ANTIOXIDANTS AND GENE REGULATION:

The Effects of Vitamins C and E on Estrogen Receptors

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RESEARCH SHOWS THAT ESTROGEN BINDING TO ITS RECEPTOR PLAYS A ROLE IN BREAST CANCER DEVELOPMENT AND THAT ANTIOXIDANTS POSSIBLY MITIGATE THIS EFFECT. PAST RESEARCH EXAMINED WHETHER VARIOUS TREATMENTS LEAD TO ACCELERATED CELL DIVISION, BUT CHARACTERIZATION AND COMPARISON OF THE EFFECTS OF DIFFERENT TREATMENTS ON GENE LEVEL EXPRESSION OF THE RECEPTOR WAS NOT ACCOMPLISHED. INITIALLY, THE EFFECT OF ANTIOXIDANTS ON THE ESTROGEN RECEPTOR EXPRESSION WAS INVESTIGATED, REVEALING THE PRESENCE OF VITAMINS C AND E. SUBSEQUENTLY, THE INDIVIDUAL EFFECTS OF VITAMINS C AND E ON NITRIC OXIDE RELEASE (A POSSIBLE CANCER REDUCTION AGENT) STIMULATED BY ESTROGEN ACTING ON THE SURFACE ESTROGEN RECEPTOR OF BREAST CANCER CELLS WAS OBSERVED. GENERALLY, VITAMIN E WAS MOST EFFECTIVE FOR IMPROVING NITRIC OXIDE RELEASE.

INTRODUCTION

Breast cancer is one of the most common cancers among women worldwide. Statistical data have shown that among American women, nearly one out of three cancers diagnosed is a breast cancer and in the year 2001, approximately 192,200 cases of breast cancer were diagnosed among women in the United States. Through many years of breast cancer research, it has been documented that estrogen plays a critical role in the development of breast cancer.[i]

Estrogens are steroid molecules and sex hormones that stimulate the development of female characteristics and sexual reproduction. The most common forms of human estrogens are 17-*beta* estradiol and estrone that are produced and secreted by the ovaries. Two of the female organs that play a central role in sexual reproduction, the breast and the uterus, are the main target organs of the estrogen molecule. Cells of the breast and the uterus have estrogen receptors (*alpha* and *beta*) that have specific sites to which only estrogen can bind. Estrogen molecules are only effective when they bind to the receptors. When estrogen molecules pass through the membrane of the cell and bind to the estrogen receptors, the shape of the receptor changes and estrogen receptor complex attaches to the estrogen response elements (EREs) in DNA, causing certain genes to become active. The active genes produce molecules of messenger RNA that influence cell activity in a variety of ways by synthesizing specific proteins.[ii] This study examines the interaction of estrogen and antioxidants on the expression of estrogen receptor genes.

Cancer is caused by mutations in growth regulatory genes. Mutations may be caused by transcription errors in DNA before the process of cell division.[iii] As these cells proliferate, the transcription errors are then carried on to the new generation of cells and may lead to uncontrollable proliferation.

Besides its role in developing female sexual characteristics, estrogen also impacts on the health of the immune system, and the body's response to the stress and the changing environment.[iv] One of the main effects of estrogen is inducing stimulation for cell proliferation.[v] Cell proliferation is estrogen's natural role but it increases a woman's chance of developing breast cancer or uterine cancer. Previous studies have demonstrated the effect of 17-*beta* estradiol on cell proliferation. In several studies, 17-*beta* estradiol enhanced the rate of cell proliferation and also decreased the level of apoptosis of breast cancer cells by inducing the bcl-2, anti-apoptosis gene .[vi] Estrogen cannot distinguish between mutated cells and healthy cells. Proliferation stimulated by estrogen can result in the proliferation of mutant cells, thereby causing cancer.[vii] To prevent estrogen from promoting cell proliferation, researchers have been developing anti-estrogen substances such as tamoxifen, and raloxifene that can block the estrogen receptor.[vii]

Currently, two types of estrogen receptors (ER) have been identified, *alpha* and *beta*. They both bind to DNA but it has been shown that in MCF-7 adenocarcinoma human breast cancer cells, ER *alpha* is the prevalent form and ER *beta* is hardly detectable. Estrogen *alpha*-receptor is also mainly involved with the development of a breast cancer.[ix]

Most research on breast cancer regarding antioxidants has focused on the effect of melatonin (pineal indoleamine), a type of an antioxidant, on the proliferation of MCF-7 breast cancer cells. Various studies have demonstrated that melatonin has an anti-proliferative effect on estrogen responsive MCF-7 breast cancer cells and also that the administration of melatonin reduces the incidence and growth rate of chemically induced mammary tumors.[x] According to the several studies, not only does melatonin inhibit the MCF-7 cell growth but it also increases cell doubling time and delays the entry of MCF-7 cells into mitosis.[xi] Furthermore, it has been shown that melatonin decreases ER bonding activity and ER mRNA expression.[xii] However, when melatonin and estradiol were treated together, the anti-proliferative effect of melatonin was reversed, demonstrating that the effect of melatonin can be counteracted by cell cycle acceleration stimulated by estradiol.[xiii]

