to hepatocyte injury (Ji et al., 2011). Non-alcoholic steatohepatitis has recently become a focus of

medical attention with the increases in the incidences of obesity and hyperlipidemia (Ayala et al., 2009). Dietary habit modification is an important factor in the treatment of NASH and may help decrease the accumulation of hepatic lipids and improve insulin resistance (Yang et al., 2011).

The development of a fatty liver occurs widely in human and animal species. It is often a response to various forms of acquired or inherited metabolic disorders (Hermier et al., 1988). It can also be a response to toxins, nutritional deficiencies/excesses, or infections. There are few natural animal populations in which to study the metabolic adaptations resulting in hepatic steatosis as well as individual responsiveness. They include the fasting suncus, lactating cow and wild migration species of birds and fishes. In these migrating species, hepatic steatosis occurs spontaneously as a consequence of energy storage before migration (Pilo and George, 1983). As a result of the complexity of the spectrum of NAFLD, animal models may provide the necessary tools to overcome variables such as genetic heterogeneity, gender differences, and environmental factors, including diet and lifestyle (Martin-Castillo et al., 2010).

Fatty Liver Syndrome

Hepatic lipidosis, or fatty liver syndrome (FLS), often precedes the development of fatty liver hemorrhagic syndrome (FLHS) in chickens (Van Elswyk et al., 1994). FLHS is characterized by obesity, reduced egg production, and hemorrhages of the liver (Stake et al., 1981). It is also characterized by an increase in the amount of fat in the liver. Chickens whose liver contains more than 5% of the wet weight of the liver as lipids are considered to have FLHS (Hermier et al., 1988).

Fatty liver occurs commonly in avian species, especially in the laying female. The sexual dimorphism observed in the incidence of hepatic lipidosis is likely related to the influence of lipogenic steroid hormones such as estradiol. When a bird starts laying eggs, there is a rise in circulating estradiol levels, followed by dramatic increase in hepatic lipogenesis. This results in hyperlipidemia (Hermier et al., 1996). The liver is the major site of lipogenesis in chickens (Pearce, 1977; Henderson and Sargent, 1981).

In female domestic poultry, hepatic steatosis is related to changes in lipid metabolism that are associated with egg formation and vitellogenesis (Hermier et al., 1988). Vitellogenesis is the accumulation of lipids, especially triglycerides, in the growing oocytes (Hermier et al., 1996). Yolk lipids are synthesized in the liver and transported through the blood to be deposited in the yolk (Redshaw and Follett, 1972). Many other organs contribute to normal ovarian function, including the liver, adipose tissue, pancreas, and the hypothalamic-pituitary axis. These same organs can also be affected by fatty liver (Chen et al., 2006).

Feed-satiated broiler breeder hens often show lipotoxicity-like symptoms (Chen et al., 2006). In females, early rapid growth and unlimited feed access leads to enhanced adult fatness (Chambers et al., 1981; Havenstein et al., 2003a,b) and poor reproductive performance (Yu et al., 1992 a,b; Robinson et al., 1993). The restriction of feed intake by broiler breeder hens to approximately 50-60% of *ad libitum* reduces the incidence metabolic diseases and improves egg production (Chen et al., 2006). Research has shown that broiler breeder hens fed *ad libitum* during the rearing period undergo sexual maturation and begin laying eggs earlier than their feed-restricted counterparts (Chen et

al., 2006). Hens fed *ad libitum* also exhibit a large drop in egg production at an earlier age and produce fewer eggs overall (Bornstein et al., 1984).

A syndrome resembling fatty liver hemorrhagic syndrome in laying hens was reproduced in 6 to 7 week old male chickens with intramuscular injections of estradiol dipropionate (Pearson and Bulter, 1978). Estradiol dipropionate has been shown to influence hepatic lipidosis (Polin and Wolford, 1977). Intramuscular administration of exogenous estradiol every 4 or 5 days (5.0 or 7.5 mg/kg body weight) induced FLHS in both Rhode Island Red and white leghorn hens (Stake et al., 1981). *In vivo* and *in vitro* studies in the estrogenized bird clearly demonstrated that estrogens stimulate fatty acid synthesis by enhancing the activity of acetyl CoA-carboxylase and fatty acid synthetase (Hermier et al., 1996).

Avian models have contributed greatly to our understanding of vertebrate lipid metabolism because they are very sensitive to dietary changes (Ayala et al., 2009). The hyper-lipidemic chicken is a potential model for nonalcoholic steatohepatitis in humans and could be used for therapeutic trials (Martin-Castillo et al., 2010). The chicken model offers technical advantages over mammalian models and may help in the development of a more effective treatment strategy (Martin-Castillo et al., 2010).

Isoflavones Effect on Fatty Liver Syndrome and Hepatic Steatosis

Epidemiologic studies have shown that the morbidity of non-alcoholic steatohepatitis (NASH) in Japan is lower than that of the United States (Yang et al., 2011). This may be associated with the high consumption of soy products in Japan. The mechanism by which soy protein decreases serum and hepatic lipids has not yet fully been established. One possibility is that it causes an increase in bile acid excretion. This

is because soy protein can act as a dietary fiber to increase bile acid excretion and increase the rate of cholesterol re-synthesis in the liver (Yang et al., 2011).

Genistein, a major soy isoflavone, has been investigated for its hypolipedemic, anti-lipogenic, antioxidant, and estrogenic effects in various biological systems (Lee et al., 2006; Salih et al., 2009). Several studies in humans and animals have indicated that consuming soy protein can improve lipid profiles, decrease hepatic fat, and decrease the incidence of fatty liver (Yang et al., 2011). Genistein supplementation may reduce fat deposition, through the activation of fatty acid β-oxidation or through the inhibition of adipogenesis and lipogenesis (Kim et al., 2010).

The inhibitory and stimulatory effects of genistein on adipose tissues are likely dose dependent and related to its multiple actions (Dang, 2009). The balance among these various actions determines the final biological effects of genistein on adipocytes. Both ERα and ERβ are expressed in adipose tissues. The effects of genistein on adipocytes can be explained only in part by its preferential binding affinity for ERβ (Dang, 2009). The effects of genistein on adipose tissues are not likely due to a lack of estrogenic activity, but rather due to the activation of other pathways that counteract genistein's estrogenic effects (Dang, 2009). The effects of genistein on adipocytes had been shown to be dose dependent and opposite to those of estradiol. At concentrations between 0.1 and 10 μM genistein dose-dependently inhibited adipogenesis. It stimulated adipogenesis at concentrations above 10 μM (Dang, 2009).

Soy protein may improve the liver function of rats with NASH by lowering the lipid levels in the blood and liver, increasing the anti-oxidative capacity, and improving insulin resistance (Yang et al., 2011). Various studies have shown that genistein reduces

the deposition of fat in the adipose tissue of mice (Lee et al., 2006). Genistein is able to regulate adipogenesis and triglyceride storage, which leads to changes in the number and volume of adipocytes (Dang, 2009). Oral administration of genistein has been shown to decrease serum levels of ALT and AST and prevent the development of inflammation in NASH model rats (Ji et al., 2011). Genistein administration has also been shown to improve liver function, stop the progression NASH, ameliorate lipid peroxidation, and decrease serum and liver inflammatory factor levels and their mRNA expressions in the liver of these rats (Ji et al., 2011).

Bone

The basic structural component for all vertebrate animals is bone. Bone is made from a collagen matrix with layers of hydroxyapatite crystals of calcium phosphate (Whitehead and Fleming, 2000). The formation of new bone during growth requires the precise alignment of the fibers within the collagen matrix. This collagen matrix is then stabilized by intermolecular cross-linking that forms the base that holds the calcium phosphate crystals (Knott et al., 1995). The collagen matrix gives the bone its high tensile strength and is the major structural part of the bone. The mineral, or calcium phosphate, portion provides the bone's compressive stiffness (Knott et al., 1995). Any defect in the collagen matrix that forms the bone could lead to bone fragility and breaking (Knott et al., 1995).

The formation of bone starts in the lower, hypertrophic zone of the bone (Whitehead, 2004). During the growth of an animal, the long bones grow in both length and width. This growth occurs through two different processes. Lengthening or longitudinal growth of the bone occurs through endochondral ossification. The widening

of the long bones occurs through intra-membranous ossification (Whitehead, 2004). Intra-membranous ossification of the endosteal surface involves the resorption of bone. This allows the bone to widen as an expanding ring with the formation of new bone on the outer surface and resorption of bone on the inner surface (Whitehead, 2004). In the early growth period, the bone ring expands rapidly. The cavity within the ring does not become completely filled with bone before the resorption of the endosteal surface starts. As the growth of the bone slows down, the speed of filling of the cavity increases (Whitehead, 2004).

Bone is an incredibly complex tissue that is regulated through multiple systems (Beck and Hansen, 2004). Healthy bone is maintained through a balance between osteoclastic and osteoblastic activities that involve estrogens. Osteoclasts are cells that resorb bone. They provide calcium to the body by mobilizing it from bone. Osteoblasts are cells that create new bone by incorporating calcium into bone (Loveridge et al., 1992). An imbalance between the osteoblast and osteoclast activity results in disturbances of bone integrity and often causes bone fragility (Beck and Hansen, 2004). Osteoblasts act on signals from cytokines. Any changes in these cytokine signals to the osteoblasts could lead to abnormal metabolism and the formation of defective fibers leading to an increase in bone fragility (Knott et al., 1995).

There are two main types of bone that provide structural integrity. These are cortical and cancellous (or trabecular) bone. Both types of bone are forms of lamellar bone (Whitehead and Fleming, 2000). Trabecular bone is formed through osteoclast resorption followed by osteoblastic bone formation (Whitehead, 2004). A decline in structural bone components does not cause changes in the external dimensions of long

bones. This is because the resorption of cortical bone is confined to endosteal surfaces of the bone (Whitehead and Fleming, 2000). A decline in these components through the thinning of cortical bone and loss of trabecular bone integrity often results in bones that are weaker and more susceptible to fracture (Whitehead and Fleming, 2000).

Medullary Bone

Although the basic structure of all vertebrate animals is bone, there are differences between the bones of various types of animals. Avian bone contains both cortical and trabecular bone. It also contains a third type of non-structural bone that is formed when a hen reaches sexual maturity. This non-structural bone is called medullary bone (Whitehead and Fleming, 2000). Medullary bone is unique to birds and crocodilians. At the onset of sexual maturity, the function of osteoblasts changes from forming cortical bone to producing the woven medullary bone (Whitehead, 2004). It is believed that the purpose of medullary bone is to provide a labile source of calcium for eggshell formation. It is deposited on the surfaces of structural bone and in spiclues within the medullary cavities, especially in the leg bones (Whitehead, 2004).

