

**Soy Isoflavone Supplementation Does Not Alter Lymphocyte
Proliferation and Cytokine Production In Postmenopausal Women.**

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Thesis Submitted to the Faculty of Virginia Polytechnic Institute and State
University in Partial Fulfillment of the Requirement for the Degree of

MASTER OF SCIENCE
IN
HUMAN NUTRITION, FOODS, AND EXERCISE

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April 30, 2001

Blacksburg, VA

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Human Nutrition, Foods and Exercise

ABSTRACT

A growing body of evidence has demonstrated that soy isoflavone consumption may protect against the development of various chronic diseases. This protection could be linked to isoflavone-induced alterations in immune function. However, recent *in vitro* and animal studies suggest that soy isoflavones may either enhance or suppress immunocompetence, depending upon the isoflavone concentration, target tissue, and a number of other factors. To date, no study has investigated the effect of dietary soy isoflavone supplementation on immune parameters *in humans*. Therefore, the purpose of this double-blind, placebo-controlled, 4 wk intervention trial was to investigate whether supplementation with soy isoflavones alters indices of immune function in postmenopausal women. Twenty healthy women (50-69 yr), who were not on hormone replacement therapy, were randomly divided into 2 treatment groups. The supplemented group ($n=10$) consumed soy isoflavone tablets (100 mg/d) for 4 wk, while the control group ($n=10$) received placebo tablets. Fasting blood samples were drawn at baseline and on d 28 to assess specific immune parameters. In addition, plasma concentrations of genistein and daidzein were quantified at baseline and at the end of the intervention period. Despite high individual variability among subjects, there was a significant increase ($p<0.005$) in plasma isoflavone concentration in the supplemented group. However, all assessed immune parameters remained unchanged after supplementation and did not differ between the 2 treatment groups. In conclusion, this study suggests that short-term soy isoflavone supplementation at physiologically attainable concentrations does not alter the aforementioned immune parameters in healthy postmenopausal women. Due to the conflicting data concerning the effect of dietary soy isoflavones on immune function, further research in this area is warranted.

Acknowledgements

I would like to thank Dr. Barbara Davis who has been an amazing and wonderful mentor and a great source of inspiration during my two years here at Virginia Tech. She has always encouraged me to think independently and pursue my goals. I am really grateful to have her as my advisor and mentor. Thanks for giving me the skills and knowledge to pursue a career in research.

To my committee members, Dr. Bakhit and Dr. Denbow I would like to offer my thanks for all your wisdom and help during my thesis.

I would like to extend my thanks to Kathy Reynolds, Janet Rhinehart and Carolyn Harris for not only assisting me in the lab and teaching me all the techniques used in this study but also being a huge source of support during my thesis. Thanks to the secretaries, Sherry Terry, Sherry Saville, Sophia Leedy and Kathy Miller for all your help.

Special thanks to Nicolin Girmes-Grieco who worked alongside me on this project. I sincerely appreciate all your help and thoroughly enjoyed working with you. I would also like to thank all my friends who have been my biggest support, motivation and my family away from home.

Last but definitely not the least, I would also like to thank my parents, my sisters Debbie, Nicola, Giselle and my brother Wayne for all their love, support and encouragement.

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Chapter 1: Introduction

A number of studies have investigated the relationship between nutrition and the immune system (1). The role of immune function has become increasingly important in our understanding of the mechanisms underlying the body's ability to prevent cancer. There is increasing evidence that dietary alteration of immune function may be a key component of disease prevention (2,3).

Recent publications have emphasized the difference between countries in the incidence of many diseases, including coronary heart disease, breast cancer, endometrial and ovarian cancers as well as menopausal symptoms (4,5,6). These differences have been attributed in part to diet, amongst other characteristics. Asian women who traditionally consume a low fat, high soy diet have a four-to-six-fold lower risk of developing breast cancer as compared to their Western counter parts (7). However, when these Asian women move to Western countries and adopt the typical Western diet, their incidence of breast cancer approaches that of the Western world after two generations (7). This strongly suggests that the marked difference in breast cancer incidence between women of Asian and Western cultures is not due to genetic factors alone but is more likely to be due to environmental factors, one of which may be diet. A major difference between Asian and Western diets is the high consumption of soy foods by the Asian population (8,9). This results in dramatic differences in one class of potentially active components in soy, isoflavones. It is estimated that consumption of soy by Asians results in intakes of 25-45 mg of isoflavones per day for the average person (9,10), whereas in the U.S. and Canada average isoflavone consumption is less than 5 mg/d (9). Japan has the highest consumption of isoflavones at approximately 200 mg/d (11). Epidemiological studies (4,5) as well as animal studies (10,12) suggest that high consumption of isoflavone-rich soy food may confer a reduced risk of cancer at many sites. These observations have prompted an increased interest in soy isoflavone consumption and their potential as disease preventing agents.

Soy isoflavones are known to exert weak estrogenic activity by virtue of their structural similarity to estrogens (12,13). Since sex hormones are known to modulate the human immune response (13), it may be possible that dietary isoflavones modulate the

immune system, by acting either as estrogen agonists or antagonists. Limited data exists regarding the relationship between soy isoflavone consumption and immune function. Recent animal and *in vitro* data have suggested that soy isoflavones at high concentrations may act as protein tyrosine kinase inhibitors and thus could act as immunosuppressive agents (14). On the other hand, a number of other *in vitro* studies have investigated the immunostimulatory role of genistein and daidzein at physiologically relevant concentrations (7, 9, 12).

This controversy surrounding the immunomodulatory effect of soy isoflavones may be due to the fact that there are a number of factors that dictate the effect of soy and soy isoflavones. In addition to having weak estrogenic and antiestrogenic activities, isoflavones also possess a variety of characteristics such as antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing abilities. Thus, many of these activities could affect immune function. Soy isoflavones also act as inhibitors of a number of enzymes like protein tyrosine kinase, topoisomerase II, and others important for proper functioning of the immune system.

In October 1999, the US Food and Drug Administration authorized a new health claim suggesting that 25 g/d of soy protein has potential health benefits (15). Soy products and soy isoflavone tablets are already being widely consumed by postmenopausal women for alleviation of menopausal symptoms and prevention of osteoporosis, as well as for the prevention of some cancers and heart disease. Thus, it is important to clarify the role of soy consumption with respect to the immune function. However, there have been very few studies and virtually no human studies that have investigated this relation between soy isoflavone consumption and immune response. This study was the first *human* study that investigated this relationship. Because of the increased use of soy isoflavones by postmenopausal women, we studied the effects of supplementation with the isoflavones genistein and daidzein on parameters of immunity in this population.

Chapter 2: Literature Review

Immune response:

Immune response in humans can be generally categorized into two components, innate immunity and adaptive immunity. While innate immunity is the defense mechanism of the immune system that we are born with, acquired or adaptive immunity is the defense mechanism that the body develops as a response to a particular pathogen. Acquired immunity can further be subdivided into cell-mediated immunity and humoral immunity (16).

Immune responses are produced primarily by cells called leukocytes. Leukocytes that are particularly important in the adaptive immune response are called lymphocytes (17). Lymphocytes specifically recognize individual pathogens intracellularly or in the extracellular tissue fluids or blood. B lymphocytes or B cells form an important part of the humoral immune response providing immunity against extracellular microbes. B cells recognize these extracellular pathogens by means of a cell surface receptor and are thus stimulated to produce antibodies. These antibodies specifically recognize and bind to particular target molecules on the surface of the pathogen called the antigen. This binding causes the activation of phagocytosis, which is the engulfing and elimination of the pathogen (17). T lymphocytes are involved with cell-mediated immunity and have a variety of functions. T helper cells can interact with B cells and help them to differentiate and produce antibodies; T helper cells can also assist mononuclear phagocytes and help them to destroy intracellular pathogens (17). T cytotoxic cells, on the other hand, are responsible for destroying tumor cells or host's cells that have been infected by intracellular pathogens. The T cells recognize antigens present on the surface of these infected cells, thus bringing about destruction of the cells/tumors (17). Natural Killer (NK) cells are a third group of lymphocytes (13), they account for up to 15% of blood lymphocytes and express neither T cell nor B cell antigen receptors. The main responsibilities of NK cells are surveillance against spontaneously arising tumors, destruction of tumor cells, and restriction of tumor metastases (18). Additional functions of NK cells include killing of virus-infected cells and secretion of certain regulatory

molecules called cytokines, which are discussed below. Other cytokines like IL-12 and IL-15 are involved in the expansion and activation of NK cell populations.

All of these lymphocytes are capable of recognizing essentially only one particular antigen (18). When these cells encounter a recognizable antigen they bind to it, thus causing stimulation of the lymphocytes and induction of cell division or proliferation. Lymphocytes that have been stimulated by binding to their particular antigen, take the first step towards clonal expansion (19). In this process, an antigen binds to and stimulates a specific T or B cell population to undergo mitosis and develop into a clone of cells with the same antigenic specificity as the original parent cell, thus amplifying the ability of the immune system to eliminate the pathogen. A measure of the rate of this proliferation/expansion is a common marker of immune function.

Lymphocytes may also start to produce cytokines themselves. Cytokines are "messenger proteins" secreted by immune cells and are soluble mediators of intercellular communication (13). These proteins are involved in signaling between cells during an immune response. Over 100 different human cytokines have been identified (13). Of these, Interleukins (IL) are an important group of cytokines and are mainly produced by T cells. Presently, Interleukins 1-17 have been identified. IL-2, an important cytokine supports the continuous, exponential growth of human T cells and also acts as a differentiation molecule that promotes T cytotoxic cell activity and B cell activity (18). IL-2 induces secretion of interferon- γ (IFN γ) by T cells. IFN γ is another important cytokine that enhances NK cell activity, induces the generation of T cytotoxic cells, activates macrophages for tumor killing and antimicrobial activity and modulates the expression of class II major histocompatibility complex (MHC) molecules (18). MHC is a cluster of genes, whose products play a central role in the development of both humoral and cell-mediated immune responses. This complex also determines whether a transplanted tissue will be accepted as self (histocompatible) or rejected as foreign (histoincompatible) (20).

As stated earlier, T lymphocytes are classified into T helper and T cytotoxic cells, based on their immune response. T helper cells can be further subdivided into Th1 and Th2 cells on the basis of their cytokine production (17). The Th1 subset responds to viral infections and intracellular pathogens by secreting IL-2 and IFN γ , which activate T

cytotoxic cells and macrophages (21). The Th2 subset produces IL-4, IL-5, IL-6 and IL-10 and is effective at stimulating B cells to proliferate and produce antibodies, thus functioning to protect against free-living microorganisms (17).

Soy Isoflavones:

Soy is a rich source of nonsteroidal estrogens of the isoflavone class (22). Isoflavones are considered phytoestrogens, which are defined as naturally occurring plant-based compounds that are structurally and functionally similar to estradiol. A characteristic chemical feature of phytoestrogens is the presence of a phenolic ring that, with few exceptions, is required for binding to the estrogen receptors (Fig 1). Isoflavones can act as estrogen agonists or antagonists (23). However, a number of factors including concentration, receptor status, presence or absence of endogenous estrogens and the type of target organ or cell influences their mode of action.

