Introduction

Menopause, a healthy and normal life occurrence among women ages 40-58, is often considered a medical "condition" and is treated as a nuisance. Symptoms of the change can range from hot flashes and vaginal discomfort to fatigue and general irritability. Women more recently have been turning away from the traditional Hormone Replacement Therapy (HRT) in favor of more "natural" alternatives, such as meditation, acupuncture, vitamins, and supplements (*Menopause Practice, a Clinician's guide, 2004*). In particular these supplements are viewed as safe alternatives to HRT because they contain phytoestrogens, which are plant based and so viewed as "natural."

There are several different forms of estrogen, but the strongest and most prominent in humans is 17β-estradiol. Estrogens are primarily made in the ovaries, specifically they are believed to be produced in the egg follicle and interstitial cells, but can be secreted by the adrenal glands as well (*Estrogen, Encyc. Britannica*). While estrogens interact as a complex system with other signaling hormones, they primarily affect areas normally associated with reproduction. Among roles such as controlling the development of egg follicles, thickening the vaginal wall, and uterine contractions, estrogen also contributes largely to the general development of the female body. Figure 1 shows a chart denoting the major areas in which estrogen plays a role.

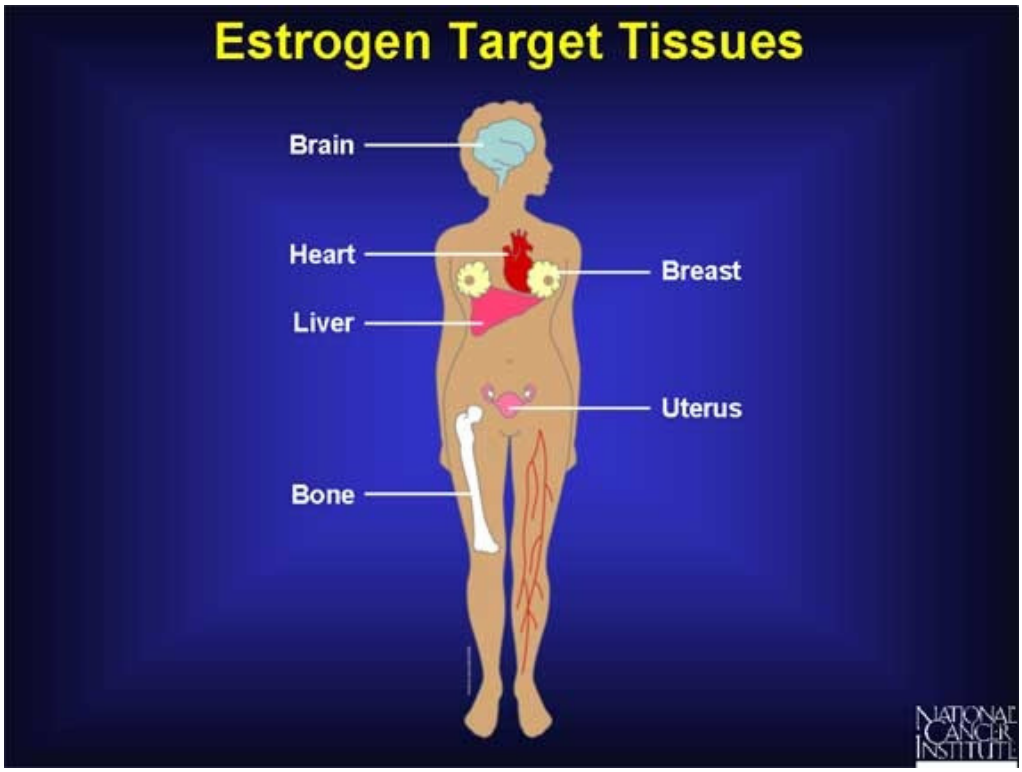


Figure 1: Diagram of the female body denoting major areas estrogen affects.(*Understanding Cancer Series, Natl. Cancer. Inst.)*

Estrogen is a steroidal molecule, and like other steroids is derived from cholesterol. The structure of estrogen can be seen in Figure 2**.** Estrogen receptors (ERs) belong to the nuclear receptor family; receptors of this type are ligand induced transcription factors. There are two types of estrogen receptors, α-ER and β-ER. The receptors have a different distribution throughout the body, which possibly indicates they have different functions. This distribution can be seen in Figure 3. It has been determined that both types of receptors reside within the nucleus of the cell, where estrogen reaches them through passive diffusion through the membrane. However due to the fact that the receptors can only be studied in disrupted cells, it cannot be determined exactly where the receptors lie (*Creighton, Thomas C. 1999*).