It is thought that the switch from the creation of structural bone to medullary bone formation is nearly total. Fluorescent marker studies have shown no indication of structural bone formation or structural bone remodeling in hens during the laying period (Hudson et al., 1993; Whitehead, 2004). This means that medullary bone is formed at the expense of structural bone (Taylor and Moore, 1954; Simkiss, 1967). Medullary bone is fundamentally weaker than structural bone and the actual contribution of medullary bone to the mechanical strength of the bone is quite small (Whitehead, 2004; Knott et al., 1995). The total effect of the replacement of structural bone with medullary bone is to

weaken the overall strength of the hen's skeleton and increase the risk of fracture (Whitehead, 2004).

The purpose of medullary bone is to provide a source of calcium for eggshell formation. It is characterized by an irregular organization of collagen fibers in its matrix, which makes it mechanically weaker than other bone types (Whitehead and Fleming, 2000). This irregular collagenous matrix differs greatly from that of the structural bone types (Knott et al., 1995). Much of the medullary bone is present in isolated spicules (Whitehead, 2004). These spicules occur within the bone marrow cavity and as a layer lining the surfaces of structural bone components (Whitehead and Fleming, 2000). The amount of medullary bone found within a particular bone varies from a partial filling around the outside of the cortical cavity to completely filling the cavity (Fleming et al., 1998a).

As the bird ages, structural bone is lost from the long bones throughout the skeleton. These include the long bones of the wings, legs, vertebrae, and keel (Whitehead, 2004). The highest content of medullary bone is usually found in the femur bones (Whitehead and Fleming, 2000). Bones that have the most structural bone replaced by medullary bone have a weakened overall integrity of the bone (Whitehead, 2004). Even though the increase in the volume of the medullary bone is large, it has little resistance to bending or compressive stress. This is because there is little connectivity between the medullary bone nodules (Knott et al., 1995). The mineral density of medullary bone is usually similar to that of cancellous bone. This means that measures of radiological density or ash content in these bones give little information relevant to actual bone strength (Whitehead and Fleming, 2000).

Medullary bone formation is stimulated by the synergistic action of estrogens and androgens that accompanies the maturation of the ovarian follicles (Dacke et al., 1993; Dawson, 2000). The amount of medullary bone builds up rapidly just prior to and during the early stages of lay. It continues to accumulate slowly over the total length of the laying period (Whitehead, 2004). This accumulation of medullary bone means that total bone content may remain constant or even increase during the laying period although the strength of the bone is declining (Whitehead, 2004).

Medullary bone is turned over at a much faster rate than structural bone during the egg-laying cycle. This is because of the increased demand for calcium for the formation of the eggshell (Whitehead, 2004). The shell is formed in the shell gland during the evening and nighttime hours. Calcium for eggshell formation is derived directly from the gut and from the mobilization of medullary bone (Dawson, 2000). The medullary bone is used as a calcium source because, during shell formation, the gut generally does not contain any calcium sources. After the egg is laid, osteoblasts replace the osteoclasts resorpting the medullary bone and begin to regenerate it (Whitehead, 2004). Osteoclasts are not specific to medullary bone so resorption can also occur at any exposed structural bone surfaces (Whitehead, 2004). When the hen stops laying eggs, the medullary bone gradually disappears and structural bone formation begins again (Whitehead, 2004).

Cage Layer Fatigue and Osteoporosis

A disease condition was noticed shortly after laying hens were housed in cages in the mid-20th century. This disease was originally termed cage layer osteoporosis (Webster, 2004). Osteoporosis is the term for a progressive decrease in the amount of mineralized structural bone that leads to bone fragility and fracture (Whitehead, 2004).

Cage layer osteoporosis is the most significant skeletal disease in mature chickens, especially in ones used for egg production (Maydea and Ernst, 2008). The name for this disease was changed from cage layer osteoporosis to cage layer fatigue since the latter term was a more accurate description (Webster, 2004).

Shortly after the use of battery cages was introduced in 1955, Couch was the first researcher to describe this disease in caged laying hens that involved bone brittleness, paralysis, and death (Whitehead and Fleming, 2000). There are 2 separate types of cage layer fatigue: peracute and acute. In the peracute disease, hens die suddenly without prior symptoms. In the acute disease, hens often collapse due to sudden paralysis of the legs (Webster, 2004). This paralysis has been found to be associated with weakened skeletons that are characterized by osteoporosis (Urist and Deutsch, 1960; Bell and Siller, 1962). Skeletal deformities are often seen and are the result of severe bone fractures due to weakened bones (Webster, 2004).

Cage layer fatigue has been described as an extreme manifestation of the osteoporosis that develops in laying hens that are kept in cages. It is associated with bone loss and fractures (Beck and Hansen, 2004). Osteoporosis in laying hens is characterized by a loss of structural bone mass and shows a thinning of cortical bone and loss of connectivity of trabecular bone (Knott et al., 1995). Osteoporosis has been determined to be the underlying cause of cage layer fatigue (Mazzuco and Hester, 2005). A major factor in determining the severity of osteoporosis that develops is the length of time that birds are in a continuously reproductive state. Severe osteoporosis is not dependent on the precise number of eggs laid, but of the length of the time laying eggs (Whitehead, 2004).

Cage layer fatigue is the most severe in hens at the end of the laying period. The bones of these hens are very brittle and break easily (Beck and Hansen, 2004).

Caged layer fatigue may be an extreme consequence of the loss of structural bone in the vertebrae, through osteoporosis. This bone loss eventually leads to spinal bone collapse and paralysis (Urist and Deutsch, 1960; Bell and Siller, 1962). Hens with severe cage layer fatigue are often unable to stand. They continue to eat and drink if they are able to reach the containers (Couch, 1955; Grumbles 1959). Affected hens could become emaciated and die if unassisted, but many recover in several days if they are placed on the floor (Webster, 2004).

Many birds showing the symptoms of cage layer fatigue have fractured thoracic vertebrae. Research suggests that these fractures often compress the spinal cord and sometimes have bone fragments that penetrate into the spinal cord. This penetration results in the degeneration of the spinal cord (Riddell et al., 1968; Webster, 2004). In a survey of end-of-lay battery hens in the UK, researchers found that 29% of birds housed in battery cages experienced one or more fractures during their lifetime (Gregory and Wilkins, 1989). During depopulation, transport, and hanging on shackles, 10% showed new fractures (Gregory et al., 1994). The humerus and ulna are the 2 bones that are most frequently broken in laying hens (Gregory et al., 1990; Mazzuco and Hester, 2005).

Bone fragility in caged layers has become a major problem in the modern poultry industry (Sahin et al., 2007). The confinement of birds in cages with limited exercise has contributed to this problem by creating a form of disuse osteoporosis (Whitehead and Flemming, 2000). Genetics may be partly to blame for the levels of cage layer fatigue seen today. Poultry companies have selected for lightweight, energetically deficient birds

that maintain a high rate of lay for a long time (Whitehead and Wilson, 1992). The selection of the modern hen to continuously produce eggs over a prolonged period makes it highly susceptible to osteoporosis (Whitehead, 2004). It is often thought that osteoporosis occurs in hens because they deplete their bones by laying so many eggs. This is not correct because hens actually consume more calcium than they need to produce eggshells (Whitehead, 2004).

Effects of Isoflavones on the Bones and Eggs of Birds

There have been few studies conducted to determine the effects isoflavones have on the skeleton. The limited amount of existing data suggests that isoflavones exert agonistic effects on bone (Weaver et al., 2001) There is no indication that soy isoflavones have a negative effect on bone (Lees et al., 2007). If isoflavones interfered with the activity of endogenous estrogens, there would likely be a negative effect on the skeleton. Substances that are capable of influencing bone resorption are of interest to researchers because of their potential value to help reduce osteoporosis (Blair et al., 1996). Soy isoflavones have been shown to increase bone mineral content and bone density in humans (Greendale et al., 2002; Fitzpatrick, 2003; Sahin et al., 2007).

Research has shown that there is a direct relationship between soy isoflavones and calcium metabolism and soy isoflavones are able to improve the absorption of calcium from the intestines (Arjmandi et al., 2002; Sahin et al., 2007; Fonseca and Ward, 2004). Research has also shown that isoflavones can have effects on bone remodeling (Blair et al., 1996). Animal studies suggest that dietary isoflavones may have beneficial effects on bone mineral density and bone turnover (Ishida et al., 1998; Fanti et al., 1998; Picherit et al., 2001).

In 2007, Sahin et al. published one of the first studies reporting on how soy isoflavones affect egg production, egg quality, and bone mineral density in poultry during the late laying period (Sahin et al., 2007). Their study involved quail reared under thermo-neutral or heat-stressed conditions that were supplemented with soy isoflavones. In the thermo neutral conditions, feed intake, egg production, and egg weight were not affected. The eggshell thickness and eggshell weight was increased with soy isoflavone supplementation (Sahin et al., 2007). In the heat stressed groups, feed intake, egg production, egg weight, eggshell thickness and weight, and Haugh unit all increased with soy isoflavones (Sahin et al., 2007). Supplemental soy isoflavones might act to enhance the rate of bone formation and egg quality in quail (Sahin et al., 2007). Soy isoflavones have been shown to decrease the concentration of intracellular calcium in osteoclasts (Yamaguchi and Gao, 1998; Kajiya et al., 2000). This may cause an increased amount of calcium to be available for the formation of new bone and eggshells (Sahin et al., 2007).