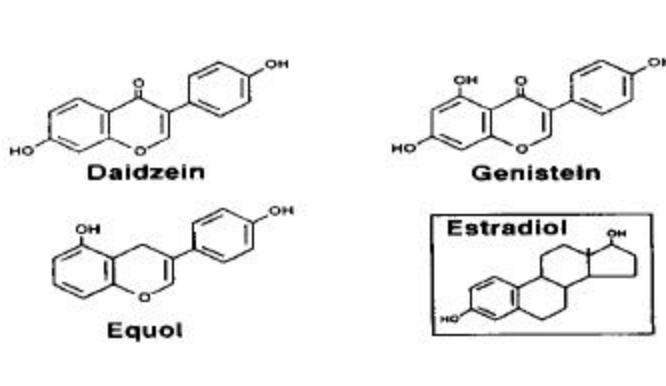


Fig 2.1: Principal isoflavone aglycones of soy, the bacterial product of isoflavone metabolism equol, and the female sex hormone estradiol (7).

Dietary soy isoflavones exist as β -glycoside conjugates in soy products (8). The two principal isoflavones present in soy are **genistin** and **daidzin**. These are metabolized by gut microflora to their respective active aglucone forms **genistein** and **daidzein**. Stomach acids may also help in this process. The sugar portion is removed from the glycosides thus converting them to their active aglucone form (12). It has also been reported that daidzein is more bioavailable than genistein due to its longer half-life in the intestine (24,25). An important isoflavone, equol is not found in soybeans but is derived from soy isoflavones through the action of intestinal bacteria. Equol has been found in

high concentrations in the portal venous blood of rats and in bile. Genistein was also found to appear rapidly in bile confirming that, in common with endogenous estrogens, isoflavones undergo biliary secretion (26-29). These findings suggest an enterohepatic circulation for isoflavones.

Along with steroid hormone metabolism (30-32), the liver plays an important role in the further metabolism of isoflavones by conjugating the aglycone with glucuronic acid and to a lesser extent, sulfuric acid (33). Conjugation is considered a detoxification step but in some cases after conjugation the biological activities of the conjugates are enhanced (54). Most studies of the metabolism of isoflavones have focused on urinary excretion. In both blood and urine, isoflavones are found primarily as conjugates. It has been found that, after a soy rich meal the urinary excretion of isoflavones can increase upto 1,000 times (12). This indicates that soy isoflavones are readily absorbed and metabolized even at high concentrations.

The incidence of breast cancer in Western countries is far greater than that observed in Asian and third world populations (7). Epidemiological studies have shown that this difference is due more to environmental factors rather than genetic differences and diet is considered one of the major contributing factors (7). In the Far East and Asian countries, soybeans and their proteins have long been staples in the diet (8,9). The incidence of hormone-dependent diseases is relatively low in these countries. Recent research has suggested that this low incidence of disease could be attributed to soy consumption (4,5,12,13).

In most Asian countries the consumption of isoflavones is estimated at 50-100 mg/d (8) and in Japan it is as high as 200 mg/d (10). Comparatively, consumption of isoflavones in Western countries is as low as 5 mg/d (8). However, soy consumption is increasing in these countries due to the availability of soy tablets and other products, as well as an increased awareness of the potential benefits of soy on human health.

Foods made from soy have varying amounts of isoflavones depending on the extent to which they have been processed. Soy foods such as tofu, soymilk, soy flour and soy nuts have isoflavone concentrations of 1.3-3.8 mg/g. Soy sauce and soybean oil have virtually no isoflavones (9,34). Soy products used as additives such as isolated soy protein have varying amounts of isoflavones depending on how they are processed. These

compositional differences may have a significant impact on the amount of isoflavones being consumed from highly processed foods by individuals in the US and may affect the metabolism and bioavailability of the isoflavones.

Soy Isoflavones and the Immune Response

Background:

As previously stated, isoflavones are considered phytoestrogens and can exert weak estrogenic and/or antiestrogenic actions in estrogen sensitive tissues. There have been a number of studies conducted to investigate the relationship between sex hormones, particularly estrogen, and the immune system (35-38). In several mammalian species, including humans, females have demonstrated higher serum immunoglobulin (Ig) levels as compared to males. In fact, in a study conducted by Terres et al., (38), it was found that females show a quantitative and qualitative enhanced capacity to produce antibodies after immunization. This difference in antibody production has been attributed to the sex hormones.

Estradiol in particular, at physiological concentrations, was found to inhibit the activity of suppressor T cells in pokeweed mitogen stimulated cultures (39). This inhibition caused an enhanced B cell differentiation that led to an increased antibody synthesis. On the other hand estradiol seems to suppress cell mediated immunity thus increasing the risk of tumor production. In a study conducted by Yohn (40), it was found that a gonadectomy in hamsters decreased the number of tumors, however when these animals were given a dose of 0.05 mg estradiol daily, the number of tumors increased. Similar observations were made in humans (41,42) where estrogen was found to decrease T-lymphocyte activation in breast cancer patients, thus suppressing immune responses against the tumors.

Also, women are more susceptible to pathological conditions like autoimmune diseases and allergies as compared to men and this has been attributed to the involvement of estrogenic hormones (43). Sex hormones, in particular estrogen have been linked to diseases like lupus, rheumatoid arthritis (44), as well as asthma (45) and dermatitis (46). Animal studies have also suggested females to be more susceptible to autoimmune

diseases like diabetes, autoimmune thyroiditis and arthritis than males. A number of animal studies have provided data showing an acceleration of the autoimmune disease lupus when lupus-resistant male mice were treated with estrogen or anti-androgens (47).

As previously stated, the soy isoflavones, genistein and daidzein share structural similarities with estradiol (Fig. 1) (12,13). Thus considering the immunomodulatory effects of estrogen, it may be possible that these dietary isoflavonoids could also influence the immune system. However, the exact mechanism by which these isoflavones may exert their influence is highly speculative.

Genistein as an estrogen agonist or antagonist:

Genistein is structurally similar to estradiol and binds weakly to the estrogen receptor, which is the intracellular mediator of estrogen action. Genistein has only about 1/1000 the estrogenic activity of estradiol (22,42), however the circulating concentration in individuals consuming a moderate amount of soy foods is nearly 1,000 fold higher than peak levels of endogenous estradiol in premenopausal women (22,43). Zava et al., (51) concluded that at low physiologically relevant concentrations (1 nM-1 µM), genistein is a potent estrogen agonist based on the fact that at these concentrations, genistein caused the induction of the estrogen-regulated antigen pS2 as well as growth stimulation of MCF-7, estrogen positive breast cancer cells. However, at higher concentrations (\geq) 10 µM genistein was found to be growth inhibitory. These data suggest that soy-derived genistein, at physiologically relevant concentrations, have estrogenic activity and thus might exert estrogenic actions *in vivo*, especially in postmenopausal women, where ovarian synthesis of estrogen has ceased. These findings were in agreement with epidemiological studies (52,53) wherein genistein intake may have exhibited estrogenic effects thus reducing the number of health problems related to estrogen deficiency in postmenopausal women.

On the other hand, there is some concern that since estrogen is a known promoter of breast cancer, genistein might have the same promotional effect in breast cancer survivors and in women at high risk of breast cancer. However, epidemiologic studies reveal that women consuming large amounts of soyfoods have a 5-10 fold lower incidence of breast cancer (8,22,52). In a case controlled study conducted by Lee et al.

(54) investigating the role of soyfood consumption and the incidence of breast cancer in Singapore, it was shown that high soy intake significantly reduced the risk of developing breast cancer in premenopausal women. This was further emphasized by animal studies that demonstrated that genistein inhibits the growth of carcinogen induced breast tumors. This can be explained by the fact that genistein exerts estrogen agonistic activity via estrogen receptors (ER). However, at high concentrations genistein exhibits an ER-independent cellular mechanism thus inhibiting cell proliferation. This was seen in a study conducted by Zava et al., (51), where at physiologically relevant concentrations ranging from 10 nM to 1 μ M, genistein showed growth promoting actions. However, at concentrations greater than 10 μ M, genistein abruptly inhibited the proliferation of ER (+) and ER (-) breast cancer cells. This higher concentration is not achievable *in vivo* and therefore its significance to prevention of breast cancer is not certain. It is clear from this study that the action of genistein on cell proliferation is dose dependent. Other factors influencing estrogenic activity of genistein include levels of endogenous estrogen present, bioavailability of the soy isoflavones as well as the target tissue (7).

Enhancement of non-specific immune function by soy daidzein:

In a study by Zhang et al., the dose dependent immunostimulatory effects of daidzein on immune function in mice were studied (55). It was found that the *in vivo* dietary administration of daidzein at 20 and 40 mg/kg daily for seven days had marked effects both on humoral as well as cell-mediated immune response in Swiss mice. Increases in the phagocytic response of peritoneal macrophages and thymus weight were reported along with an increase in the lymphocyte proportion of the peripheral blood. However at a concentration of 10 mg/kg, daidzein did not show any significant effects (55). In a recent study, genistein (5mg/d) injected into ovariectomized female rats was found to decrease thymic weight by 80% and also decreased the total thymocyte number (66). The isoflavone concentrations at which significant results were obtained were higher than those consumed in the normal diet. This immunostimulatory effect of daidzein may be attributed to the fact that estrogen receptors have been found in the immune system specifically on lymphocytes. Daidzein may bind the estrogen receptor

and thus exert estrogenic activity. However, it should be noted that this binding affinity as well as estrogenic activity is extremely weak when compared with those of estradiol.

Another plausible mechanism by which high concentrations of daidzein may affect the immune system is by suppressing prostaglandin synthesis (55). This could be true since prostaglandins regulate immune responses and are known to be immunosuppressive at high concentrations. Additional investigation of the relationship between daidzein and prostaglandin synthesis is needed.

Potentiation of lymphocyte activation by soy isoflavones:

In a 1997 study, Wang and colleagues (56) demonstrated the effects of daidzein and genistein on lymphocyte proliferation and cytokine production in murine splenocytes. It was found that daidzein, at concentrations of 0.01-10 µM, was capable of significantly increasing proliferation in response to stimulation by the mitogens Concanavalin A (Con A, p<0.01) and lipopolysaccharide (LPS, p<0.05). Similarly, significant (p<0.05) increase in the secretion of the cytokines IL-2 and IL-3 by daidzein was also observed (56). Genistein, which has a binding affinity similar to daidzein for the estrogen receptors in estrogen responsive tissues, did not have immunostimulatory action. This suggests that an estrogen dependent mechanism might not be involved in the stimulatory activity of daidzein on lymphocyte proliferation and cytokine production. Thus the exact mechanism by which daidzein and genistein exert their action on the immune system requires further investigation.