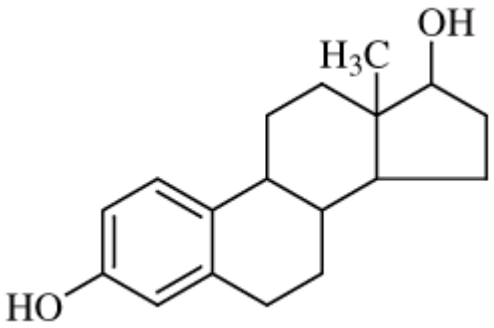


Figure 2: Molecular structure of estradiol(*Gangolli, 2005*)

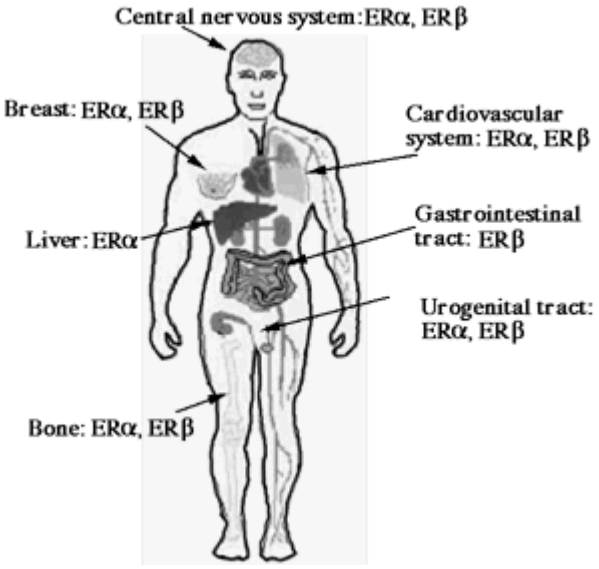


Figure 3: Locations of α and β estrogen receptors in the human body(*Estrogen Receptors and Functions, Carnegie melon*)

The structure of both receptors is divided into 6 functional domains. Overall there is a 44% amino acid consensus between the two types of receptors. The A/B domain contains the Activation Function 1 (AF-1) sequence. AF-1 binds coactivators such as growth factors. The C

domain is 88% conserved between the α-ER and β-ER, and contains the DNA binding domain. The D region, commonly referred to as the hinge region, links the domains together. The E/F domain is responsible for dimerization, and also contains both the ligand binding site and the AF-2 site. The AF-2 site interacts with a sequence known as the nuclear receptor interaction domain, or NR region on coactivators. The sequence of this region is LXXLL, where L is leucine and X is any amino acid (*Estrogen Receptors and Functions, Carnegie Melon*). The significant differences in amino acid sequences of the AF sites and ligand binding sites between the two types of receptors leaves the possibility of different coactivator interactions.

The mechanism of ligand binding to the estrogen receptor is sometimes referred to as the "mousetrap model." The secondary structure of the receptor consists of 12 alpha helixes, and the tertiary structure has the helixes organized to create a hydrophobic pocket for the polar ligand (estrogen) to bind. When the ligand binds, an allosteric change forces helix 12 to close over the pocket and "trap" the ligand inside the hydrophobic region. This movement causes a hydrophobic groove to be created on the surface of the receptor, and allows the NR region of coactivators to bind. This optimal position of helix 12 is known as the agonist position (*Creighton, Thomas C. 1999*).

The receptor can bind nearly any molecule with an aromatic ring structure. This is due predominantly to the large size of the hydrophobic cavity—it is nearly twice that of the volume of 17β-estradiol (*Brzozowski et al., 1997*). Some molecules prevent helix 12 from trapping the ligand, and instead cause the helix to lie in a hydrophobic grooved formed by helix 3 and 5. This does not properly display the AF-2 region of the receptor, and very little if any coactivators can bind. Molecules such as this are known as estrogen receptor modulators (SERMS) and are thought to play an important role in regulation of receptor activity. The complimentarity between helix 12 and the hydrophobic groove that is formed indicates that the antagonist position is not just an intermediate or accidental conformation(*Brzozowski et al., 1997*).

Molecules that bind the receptors in the agonist conformation stimulate transcription of estrogen regulated genes, while those that bind in the antagonist position inhibit their transcription. The receptors bind DNA as homo dimers at sites known as estrogen response elements (EREs) with the consensus sequence 5'GGTCAnnnTGACC-3', where n is any nucleotide (*Paech et al.1997*). The EREs are very sensitive and the receptor binding is very specific, as there is only a 2 nucleotide difference between EREs and the hormone response elements for glucocorticoid, progesterone, and androgen receptors (*Creighton, Thomas C. 1999*). The binding of the receptor to the EREs directly stimulates transcription of the gene by recruiting the necessary proteins required to start the process.