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A Comparison of the Effects of Estradiol and the Soy Phytoestrogen Genistein on Liver Lipid Content of Chickens

Abstract

Abnormal liver lipid accumulation, known as fatty liver or hepatosteatosis, is associated with many factors including plasma estrogen levels, excessive alcohol consumption, metabolic derangements, and obesity. Research evidence suggests that soy phytoestrogens may have a protective effect against liver lipid accumulation induced by estrogens in mammals. Some chicken breeds are prone to excess lipid accumulation in the liver associated with high endogenous estrogen levels. Experimentally, exogenous estrogens have been used to induce hepatic lipid accumulation in chickens. A series of experiments was performed to determine if genistein, one of the most common phytoestrogens, would have a protective effect against liver lipid accumulation induced by exogenous estrogen. Three experiments were performed using different types and ages of chickens; aged hens, mature hens, and male broiler chicks. Birds were randomly selected and divided into six treatment groups. All treatments doses were dissolved in dimethyl sulfoxide (DMSO) with sesame seed oil as a carrier. Genistein doses were given by a daily oral gavage for fourteen days. Estrogen doses were given by injection in the subcutaneous tissue in the back of the neck three times during each experiment. There were no significant differences in most of the items measured. There was a significant

treatment difference in the liver weight (p=0.035) and relative liver weight (p=0.01) for the experiment with male broiler chicks. The amount of genistein in plasma and liver samples was measured by HPLC. There was a significant difference in the amount of genistein in the plasma of the aged hens after treatment with genistein (p=0.01). There was also a significant difference in the amount of genistein in the liver of the aged hens (p=0.003) and male broiler chicks (p=0.005) after treatment with genistein. In the current study, genistein appears to have had little effect on liver lipid accumulation. Exogenous estrogen was used to induce heavy liver lipid accumulation, but failed to induce this accumulation in all of the experiments. Because of this, it was difficult to determine if genistein had a protective effect on the liver.

Keywords: Genistein, Liver, Estrogen, Lipid, Chicken, Phytoestrogen

Introduction

The development of an abnormal fatty liver occurs widely in many vertebrates, including humans. It is often a response to various forms of acquired or inherited metabolic disorders (Hermier et al., 1988). Increased liver lipid accumulation also occurs commonly in avian species, especially in the female laying chicken. This higher incidence of hepatic lipidosis in mature female chickens is due to the influence of lipogenic steroid hormones such as estradiol (Ayala et al., 2009). At the onset of lay, there is a rise in plasma estradiol levels, followed by dramatic increase in hepatic lipogenesis and subsequent accumulation of lipid in the liver. Because of this, avian models have contributed greatly to the understanding of vertebrate lipid metabolism (Ayala et al., 2009). Exogenous estrogens have been used experimentally to induce fatty

liver or hepatic lipidosis in Rhode Island Red and White Leghorn laying hens (Stake et al., 1981) and in 6 to 7 week old male chickens (Pearson and Butler, 1978). The hyper-lipidemic chicken has been proposed as a useful model for the study of the causes and prevention of hepatic lipidosis and steatohepatitis (Martin-Castillo et al., 2010).

Epidemiologic studies have shown that the incidence of non-alcoholic steatohepatitis (NASH) in Japan is lower than that of the United States. This may be associated with the high consumption of soy products in Japan (Yang et al., 2011). The mechanism by which dietary soy intake decreases serum and hepatic lipids is not yet been fully established. The phytoestrogen genistein, a major soy isoflavone, has been investigated for its hypolipedemic, anti-lipogenic, antioxidant, and estrogenic/anti-estrogenic effects in various biological systems (Lee et al., 2006; Salih et al., 2009). Oral doses of genistein have prevented the development of inflammation in non-alcoholic steatohepatitis model rats (Ji et al., 2011). The inhibitory and stimulatory effects of genistein on adipose tissues appear to be dose dependent and related to its multiple estrogenic and anti-estrogenic actions (Dang, 2009). The balance among these various actions determines the final biological effects of genistein on adipocytes.

Variations of the avian model of liver lipid acccumulation were used to investigate the effects of the soy phytoestrogen genistein on the development of estrogen-induced hepatic lipid accumulation. Three studies were conducted with various types and ages of chickens. Subcutaneous injections of exogenous estrogen were used to induce liver lipid accumulation in the studies and oral doses of genistein were administered to determine it's protective effect on the liver.

Materials and Methods

Animal Treatments and Doses

This study involved three experiments utilizing different ages and types of chickens: (Aged Hens) 24 five-year-old laying hens, (Mature Hens) 36 two-year-old laying hens, and (Male Chicks) 48 one-week-old male commercial broiler chicks. Birds were randomly assigned to each of six treatment groups (Table 1) and fed appropriate standard diets *ad libitum*. All animal use was approved by the Auburn University Institutional Animal Care and Use Committee.

Table 1. Treatment Doses.

Study	Treatment	Estradiol Dose[1]	Genistein Dose[2]
Aged Hens	Sham Control	0 mg/kg body weight	0 mg/kg body weight
	Genistein Control	0 mg/kg body weight	20 mg/kg body weight
	Estrogen Control	7.5 mg/kg body weight	0 mg/kg body weight
	Low Genistein	7.5 mg/kg body weight	10 mg/kg body weight
	Medium Genistein	7.5 mg/kg body weight	15 mg/kg body weight
	High Genistein	7.5 mg/kg body weight	20 mg/kg body weight
Mature Hens[3]	Sham Control	0 mg/kg body weight	0 mg/kg body weight
	Genistein Control	0 mg/kg body weight	20 mg/kg body weight
	Estrogen Control	13 mg/kg body weight	0 mg/kg body weight
	Low Genistein	13 mg/kg body weight	10 mg/kg body weight
	Medium Genistein	13 mg/kg body weight	15 mg/kg body weight
	High Genistein	13 mg/kg body weight	20 mg/kg body weight
Male Chicks	Sham Control	0 mg/kg body weight	0 mg/kg body weight
	Genistein Control	0 mg/kg body weight	20 mg/kg body weight
	Estrogen Control	7.5 mg/kg body weight	0 mg/kg body weight
	Low Genistein	7.5 mg/kg body weight	10 mg/kg body weight
	Medium Genistein	7.5 mg/kg body weight	15 mg/kg body weight
	High Genistein	7.5 mg/kg body weight	20 mg/kg body weight

[1] Dose given by 3 subcutaneous injections in the back of the neck.
[2] Dose given by daily oral gavage.
[3] The study with Mature Hens received a different estrogen dose than other studies.

As appropriate for the assigned treatment group, genistein doses were given by a daily oral gavage for fourteen days. Estradiol dipropionate doses were given on days 0, 5, and 10 by injection in the subcutaneous tissue in the back of the neck. Daily oral applications were carried out using a 1 ml tuberculin syringe fitted with a 16 gauge, 2.5 cm gavage needle. The estradiol doses were given with a 1 ml tuberculin syringe and a 21-gauge, 1 in. needle. All treatments doses were dissolved in the same amount of dimethyl sulfoxide (DMSO) then mixed with sesame seed oil as a carrier. Treatment groups that received blank doses (0 mg/kg) simply received the DMSO/sesame seed oil vehicle.

Plasma and Liver Sample Collection

Body weights of all birds were recorded at the beginning and at the end of the experiment in order to calculate the change in body weight per bird over the course of the study. Blood was collected from the hens on days 0 and 15 from the ulnar vein into heparinized tubes and placed on ice. Blood was not collected from the male chicks at day 0 because an adequate volume of blood could not be collected from chicks of that size. Tubes of blood were centrifuged at 1500 g for fifteen minutes at 4°C. Plasma was collected, dispensed into tubes and stored in a -20°C freezer.

On day 15, birds were euthanized by CO_2 asphyxiation and necropsied. Livers were collected from each bird and weighed. Relative liver weight was calculated as a percentage of final body weight. A portion of each liver was placed in physiological saline (0.85% saline) and stored in a refrigerator for HPLC analysis and lipid determination.

HPLC Genistein Measurement

The amount of genistein contained in the plasma of the birds was determined by high performance liquid chromatography (HPLC) (Appendix 1). This protocol was modified from the method created by Thomas et al., 2001. Genistein in the plasma and liver samples was hydrolyzed with *Helix pomatia* glucuronidase/sulfatase to convert all genistein forms to the aglycone form to allow for the measurement of total genistein. The samples were then extracted with ether and reconstituted in a methanol-ammonium formate mixture with hexane. The reconstituted samples were removed from under the hexane layer and subjected to HPLC analysis.

Liver Lipid Measurements

Liver samples were ground in a glass cell homogenizer with an equal portion of deionized water. The proportion of dry matter was determined by dividing part of the homogenized aliquot of sample between two weighed aluminum pans. The pans were placed in a drying oven at 105-110°C for 16 hours. The pans were then removed from the oven and placed in a desiccator to cool. The pans were then weighed to determine the dry mass and the amount of dry mass relative to total liver wet mass.

The lipid concentration in each sample was determined by the Folch method. Homogenized liver samples were divided between two test tubes. Each tube received an addition of 20 ml of a 2:1 chloroform:methanol (v/v) mixture. The tubes were shaken for 1-2 minutes to mix the samples prior to centrifugation at 2000g for 30 minutes. After centrifugation, the upper methanol:water layer was removed and discarded. The lower layer of chloroform was carefully removed and placed into a previously weighed aluminum pan. The chloroform was evaporated from the samples using a steam bath prior

to drying in a drying oven at 105-110°C for 2 hours. The pans were then removed from the oven and placed in a desiccator to cool. The pans were weighed to determine the lipid amount. The percent lipid concentration was determined by dividing lipid mass by the weight of the dry mass and then multiplying by 100.

Statistical Analysis

Statistical relationships were evaluated using SAS statistical software (SAS Institute, 2002). The General Linear Model (GLM) and Tukey's Studentized Range (HSD) Test were conducted to determine any statistical differences. Statistical differences were determined to be significant at a P value of 0.05 or less.

Results

Body Weights

The initial and final body weights for each treatment are presented in Table 2. Initial body weights were not different for the birds within each experiment. There were no significant differences in final body weights between treatments within each experiment. The change in body weights between the initial and final weighings is presented in Table 2. Body weight increased in the male broiler chicks because the animals used were a fast growing broiler strain. However, there were no significant differences in body weight due to treatment.

Liver Weight and Relative Liver Weight

There were no significant treatment differences in the liver weights or relative liver weights for either experiment involving hens (Table 3). In the experiment involving male chicks, birds in the Low Genistein treatment had significantly heavier livers (P=0.0349) and relative liver weights (P=0.0083) than the Sham Control treatment, but

were not different from any other treatment. In all three experiments, birds receiving the estradiol doses had heavier livers than birds not receiving estradiol doses. The Sham Control treatments had the lowest liver weights for each study.