Effect of genistein on IL-1 β promoter activity:

A recent study has suggested that estradiol can enhance IL-1 β production in a model monocytic cell line (hER+IL-1 β -CAT+) and that this occurs at the transcriptional level (57). Since genistein is known to be an estrogen agonist, an *in vitro* study was conducted by Ruh and colleagues (57) to examine this effect of genistein on IL-1 β promotor activity. Genistein was found to enhance IL-1 β promoter activity but at a very low potency (57). The low potencies may be due to the fact that the immune system might not be as sensitive to environmental estrogens as the tissues and organs of the

reproductive systems. However the effect of genistein on cytokine gene expression in humans still remains to be studied.

Genistein modulates Natural Killer (NK) cell activity:

Natural Killer (NK) cell activity is an important immune parameter, acting against tumor development and progression as well as against infectious agents. NK cell activation is dependent on tyrosine kinase activity and genistein is a known inhibitor of tyrosine-specific protein kinase (14). In a study conducted by Zhang et al.(57), genistein, at physiologically relevant concentrations (0.1-0.5 μ mol/L), and daidzein and genistein glucuronides at levels of 0.1-10 μ mol/L significantly ($p<0.05$) enhanced NK cell-mediated K562 cancer cell killing by human peripheral blood cells. In addition, this stimulatory activity of genistein (<0.5 μ mol/L) and the isoflavone glucuronides (0.1-10 μ mol/L) were further enhanced in the presence of IL-2 (0.31 ng/well). However, at concentrations >0.5 μ mol/L genistein was found to inhibit NK cytotoxicity significantly ($p<0.05$).

Genistein is a specific inhibitor of tyrosine specific kinase:

As noted above, genistein has the ability to inhibit breast cancer cell growth (51). This inhibition may be due to the inhibition of the enzyme tyrosine kinase (14) by genistein. Tyrosine-specific protein kinase (PTK) activity is associated with cellular receptors for several growth factors such as epidermal growth factor, platelet derived growth factor, insulin and insulin-like growth factor (14). Genistein was found to inhibit the activities of epidermal growth factor receptor PTK activity leading to the formation of non-productive enzyme-substrate complexes. Since the binding of growth factors to their receptors at the plasma membrane is essential for cell growth and differentiation, PTK inhibition by genistein can inhibit cell proliferation and transformation (14, 59). In a study conducted by Traganos et al. (60), a protein kinase inhibitor (H7) blocked normal human lymphocyte stimulation and induced apoptosis of both normal lymphocytes and leukemia cells. In addition to cell growth, tyrosine phosphorylation is also involved in NK cell activity and cytokine production (61). Thus, inhibition of protein tyrosine kinase could be a potential mechanism by which genistein exerts immunomodulatory effects.

Potential immunosuppressive actions of soy isoflavones:

There is considerable data suggesting the role of protein tyrosine kinase (PTK) inhibitors as useful immunosuppressive agents (60, 61). As noted above, genistein is a potent PTK inhibitor and it also functions in inhibiting DNA topoisomerase II, another important enzyme in immune function. Genistein was found to inhibit IFN γ production in mouse Th1 cells and IL-4 in Th2 cells in a dose dependent manner (58). This could be via inhibition of tyrosine phosphorylation, therefore inhibiting cytokine production. Additional data have also suggested that genistein might inhibit normal and malignant mononuclear cells in large animals and humans through the inhibition of DNA topoisomerase II synthesis (62, 63). Also, in a recent study, O'Connor and colleagues fed Lewis rats diets containing high doses of genistein (5 mg/g isoflavones) as an immunosuppressive drug to delay rejection of rat cardiac allografts (64). In another study, genistein (5 mg/day) injected into ovariectomized female mice was found to decrease the proportions of both CD4+ and CD4+CD8+ cells (65). In the same study, thymocyte number was greatly reduced in genistein-injected mice as compared to controls (65). As previously mentioned, Zhang et al. (58), observed that at *in vitro* concentrations greater than 0.5 μ mol/L genistein was found to inhibit NK cytotoxicity significantly ($P<0.05$).

However, it is important to remember that most of the above studies were conducted in animals or *in vitro* and the dose of isoflavones administered was much higher than the normal physiologically relevant doses.

Other mechanisms of action of soy isoflavones:

In addition to the above mechanisms of action of soy isoflavones, *in vitro* studies indicate that these compounds could act through a variety of additional cellular mechanisms. Genistein as well as the other flavonoids have shown potent antioxidant properties (66) that would prevent free radical formation, DNA damage and ultimately carcinogenesis. At very high concentrations, *in vitro* studies have found genistein to induce apoptosis and to inhibit angiogenesis (63) and topoisomerase II (63), all of which are involved with increased cell growth and thus might be related to immune function. Genistein has also been reported to inhibit aromatase and 17 β -hydroxysteroid oxidoreductase, enzymes responsible for converting peripheral androgens to estrogens

and estrone to estradiol, respectively. This could be an additional mechanism by which soy isoflavones carry out their actions.

However, it has been observed that the concentrations required to carry out the above functions *in vitro* ($>100 \mu\text{M}$) far exceed the highest concentration of isoflavones that can be achieved *in vivo* ($0.1\text{-}1.1 \mu\text{M}$) even after heavy consumption of soy foods (62-66). This would suggest that these mechanisms might not be responsible for the isoflavone activity *in vivo*, unless the cells have some mechanism to concentrate the isoflavones, or if over expression of the above growth regulators renders them more sensitive to the effects of the isoflavones.

Most of the data studying this controversial relationship between soy isoflavones and immune function comes from animal studies or *in vitro* studies. To date virtually no human study has investigated this association. Since soy isoflavone consumption is on the rise it was important to study the effect of soy isoflavone consumption on immune function in humans. Therefore, we conducted an *in vivo* study to determine the effect of soy isoflavone consumption (100 mg/d for 4 wk) on specific immune parameters in postmenopausal women.

Chapter 3: Soy Isoflavone Supplementation Does Not Alter Immune Parameters in Postmenopausal Women

Abstract

A growing body of evidence has demonstrated that soy isoflavone consumption may protect against the development of various chronic diseases. This protection could be linked to isoflavone-induced alterations in immune function. However, recent *in vitro* and animal studies suggest that soy isoflavones may either enhance or suppress immunocompetence, depending upon the isoflavone concentration, target tissue, and a number of other factors. To date, no study has investigated the effect of dietary soy isoflavone supplementation on immune parameters *in humans*. Establishing whether isoflavones effect immunity in aging adults is particularly relevant since compromised immune function has been observed in this population. Therefore, the purpose of this double-blind, placebo-controlled, 4 wk intervention trial was to investigate whether supplementation with soy isoflavones alters indices of immune function in postmenopausal women. Twenty healthy women (50-69 yr), who were not on hormone replacement therapy, were randomly divided into 2 treatment groups. The supplemented group ($n=9$) consumed soy isoflavone tablets (100 mg/d) for 4 wk, while the control group ($n=9$) received placebo tablets. Fasting blood samples were drawn at baseline and on d 28 to assess circulating lymphocyte distribution, lymphocyte proliferation, cytokine production, and NK cell cytotoxicity. In addition, plasma concentrations of genistein and daidzein were quantified at baseline and at the end of the intervention period. Despite high individual variability among subjects, there was a significant increase ($p<0.005$) in plasma isoflavone concentration in the supplemented group. However, all assessed immune parameters remained unchanged after supplementation and did not differ between the 2 treatment groups. In conclusion, this study suggests that short-term soy isoflavone supplementation at physiologically attainable concentrations does not alter the aforementioned immune parameters in healthy postmenopausal women.

Keywords: Isoflavones, genistein, daidzein, postmenopausal, lymphocytes, natural killer cells

Abbreviations: Con A, Concanavalin A; IFN γ , interferon- γ ; IL-2, interleukin-2; NK, natural killer; PBS, phosphate buffered saline; PTK, protein-tyrosine kinase.

Running Title: Isoflavones and Human Immune Function

Introduction

It is well documented that immune function declines in aging. For example, during aging both males and postmenopausal women show declines in anti-viral responses(1). Also, aging has been associated with reduced lymphocyte proliferation (2) and high levels of pro-inflammatory cytokines (3). In an effort to counteract the negative consequences of aging, nutritionists have investigated the effect of specific dietary components on various aspects of immunity. For example, vitamin E supplementation has been found to enhance cell mediated immunity in healthy elderly populations (4). Continued investigation of the impact dietary constituents have on immune function, especially in aging individuals, is important.

Epidemiological data have suggested that consumption of soyfoods, rich in the phytoestrogens genistein and daidzein may have significant health benefits (5). Soy consumption has been associated with a decreased risk of cardiovascular disease (6), and may be beneficial in preventing the bone loss that leads to osteoporosis (7). Women also consume soy isoflavones to alleviate symptoms related to menopause (8). In addition to having weak estrogenic and antiestrogenic activities, isoflavones also possess characteristics such as antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing abilities that may modulate immunity (9).

To date, limited studies have investigated the relationship between soy isoflavone consumption and immune function. However, data do exist suggesting isoflavones may either act to stimulate or suppress immunity depending on the dose and route of administration. In an *in vitro* study conducted by Wang et al. (10), daidzein at concentrations of 0.01-10 μ M significantly ($p<0.05$) increased lymphocyte proliferation as well as secretion of cytokines IL-2 and IL-3 in murine splenocytes in response to Con A and lipopolysaccharide (LPS) stimulation. Genistein alone at similar concentrations did not have any immunostimulatory action (10). Since both isoflavones have similar estrogen receptor-binding affinities in estrogen responsive tissues (10), the findings of this study suggest that an estrogen-dependent mechanism might not be involved in the stimulatory activity of these isoflavones on lymphocyte proliferation and cytokine production. However, a combination of genistein (1 μ M) and daidzein (0.01 μ M) had a

greater effect on murine splenocyte cultures than daidzein alone, suggesting that more than one mechanism might be involved (10). Another *in vitro* investigation showed that, genistein significantly ($p<0.05$) stimulated natural killer (NK) cell-induced K562 cancer cell killing at a concentrations range of 0.1-0.5 μ M (11). However, at higher doses (5-50 μ M) genistein significantly ($p<0.05$) inhibited NK cell-mediated cytotoxicity. Thus, genistein may act as a potential immunosuppressive agent at pharmacological concentrations. Since activation of NK cells is in part dependent on tyrosine phosphorylation (12), genistein, a known protein tyrosine kinase (PTK) inhibitor (13), may suppress NK cell activity via this mechanism. In addition, a recent study investigating the immunosuppressive action of isoflavones reported a decrease in total number of thymocytes as well as proportions of CD4 $^{+}$ and CD4 $^{+}$ CD8 $^{+}$ cells in genistein-injected (5mg/d) ovariectomized female mice (14). Data from O'Connor and colleagues (15) demonstrate that administration of a diet containing high doses of the soy isoflavones (5mg/g isoflavones) may act as an immunosuppressive agent to delay rejection of rat cardiac allografts. These conflicting observations regarding the immunomodulatory effect of soy isoflavones may be due to the fact that the mechanistic actions of isoflavones are highly sensitive to the amount, duration and route of administration of isoflavones.