As females reach the age of menopause their ovaries slow the production of estradiol, and the primary hormone in the body becomes estrone. Estrone has a lower affinity for receptors, so is

viewed as a weaker estrogen. This switch is what creates so many symptoms of discomfort for menopausal women (*Menopause Online*). Hormone replacement therapy eases much of the discomfort of the switch by replenishing women's supplies of estradiol.

HRT works by supplementing the female body's decreasing supply of hormones with either estradiol or an estradiol and progesterone combination. HRT was practiced widely until recent studies showed numerous adverse effects directly resulting from HRT. For example, even though the risk for colorectal cancer and bone fractures were markedly decreased in women who used the therapy, there were increases in risk of breast cancer, stroke, heart attack and blood clots (*US. Dept. of Health and Human Services, 2005*).

The reason behind the increased risk of cancers surrounding the use of HRT is related to the primary function of estrogen to increase cell proliferation. Tissues that have increased proliferation due to estrogen are breast and uterine; consequently breast and uterine cancer are the most common cancers resulting from HRT. Although estrogen does not directly cause cancer, it greatly increases the chances of anyone with DNA mutations in estrogen responsive tissues of developing cancer(*Natl. Cancer Inst.*). Many cancers are also estrogen responsive, which means HRT would increase the proliferation rate of cancer already in existence.

Phytoestrogens are not produced in the human body, but will still bind to estrogen receptors, just at a lower affinity than does estrogen. While there is anecdotal evidence that attest products alleviate the symptoms associated with menopause, there is little research on their effects on cell proliferation. To address this question, Caron (2007) investigated the effects of Promensil, an over the counter supplement made up of phytoestrogens and marketed as an alternative to HRT, on the proliferation of MCF-7 cells. MCF-7 (ATCC# HTB-22) is an estrogen responsive breast epithelial cell line that has been used as a model system to study estrogen responsiveness for many years (*Berthois et al. 1986*). While Caron's work suggested that Promensil decreases the growth rate of MCF 7 cells and increases the expression of caspase-3, an apoptosis related protein, the MCF7 cells used in the study appeared to be minimally or non responsive to estrogen.

Hamelers *et al.* in 2003 investigated the variable response of various strains of MCF-7 cells to estrogen. They suggested that insulin-like growth factor 1(IGF 1) increases the estrogen responsiveness of MCF-7 cells. This may also explain increasing estrogen responsiveness at higher passage numbers, due to selection for cells that have a growth advantage as a result of endogenous production of IGF for autocrine signalling.

Research presented here attempts to restore estrogen responsiveness to the MCF-7 cells used by Caron. This is necessary to validate the results of the original study suggesting that certain phytoestrogens may actually have a protective role in the development of breast cancer. In

order to determine the best conditions for estrogen responsiveness, effects of both the passage number and addition of IGF have been explored in this study.

Materials and Methods

Culture Conditions

MCF7 cells were maintained in either T-25 or T-75 flasks. They were incubated at 37°C, 5% CO_2 and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). Cells were given fresh media every 3 days, and split when at least 80% confluent.

Passaging

Cells were passaged using trypsin EDTA (2.5mg/l Trypsin, 0.2g/l EDTA)minutes until loosened enough to be resuspended. They were then resuspended in DMEM with 10% FBS and split into 2 or more T-25 (or T-75) flasks in fresh media. Cells were used either from a fresh split, or from a stock frozen in medium containing 10% FBS and 10% DMSO.

Estradiol Treatment

Cell concentrations were determined via hemocytometer counts and plated at an average cell count of $3x10^5$ cells/ml.

A 1:2 dilution series of β-estradiol in 100% ethanol at starting concentration $1x10^{-7}$[M] was prepared. The dilutions were as follows:$2x10^{-5}$, $1x10^{-5}$, $5x10^{-6}$, $2.5x10^{-7}$, and 1.25^{-6}[M]. For treatment of cells, 5µl of the appropriate solutions were added to the wells.

Cells were plated in 1ml of phenol red free DMEM supplemented with 10% DCC/charcoal stripped FBS in either 6, 12 or 24 well plates. 12 hours after plating, estradiol was added to the cells as shown in Tables 1, 2, and 3. As controls cells were plated both with and without 0.5% ethanol (5µl of 100% ethanol). 24 hours after estradiol treatment cells were frozen for immunoblotting or used in an MTS assay of cell proliferation.

Table 1: Estradiol Treatment For a 12 well plate. Concentrations of estradiol indicate final concentration present in 1ml media containing cells. The well containing only media is to serve as a blank for the MTS assay.