Genistein Content of the Liver

There were significant differences in the amount of genistein contained in liver samples taken after the birds received treatment doses for the experiments involving the aged hens ($P<0.0001$) and the male chicks ($P=0.0051$) (Table 3). There were no significant differences in the amount of genistein in the liver of the experiment with the mature hens. In the experiment involving aged hens, birds in the Sham Control, Estrogen Control, Low Genistein, and Medium Genistein treatments had significantly less genistein in the livers than the Genistein Control and High Genistein treatments. In the experiment with the male broiler chicks, birds in the Sham Control treatment had significantly less genistein than either the High Genistein or Genistein Control treatments. The Estrogen Control, Low Genistein, and Medium Genistein treatments were intermediate. In all three experiments, there appeared to be a dose-dependent response to genistein. The liver samples of the male broiler chicks contained more genistein (10.2-10.8 ng genistein/g liver) than the mature hens (9.2-10.2 ng genistein/g liver) or the aged hens (2.4-3.6 ng genistein/g liver).

Genistein Content of the Plasma

The amount of genistein in the plasma was determined by HPLC. There were no significant differences between treatment groups, in the amount of genistein in the plasma prior to receiving treatment doses, for either experiment involving hens (Table 4). The amount of genistein in the plasma was not determined in the experiment involving

male chicks because it was impossible to collect the necessary amount of blood from the chicks and have them survive to be included in the research.

There were significant differences between treatment groups, in the amount of genistein in the plasma after treatment doses were given, in the experiment involving the aged hens (P=0.0076) (Table 4). There were no significant treatment differences in either of the other two experiments. In the experiment with aged hens, birds in the Estrogen Control treatment group had significantly less genistein in the blood than the Genistein Control and High Genistein treatments. The Sham Control, Low Genistein, and Medium Genistein treatments were not significantly different from any of the other treatments. This result was expected because the Genistein Control and High Genistein treatments received the same dose of genistein, which was also the highest dose of genistein.

There were significant differences between treatment groups, in the change in the amount of genistein in the plasma between the pre-treatment and post-treatment measurements, for the experiment involving the aged hens (P=0.0013) (Table 4). In the experiment with aged hens, birds in the Estrogen Control treatment group had significantly less change in genistein in the plasma than the Genistein Control and High Genistein treatments. The High Genistein Treatment had significantly more change in plasma genistein level than the Estrogen Control, Sham Control, and Low Genistein treatments.

Liver Dry Matter and Liver Dry Matter as a Percentage of Liver Weight

There were no significant treatment differences in liver dry matter mass or liver dry matter weight as a percentage of liver weight in any of the experiments (Table 5). Animals receiving the High Genistein treatment tended to have less liver dry matter than

animals receiving the Low Genistein and Medium Genistein treatments. For all experiments, the High Genistein treatments had less relative liver dry matter suggesting that there was more water or lipid in the liver.

Liver Lipid Weight and Relative Liver Lipid Weights

There were no significant treatment differences in liver lipid weight, liver lipid weight as a percentage of liver weight, or liver lipid weight as a percentage of liver dry matter weight for any of the experiments (Table 6). Animals receiving both estrogen and genistein doses tended to have less liver lipid weight than animals in the Estrogen Control treatment not receiving genistein doses. This is consistent with genistein having a protective effect against liver lipid accumulation.

Discussion

Several studies have shown that genistein regulates different genes than estrogens (Penza et al., 2006; Rimbach et al., 2008). Unlike estradiol, genistein can have both estrogenic and anti-estrogenic effects in animal systems. Because of this, it is likely that genistein utilizes both estrogen receptor and non-estrogen receptor mediated pathways (Dang, 2009). When genistein is given at a wide concentration range, it affects adipogenesis in a biphasic dose-dependent way. Genistein has exhibited an inhibition of adipogenesis at low concentrations and an enhancement of adipogenesis at high concentrations (Dang et al., 2003).

Genistein has been shown, in many studies, to induce pleiotropic estrogenic, anti-estrogenic, and enzyme inhibiting effects. These effects vary depending on the dose of genistein given, the target species, its reproductive status, and the target tissue or cells (Dang, 2009). The various pleiotropic effects induced by genistein have been observed in

the same concentration ranges. Because of this, it is possible that these pleiotropic effects have an influence on each other. If this were the case, the balance of the various pleiotropic effects of genistein would determine the final biological effects genistein has on animals (Dang, 2009).

An adipose tissue-specific decrease in body weights has been reported for rodent studies of injected genistein doses (Naaz et al., 2003). In the two present experiments involving hens, many animals tended to lose weight during the study. Static or declining body weight with age is common in commercial strain laying hens as used in this study. In the experiment involving the aged hens, the two treatment groups that gained weight experienced some mortality amongst the lighter hens. This suggests that the mortality skewed the body weight data and caused the trend towards an increase in average body weight.

Liver size and weight can change because of an overall change in liver size, altered accumulation of fat or other components, or changes in water content. Estrogens are known to increase liver lipid content in most species studied. Estrogenic isoflavones such as genistein would be expected to also increase liver lipid content if they were indeed functioning as estrogens. However, genistein appears to function both as an estrogen or an anti-estrogen depending on the dose, target tissue, or species receiving the genistein. These pleiotropic effects of genistein have been seen in a number of studies.

Research on rats has shown that animals fed a diet supplemented with isoflavones had significantly lower body weights and liver weights than animals not supplemented with isoflavones (Davis et al., 2005). Genistein has been reported to regulate adipogenesis and triglyceride storage in some animals. This regulation leads to changes

in the number and volume of adipocytes (Dang, 2009). In studies on rats, adipogenesis was inhibited with genistein doses between 0.1 and 10 μM and was stimulated with genistein doses above 10 μM (Dang, 2009).

Research has shown that dietary intake of genistein at 40 mg/day reduced hepatic lipogenesis and serum lipid levels in rats (Takahashi et al., 2009). In the current study, the experiments involving hens used nearly this same amount of genistein in the High Genistein and Genistein Control treatments. The only significant difference in relative liver weights was seen in the study with the male chicks. This may be because of the types of animals used in the studies. The effects of genistein are known to vary between types of animals. The liver's ability to accumulate lipids may also have a large variation between animal types. Genistein appears to affect lipid accumulation differently than estrogen. Genistein has been shown to inhibit adipose cell numbers and adipogenesis at low concentrations and stimulate it at high concentrations (Dang, 2009).

Humans and domestic animals are frequently exposed to genistein through the consumption of soy-based food products. Plasma concentrations of genistein have been shown to increase in a dose dependent manner in response to genistein administration in animals and humans (Dang, 2009). The levels of genistein in the plasma of humans consuming a diet high in soy products have been shown to be between 2.5 and 4 μM/L (675 and 1081 ng/ml) (Chen and Donovan, 2004; Dang and Lowik, 2005; McClain et al., 2006). It was expected that there would be no significant differences in the pre-treatment plasma genistein levels because the plasma was taken from the animals prior to receiving the treatment doses. The pre-treatment plasma samples of the young hens contained more genistein (3.4-3.5 ng genistein/ml plasma) than the old hens (1.80-1.84 ng genistein/ml

plasma). This may be due to variations in the amount of genistein in the soybean meal in the diets or in the reproductive state of these animals.

Research suggests that the type of animal could play a major part in the amount of genistein found in the plasma. The content of genistein in the plasma of human women was 11 times higher than the content in female rats at 4 hours after a single administration of genistein at a dose of 1 mg/kg body weight (Gu et al., 2006). The amount of genistein in the plasma of the animals in the current study is much lower than reported in human studies. This difference could be due to metabolic differences between humans and chickens or due to sampling times. The plasma of the chickens in the current study was collected nearly 24 hours after the last treatment dose was received and may have decreased from peak levels.

In all three experiments, there was a dose-dependent response to genistein in the post-treatment plasma and liver samples, although it only reached significance in the experiment involving the aged hens. The post-treatment plasma samples of the male chicks contained more genistein (5.7-6.4 ng genistein/ml plasma) than the young hens (3.3-3.9 ng genistein/ml plasma) or the mature hens (1.5-1.9 ng genistein/ml plasma). The post-treatment liver samples of the male broiler chicks contained more genistein (10.2-10.8 ng genistein/g liver) than the young hens (9.2-10.2 ng genistein/g liver) or the old hens (3.6-2.4 ng genistein/g liver). The variations in plasma genistein levels seen in the current study could be due to the amount of feed consumed by the rapidly growing male chicks or due to the ability of the liver to process genistein.

In this study, exogenous estrogen did not induce as much liver fat accumulation as expected. Previous research showed that intramuscular injection of exogenous estradiol

every 4 or 5 days (5.0 or 7.5 mg/kg body weight) induced fatty liver-hemorrhagic syndrome (FLHS) in both Rhode Island Red and White Leghorn hens (Stake et al., 1981). In the current studies, three subcutaneous injections of estrogen diproprionate of 7.5 or 13 mg/kg body weight did not induce heavy liver lipid accumulation. The original research in this area was performed in the late 1970's and early 1980's. Since then, commercial poultry breeders have selected for animals resistant to developing fatty liver, which may have reduced the genetic susceptibility to developing FLHS.

Research involving geese has shown that induction of a fatty liver is not possible in all breeds or species (Hermier et al., 1991). The breed-related differences may occur because of genetic differences in liver lipid metabolism or the birds' ability to channel fatty acids towards lipoprotein assembly and secretion (Hermier et al., 2003). These breed related differences may also occur in chickens. In research performed by Stake et al., Rhode Island Red chickens showed severe effects of FLHS, while no White Leghorn chickens showed effects. This may indicate a major breed difference in the response to exogenous estradiol in chickens. Thus, it is possible genetic resistance may be the reason that the doses used in the current experiment did not induce as great a degree of liver lipid accumulation as seen in previous studies.

Immature male chickens have also been used in research on FLHS. Administration of exogenous estrogens has been used to reproduce the typical hyperlipidemia seen in laying hens (Courtney et al., 1988; Kudzma et al., 1973; Luskey et al., 1974; Manning et al., 1989; Pearce and Balnave, 1975). In one example, intramuscular injections of estrogen diproprionate were used to reproduce a syndrome resembling fatty liver hemorrhagic syndrome in 6 to 7 week old male chickens (Pearson

and Bulter, 1978). The current study used three subcutaneous injections of 7.5 mg estrogen diproprionate/kg body weight. This treatment dose was insufficient to induce heavy lipid accumulation in the liver in the male broiler chicks.