Further justifying the need for determining the effect of isoflavone supplementation on immune function comes from a recent report of the synthetic isoflavone derivative, ipriflavone (16). In this study, a significant number of postmenopausal women consuming this supplement (600 mg/d) for 12 mo were observed to have lymphocytopenia within 6 mo of treatment. This decrease in average lymphocyte numbers returned to normal within 24 mo in 81% of the patients, while the remaining 19% were followed up until the counts returned to normal. Thus, results from this trial further emphasize the need to clarify the role between isoflavone supplementation and immune function.

In October 1999, the US Food and Drug Administration authorized a new health claim suggesting that 25 g/d of soy protein can reduce coronary heart disease(17). Soy products and soy tablets may already be widely consumed by postmenopausal women for the prevention of some cancers, heart disease and osteoporosis as well as alleviation of

menopausal symptoms. In fact, according to the 2001 Soyfoods Guide, soy food sales in the United States have increased dramatically from \$2 million to just over \$2 billion during the past two decades (18). Thus, it is important to clarify the role of soy consumption with respect to immune function in this population. However, virtually no human data on this relationship between soy consumption and immune response is available. Therefore, the objective of this *in vivo* study was to determine the effects of dietary soy isoflavone supplementation on lymphocyte distribution and proliferation, cytokine production and NK cell cytotoxicity in healthy postmenopausal women.

Materials and Methods

Subjects:

Postmenopausal women aged 50-69 y were recruited to participate in this study. Exclusion criteria for selection included unusual dietary habits, regular consumption of soy rich foods; high alcohol consumption; last menstrual cycle less than two years ago; history of disease; estrogen replacement therapy; use of any tobacco products and use of drugs, dietary- and/or herbal supplementation known to influence immune function. All subjects were free-living and maintained their normal dietary habits and physical activities. Approval for the use of human subjects was granted from the Virginia Tech Institutional Review Board and all subjects provided informed consent.

Experimental Design:

In this double-blind, placebo-controlled intervention trial, all subjects were randomly assigned to one of two treatment groups. The experimental group ($n=9$) consumed two 50 mg soy isoflavone tablets/d (NovaSoy, Arthur Midland, Decatur, IL) for 28 d, while the control group ($n=9$) received similar looking placebo tablets. The isoflavone tablets provided a total of 55-60 mg/d of isoflavone aglycones, which is representative of the amount typically consumed in Asian societies (19, 20). To monitor compliance, subjects initialed a calendar each day following ingestion of the tablets and they were contacted by phone during the study. In addition, all participants completed 3-day dietary intake records twice during the intervention period to confirm usual dietary habits.

Fasting blood samples (24 ml) were collected from each subject on d 1 (pre) and d 28 (post) of the trial and used for determination of plasma isoflavone concentration, distribution of circulating lymphocytes, lymphocyte proliferation in response to mitogen, cytokine production by stimulated lymphocytes and NK cell cytotoxicity.

Determination of Isoflavone Concentration in Plasma

High Performance Liquid Chromatography (HPLC) was used for the determination of the soy isoflavones, daidzein and genistein in plasma according to the method of Xu et al. (21). Plasma (500 µl) was diluted in 10 ml of 80% methanol and centrifuged at 3000 x G for 10 min at 10°C. After centrifugation, the supernatant was collected in an evaporator flask and the pellet was resuspended in 5 ml methanol and centrifuged as above. Following centrifugation, the combined methanol extracts were evaporated and then dissolved in 5 ml of 1 M sodium acetate. Glucuronidase/sulfatase (50 µl H2 Type, Sigma G0876) was added and samples were incubated overnight at 37°C. The isoflavones from this prepared hydrolysate were extracted on a C18 SPE reverse phase column (Fisher Scientific, Pittsburgh, PA) and then separated on a Luna C18 250 x 4.6 mm column (Phenomenex, Torrance, CA), with 15% HPLC-grade methanol in HPLC grade water as the mobile phase. All samples were run in duplicates and fluorescein prepared in 80 % methanol was used as the internal standard. A standard curve was prepared using a series of genistein standards (200, 500, 1000 and 1200 ng) dissolved in 80% HPLC methanol and assayed to quantify the isoflavones.

Determination of Lymphocyte Distribution:

Fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD4 (clone SK3, T-helper cells), FITC-labeled mouse anti-human CD3 (clone SK7, pan-T cells), phycoerythrin (PE)-labeled mouse anti-human CD8 (clone SK1, T-cytotoxic cells), PE-labeled mouse anti-human CD19 (clone 4G7, pan-B cells) and PE-labeled mouse anti-human CD16+CD56 (clones B73.1 and MY31, NK cells) monoclonal antibodies (mAb) were used to label lymphocytes in 100 µl whole lysed blood (Becton Dickinson, San Jose, CA). Cells were double-stained with CD4 and CD8, CD3 and CD19, and CD3 and CD16+CD56 mAbs in duplicate. CD-Check Plus (Becton Dickinson, San Jose, CA) was used as a control. Cells were resuspended in 0.5 ml of 1.0 % paraformaldehyde and kept on ice until analysis the next morning. A total of 10,000 cells/sample was analyzed using an Epics XL flow cytometer (coulter Electronics, Hialeah, FL) at the Virginia Tech Flow Cytometry Lab. Lymphocyte populations were assessed by forward and side light scatter and subsequent analyses were made by gating this population. Results generated from

this analysis were expressed as percentages of gated lymphocytes staining positively for each antibody.

Lymphocyte Isolation:

Peripheral blood mononuclear cells were isolated using the double density Ficoll separation method (22). Heparanized blood (8 ml) from each subject was drawn into Vacutainer BD tubes containing Ficoll Paque reagent (BD Pharmingen, San Diego, CA). After centrifugation for 30 min, the lymphocytes were removed from the interface, washed twice with sterile PBS and resuspended in complete media (RPMI supplemented with 2 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin). An aliquot of the cell suspension was counted using a haemocytometer in the presence of Tryphan Blue dye and the isolated lymphocytes were used for proliferation, cytokine and natural killer cell cytotoxicity assays.

Lymphocyte Proliferation:

The ability of isolated lymphocytes to proliferate in response to the T-cell mitogen, Concanavalin A (Con A) was measured using the ^3H -thymidine incorporation assay (23). Briefly, isolated lymphocytes from each subject were seeded into 96-well plates (1×10^6 cells per well) in complete media plus 10% autologous serum and incubated with 0, 2.5 or 5.0 µl/ml Con A. Following incubation at 37°C and 5 % CO_2 for 68 h, 20 µl of ^3H -thymidine (50 µCi/ml) were added to the cells and incubated for an additional 4 h at 37°C . Cells were collected onto a glass fibre filter using a cell harvester (Skatron, Sterling, VA) and the incorporated radioactivity was measured using a liquid scintillation counter (Beckman LCS 6500). The results were obtained as counts per minute (cpm) and the effect of soy isoflavones on lymphocyte function was reported as the stimulation index calculated as the average counts per minute (cpm) of mitogen stimulated cultures minus the cpm of cultures without mitogen (baseline cpm) divided by the baseline cpm.

Analysis of IL-2, and IFN γ Protein expression:

Interleukin-2 (IL-2) and Interferon- γ (IFN γ) production in response to Con A stimulation was measured in cell culture supernatant. Isolated lymphocytes (7.5×10^5

cells/well) were seeded into 24 well plates in complete medium plus 10% autologous serum containing 0 or 10 µg/ml Con A. Following incubation at 37°C for 48 h, cell free supernatants were stored at -80°C until assayed. Enzyme linked immunosorbent assay (ELISA) analysis (OptEIA, Pharmingen, San Diego, CA) was used to assess cytokine protein concentration in cell culture supernatant.

Natural Killer Cell-Mediated Cytotoxicity:

NK cell-mediated cytotoxicity was determined according to the procedure of Korzeniewski and Callewaert (24) using the CytoTox 96 Kit (Promega Corporation, Madison, WI). An aliquot (100 µl) of K562 (target) cells (American Type Culture Collection, Manassas, VA) were maintained in Iscove's medium prepared with 10 % FBS, 4 mM glutamine, 100 µg/ml Penicillin and 100 units/ml Streptomycin. Effector-to-target cell ratios were adjusted to 12.5:1 (2.5×10^5 : 2×10^4) and 25:1 (5×10^5 : 2×10^4) and plated in quadruplicate in 96-well, NUNC v-bottom culture plates (Fisher Scientific) containing effector cells (2.5×10^5 and 5×10^5) and target cells (2×10^4). Following incubation for 6 h at 37°C in a humidified CO₂ incubator (NuAire, Plymouth, MN), lactate dehydrogenase (LDH) release by lysed target cells was measured spectrophotometrically at 490 nm using a Ceres 900 microtiter plate reader (Bio-Tek Instrument, Inc., Winooski, VT). Percent cytotoxicity was calculated using the mean for each quadruplicate in the following equation: $[(E - LS - TS) / (TM - TS)] \times 100$, where E = experimental wells, LS = lymphocyte spontaneous LDH release, TS = target spontaneous LDH release, and TM = target maximum LDH release.

Statistical Analysis:

Statistical analyses were performed in collaboration with the Statistical Consulting Center at Virginia Tech. Changes in the immune parameters measured were statistically analyzed between the two treatment groups and over time using a repeated measure ANOVA (Sigma Stat, SPSS Inc., Chicago, IL). Mean differences were considered to be significant at p<0.05. All data are expressed as mean ±SEM.

Results

Subjects

Participant information is given in the Table 1. All female subjects recruited for this study were Caucasian females, age 50-69 y. Mean ages for the Suppl group (59.0 ± 1.7 y) and the Ctrl group (57.1 ± 2.0 y) were similar (Table 3.1). All participants were considered healthy and had been postmenopausal for ≥ 2 years.

According to the 3-day dietary records, all 18 subjects avoided eating any products containing soy isoflavones. Subject compliance was high, as reflected by the biochemical assessment of plasma isoflavone concentrations as well as the initialed calendars and phone interviews. The plasma concentration of the primary soy isoflavone, genistein, was significantly ($P < 0.005$) higher in the supplemented group compared to the control group after 28 d of soy treatment (Table 3.1). None of the women who participated in the study reported any adverse effects from the soy isoflavone supplementation.

Table 3.1: Subject Characteristics

Age (y)	N	Age (y)	Years Menopausal	Plasma Genistein (nmol/L) [†]	Plasma Daidzein (nmol/L) [†]
Control	9	57.1 ± 2.0	6.6 ± 2.7	ND	ND
Supplemented	9	59.0 ± 1.7	7.1 ± 2.1	$402.4 \pm 121.0^*$	ND

Values are expressed as mean \pm SEM. ND= none detected

[†] N=8 for plasma isoflavone concentration data.

*Values are significantly different between the control group and supplemented group at $P < 0.005$.