	1	2	3	4
A	$3x10^5$cells	$3x10^5$cells	$3x10^5$cells,5µl 100% ethanol	1ml media
B	$3x10^5$cells, $1x10^{-7}$M estradiol	$3x10^5$cells, $5x10^{-8}$ M estradiol	$3x10^5$cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$M estradiol
C	$3x10^5$cells,$1x10^{-7}$M estradiol	$3x10^5$cells,$5x10^{-8}$ M estradiol	$3x10^5$cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$M estradiol

Table 2: Estradiol Treatment For a 24 well plate Concentrations of estradiol indicate final concentration present in 1ml media containing cells. The well containing only media is to serve as a blank for the MTS assay.

	1	2	3	4	5	6
A	1ml media	$3x10^5$cells, $1x10^{-7}$M estradiol	$3x10^5$cells, $5x10^{-8}$ M estradiol	1ml cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$ M estradiol	$3x10^5$cells, 6.25^{-9} M estradiol
B	$3x10^5$cells, 5µl 100% ethanol	$3x10^5$cells, $1x10^{-7}$M estradiol	$3x10^5$cells, $5x10^{-8}$ M estradiol	1ml cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$ M estradiol	$3x10^5$cells, 6.25^{-9} M estradiol
C	$3x10^5$cells	$3x10^5$cells, $1x10^{-7}$M estradiol	$3x10^5$cells, $5x10^{-8}$ M estradiol	1ml cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$ M estradiol	$3x10^5$cells, 6.25^{-9} M estradiol
D	$3x10^5$cells	$3x10^5$cells, $1x10^{-7}$M estradiol	$3x10^5$cells, $5x10^{-8}$ M estradiol	1ml cells, $2.5x10^{-8}$ M estradiol	$3x10^5$cells, $1.25x10^{-9}$ M estradiol	$3x10^5$cells, 6.25^{-9} M estradiol

Table 3: Estradiol Treatment For a 6 well plate

	1	2	3
A	1ml cells, 5µl 100% ethanol	$3x10^5$cells, 5µl $1x10^{-7}$M estrogen	$3x10^5$cells, 5µl $5x10^{-8}$ M estrogen
B	$3x10^5$cells, 5µl $2.5x10^{-8}$ M estrogen	$3x10^5$cells, 5µl $1.25x10^{-9}$ M estrogen	$3x10^5$cells, 5µl 6.25^{-9}M estrogen

IGF Treatment

Cells grown with IGF were plated in the same manner except they were exposed to concentrations of 2ng/ml IGF-1(Sigma, St. Louis, MO) at the same time as the estrogen treatments. For IGF-1 studies, cells from multiple passages were plated on a single plate and treated with IGF-1 simultaneously.

MTS Assay

The Cell Titer96© AQ_{ueous} Non-Radioactive Cell Proliferation Assay was purchased from Promega. The assay contains a tetrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and an electron coupling reagent PMS (phenazine methosulfate). The MTS is converted by the dehydrogenase enzymes found in metabolically active cells into formazan, a substance soluble in culture media. The absorbance of the cultures is then read at 490 nm and is directly proportional to the amount of metabolic activity of the cells—hence it should be an accurate measure of cell viability(*Technical Bulletin, Promega 2007*).

To perform the MTS assay 20µl of the MTS solution was added to each well of the plates (either the 12 or 24 well plates) and incubated at 37°C for approximately 2 ½ hours. This occurred 24 hours after the estradiol treatment. The plates were then read in the plate reader at 490 nm and absorption values taken, and the average reading of data was taken for plates with only one passage number.

Immunoblotting

To prepare the cells for immunoblotting, the plates were rinsed in PBS (10mM sodium phosphate, 150mM NaCl, pH 7.4) and frozen at -80°C. The plates were then scraped in 100µl cold PBS and a Bradford Assay was performed. In this manner the total protein concentrations could be normalized among samples.

A standard SDS-PAGE was run with pre-cast Pierce Precise Protein 10 well 12% gels for approximately 40 minutes at 250 Volts in running buffer (100 mM Tris, 100 mM HEPES, 1% SDS, pH 8.0). The proteins were then transferred to a MilliporePVDF membrane using semi-dry blotting technique which involved wetting the membrane with methanol then using a semi-dry transfer buffer (25mM Tris, 250 mM glycine, 15% methanol) and running the blotting apparatus at 20 Volts for 30 minutes. The membrane was then blocked in 5% non-fat dry milk in PBS for 10 minutes, then washed in TBS (10mM Tris HCL, 150 mM NaCl, pH 7.4). The membrane was incubated in PCNA mouse antibody (purchased from Santa Cruz Biotechnology, used at a 1:200 dilution) overnight at 4°C, then washed for at least 10 minutes each in TBS, TBS+ 10% Tween, and TBS again. Goat anti-mouse (purchased from Santa Cruz Biotechnology, used at 1:1500 dilution) was incubated on the membrane overnight at 4°C. The membrane was washed for at least 10 minutes each in TBS, TBS+ 10% Tween, and TBS, and was developed using NBT/BCIP+Tetramisol.