In the current study, genistein appears to have had little effect on liver lipid accumulation. Exogenous estrogen was used to induce heavy liver lipid accumulation, but failed to induce this accumulation in all of the experiments. Because of this, it was more difficult to determine if genistein had a protective effect on the liver. It was noted that the Medium Genistein treatment showed more effects that the other treatments. This is likely due to the dose-dependent and pleiotropic effects that have been reported for genistein.

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Table 2. Starting, Final, and Change in Body Weight.

Study	Treatment	Starting N	Starting Body Weight (kg)	P value	Final N	Final Body Weight (kg)	P value	Body Weight Change (kg)	P value
Aged Hens	Sham Control	4	1.772 ± 0.081	0.1835	4	1.725 ± 0.108	0.6827	-0.048 ± 0.083	0.2383
	Genistein Control	4	1.592 ± 0.238		2	1.708 ± 0.426		+0.117 ± 0.187	
	Estrogen Control	4	1.932 ± 0.141		4	1.823 ± 0.182		-0.110 ± 0.127	
	Low Genistein	4	1.753 ± 0.151		4	1.692 ± 0.166		-0.061 ± 0.118	
	Medium Genistein	4	1.868 ± 0.163		3	1.889 ± 0.155		+0.021 ± 0.155	
	High Genistein	4	1.746 ± 0.155		4	1.701 ± 0.154		-0.046 ± 0.042	
Mature Hens	Sham Control	6	2.114 ± 0.247	0.8829	6	2.029 ± 0.341	0.8871	-0.085 ± 0.138	0.1573
	Genistein Control	6	2.019 ± 0.310		6	1.962 ± 0.290		-0.057 ± 0.067	
	Estrogen Control	6	2.160 ± 0.271		6	2.131 ± 0.226		-0.029 ± 0.069	
	Low Genistein	6	2.057 ± 0.179		6	2.021 ± 0.176		-0.029 ± 0.043	
	Medium Genistein	6	2.173 ± 0.312		6	2.058 ± 0.316		-0.115 ± 0.026	
	High Genistein	6	2.113 ± 0.148		6	2.110 ± 0.167		-0.003 ± 0.068	
Male Chicks	Sham Control	8	0.166 ± 0.020	0.9997	8	0.930 ± 0.072	0.5748	+0.764 ± 0.060	0.5748
	Genistein Control	8	0.166 ± 0.022		8	0.909 ± 0.010		+0.743 ± 0.010	
	Estrogen Control	8	0.166 ± 0.022		8	0.868 ± 0.097		+0.702 ± 0.089	
	Low Genistein	8	0.168 ± 0.029		8	0.895 ± 0.013		+0.727 ± 0.010	
	Medium Genistein	8	0.169 ± 0.021		8	0.874 ± 0.010		+0.705 ± 0.094	
	High Genistein	8	0.168 ± 0.013		8	0.841 ± 0.010		+0.673 ± 0.010	

[1] Values shown are treatment averages with standard deviation.

Table 3. Liver Weight, Liver Weight as a Percentage of Final Body Weight, and Liver Genistein Levels.

Study	Treatment	N	Liver Weight (g)	P value	Liver Percent (%)	P value	Genistein (ng/g)	P value
Aged Hens	Sham Control	4	49.241 ± 20.152	0.5337	2.851 ± 1.803	0.4413	4.735 ± 0.158^{C}	<0.0001
	Genistein Control	2	50.172 ± 29.624		2.809 ± 1.034		7.510 ± 0.778^{A}	
	Estrogen Control	4	65.729 ± 5.602		3.614 ± 0.199		4.705 ± 0.195^{C}	
	Low Genistein	4	63.007 ± 21.236		3.673 ± 0.870		5.400 ± 0.489^{BC}	
	Medium Genistein	3	72.709 ± 10.697		3.887 ± 0.817		5.840 ± 0.295^{B}	
	High Genistein	4	65.229 ± 18.683		3.793 ± 0.826		7.440 ± 0.652^{A}	
Mature Hens	Sham Control	6	51.085 ± 12.431	0.4224	2.506 ± 0.299	0.1599	9.090 ± 0.935	0.1260
	Genistein Control	6	60.412 ± 10.745		3.104 ± 0.578		10.087 ± 0.740	
	Estrogen Control	6	62.990 ± 9.705		2.962 ± 0.416		9.090 ± 0.859	
	Low Genistein	6	56.448 ± 7.274		2.793 ± 0.279		9.543 ± 0.575	
	Medium Genistein	6	56.656 ± 9.444		2.782 ± 0.506		9.647 ± 0.928	
	High Genistein	6	55.077 ± 10.172		2.598 ± 0.311		10.033 ± 0.575	
Male Chicks	Sham Control	8	29.866 ± 3.738^{B}	0.0349	3.209 ± 0.297^{B}	0.0083	10.083 ± 0.467^{B}	0.0051
	Genistein Control	8	30.937 ± 3.523^{AB}		3.412 ± 0.260^{AB}		10.788 ± 0.575^{A}	
	Estrogen Control	8	31.242 ± 3.664^{AB}		3.640 ± 0.628^{AB}		9.995 ± 1.080^{B}	
	Low Genistein	8	36.599 ± 4.867^{A}		4.178 ± 0.902^{A}		10.603 ± 0.208^{AB}	
	Medium Genistein	8	35.648 ± 6.690^{AB}		4.078 ± 0.578^{AB}		10.688 ± 0.243^{AB}	
	High Genistein	8	33.740 ± 5.218^{AB}		4.044 ± 0.645^{AB}		10.770 ± 0.430^{A}	

[A-C] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).

[1] Values shown are treatment averages with standard deviation.

Table 4. Starting Amount, Final Amount, and Change in the Amount of Genistein in the Plasma.

Study	Treatment	N	Starting Genistein (ng/ml)	P value	Final Genistein (ng/ml)	P value	Change in Genistein (ng/ml)	P value
Aged Hens	Sham Control	4	2.120 ± 0.314	0.9815	2.203 ± 0.151^{AB}	0.0076	0.083 ± 0.181^{BC}	0.0013
	Genistein Control	2	2.228 ± 0.339		2.605 ± 0.247^{A}		0.425 ± 0.106^{AB}	
	Estrogen Control	4	2.163 ± 0.249		2.098 ± 0.064^{B}		-0.065 ± 0.189^{C}	
	Low Genistein	4	2.203 ± 0.138		2.295 ± 0.260^{AB}		0.093 ± 0.133^{BC}	
	Medium Genistein	3	2.173 ± 0.159		2.460 ± 0.101^{AB}		0.273 ± 0.108^{ABC}	
	High Genistein	4	2.120 ± 0.090		2.563 ± 0.145^{A}		0.443 ± 0.066^{A}	
Mature Hens	Sham Control	6	3.417 ± 0.263	0.9522	3.280 ± 0.304	0.1667	-0.137 ± 0.278	0.5372
	Genistein Control	6	3.482 ± 0.370		3.881 ± 0.522		0.400 ± 0.853	
	Estrogen Control	6	3.405 ± 0.548		3.262 ± 0.485		-0.143 ± 0.552	
	Low Genistein	6	3.545 ± 0.364		3.665 ± 0.482		0.120 ± 0.686	
	Medium Genistein	6	3.358 ± 0.313		3.683 ± 0.421		0.325 ± 0.369	
	High Genistein	6	3.532 ± 0.436		3.848 ± 0.737		0.317 ± 0.907	
Male Chicks	Sham Control	8	Not Calculated		5.790 ± 0.290	0.1860	Not Calculated	
	Genistein Control	8	Not Calculated		6.445 ± 0.793		Not Calculated	
	Estrogen Control	8	Not Calculated		5.671 ± 0.748		Not Calculated	
	Low Genistein	8	Not Calculated		6.120 ± 0.850		Not Calculated	
	Medium Genistein	8	Not Calculated		6.293 ± 0.830		Not Calculated	
	High Genistein	8	Not Calculated		6.424 ± 0.769		Not Calculated	

[A-C] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).

[1] Values shown are treatment averages with standard deviation.

Table 5. Liver Dry Matter Weight and Liver Dry Matter as a Percentage of Liver Weight.

Study	Treatment	N	Dry Matter Weight (g)	P value	Dry Matter Percent (%)	P value
Aged Hens	Sham Control	4	0.092 ± 0.063	0.8053	2.265 + 1.550	0.7677
	Genistein Control	2	0.077 ± 0.009		2.075 + 0.000	
	Estrogen Control	4	0.092 ± 0.021		2.376 + 0.558	
	Low Genistein	4	0.094 ± 0.055		2.433 + 1.456	
	Medium Genistein	3	0.134 ± 0.102		3.500 + 2.556	
	High Genistein	4	0.074 ± 0.034		1.869 + 0.894	
Mature Hens	Sham Control	6	0.316 ± 0.114	0.5474	7.913 ± 2.915	0.4629
	Genistein Control	6	0.385 ± 0.055		9.928 ± 1.463	
	Estrogen Control	6	0.389 ± 0.104		9.958 ± 2.581	
	Low Genistein	6	0.385 ± 0.043		9.869 ± 1.078	
	Medium Genistein	6	0.364 ± 0.061		9.423 ± 1.731	
	High Genistein	6	0.332 ± 0.099		8.554 ± 2.472	
Male Chicks	Sham Control	8	0.212 ± 0.038	0.8975	5.780 ± 0.849	0.6862
	Genistein Control	8	0.185 ± 0.051		4.629 ± 1.324	
	Estrogen Control	8	0.210 ± 0.065		5.407 ± 1.713	
	Low Genistein	8	0.220 ± 0.082		5.794 ± 2.149	
	Medium Genistein	8	0.213 ± 0.067		5.642 ± 1.816	
	High Genistein	8	0.197 ± 0.072		5.070 ± 1.771	

[1] Values shown are treatment averages with standard deviation.

Table 6. Liver Lipid Weight, Liver Lipid as a Percentage of Liver Weight, and Liver Lipid as a Percentage of Liver Dry Matter Weight.