Peripheral Lymphocyte Distribution

Flow cytometric analyses showed that 4 wk dietary soy isoflavone supplementation had no significant effect on peripheral lymphocyte composition. These data are shown in Table 3.2 and Figures 3.1-3.3. Circulating lymphocyte subpopulations were similar between the two groups at baseline and did not change significantly in response to the isoflavone treatment. Mean percentages for all measured lymphocyte subpopulations fell within normal ranges for healthy adult females.

Table 3.2: Distribution of circulating lymphocyte subpopulations expressed as percentages of total gated lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavone tablets (Suppl).

Cell Type	Ctrl Pre (%)	Ctrl Post (%)	Suppl Pre (%)	Suppl Post (%)
CD3 ⁺ CD4 ⁺	43.4 ± 2.4	44.9 ± 2.4	43.8 ± 1.8	49.0 ± 4.3
CD3 ⁺ CD8 ⁺	26.2 ± 1.9	24.1 ± 1.8	25.0 ± 2.3	24.7 ± 2.1
CD3 ⁺	64.5 ± 2.6	65.0 ± 2.6	68.3 ± 2.8	65.8 ± 3.1
CD19 ⁺	10.8 ± 1.1	10.5 ± 1.1	9.3 ± 1.0	9.1 ± 1.0
CD16 ⁺ CD56 ⁺	12.7 ± 2.1	11.4 ± 1.8	10.3 ± 1.8	10.6 ± 2.2
CD3 ⁺ CD4 ⁺ /CD3 ⁺ CD8 ⁺	1.7 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.3
CD3 ⁺ /CD19 ⁺	6.8 ± 2.6	7.4 ± 2.6	8.4 ± 2.8	8.6 ± 3.0

Values are mean ± SEM, p>0.05 between groups and time.

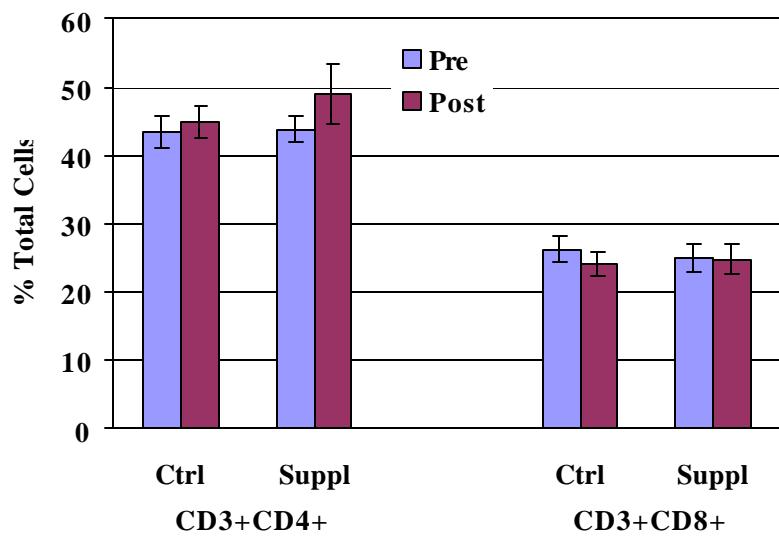


Figure 3.1: Distribution of $CD4^+$ (T-helper) and $CD8^+$ (T-cytotoxic) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are mean \pm SEM, $p>0.05$ between groups.

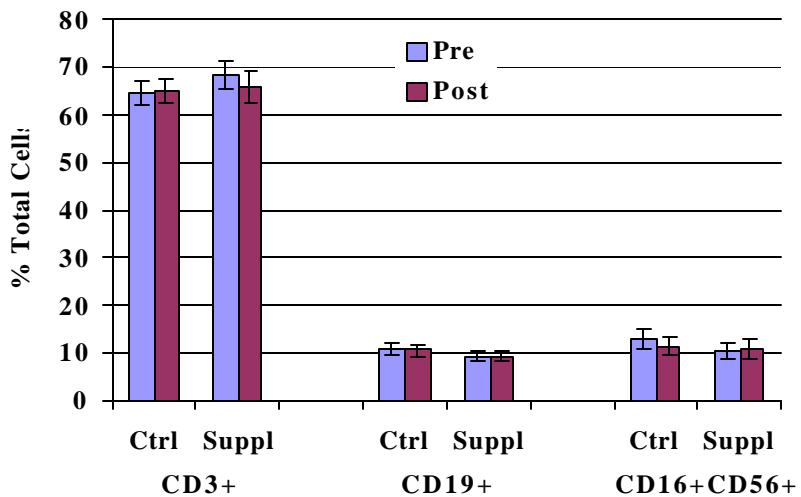


Figure 3.2: Distribution of $CD3^+$ (pan-T), $CD19^+$ (pan-B) and $CD16^+, CD56^+$ (natural killer) cells expressed as percentages of total gated circulating lymphocytes at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=9) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are mean \pm SEM, p>0.05 between groups.

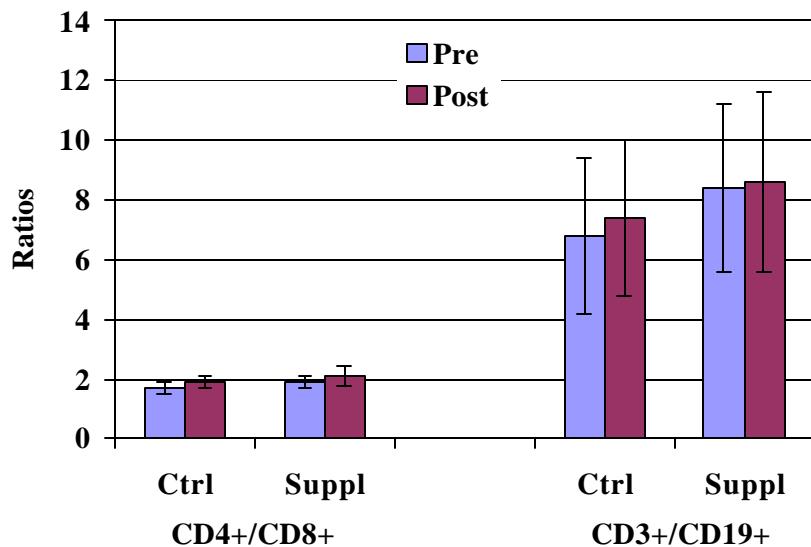


Figure 3.3: Ratio of T-helper/T-cytotoxic ($CD3^+CD4^+/CD3^+CD8^+$) cells and pan-T/pan-B ($CD3^+/CD19^+$) cells at baseline (pre) and after 4 wk (post) supplementation with placebo (Ctrl; n=7 for $CD3^+CD4^+/CD3^+CD8^+$, n=9 for $CD3^+/CD19^+$) or 100 mg/d soy isoflavone tablets (Suppl; n=9). Values are mean \pm SEM, p>0.05 between groups.

Lymphocyte Proliferation

Measurement of ^{3}H -thymidine incorporation in Con A-stimulation of lymphocyte cultures was used to study proliferation changes in response to soy isoflavone supplementation. Data are expressed as stimulation index, which is calculated as the average counts per minute (cpm) of mitogen stimulated cultures minus the cpm of cultures without mitogen (baseline cpm) divided by the baseline cpm. Proliferative ability of lymphocytes in response to the mitogen was unaltered after soy supplementation. At Con A concentrations of 2.5 $\mu\text{l}/\text{ml}$, the mean stimulation index for the Ctrl (pre=85.60; post=78.71) and Suppl (pre=70.92; post=70.35) groups did not differ significantly (Fig 3.4). An increase in the stimulation index was observed at the higher Con A concentration (5.0 $\mu\text{l}/\text{ml}$), however the indices did not differ significantly between the Ctrl (pre=134.71; post=105.76) and Suppl (pre=90.30; post=101.79) groups (Fig 3.4).

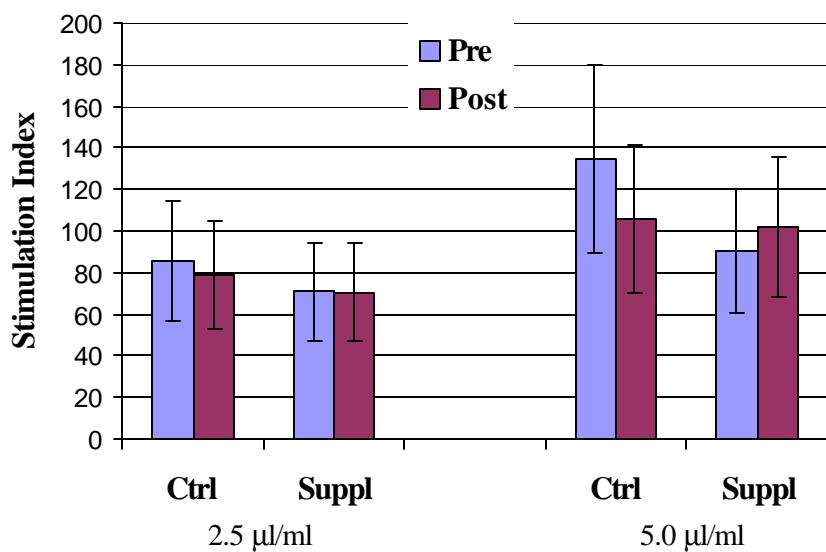
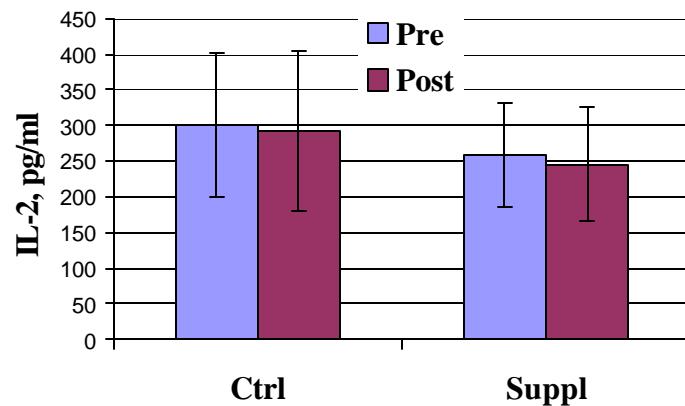


Figure 3.4: ^{3}H thymidine incorporation by Con A stimulated lymphocytes isolated from subjects (n=9/group) at baseline (pre) and 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavones (Suppl) for 4 wk. Values are mean \pm SEM, p>0.05 between groups.

Cytokines IL-2 and IFN γ Production:

Concentrations of IL-2 and IFN γ produced by Con A stimulated lymphocytes did not differ significantly between the two treatment groups after 4 wk of soy supplementation. Mean baseline IL-2 concentrations for Ctrl (n=6) and the Suppl (n=7) groups were; 301.18 pg/ml and 258.83 pg/ml respectively, while the corresponding post-intervention levels were 292.19 pg/ml and 244.81 pg/ml respectively (Fig 3.5A). IFN γ concentrations for the Ctrl group (n=5) were pre=1053.84 pg/ml; post=754.75 pg/ml and for the Suppl group (n=5) IFN γ levels were pre=923.83 pg/ml; post=642.13 pg/ml (Fig 3.5B).