Results

Figure 4 shows the proliferation effects of estrogen on cells at different passage number as determined by the MTS assay While there is some indication that the cells at lower passage numbers had fewer cells, the pattern is not consistent across passage number. Additionally, there is no clear change in cell number with increasing estrogen concentration across the range tested.

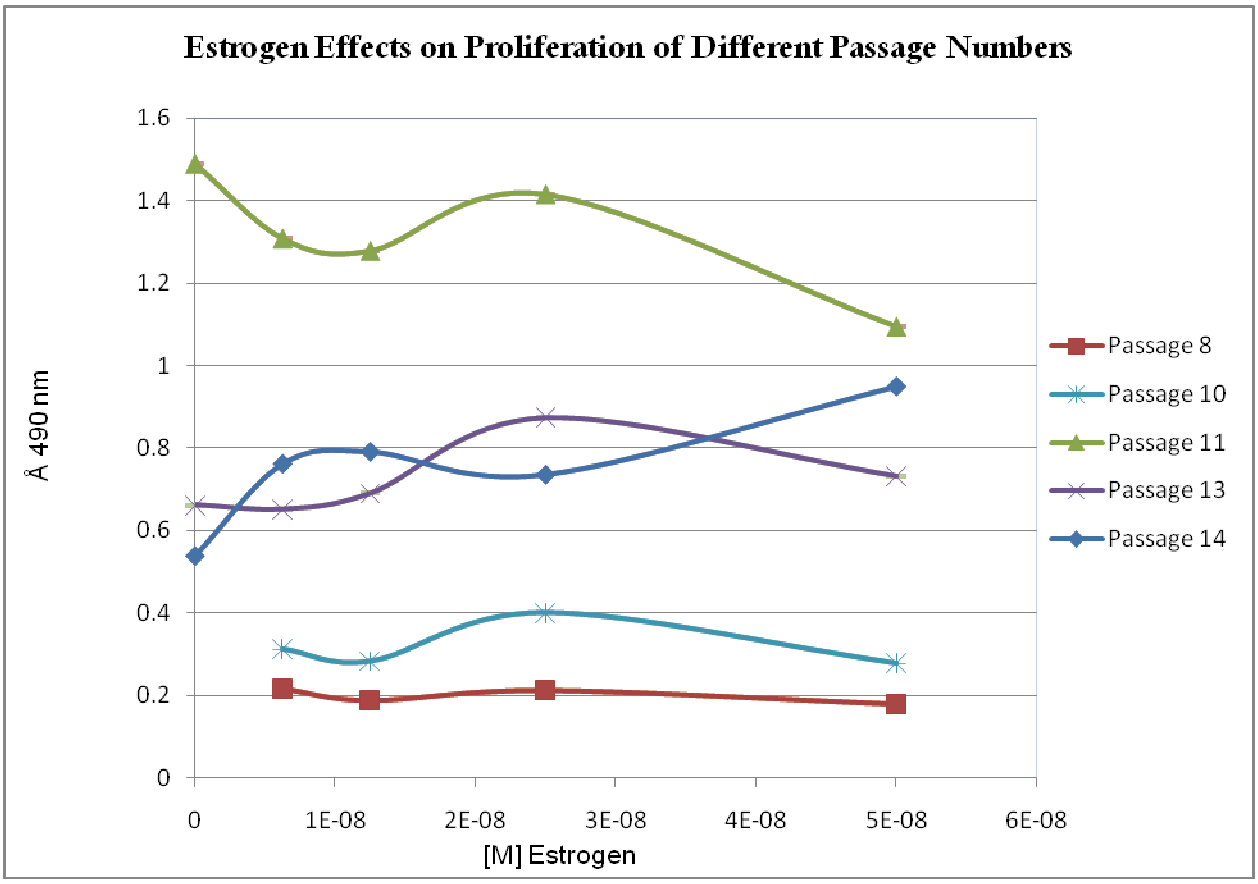


Figure 4. Effects of estrogen on proliferation for cells from different passage numbers. Cells were cultured as decscribed and treated with estrogen. 24 hours later, cell number was determined at Å490 using an MTS assay. N=1, each data points represent an average of 2 or 3 replicate samples..

Figure 5 shows the effects as determined by the MTS assay of estrogen addition on cells at different passage number treated with IGF. (Data for Figures 4 and 5 can be found in the Appendix as Tables 1-6) Again, there is no clear pattern of estrogen responsiveness across the concentration range tested . However, unlike in Figure 5, there is no indication of differences in cell number that correlate with passage number.