Study	Treatment	N	Lipid Weight (g)	P value	Liver Lipid Concentration (%)	P value	Dry Matter Lipid Concentration (%)	P value
Aged Hens	Sham Control	4	0.040 ± 0.041	0.5711	0.069 ± 0.048	0.6598	38.307 + 17.372	0.4275
	Genistein Control	2	0.026 ± 0.014		0.053 ± 0.003		33.336 + 13.639	
	Estrogen Control	4	0.036 ± 0.013		0.054 ± 0.019		38.339 + 6.490	
	Low Genistein	4	0.046 ± 0.042		0.065 ± 0.041		43.021 + 17.297	
	Medium Genistein	3	0.085 ± 0.077		0.115 ± 0.094		57.440 + 11.757	
	High Genistein	4	0.033 ± 0.020		0.060 ± 0.046		42.477 + 11.722	
Mature Hens	Sham Control	6	0.081 ± 0.049	0.0807	0.151 ± 0.053	0.1076	25.211 ± 8.107	0.1068
	Genistein Control	6	0.128 ± 0.050		0.209 ± 0.574		32.610 ± 9.116	
	Estrogen Control	6	0.178 ± 0.088		0.276 ± 0.105		44.726 ± 16.086	
	Low Genistein	6	0.129 ± 0.050		0.237 ± 0.118		33.878 ± 12.548	
	Medium Genistein	6	0.108 ± 0.032		0.189 ± 0.037		29.948 ± 9.203	
	High Genistein	6	0.105 ± 0.041		0.188 ± 0.042		32.673 ± 9.943	
Male Chicks	Sham Control	8	0.060 ± 0.013	0.8769	0.205 ± 0.053	0.5662	29.031 ± 6.497	0.3247
	Genistein Control	8	0.062 ± 0.029		0.208 ± 0.111		33.386 ± 12.341	
	Estrogen Control	8	0.074 ± 0.036		0.233 ± 0.091		36.050 ± 11.195	
	Low Genistein	8	0.063 ± 0.029		0.167 ± 0.064		28.564 ± 7.080	
	Medium Genistein	8	0.061 ± 0.026		0.171 ± 0.069		29.188 ± 10.528	
	High Genistein	8	0.071 ± 0.031		0.207 ± 0.082		38.391 ± 14.499	

[1] Values shown are treatment averages with standard deviation.

The Effects of Genistein on the Bone Quality of Laying Hens

Abstract

Research has suggested that the primary soy phytoestrogen, genistein, may help alleviate osteoporosis in women by increasing bone density through its estrogenic action. It is unknown whether genistein may improve bone density in aging laying hens or actually contribute to the problem through negative interactions with the hen's endocrine system. The objective of this study was to characterize and quantify the effects of the genistein on bone physiology in aged hens. Sixty White Leghorn laying hens, which were 3 years old and had never been through an induced molting process, were randomly selected and divided into 4 treatments: Sham Control, Low Genistein (10 mg genistein/kg body weight), Medium Genistein (15 mg genistein/kg body weight), and High Genistien (20 mg genistein/kg body weight). Doses were given by subcutaneous injection in the back of the neck every other day for 8 weeks. There were significant differences between the final body weights ($p=0.0432$), wet femur weights ($p=0.001$), dry femur weights ($p=0.002$), relative dry femur weights ($p=0.01$), ashed femur weights ($p=0.0002$), relative ashed femur weights ($p=0.006$), dry femur weights percent of wet femur weights ($p=0.003$), ashed femur weights percent of wet femur weights ($p=0.0004$), and ashed femur weights as a percentage of dry femur weights ($p=0.004$) of the birds belonging to the High Genistein and Medium Genistein treatments. The femurs from birds in the High

Genistein treatment group required significantly more force (kg) to break than the femurs of birds in the Medium Genistein treatment (p=0.009). Vertebrae from the Sham Control treatment group required more force (kg) to cause a break (p=0.0001) than samples from birds in either the Medium or High Genistein treatment groups. In this study, genistein appeared to have a positive effect on femur strength and a negative effect on vertebrae strength. This suggests that genistein has different effects on various types of bone. The results of this study suggest that the High Genistein treatment (20 mg/kg body weight) has a beneficial effect on femur quality of aged laying hens.

Keywords: Genistein, Hen, Bone, Phytoestrogen, Soy, Osteoporosis

Introduction

Osteoporosis is characterized by a progressive decrease in the amount of mineralized structural bone, and, therefore leads to bone fragility and fracture (Whitehead, 2004). Cage layer fatigue is an extreme form of disuse osteoporosis that affects laying hens kept in cages. The effects of cage layer fatigue are most severe in hens at the end of the laying period. Bones from these hens are very brittle and break easily (Beck and Hansen, 2004). Cage layer fatigue is the most significant skeletal disease found in mature chickens (Mayedea and Ernst, 2008). A major factor in the severity of osteoporosis that develops is the length of time that birds are in a continuously reproductive state. The amount of time, not the precise number of eggs laid, is the most important factor in the severity of the condition (Whitehead, 2004).

Bone development and formation changes over the lifespan of a chicken. At the onset of sexual maturity, the function of osteoblasts changes from forming cortical bone to producing medullary bone (Whitehead, 2004). This means that medullary bone is

formed at the expense of the structural cortical bone (Taylor and Moore, 1954; Simkiss, 1967). Medullary bone is a woven, non-structural bone that is unique to birds and crocodilians (Whitehead and Fleming, 2000). It is believed that the purpose of medullary bone is to serve as a source of calcium for eggshell formation.

Medullary bone is fundamentally weaker than cortical bone and contributes little to the mechanical strength of the bone (Whitehead, 2004; Knott et al., 1995). When structural bone is replaced with medullary bone, the overall strength of the hen's skeleton is weakend and there is an increased risk of fracture (Whitehead, 2004). The amount of medullary bone builds up rapidly just prior to and during the early stages of lay. It then continues to accumulate slowly over the total length of the laying period (Whitehead, 2004). As the bird ages, structural bone is continually lost from the long bones of the wings, legs, vertebrae, and keel (Whitehead, 2004). The highest content of medullary bone is usually found in the femurs (Whitehead and Fleming, 2000). Bones that have more structural bone that is replaced by medullary bone are weaker than other bones (Whitehead, 2004).

Few studies have been performed to determine the effects of isoflavones on the skeleton. Based on a limited amount of data, research suggests that isoflavones exert positive effects on bone (Weaver et al., 2001; Lees et al., 2007). Soy isoflavones have been shown to increase bone mineral content and bone density in humans (Greendale et al., 2002; Fitzpatrick, 2003; Sahin et al., 2007). Research has also shown that there is a direct relationship between soy isoflavones and calcium metabolism and that soy isoflavones are able to improve the absorption of calcium from the intestines (Arjmandi et al., 2002; Fonseca and Ward, 2004). This increased intestinal absorption of calcium

could help to contribute to the bone-conserving effects of soy (Omi et al., 1994). Animal studies suggest that dietary isoflavones may have beneficial effects on bone mineral density and bone turnover (Ishida et al., 1998; Fanti et al., 1998; Picherit et al., 2001). Research has also shown that isoflavones can affect bone remodeling (Blair et al., 1996).

Over the years, poultry companies have selected for lightweight birds that lay a lot of eggs for a long period of time (Whitehead and Wilson, 1992). The selection of the modern hen to continuously produce eggs over a prolonged period makes it highly susceptible to osteoporosis (Whitehead, 2004). This suggests that the egg laying hen would be an ideal model for research on osteoporosis. Research was performed to determine the effect of genistein on bone quality in laying hens that had not experienced an induced molting process. Based on previous animal and human research, it was expected that genistein would have a beneficial effect on bone quality in these laying hens.

Materials and Methods

Animal Treatments and Doses

For this experiment, 60 white leghorn-laying hens that were three years old and had never been put through a molting process were obtained from stock kept at the Auburn University Poultry Research Unit. All animal use was approved by the Auburn University IACUC. Fifteen birds were randomly assigned to each of four treatment groups (Table 14). As appropriate for the assigned treatment group, doses were given every other day by subcutaneous injection in the fatty tissue in the back of the neck over eight weeks. Doses were given with a 1 ml tuberculin syringe and a 21-gauge, 1 in. needle. All treatments doses were dissolved in the same amount of dimethyl sulfoxide (DMSO) then

mixed with sesame seed oil as a carrier. Treatment groups that received blank doses (0 mg/kg) simply received the DMSO/sesame seed oil vehicle.

Table 7. Treatment Doses.

Treatment	Genistein Dose[1]
Sham Control	0 mg/kg body weight
Low Genistein	10 mg/kg body weight
Medium Genistein	15 mg/kg body weight
High Genistein	20 mg/kg body weight

[1] Dose given by subcutaneous injections in the back of the neck.

Plasma and Bone Sample Collection

Body weights of all birds were recorded at the beginning and end of the experiment. Change in body weight per bird over the course of the study was calculated from the body weights. Blood was collected from the hens on days 0 and 15 from the ulnar vein into heparinized tubes and placed on ice. Tubes of blood were centrifuged at 1500 g for fifteen minutes at 4°C. Plasma was collected, dispensed into tubes and stored in a -20°C freezer. On day 60, birds were euthanized by CO_2 asphyxiation and necropsied. Femurs and vertebrae were collected from each bird and stored in a -20°C freezer until analysis.

HPLC Genistein Measurement

The amount of genistein contained in the plasma of the birds was determined by high performance liquid chromatography (HPLC) (Appendix 1). This protocol was modified from the method created by Thomas et al., 2001. Genistein in the plasma and liver samples were hydrolyzed with *Helix pomatia* glucuronidase/sulfatase to convert all genistein forms to the aglycone form to allow for the measurement of total genistein. The

samples were then extracted with ether and reconstituted in a methanol-ammonium formate mixture with hexane. The reconstituted samples were removed from under the hexane layer and subjected to HPLC analysis.

Femur Moisture Measurements

Femurs collected from all treatments were allowed to thaw overnight under refrigeration. All muscle tissue was removed. One femur from each bird was weighed and the wet femur weight and relative wet femur weight were recorded. The femurs were then placed in a drying oven for 48 hours at 50°C. Once removed from the drying oven, the femurs were placed in a desiccator and allowed to cool to room temperature. The dry weights of the femurs were recorded and the relative dry femur weights were calculated.

The femurs were then placed in crucibles and ashed at 600°C for 18 hours. The femurs were ashed to determine if any of the treatments had an effect on the mineral content in the femur. The ashed femurs were then placed in a desiccator and allowed to cool to room temperature. The femur ash weights were recorded and the relative femur ash weights were calculated. The dry femur weight percent of wet femur weight, ashed femur weight percent of wet femur weight, and ashed femur weight percent of dry femur weight were also calculated.