(A)



(B)

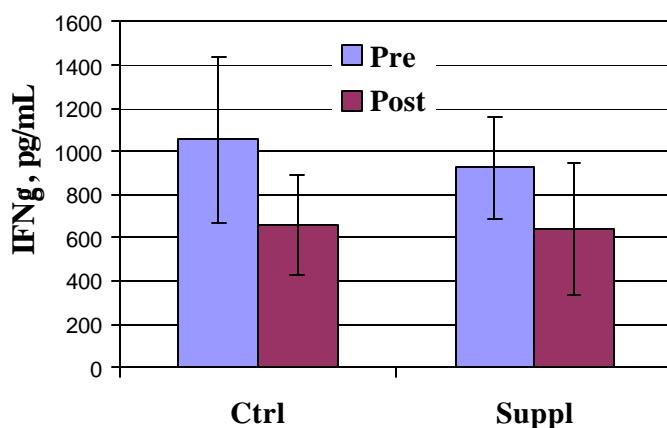


Figure 3.5: Cytokine production by Con A stimulated (10 μ g/ml) lymphocytes isolated from subjects at baseline (pre) and 4 wk (post) supplementation with placebo (Ctrl) or 100 mg/d soy isoflavone tablets (Suppl) for 4 wk. (A) IL-2 (Ctrl n=8; Suppl n=6) and (B) IFN γ (n=5/group) concentrations were assessed by ELISA. Values are mean \pm SEM, p>0.05 between groups.

Natural Killer Cell Activity

No significant differences were observed in NK cell-mediated cytotoxicity in response to soy isoflavone supplementation for 4 wk. NK (effector) cell activity was based on LDH release by K562 (target) cells at 25:1 and 12.5:1 effector to target cell ratios. At the higher ratio (25:1), mean NK cell cytotoxicity was $24.6 \pm 2.9\%$ and $26.1 \pm 3.6\%$ (pre) and $22.5 \pm 2.1\%$ and $19.5 \pm 3.0\%$ (post) for the Ctrl and Suppl group, respectively (Fig 3.6). At the lower ratio (12.5:1), mean % cytotoxicity was $16.9 \pm 3.8\%$ and $17.1 \pm 5.4\%$ (pre) and $13.3 \pm 2.9\%$ and $12.4 \pm 5.3\%$ (post) for the Ctrl and Suppl group, respectively (Fig 3.6).

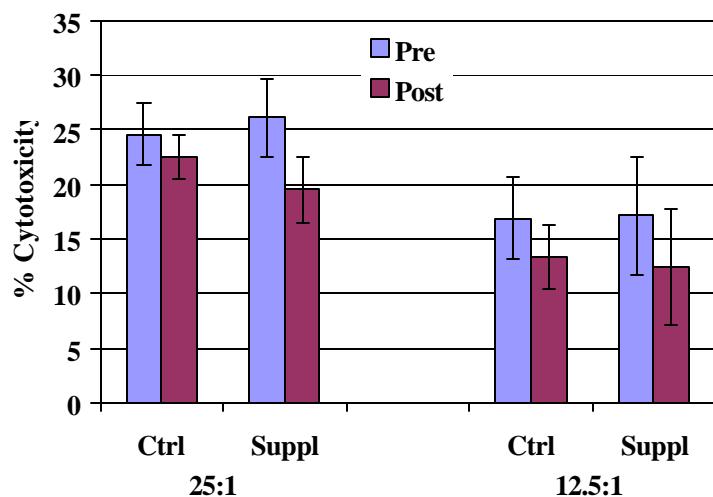


Figure 3.6: Percent cytotoxicity of circulating natural killer cells based on lactate dehydrogenase release by target K562 cells at baseline (pre) and after 4 wk (post) supplementation with placebo tablets (Ctrl; n=9 for 25:1, n=7 for 12.5:1) or 100 mg/d soy isoflavone tablets (Suppl; n=8 for 25:1, n=5 for 12.5:1). The ratios 25:1 and 12.5:1 represent the two effector to target cell ratios used. Values are mean \pm SEM, p>0.05 between groups.

Discussion

A growing body of evidence suggests that nutrition and immune function are closely related. In recent years the soy isoflavones, genistein and daidzein, have received increasing attention due to their potential to prevent a variety of cancers, cardiovascular disease, osteoporosis and their role in alleviating menopausal symptoms. These protective actions have been attributed in part to the weak estrogenic activity of isoflavones as well as estrogen-independent mechanisms such as antioxidant, antiproliferative, anti-inflammatory, enzyme inhibitory and differentiation-inducing properties. Yet, to date, research examining the effects of soy isoflavone supplementation on immune parameters in humans is limited. Since aging has been associated with decreased immunocompetence, we studied the effects of soy isoflavones on cell-mediated immunity in postmenopausal females. In this study, we found no difference in the distribution of circulating lymphocytes, the *in vitro* proliferative ability of lymphocytes in response to Con A stimulation, the production of cytokines, and the activity of natural killer cells after 4 wk of soy isoflavone supplementation at 100 mg/d in healthy postmenopausal women.

The results of several *in vitro* and animal studies have suggested enhanced immune function in response to soy isoflavone supplementation. In an *in vitro* study conducted by Wang and colleagues (10), daidzein at physiologically relevant concentrations (0.01-10 µM) in culture medium potentiated lymphocyte activation and increased IL-2 and IL-3 production in mitogen-activated murine lymphocyte cultures. However, in our study, 100 mg/d of soy isoflavone supplementation, which resulted in mean plasma genistein concentration of 0.4 µM and no detectable plasma daidzein concentration, did not significantly alter lymphocyte proliferation or cytokine IL-2 and IFN- α production in response to stimulation by the mitogen Con A. In an earlier *in vivo* study (25), the same researchers reported an enhancement of lymphocyte activation in response to high doses of daidzein supplementation (20-40 mg/kg/d) in mice. However, this immunostimulatory effect of daidzein was not observed at the physiologically relevant concentration of 10 mg/kg (25). A major difference between the previous studies and our *in vivo* human study was that the former studies used daidzein exclusively, whereas the soy isoflavone tablets used in this study mimicked the natural isoflavone composition of soybeans. In addition, unlike our study, the study of Wang and

colleagues (10) was *in vitro* and thus differences in bioavailability of the isoflavones could have been a decisive factor in determining our results.

In this study, 4 wk of soy isoflavone supplementation did not affect NK cell-mediated K562 cancer cell killing. These findings were in contrast to data obtained by Zhang and colleagues (11), where genistein significantly ($p<0.05$) stimulated human NK cell-induced K562 cell lysis at a concentration range of 0.1-0.5 μ M. Yet at higher concentrations (5-50 μ M) significant ($p<0.05$) inhibition of NK cell-mediated cytotoxicity was observed. Thus, at pharmacological doses, genistein may actually exert immunosuppressive effects in humans. Although the mean genistein concentration of 0.4 μ M among our supplemented subjects falls between the two lower concentrations (0.1 and 0.5 μ M) used by Zhang and coworkers (11), the lymphocytes in our lab had no direct exposure to soy isoflavones during their 6 h incubation with K562 cancer cells. Therefore, if genistein is indeed capable of potentiating NK cell activity, as proposed by Zhang and colleagues, its effect seems to be immediate. Our results suggest that this potential effect may not be sustainable after the exposure of lymphocytes to genistein has ceased. Alternatively, isoflavones may be affecting the target cells in the culture conditions utilized in the *in vitro* study by Zhang (11).

The proposed immunomodulatory actions of genistein seem to be highly dose-dependent, since at high concentrations of this isoflavone (5-50 μ M), Zhang and colleagues (11) observed significant ($p<0.05$) inhibition of NK cell-mediated cytotoxicity . Thus, at pharmacological doses, genistein may actually exert immunosuppressive effects in humans. Further evidence that soy isoflavones may suppress immune function comes from two recent animal studies. O'Connor and colleagues (15) fed Lewis rats diets containing high doses of soy isoflavones (5 mg/g isoflavones) as immunosuppressive agent to delay rejection of rat cardiac allografts. In addition, genistein (5 mg/d for 21 d) intravenously injected into ovariectomized female mice resulted in an 80% reduction of thymic weight, while genistein injection at 2 mg/d for only 7 d led to a 70% decrease in thymic weight (14). The researchers also found that the proportions of thymic CD4⁺ and CD4⁺CD8⁺ cells decreased in genistein treated mice compared to the control animals (14). These immunosuppressive actions of genistein may partly be explained by its role as a specific PTK inhibitor, since the activation of NK cell cytotoxicity and cytokine production (12) are both dependent on tyrosine phosphorylation. As previously mentioned, we report no significant changes in the specific

immune parameters, including NK cell activity and cytokine production. In all of the above studies, the inhibitory activity of genistein was observed only at concentrations substantially higher than the plasma genistein levels (0.1-1.1 μ M) obtained in our study. Furthermore, the routes of isoflavone administration used in the previous studies were direct isoflavone injection or consumption of diets containing high isoflavone soy proteins (5 mg/g isoflavones) (15). On the contrary, in the present study, subjects ingested tablets containing concentrations of isoflavones (100 mg/d) typically found in Asian soy rich diets (19,20). Thus, route of administration and dose could have contributed to the observed results. Additionally the differences in individual bioavailability of dietary soy isoflavones may have been an important factor in our study. Since our study was in an *in vivo* environment, a number of factors could have influenced the metabolism and uptake of the ingested isoflavones. In fact, on measuring plasma isoflavones we found variations in circulating genistein levels from 0.1-1.1 μ M. In addition to being dose-dependent, isoflavone-mediated activities are further influenced by a number of other factors, such as endogenous estrogen levels. The fact that we did not measure plasma concentrations of 17 β -estradiol may be a potential limitation of this study. However, since our subject population consisted of postmenopausal women, we did not foresee differences in estrogen levels as being a confounding factor.

In conclusion, data from our study revealed that 28 d of soy supplementation did not alter selected immunologic parameters in healthy postmenopausal women. This is important information in light of the recent data regarding the ability of the synthetic isoflavone derivative, ipriflavone, to decrease the number of circulating lymphocytes in postmenopausal women consuming this drug for 6 mo (16). Thus, further studies documenting the long-term effect of soy isoflavone supplementation on immunity is warranted.

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Chapter 4: Results

Subject Characteristics and Plasma Soy isoflavone levels:

The participants recruited for this study were Caucasian, postmenopausal women ranging in age from 50 to 69 y. Of the 21 subjects, one participant did not complete the study due to a scheduling conflict with the post-intervention blood draw. Also, on determination of plasma isoflavones levels, two subjects were found to have high plasma isoflavone levels at baseline and were therefore excluded from data analysis. Thus, a total of 18 subjects (n=9/group) were included in the final analyses.

Subject characteristics are listed in Table 4.1. Based on a health questionnaire completed by each subjects, all participants were considered generally healthy and had been postmenopausal for ≥ 2 y. Compliance was assessed by monitoring initialed calendars completed by subjects and through regular phone interviews conducted during the supplementation period. Also, biochemical assessment of plasma isoflavones further confirmed subject compliance. According to the 3-d dietary records, all 18 subjects avoided eating any products containing soy isoflavones.