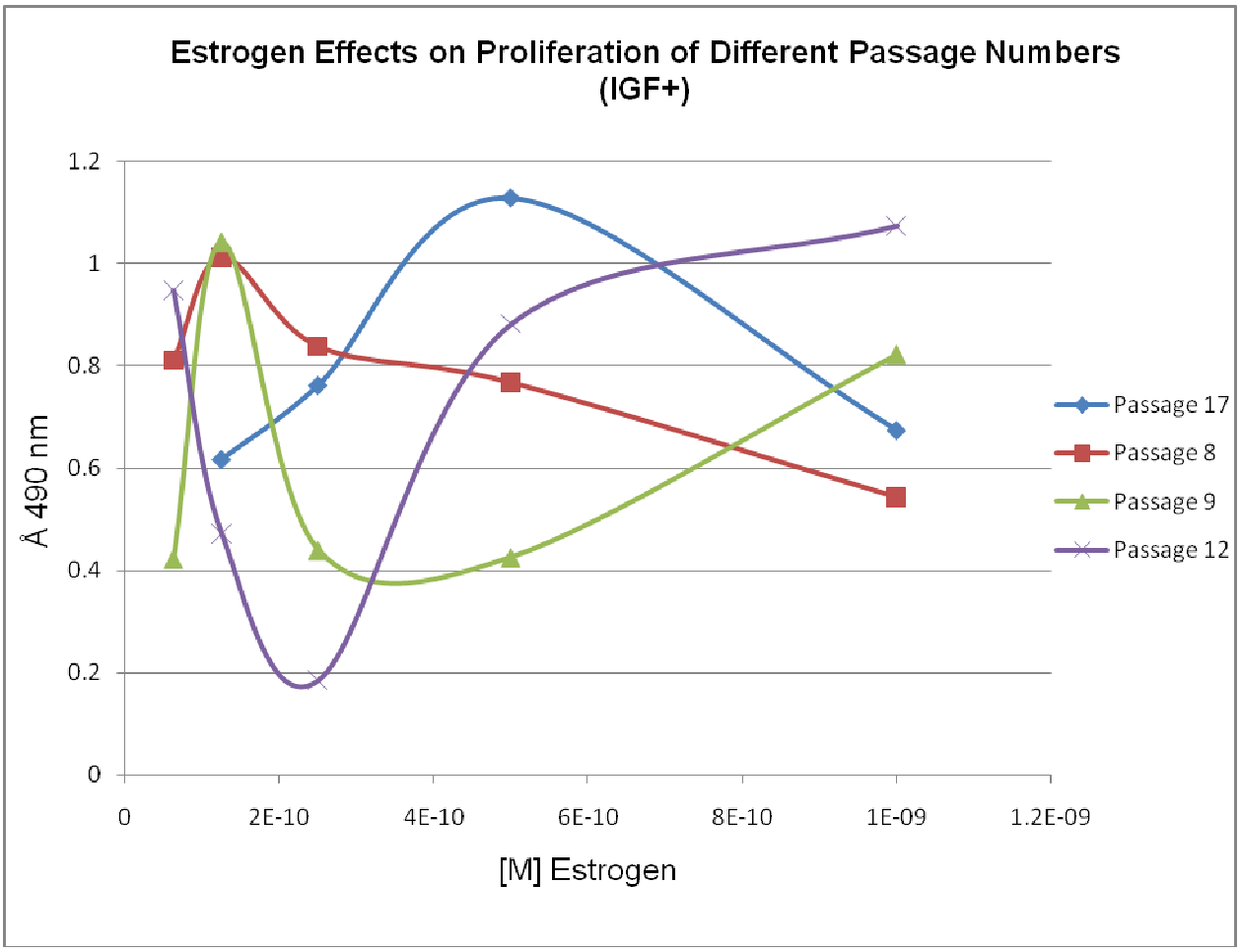
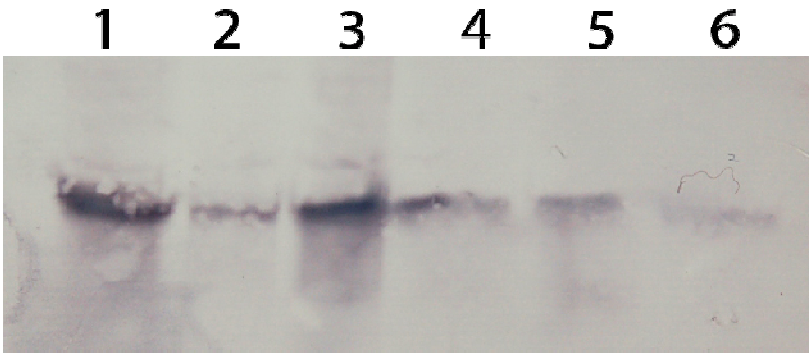


Figure 5. Effects of estrogen on proliferation for cells from different passage numbers treated with X M IGF-1. Cells were cultured as described and treated with estrogen and IGF. 24 hours later, cell number was determined at Å490 using an MTS assay. N=1, each data points represents a single sample.