Bone Breaking Strength Measurements

The dry femurs were used to determine the bone breaking strength. Dry vertebrae were also used. To dry the vertebrae, one vertebra from each bird was removed from the freezer and thawed overnight under refrigeration. All muscle tissue was removed. The vertebrae were then dried for 48 hours at 50°C. Once removed from the drying oven, the vertebra were placed in a desiccator and allowed to cool to room temperature. A TA.XT2

texture analyzer machine was used to measure the force necessary to break the bones. The femurs were broken at the middle of the bone. The vertebrae were tested with the machine platforms at a distance of 12.7 mm apart.

Statistical Analysis

Statistical relationships were evaluated using SAS statistical software (SAS Institute, 2002). The General Linear Model (GLM) and Tukey's Studentized Range (HSD) Test were conducted to determine any statistical differences. Statistical differences were determined to be significant at a P value of 0.05 or less.

Results

Body Weight

There were no significant differences in the starting body weights or change in body weights during treatment for any treatment groups (Table 8). The birds in the High Genistein treatment tended to be heavier than birds in the other treatments and were the only treatment to gain weight during the study. The birds in the Medium Genistein treatment tended to have the lowest body weights. There was a significant difference in the final body weights between birds in the Medium Genistein and High Genistein treatments (P=0.0432) (Table 8). Birds in the Medium Genistein treatment had a lower final body weight than those in the High Genistein treatment.

Genistein Content of the Plasma

The amount of genistein in the plasma was determined by HPLC. There were no significant differences between treatment groups in the amount of genistein in the plasma prior to or after receiving treatment doses (Table 9). Genistein was expected to be found plasma of the birds prior to receiving the treatment doses because their feed contained

soybean meal. The pre-treatment plasma samples ranged from 3.32 to 3.46 ng genistein/ml plasma. Although there were no significant differences in the amount of genistein in the post-treatment samples, there was a numeric dose-dependent response to genistein. The birds in the High Genistein treatment had the highest plasma genistein concentration followed by those in the Medium Genistein, Low Genistein, and Sham Control treatments respectively. The concentration of genistein in the post-treatment plasma ranged from 3.35 to 3.78 ng genistein/ml plasma.

Femur Wet Weight and Relative Femur Wet Weight

There was a significant difference in the wet weight of the femurs between treatment groups (Table 10). Birds in the Sham Control and Medium Genistein treatments had significantly lower femur weights than those in the High Genistein treatment (P=0.0010). The wet femur weight of birds in the Low Genistein treatment was not significantly different from those of birds in any other treatment group. There were no significant differences in femur weight as a percentage of the final body weight for any of the treatment groups (Table 10). Although not significant, the birds in the Sham Control and Medium Genistein treatments had lower relative wet femur weights than those of the Low Gensitien and High Gensitein treatments.

Femur Dry Weight and Relative Femur Dry Weight

There were significant differences in the dry weight of the femurs and dry weight of the femur as a percentage of the final body weight between treatment groups (Table 11). This indicates that the differences are due to changes in the bone, but not in the moisture content of the bone. The dry femur weights of the birds in the Sham Control and Medium Genistein treatments were significantly lower than those of birds in the High

Genistein treatment (P=0.0002). The dry femur weights of the birds in the Low Genistein treatment were not significantly different from those in any other treatment group. The dry femur weight as a percentage of final body weight of birds in the Sham Control and Medium Genistein treatments were significantly lower than those in the High Genistein treatment (P=0.0115), with those in the Low Genistein treatment not being significantly different from any other treatment.

Femur Ashed Weight and Relative Femur Ashed Weight

There were significant differences in the ashed femur weights and the ashed femur weight as a percentage of final body weight between treatment groups (Table 12). This indicates changes to the mineralization of the bone based on the various treatment doses. The birds in the High Genistein treatment had significantly heavier ashed femur weights than those in the Sham Control and Medium Genistein treatments (P=0.0002). The ashed femur weights of birds in the Low Genistein treatment were not significantly different from any other treatment group. Birds in the High Genistein treatment had significantly heavier relative ashed femur weights than those in the Sham Control and Medium Genistein treatments (P=0.0058). The relative ashed femur weight for birds in the Low Genistein treatment was not significantly different from those in any other treatment.

Femur Dry Weight and Femur Ashed Weight as a Percentage of Femur Wet Weight

There was a significant difference in the dry femur weight as a percentage of wet femur weight and in the ashed femur weight as a percent of the wet weight of the femur between treatment groups (Table 13). The birds in the Sham Control and Medium Genistein treatments had dry femurs that were a significantly lower percentage of wet

femur weight than those in the High Genistein treatment (P=0.0003). Birds in the Sham Control and Medium Genistein treatments had ashed femurs that were a significantly lower percentage of the wet femur than those in the High Genistein Treatment (P=0.0004).

Femur Ashed Weight as a Percent of Femur Dry Weight

There was a significant difference in the ashed femur weight as a percentage of dry femur weight between treatment groups (Table 13). Birds in the Sham Control and Medium Genistein treatments had ashed femur weights that were a significantly lower percentage of dry femur weight than those in the High Genistein treatment group (P=0.0044). The birds in the High Genistein treatment (20 mg/kgbody weight) consistently had significantly different bone parameters than birds in the Medium Genistein treatment (15 mg/kg body weight), but not birds in the Low Genistein treatment (10 mg/kg body weight).

Bone Strength

The strength of the bones was tested with a texture analyzer to determine the amount of force necessary to break the bone. There was a significant difference in force required to break both the femurs and vertebrae between treatment groups (Table 14). The femurs from birds in the High Genistein treatment required significantly more force to break than femurs from birds in the Medium Genistein treatment (P=0.009). There were no other significant differences between treatment groups. Birds in the Sham Control required more force to break the vertebrae than those in the High Genistein or Medium Genistein treatments (P=0.001). The birds in the Low Genistein treatment required more force to break the vertebrae than birds in the Medium Genistein treatment.

Based these results, genistein appears to have a negative effect on the strength of the vertebrae.

Discussion

There are few studies investigating the effects of the soy phytoestrogen genistein on bone strength in animals. This is the first study to investigate the effect of genistein on the bone quality of laying hens during late egg production. The ability of isoflavones to prevent bone loss or increase bone mineralization varies between studies. This variation could be attributed to the pleiotropic effects of the selected doses. Doses that were too low or too high may result in only partial bone-sparing effects (Branca, 2003). Research has also shown that there are large differences in bone composition parameters between species. Femur ash content has been shown to be similar in human, dog, and pig cortical bone. However, previous studies have shown that there there is a slightly greater femur ash content in cows, sheep, and chickens. The rat has been shown to have the highest amount of femur ash content (Aerssens et al., 1998). Because of this variation, it is difficult to compare research between species.

In the current study, genistein treatments had little effect on body weight. This was consistent with research involving ovarectomized Sprague-Dawley rats, where no significant differences were reported in the weights of genistein treated and control animals (Blair et al., 1996) or in animals injected with a vehicle or genistein at 1, 5 or 25 μg/g body weight for 21 days (Fanti et al., 1998). Research in humans has shown that soy isoflavones can increase bone mineral content and bone density (Greendale et al, 2002; Fitzpatrick, 2003). This increase may be based on the ability of isoflavones to inhibit the formation and activity of osteoclasts (Dang and Lowik, 2005). In the current study, the

High Genistein treatment had slightly heavier wet femur weights and relative wet femur weights than the other treatments. This result is consistent with a study involving Japanese quail, which showed that supplementing the diet with soy isoflavones enhanced the rate of bone formation (Sahin et al., 2007).

Supplementation with soy isoflavones has been shown to significantly improve bone mineral density in Japanese quail (Sahin et al., 2007). Research involving ovarectomized rats showed that animals treated with 44μM/day of genistein for 30 days had significantly heavier dry femoral weight than the control group (Blair et al., 1996). Also, rats receiving injections of genistein at 5 μg/g body weight/day for 21 days had more bone mineral density than animals receiving genistein injections of 1 or 25μg/g body weight (Fanti et al., 1998). This suggests that the effects of genistein are very dose dependent. The results of the current study showed that the Medium Genistein treatment (15 mg/kg body weight) had less of an effect on bone than the High Genistein (20 mg/kg) and Low Genistein (10 mg/kg body weight) treatments. This is consistent with genistein showing dose dependent effects.

Soy isoflavones have been shown to decrease the concentration of intracellular calcium in osteoclasts (Yamaguchi and Gao, 1998; Kajiya et al., 2000). This would cause an increase in the amount of calcium available for new bone formation (Sahin et al., 2007). Research on ovariectomized rats has shown that consumption of high amounts of isoflavones can increase bone mineral content (Greedale et al., 2002; Fitzpatrick, 2003). Rats treated with 44 μM/day of genistein for 30 days had significantly heavier ashed femur weight than the control group (Blair et al., 1996). The results of the current study

are consistent with previous research with the treatment receiving the most genistein having the heaviest ashed and relative ashed femur weights.

Humans and domestic animals are frequently exposed to genistein through the consumption of soy-based food products. Normal human consumption of soy based food results in plasma genistein concentrations lower than 4 uM (1080 ng/ml) (Dang and Lowik, 2005). Depending on the diet, concentrations can vary by more than 100-fold (Branca, 2003). The genistein concentration in the plasma samples of the hens ranged from 3.32 to 3.46 ng/ml pre-treatment to 3.35 to 3.78 ng/ml post treatment. The amount of genistien in the pre-treatment samples is likely due to genistein contained in the corn/soy bean meal based diet of the hens. The amount of genistein in the plasma of the animals in the current study is much lower than reported in human studies. This difference could be due to metabolic differences between humans and chickens or due to sampling times. The plasma of the hens in the current study was collected nearly 24 hours after the last treatment dose was received.

During the lifespan of a chicken, structural bone is lost from various parts of the body and is replaced with medullary bone. These parts include the bones of the wing, legs, vertebrae, and keel (Whitehead, 2004). Research has shown that the highest concentration of medullary bone is located in the femurs (Whitehead and Fleming, 2000). Research involving ovariectomized rats showed significantly higher right femur bone densities and lumbar vertebra bone densities in animals receiving soy protein supplementation (Arjmandi et al., 1996). Based on the results of the current study, the High Genistein treatment may have increased the strength of the femur by replenishing structural bone that was lost. This is supported by the fact that the High Genistein

treatment had the heaviest wet femur weights and required the most force to break. Unfortunately, the Sham Control required significantly more force to break the vertebra compared to the genistein treatments. This suggests that genistein has a negative effect on vertebral strength.