The concentrations of plasma genistein as determined by HPLC were significantly increased ($p<0.005$) in the supplemented group as compared to the control group after 28 d of isoflavone consumption. The concentration of circulating genistein in the supplemented group was within the range of 0.1-1.1 μM with a mean concentration of 0.4 μM , while no detectable levels of daidzein were observed in this group. Also, subjects consuming placebo tablets (Ctrl group) did not have detectable plasma levels of both genistein and daidzein. Individual concentrations of plasma genistein after 4 wk of soy isoflavone supplementation have been listed in Table 4.2. None of the women who participated in the study reported any adverse effects from the soy isoflavone supplementation.

Table 4.1: Subject Characteristics

Group	N	Age (y)	Years Menopausal	Plasma Genistein (nmol/L) [†]	Plasma Daidzein (nmol/L) [†]
Control	9	57.1 ± 2.0	6.6 ± 2.7	0.0 ± 0.0	0.0 ± 0.0
Supplemented	9	59.0 ± 1.7	7.1 ± 2.1	402.4 ± 121.0*	0.0 ± 0.0

Data are expressed as mean ± SEM.

[†] N=8 for plasma isoflavone concentration data.

*Values are significantly different between the control group and supplemented group at p<0.005.

Table 4.2: Plasma genistein concentrations of individual subjects after supplementation with 100 mg/d of soy isoflavone tablets for 4 wk.

Subject #	Plasma Genistein (nmol/L)
1	532.94
5	190.50
7	166.24
9	260.55
11	1198.21
12	360.23
14	301.85
18	208.88

Isolation of lymphocytes from whole blood:

Lymphocytes were isolated using the Ficoll Paque density gradient method using vacutainer BD tubes. Total number of lymphocytes obtained from 16 ml of whole blood was determined by counting with a hemocytometer using Trypan Blue dye. After counting, the isolated lymphocytes were aliquoted for use in the lymphocyte proliferation assay (1×10^6 cells/well), natural killer cell cytotoxicity assay (2.5×10^5 cells/well, 5.0×10^5 cells/well) and cytokine production assay (7.5×10^5 cells/well). Total number of lymphocytes isolated from 16 ml of whole blood from individual subjects is listed in Table 4.3. In cases where the number of isolated lymphocytes was insufficient, only one concentration of cells was used for the NK cytotoxicity assay.

Table 4.3: Total number of lymphocytes isolated from 16 ml of blood from individual subjects.

Subject	Pre-treatment	Post-treatment
	Total no. of cells*	Total no. of cells*
1	23.04×10^6	20.74×10^6
2	16.4×10^6	16.42×10^6
3	12.91×10^6	10.03×10^6
4	23.23×10^6	15.12×10^6
5	10.8×10^6	14.33×10^6
6	6.19×10^6	13.06×10^6
7	13.85×10^6	14.5×10^6
8	14.33×10^6	16.7×10^6
9	10.4×10^6	11.09×10^6
10	14.83×10^6	17.64×10^6
11	12.41×10^6	10.14×10^6
12	20.25×10^6	22.23×10^6
13	22.56×10^6	25.77×10^6
14	24.0×10^6	12.59×10^6
15	14.69×10^6	7.15×10^6
16	17.85×10^6	9.52×10^6
18	17.06×10^6	20.69×10^6
19	28.22×10^6	19.69×10^6
20	33.41×10^6	16.86×10^6
21	18.1×10^6	14.04×10^6

*total no of cells in 16 ml whole blood

Lymphocyte Proliferation:

Measurement of ^{3}H -thymidine incorporation in Con A-stimulated lymphocyte cultures was used to study changes in proliferative ability of T cells in response to soy isoflavone supplementation. Data was expressed as stimulation index which is the average counts per minute (cpm) of mitogen stimulated cultures minus the cpm of cultures without mitogen (baseline cpm) divided by the baseline cpm. Proliferation of stimulated lymphocytes remained unaltered after soy supplementation. At Con A concentrations of 2.5 $\mu\text{l}/\text{ml}$, the mean stimulation index for the Ctrl (pre=85.60; post=78.71) and Suppl (pre=70.92; post=70.35) groups did not differ significantly (Fig.4.1). An increase in the stimulation index was observed at the higher Con A concentration (5.0 $\mu\text{l}/\text{ml}$), however the indices did not differ significantly between the Ctrl (pre=134.71; post=105.76) and Suppl (pre=90.30; post=101.79) groups (Fig.4.1).

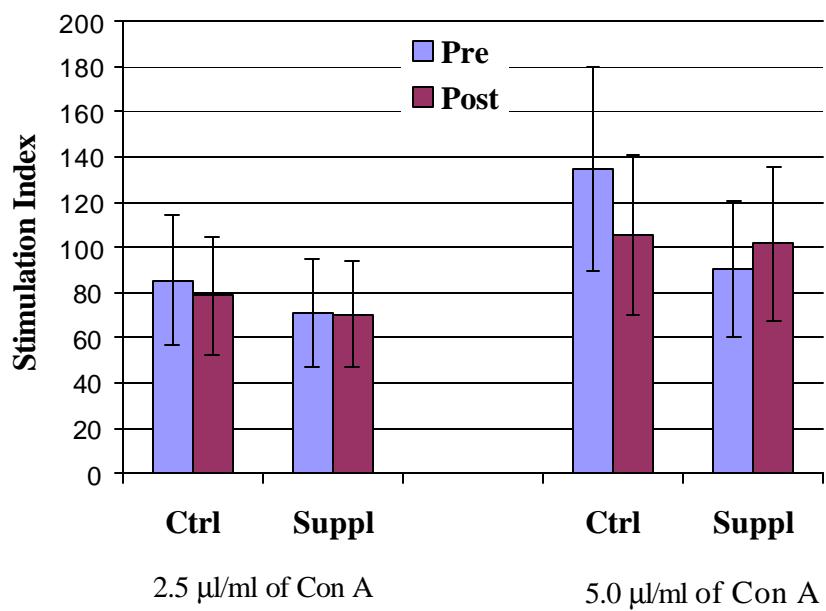
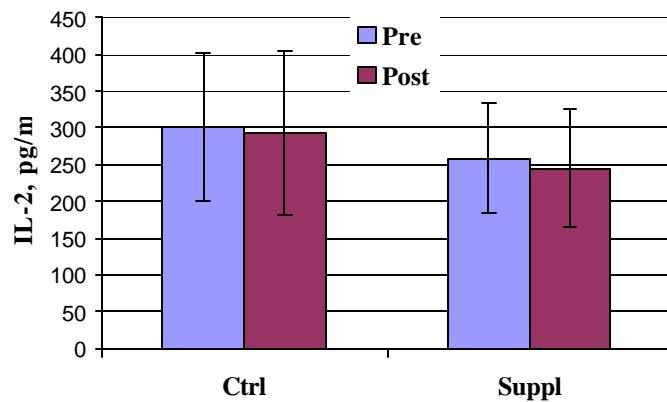


Figure 4.1: ^{3}H thymidine incorporation by Con A stimulated lymphocytes isolated from subjects ($n=9/\text{group}$) consuming placebo (Ctrl) or 100 mg/d soy isoflavones (Suppl) for 4 wk. No significant difference were observed between groups at either concentration of mitogen; values are means \pm SEM.

Cytokines IL-2 and IFN γ Production:

Concentrations of IL-2 and IFN γ produced by Con A stimulated lymphocytes did not differ significantly between the two treatment groups after 4 wk of soy supplementation. Mean baseline IL-2 concentrations for Ctrl (n=6) and the Suppl (n=7) groups were; 301.18 pg/ml and 258.83 pg/ml respectively, while the corresponding post-intervention levels were 292.19 pg/ml and 244.81 pg/ml respectively (Fig.4.2A). IFN γ concentrations for the Ctrl group (n=5) were pre=1053.84 pg/ml; post=754.75 pg/ml and for the Suppl group (n=5) IFN γ levels were pre=923.83 pg/ml; post=642.13 pg/ml (Fig. 4.2B).

(A)



(B)

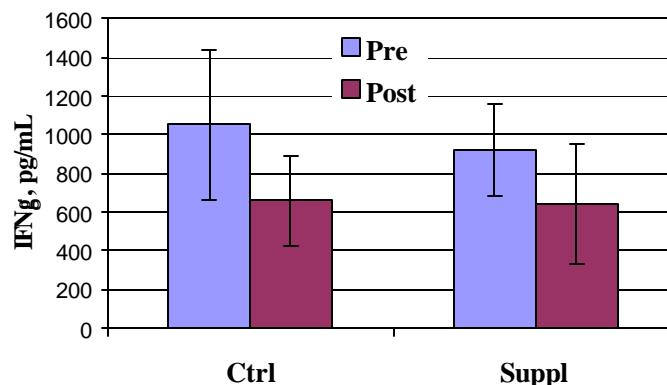


Figure 4.2: Cytokine production by Con A stimulated (10 μ g/ml) lymphocytes isolated from subjects consuming placebo (Ctrl) or 100 mg/d soy isoflavones (Suppl) for 4 wk. (A) IL-2 (Ctrl n=8; Suppl n=6) and (B) IFN γ (n=5/group) concentrations were assessed by ELISA. No significant differences were observed between groups; values are means \pm SEM

Chapter 5: Conclusion

There are limited data documenting the relationship between soy isoflavone consumption and human immune function. In recent times, the soy isoflavones, genistein and daidzein, have received increasing attention due to their potential activity in preventing a variety of cancers, cardiovascular disease, osteoporosis as well as their role in alleviating menopausal symptoms. These protective actions are in part attributed to the weak estrogenic activity isoflavones possess by virtue of their structural similarity to 17 β -estradiol. Gonadal hormones, and in particular estrogen have been associated with enhanced immune function in women. Other characteristics like antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing abilities might also contribute to the ability of soy isoflavones to modulate immune response.

In an *in vitro* study conducted by Wang and colleagues (56), the presence of daidzein (0.01-10 μ M) in culture medium caused increased lymphocyte activation in mitogen-activated murine lymphocyte cultures. In the same study, the researchers also reported a significant increase in production of cytokines (IL-2 and IL-3) in response to daidzein treatment (0.01-10 μ M). However, in our study, 100 mg/d of soy isoflavone supplementation which resulted in mean plasma genistein concentrations of 0.4 μ M did not significantly alter lymphocyte proliferation or cytokine IL-2 and IFN γ production in response to stimulation by the mitogen Con A. A major difference between the previous studies and our *in vivo* human study was that the former studies used daidzein exclusively, whereas the soy isoflavone tablets used in this study mimicked the natural isoflavone composition of soybeans. Also, Wang and coworkers (56) performed an *in vitro* study using mice cells, as compared to our *in vivo* human study, thus bioavailability could be have been significant influencing factor in our study. Also, physiological factors like other immune cells, presence of compounds that might interfere with isoflavone metabolism etc. present in the *in vivo* environment might play a role in predicting lymphocyte potentiation and cytokine action. These factors so not have to be accounted for in an *in vitro* medium. In an earlier *in vivo* study (55), the same researchers reported an enhancement of lymphocyte activation in response to daidzein supplementation in

mice. However, the doses fed to the animals in this study (20-40 mg/kg) were much higher than the physiologically relevant concentrations (100 mg/d) used in our study.