Figure 6 shows the PCNA immunoblot results for passage 13 cells grown in the presence of IGF-1 and estradiol treated (Data from the Bradford Assay used to normalize loading is shown in the Appendix, Table 7). For all of the cells treated with estrogen at varying concentrations, the level of PCNA is increased over the untreated control. However, the intensity of the band does not increase in relation to estrogen concentration across the range tested. In fact, the response seems greatest at the lowest concentration of estrogen, and appears to decrease somewhat as estrogen concentration increases.



Lane 1 $6.25x10^{-9}$ M estrogen

Lane 2 $1.25x10^{-8}$ M estrogen

Lane 3 $2.5x10^{-8}$ M estrogen

Lane 4 $5x10^{-8}$ M estrogen

Lane 5 $1x10^{-7}$M estrogen

Lane 6 0M estrogen

Figure 3: Passage 13 Immunoblot Cells were cultured as described in the presence of X M IGF-1 and treated with estrogen at the indicated concentrations. Immunoblotting was carried out using an anti-PCNA antibody.

Discussion

Figure 4 shows that there is no obvious correlation between the estrogen concentrations and proliferation without the addition of IGF. There is a slight relationship between the passage numbers and the viable cell number however. As can be seen, passage numbers 8 and 10 have fewer cells at all concentrations of estrogen than cells from higher passage numbers. Since all the cells were seeded at approximately the same density, this may be a reflection of increased growth rate at increased passage number as reported by Hamelers. Perhaps the reason for seeing such a higher density of cells at higher passage numbers is because the cells of higher passage numbers have selected for cells that produce their own IGF.

With the addition of IGF, there still appears to be no correlation between either estrogen amounts and proliferation or passage number and proliferation. Figure 5 shows the cell passage number has even less of an effect on the overall proliferation, which does support the theory that IGF differs between higher and lower passage numbers. If the amount of IGF added helps to normalize the relative amounts of IGF present between passage numbers, then of course there would not be a major difference. As for the effects of estrogen, there appears to be no general correlation in any of the passage numbers, because the response may already be maximal across the concentration range used.

Another possibility is that the plate reader was not accurate. The plate reader was set indefinitely to read 96 well plates, so in order to read data from the 24 well plates, the media from the MTS assay had to be transferred into 96 well plates. The 12 well plates fit in the reader, but the placement of the 96 well sensors in each of the 12 wells had to be determined

and was probably not very accurate at all. In addition, repeat trials of the same plates read resulted in erratic results, when no change had been made except for the passing of several seconds. Visual evidence suggests the MTS assay worked perfectly, but given the inadequacies of the plate reader it is safe to say the error lies there.

In the immunoblot(Figure 6) there appears to be some correlation to the amount of estrogen added. Lane 6 contains no estrogen and shows the weakest band, this is consistent with expected results. There appears to be a general darkening of bands with decreasing amounts of estrogen, except for in lane 2. It is possible this shows the beginnings of a relationship, but more data needs to be collected before any conclusions can be drawn.

It has been shown that phenol red is structurally similar to estrogen. Although the binding capacity of phenol red to the estrogen receptor is 0.001% that of estradiol's, the concentration of phenol red in DMEM medium is 45μM, which is far more concentrated than the amounts of estrogen added (*Berthois et al. 1986*). It is possible that the prolonged periods in phenol red containing media prior to experimental conditions contributed to the lack of an effect. Perhaps the increased IGF, in addition to the prolonged period spend in phenol red containing media both contributed to the increase in growth rate of higher passage cells. It was also observed that cells at higher passage number reached confluence faster than those from lower passage number, and this is in keeping with the findings.

Future Experiments

With the current risk of cancer caused by HRT, it is imperative that more studies be done to confirm that phytoestrogens do inhibit proliferation. In order to confirm previous findings specific to the strain of MCF-7 cells used, they must be shown to have the characteristic response to estrogen. This could most likely be done by repeating the previous experiments but using phenol red free media and DCC/charcoal stripped FBS for the entire course of cell culture. If that proves to be too impractical, then they could be grown in those conditions for only several days. If this fails to work, then growing cells in hormone and phenol red free, but with the addition of 2 ng of IGF may induce estrogen responsiveness of the cells. Most likely the MTS assay will prove to be unreliable and it would probably be more efficient to rely on immunoblotting for PCNA as a measurement of cell proliferation. In addition to the change in growing conditions, perhaps future experiments could include a broader range of estrogen concentrations. This could help make any minor differences in responses more apparent.