Research showing biphasic dose-dependent responses to genistein suggests that isoflavones may have an influence on bone formation as opposed to bone resorption (Branca, 2003). These biphasic dose-dependent effects of genistein are likely the result of concurrent activation of estrogen receptors and peroxisome proliferator-activated receptors. These two receptor types can produce different actions in the same cell and tissue systems (Dang and Lowik, 2005). Research on ovariectomized rat models has shown that genistein given at lower doses (0.5mg/day) tended to increase femur bone retention. Higher doses of genistein (1.6 mg/day and 5 mg/day) were less effective in retention of femur bone (Anderson et al., 1998). Biphasic dose-dependent responses have also been seen in other rodent studies with doses between 0.1 and 5 mg/day (Branca, 2003; Setchell and Lydeking-Olsen, 2003).

Effects of the High Genistein treatment was significantly different from that of the Medium Genistein and Sham Control treatments in all measurements except bone breaking force, but was not different from the Low Genistein treatment. These results suggest that the High Genistein treatment (20 mg/kg body weight) has a beneficial effect on bone quality of aged laying hens. Neither the Low Genistein treatment (10 mg/kg body weight) nor the Medium Genistein treatment (15 mg/kg body weight) showed a significant positive effect on the bone as compared to the Sham Control. The Medium Genistein treatment actually had effects that were similar to the Sham Control and

numerically lower than the Low Genistein. This suggests that 15 mg of genistein/kg body weight has a negative effect on bone quality in aged laying hens.

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Table 8. Starting, Final, and Change in Body Weight.

Treatment	N	Starting BW (kg)	P value	Final BW (kg)	P value	Change in BW (kg)	P value
Sham Control	15	1.810 ± 0.231	0.2448	1.809 ± 0.211^{AB}	0.0432	- 0.001 ± 0.094	0.1758
Low Genistein	15	1.836 ± 0.185		1.817 ± 0.220^{AB}		- 0.019 ± 0.113	
Medium Genistein	15	1.742 ± 0.192		1.729 ± 0.164^{B}		- 0.013 ± 0.074	
High Genistein	15	1.900 ± 0.235		1.960 ± 0.266^{A}		+0.060 ± 0.117	

$^{A-B}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).

[1] Values shown are treatment averages with standard deviation.

Table 9. Starting, Final, and Change in Plasma Genistein Levels.

Treatment	N	Starting Genistein (ng/ml)	P value	Final Genistein (ng/ml)	P value	Change in Gensitein (ng/ml)	P value
Sham Control	15	1.810 ± 0.231	0.2448	1.809 ± 0.211^{AB}	0.0432	- 0.001 ± 0.094	0.1758
Low Genistein	15	1.836 ± 0.185		1.817 ± 0.220^{AB}		- 0.019 ± 0.113	
Medium Genistein	15	1.742 ± 0.192		1.729 ± 0.164^{B}		- 0.013 ± 0.074	
High Genistein	15	1.900 ± 0.235		1.960 ± 0.266^{A}		+0.060 ± 0.117	

$^{A-B}$ Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).

[1] Values shown are treatment averages with standard deviation.

Table 10. Femur Wet Weight and Relative Femur Wet Weight.

Treatment	N	Wet Femur Weight (g)	P value	Relative Weight (%)	P value
Sham Control	15	7.134 ± 0.903^{B}	0.0010	0.411 ± 0.046	0.3510
Low Genistein	15	7.611 ± 0.668^{AB}		0.436 ± 0.044	
Medium Genistein	15	6.958 ± 0.924^{B}		0.419 ± 0.043	
High Genistein	15	8.177 ± 0.860^{A}		0.437 ± 0.045	

[A-B] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 8.

Table 11. Femur Dry Weight and Relative Femur Dry Weight.

Treatment	N	Dry Femur Weight (g)	P value	Relative Weight (%)	P value
Sham Control	15	4.990 ± 0.973^{B}	0.0002	0.277 ± 0.047^{B}	0.0115
Low Genistein	15	5.515 ± 0.814^{AB}		0.305 ± 0.041^{AB}	
Medium Genistein	15	4.751 ± 0.777^{B}		0.275 ± 0.037^{B}	
High Genistein	15	6.184 ± 0.919^{A}		0.318 ± 0.047^{A}	

[A-B] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 8.

Table 12. Femur Ashed Weight and Relative Femur Ashed Weight.

Treatment	N	Ashed Femur Weight (g)	P value	Relative Weight (%)	P value
Sham Control	15	2.669 ± 0.686^{B}	0.0002	0.148 ± 0.033^{B}	0.0058
Low Genistein	15	3.003 ± 0.653^{AB}		0.165 ± 0.030^{AB}	
Medium Genistein	15	2.470 ± 0.592^{B}		0.143 ± 0.030^{B}	
High Genistein	15	3.522 ± 0.611^{A}		0.182 ± 0.034^{A}	

[A-B] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Body weight values used for calculation obtained from Final BW in Table 8.

Table 13. Femur Dry Weight Percent of Femur Wet Weight, Femur Ashed Weight Percent of Femur Wet Weight and Femur Ashed Weight Percent of Femur Dry Weight.

Treatment	N	Dry % of Wet Weight	P value	Ashed % of Wet Weight	P value	Ashed % of Dry Weight	P value
Sham Control	15	69.481 ± 5.309^{B}	0.0003	36.986 ± 5.143^{B}	0.0004	53.031 ± 3.510^{B}	0.0044
Low Genistein	15	72.152 ± 4.988^{AB}		39.091 ± 5.383^{AB}		53.970 ± 4.023^{AB}	
Medium Genistein	15	68.046 ± 2.978^{B}		35.159 ± 4.402^{B}		51.565 ± 4.826^{B}	
High Genistein	15	75.401 ± 4.910^{A}		42.945 ± 4.483^{A}		56.860 ± 3.101^{A}	

[A-B] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).
[1] Values shown are treatment averages with standard deviation.
[2] Values used for calculation obtained from Femur Wet Weight in Table 10.
[3] Values used for calculation obtained from Femur Dry Weight in Table 11.

Table 14. Bone Breaking Strength.

Treatment	N	Femur (kg force)	P value	Vertebrae (kg force)	P value
Sham Control	15	25.752 ± 11.131[AB]	0.0090	33.031 ± 6.291[A]	0.0001
Low Genistein	15	31.305 ± 11.848[AB]		28.477 ± 9.868[AB]	
Medium Genistein	15	23.248 ± 8.692[B]		20.170 ± 6.101[C]	
High Genistein	15	35.348 ± 8.987[A]		25.427 ± 6.559[BC]	

[A-B] Values in columns not followed by the same superscript differ significantly ($P \leq 0.05$).

[1] Values shown are treatment averages with standard deviation.

Appendix 1: Protocol for Determining Total Isoflavones in Plasma and Liver with HPLC

Day 1

1. Freshly mix up the β-glucuronidase/sulfatase mixture by adding 0.15 g ascorbic acid, and 500 μl of β-glucuronidase/sulfatase from *Helix pomatia* to 10 ml of 0.2 *M* acetate buffer, pH 4.0.
2. Transfer aliquots of 250 μl of plasma to a 10 ml glass disposable centrifuge tube.
3. Treat with a 0.5 ml of the β-glucuronidase/sulfatase mixture to hydrolyze glucuronide and sulfate conjugates of genistein, daidzein, and glycitein.
4. Add 0.75 ml of 0.2 *M* ammonium acetate buffer to completely hydrolyze the plasma samples.
5. Cap the tubes and heat overnight (15-18 hours) at 37°C.

Day 2

1. Remove tubes from heat and cool to room temperature.
2. Add 200 μl of a 50 μg/ml solution of 4-hydroxybenzophenone in methyl tert.-butyl ether (internal standard solution) to each tube.
3. Extract the plasma sample by adding 6ml of methyl tert.-butyl ether.
4. Mix on an end-over-end mixer for 30 minutes.
5. Centrifuge at 2000g for 10 minutes at 4°C.
6. Transfer the ether layer to a ciliated glass culture tube (Ciliate tubes with SigmaCote).
7. Concentrate to dryness at 45-50°C under nitrogen.
8. Add 1 ml of hexane to remove the lipids.
9. Reconstitute the extract in 350 μl of methanol-0.05 *M* ammonium formate, pH 4.0 (20:80, v/v).
10. Mix on a vortex mixer as needed for reconstitution.
11. Transfer at least 250 μl of reconstituted material from under the hexane level to an auto-sampler vial for HPLC analysis.

HPLC Method

Column:

Flow Rate:

Detection: UV diode array at 259 nm

Inject: 40 μl per sample

Solvent A: 0.05 *M* ammonium formate, pH 4.0

Solvent B: methanol-acetonitrile (50:50, v/v)

Run time: 19 minutes

HPLC Gradient

- Start at 100% A. (0.0)
- Hold acquisition for 3 minutes. (minutes 0.0 – 3.0)
- Change to 40% B over 0.5 minutes. (minutes 3.0 – 3.5)
- Hold at 40% B for 11 minutes. (minutes 3.5 – 14.5)
- Change to 80% B over 1 minute. (minutes 14.5 – 15.5)
- Hold at 80% B for 3 minutes. (minutes 15.5 – 18.5)

Appendix 2: Protocol for Determining the Dry Matter Content of the Liver

1. Mix 5 g of liver tissue with 5 ml of distilled water.
2. Homogenize with a glass homogenizer.
3. Pipette 4 ml of the mixture into each of two weighed aluminum pans.
4. Dry the pans overnight (around 16 hours) in an oven at 105-110°C.
5. Cool samples in a desiccator.
6. Weigh the aluminum pans and determine the sample amount.

Appendix 3: Protocol for Determining Lipid Content of Liver Tissue

1. Mix 5 g of liver tissue with 5 ml of distilled water.
2. Homogenize with a glass homogenizer.
3. Pipette 4 ml of the mixture into each of two glass test tubes.
4. Add 20 ml of a chloroform-methanol (2:1 v/v) solution to each tube.
5. Mix each tube vigorously for 2 minutes on a vortexer.
6. Centrifuge for 10 minutes at 2000g at 4°C.
7. Remove the methanol-water upper layer and discard.
8. Gently tilt the tube and remove the lower chloroform layer.
9. Place the chloroform layer into a weighed aluminum pan.
10. Evaporate to dryness over a steam bath.
11. Dry samples in a 105-110°C oven for 2 hours.
12. Cool samples in a desiccator.
13. Weigh the aluminum pans and determine the sample amount.