We used medium supplemented with 10 % autologous serum to culture lymphocytes during the proliferation and cytokine assays. The presence of autologous serum rather than fetal bovine serum (normally used) would help to maintain the lymphocytes in their respective environments (\pm isoflavones). However, the concentration of isoflavones in the culture medium plus 10% autologous serum would be 10-fold less than in the circulation and considerably less than the concentrations used in the *in vivo* studies with mice.

Additional studies have also investigated other immune parameters in response to isoflavone treatment. Zhang and colleagues (58) reported an increase in NK cell activity in response to low levels of soy isoflavone treatment *in vitro* and a decrease in NK cell cytotoxicity at higher concentrations. Thus, it is evident that these actions of soy isoflavones are dependent on a concentration of isoflavones as well as route of administration.

Tyrosine phosphorylation has been associated with NK cell activity and cytokine production, therefore, the inhibitory action of genistein on protein tyrosine kinase could render it a potential immunosuppressive agent. Recent data have supported the use of genistein as an immunosuppressive agent in animal studies. Genistein was also found to inhibit IFN γ production in mouse Th1 cells and IL-4 in Th2 cells in a dose dependent manner (59). In addition, genistein (5 mg/d for 21 d) injected into ovariectomized female mice, was found to decrease thymic weight by 80%, total thymocyte number by 50% and also decreased proportions of both CD4+ and CD4+CD8+ cells (66). In another study, O'Connor et al., fed rats diets containing high doses of genistein (5 mg/g isoflavone) as an immunosuppressive drug to delay rejection of rat cardiac allografts (65). In all of the above studies, the inhibitory activity of genistein was observed only at concentrations substantially (5mg/d isoflavone supplementation) higher than the physiologically achievable concentrations of 0.1 - 1.1 μ M obtained in our study. As mentioned previously, we report no changes in the specific immune parameters (lymphocyte proliferation and cytokine production) studied. In the above studies the mode of administration of the soy isoflavones was by injection or by consumption of soy foods, while our subjects ingested

isoflavone tablets, this could lead to potential differences in bioavailability of isoflavones. In addition, in a parallel study conducted in our lab, no significant differences in lymphocyte distribution or NK cell activity was observed between the two treatment groups in response to dietary soy isoflavone supplementation in humans. Plasma genistein concentrations were significantly ($p<0.005$) higher in the supplemented group after 4 wk of soy consumption, however analysis revealed high individual variability in plasma isoflavone concentration among subjects and thus bioavailability could be a significant factor that could influence isoflavone activity. Thus further research is warranted to clarify this relationship between soy consumption and immune function.

A potential limitation of this study may be the variability in bioavailability observed among the individual subjects of the supplemented group. Therefore, our smaller subject number restricted us from monitoring changes in immune parameters in relation to various isoflavone concentrations. Also, endogenous estrogen levels may affect soy isoflavone action (7). Thus, the fact that we did not measure plasma concentrations of 17β -estradiol may be a potential limitation of this study. However, since our subject population consisted of women, who had been postmenopausal for >2 years, circulating estrogen levels would be negligible. Furthermore, study subjects were not obese and therefore would not have excessive estrogen production from adipose tissue. Soy isoflavone action may be modulated/affected by the presence of other compound present in natural soy product. The fact that we used isoflavone tablets rather than whole soy food may have been a modulating factor of the observed isoflavone action. However, our study was designed to determine whether genistein and daidzein themselves would affect immune function and therefore, it was necessary to use soy isoflavones rather than soy foods.

Chapter 6: Future Research

In recent years, soy supplementation has become increasingly popular due to its potential health benefits. However, the effect of soy consumption on the human immune function is still unclear. This was the first human study to investigate the relationship between normal levels of isoflavone supplementation and certain immune parameters. In this study, soy supplementation did not alter the specific immune parameters studied. Since the immunomodulatory effect of soy is highly dependent on isoflavone concentrations, future studies investigating various dosages of soy supplementation would be informative. Conducting our study for a longer period of time might also be beneficial since an interesting decreasing trend was observed for certain immune parameters. Also, endogenous estrogen level can modulate soy actions, thus looking at populations where estrogen levels might interfere with isoflavone activity could prove informative. However, since our subject population consisted of postmenopausal women, we did not foresee differences in estrogen levels as being a confounding factor. Also, a crossover study design with the various subject population groups might eliminate differences due to individual bioavailability. Also, in these future studies, the subject number should be increased thus accounting for the high variability that has been observed in individual isoflavone bioavailability. This would help to monitor changes in various immune parameters based on a population showing increasing plasma isoflavone concentrations.

Appendix A: Material and Methods

Determination of Isoflavone concentration in plasma:

High Performance Liquid Chromatography (HPLC) was used for the determination of the soy isoflavones, daidzein and genistein in plasma (24). In this analysis, 500 µl of plasma were diluted in 10 ml of 100% methanol and 10 µl internal standard was added (fluorescein prepared in 80% methanol), followed by centrifugation at 3000xG for 10 min at 10°C. After centrifugation, the supernatant was poured off into a rotary evaporator flask, the plasma residue that remained was resuspended in 5 ml of 100% methanol and centrifuged again. The supernatant was added to the evaporator flask and a third washing of the plasma residue was carried out as described above. After washings, the combined methanol extracts were allowed to evaporate and 7 ml of 1M sodium acetate (pH 5.5) was added to obtain the entire residue from the flask. This was then transferred to a labeled glass tube with a teflon liner cap. Glucuronidase/sulfatase (50 µl H2 Type, Sigma G0876) was added to the tubes and samples were incubated overnight at 37°C in a dry heating block. The isoflavones from this prepared hydrolysate were extracted on a C18 SPE reverse phase column (Fisher Scientific, Pittsburg, PA). The extracted isoflavones were eluted out into 15 ml conical centrifuge tube using 2 ml of 100% HPLC grade methanol. The collected isoflavone/methanol fraction was then taken to dryness under nitrogen in a heated water bath (40°C). The dried sample was redissolved in 150 µl of 80% HPLC grade methanol and 100 µl of the sample was then injected into the HPLC column (Luna C18 with guard cartridge, Phenomenex, Torrance, CA) for analysis. The mobile phase included 15% HPLC-grade methanol in HPLC grade water. A standard curve was prepared using a series of genistein standards (200, 500, 1000 and 1200 ng) from a stock solution (1 mg/10 ml) prepared in 80% methanol and assayed to quantify the isoflavones.

Lymphocyte Isolation:

Peripheral blood mononuclear cells consisting of monocytes and lymphocytes were isolated using the double density Ficoll separation method (67). Heparanized blood (8 ml) from each subject was drawn into Vacutainer BD tubes containing Ficoll Paque

reagent (BD Pharmingen, San Diego, CA). After centrifugation for 30 min at 1800 x G at 19 °C, the lymphocytes layer from the interface was aspirated and transferred into a new 15 ml centrifuge tubes using a pasteur pipette. The upper portion of the gel in the Vacutainer BD tubes, which contained residual lymphocytes, was then washed with 2 ml of sterile phosphate buffered saline (PBS). Using a pasteur pipette, the PBS layer was removed and added to the 15 ml tube containing the lymphocyte layer. PBS (12 ml) was then added to the isolated lymphocyte layer followed by centrifugation at 300xG for 15 min at 19 °C. After centrifugation, the supernatant was aspirated out and the pellet was subjected to washing with 10 ml PBS followed by centrifugation at 300xG for 10 min. After centrifugation, the pellet from each tube was resuspended in 12 ml of complete medium (RPMI containing 2mM glutamine, 100 µg/ml penicillin and 100 units/ml streptomycin). An aliquot (100 µl) of the cell suspension was then counted on the haemocytometer using Tryphan Blue dye (50 µl) as the staining agent. The isolated lymphocytes were then aliquoted for use in the proliferation assay, cytokine assay and natural killer cell cytotoxicity assay.

Lymphocyte Proliferation:

The isolated lymphocytes were stimulated to proliferate by culturing them in media containing the T cell mitogen, Con A. The ability of these lymphocytes to proliferate in response to the mitogen was measured using the standard ^3H -thymidine incorporation assay (14). In this assay, the isolated lymphocytes from each subject were suspended in complete medium supplemented 10% autologous serum. The cells were then seeded into 96-well plates at a concentration of 1×10^6 cells per well (100 µl) and incubated with different concentrations (2.5 µl/ml and 5.0 µl/ml) of Con A. After a 68 h incubation at 37°C and 5% CO₂, 20 µl of ^3H -thymidine (50 µCi/ml) was added to each well and the cells were incubated for an additional 4 h at 37°C. After incubation, the cells were collected onto filters using a cell harvester (Skatron, Sterling, VA) and the incorporated radioactivity was measured using a liquid scintillation counter (Beckman LCS 6500).

The results were obtained as counts per minute (cpm) and the effect of soy isoflavones on lymphocyte function was reported as the stimulation index which was calculated as follows:

$$\text{Stimulation index} = \frac{\text{baseline cpm} - \text{supplemented cpm}}{\text{baseline cpm}} (\text{corrected cpm})$$

Analysis of IL-2, and IFN γ Protein expression:

To measure concentrations of IL-2 and IFN γ , the isolated lymphocytes were seeded into 24-well plates at a concentration of 7.5×10^5 suspended in complete medium supplemented with 10% autologous serum. The cells were treated with 10 $\mu\text{g/ml}$ Con A and incubated for 48 h. Following incubation, cell free supernatants was removed and stored at -80°C until assayed. Enzyme linked immunosorbent assay (ELISA) analysis (Pharmingen, San Diego, CA) was used to assess cytokine IL-2 and IFN α protein concentration in cell culture supernatant. For the ELISA analysis, the samples and reagents were brought to room temperature ($18\text{-}25^\circ\text{C}$) prior to use. Specific standards (100 μl of IL-2 or IFN γ), sample and control were added to each well o f the ELISA plate. The plate was then sealed and incubated for 2 h at room temperature. After incubation, the contents of the plate were decanted and the wells were washed with 300 μl of wash buffer. Following washing, the plate was blotted on absorbent paper to remove any residual buffer, and then 100 μl of working detector was added to each well. The plate was sealed once again and incubated for 1 h at room temperature. The plate was washed again using wash buffer and 100 μl of substrate solution was added to each well. The wash buffer and working detector were prepared as per the directions provided in the kit. The plate was incubated for 30 min at room temperature in the dark. Finally, 50 μl of stop solution was added to each well and the plate was read at 450 nm (CERES 900 Hdi, Bio Tek Instruments Inc., Winooski, VT) within 30 min of stopping the reaction.

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