If the desired response of the MCF-7 cell line is unable to be achieved, then it may be worthwhile to repeat the entire experiment starting with the effects of Promensil on a different estrogen responsive breast epithelial cell line. There are a wide variety of cell lines available from the ATCC that would be suited to this experiment. Even if the MCF-7 line does prove to be

estrogen responsive, it would eventually be productive to investigate alternative lines to search for a general trend.

Genistein is the active and most prominent ingredient of Promensil, according to the producers; however it was found to contain a number of unidentified substances. In order to fully understand the effects Promensil has on MCF7 cells, the components must be studied both individually and in different combinations. This would lead to a myriad of experiments, given the large variety of components that make up the supplement. Ideally the exact components that induce apoptosis in the cells should be isolated for further study.

This study has so far focused on only one of the many supplements available over the counter. In the future it would be valuable to broaden the horizon to other brands. Most likely the proportions of phytoestrogens in different brands are different; it would be interesting to see a correlation between these differences and the effects on cell proliferation.

The effects of having different proportions of both 17β-estradiol and Promensil present should be studied. In no instance is 17β-estradiol entirely non-existent in the body, so experiments *in vivo* should reflect this. Eventually it would also be important to investigate the effects of estrone combined with Promensil, since estrone becomes the predominant form of estrogen in the body following completion of menopause.

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Appendix

Table 1. Passage 14 cells Å490, treated with estrogen and no IGF. Contents of wells is located in Table 2 of report.

	1	2	3	4	4	5
A	0	1.342	1.305	1.103	0.997	0.71
B	0.69	0.724	0.384	0.482	0.486	0.374
C	0.565	0.737	0.659	0.647	0.635	0.609
D	0.868	0.996	0.594	0.932	0.93	0.459
	AVG	0.94975	0.7355	0.791	0.762	0.538

Table 2. Passage 11 cells Å490, treated with estrogen and no IGF. Contents of wells is located in Table 2 of report.

	1	2	3	4	5	6
A	0	0.364	1.933	1.253	1.348	1.532
B	0.976	1.402	1.318	1.452	1.271	1.254
C	1.654	1.562	1.025	1.175	1.416	1.614
D	1.43	1.054	1.39	1.238	1.202	1.562
	AVG	1.0955	1.4165	1.2795	1.30925	1.4905

Table 3. Passage 13 cells Å490, treated with estrogen and no IGF. Contents of wells is located in Table 2 of report.

	1	2	3	4	5	6
A	0	1.298	0.766	0.71	0.429	1.142
B	0.334	0.415	0.496	0.416	0.471	0.419
C	0.883	0.962	1.349	0.892	1.045	0.762
D	0.406	0.25	0.884	0.742	0.659	0.324
	AVG	0.73125	0.87375	0.69	0.651	0.66175

Table 4. Passage 10 cells Å490, treated with estrogen and no IGF. Contents of wells is located in Table 3 of report.

	1	2	3	4
A	0.445	0.237	0.399	0
B	0.350	0.445	0.322	0.400
C	0.205	0.355	0.243	0.224
Avg	0.278	0.400	0.283	0.312

Table 5. Passage 8 cells Å490, treated with estrogen and no IGF. Contents of wells is located in Table 3 of report.

	1	2	3	4
A	0.469	0.299	0.448	0
B	0.181	0.226	0.200	0.188
C	0.177	0.199	0.176	0.245
Avg	0.179	0.213	0.188	0.217

Table 6. Å490 data for cells treated with IGF in addition to estrogen. Omitted data indicates position of well containing only media to serve as a blank.

[M] estrogen	Passage 17	Passage 8	Passage 9	Passage 12
$1x10^{-7}$	0.673	0.542	0.822	1.074
$5x10^{-8}$	1.128	0.767	0.426	0.882
$2.5x10^{-8}$	0.761	0.838	0.44	0.185
$1.25x10^{-9}$	0.616	1.013	1.041	0.472
6.25^{-9}	---	0.811	0.423	0.948
0	0.397	0.509	0.817	0.98

Table 7. Protein concentrations obtained from estrogen and IGF treated passage 13 cells in preparation for the Bradford assay.

[M] estrogen	mg/ml protein
1x10-7	8.77
5x10-8	16.91
2.5x10-8	9.68
1.25x10-9	15.77
6.25-9	16.91
0	12.18