In addition, the effects of other types of antioxidants such as carotenoids, and retinoic acid on MCF-7 cell growth also have been widely studied. These antioxidants have been shown to have an inhibitory effect on the ER positive MCF-7 cell growth but not on ER negative MDA-MB-231 breast cancer cells.[xiv] On the other hand, it has been reported that vitamin E, selenium, and palm oil tocotrienols have been shown to be effective anti-proliferative agents in both ER negative and positive cells breast cancer cells.[xv] Unlike previous studies where researchers observed the effect of antioxidants on the proliferation of the cells, in the first part of this study, the effect of the antioxidants ascorbic acid (vitamin C) and *alpha* tocopheral (vitamin E) on the gene expression of estrogen receptor will be observed.

Another part of this study is to investigate whether ascorbic acid and *alpha* tocopherol can affect the nitric oxide release from the surface receptor of ER positive MCF-7 cells. Several studies have found the existence of surface estrogen receptors in human peripheral monocytes and the ganglionic nervous system of Mytilus edulis, the common mussel. These investigations also demonstrated that 17-*beta* estradiol stimulates nitric oxide (NO) release by binding to the cell surface estrogen receptor.[xvi] Nitric oxide is a free radical that scavenges other free radicals and controls the activation state of various tissues such as immune cells, thereby helping to maintain appropriate levels of cellular activity. NO has also been shown to play a host defense against tumors, viruses and bacteria. In addition, it has been shown that free radicals promote cancer by damaging DNA. Since NO release is one of estrogen receptors' critical functions, investigating the effect of antioxidants on NO release by surface estrogen receptors on MCF-7 cells is essential. However, it has been found out that while NO plays an important role in numerous physiological and pathophysiological conditions, excessive concentrations of NO may lead to a tissue damage and organ dysfunction.[xvii] In the second phase of this experiment, cells are pre-treated with ascorbic acid and *alpha* tocopherol, and then the NO release triggered by 17-*beta* estradiol is measured by using NO-specific amperometric probe.

MATERIALS AND METHODS

GENE EXPRESSION

CELL CULTURING

A flask with MCF-7 cells (American Type Culture Collection, ATCC), RPMI 1640 10 percent Media (GIBCO, Invitrogen, Carlsbad, CA) and Tripsin (SIGMA, St Louis, MO) were prepared. From the original flask with MCF-7 cells, the media was removed, and then washed with 2ml of trypsin. Three ml of trypsin was added to the flask containing MCF-7 cells, and the flask was tilted back and forth so the trypsin would cover the whole surface with MCF-7 cells. The flask was then sat still for 5 to 15 minutes until all the cells detach from the flask surface. When all the cells were detached, 7ml of media was added to neutralize the Tripsin. Remaining cells were rinsed with phosphate buffered saline (PBS) by using a pipet. Everything in the flask was transferred to a 15ml tube and then centrifuged for 3 minutes at 1000 rpm. After the tube was centrifuged, the liquid in the tube was decanted. The cell pellet in the tube was then resuspended by adding 10ml of RPMI media. The cell pellet and the media were pipetted up and down until cell pellet was completely broken. 1 X 106 cells was added to each well of two six wells plates in 2 ml RPMI media and then incubated in a 37 oC incubator.

PREPARING SOLUTIONS

Ascorbic acid (vitamin C) stock solution with 1M concentration was prepared by adding 0.18g of Ascorbic acid (SIGMA, St Louis, MO) to 1ml of PBS (Phosphate Buffer Saline) (GIBCO, Invitrogen, Carlsbad, CA), and then 10ìl was added to 990ul of PBS, to make 10mM solution.

Alpha tocopherol solution (vitamin E) (SIGMA, St Louis MO) was prepared next. 1M stock solution was made by adding 0.43g to 1ml of ethanol. 50ìl from the1M stock solution was added to 950ìl of ethanol for concentration of 50mM. Again 10ìl of the solution was added to 90ìl of PBS for concentration of 5mM.

Estrogen solution was also prepared. 10-3 M stock solution was prepared by adding 0.003g of 17-, estradiol (SIGMA, St Louis MO) to 10ml of ethanol. 10ìl of the solution was then added to 990ìl PBS for concentration of 10-5 M.

CELL TREATMENT

Two six wells plates were treated in the following order. First two wells were untreated as controls. Next two wells were treated with Ascorbic acid. 20ìl of 10mM Ascorbic acid solution was added to these wells for final concentration of 100ìM for each well. Same procedure was repeated for next two wells but 17-beta estradiol solution was added with Ascorbic acid. 20ìl of the 10-5 M 17-beta estradiol solution was added the wells for final concentration of 10-7 M along with 20ìl of 10mM Ascorbic acid solution.

After treating first plate, second plate was treated with combinations with alpha tocopherol solution. First two wells were treated with alpha tocopherol solution. 20ìl of 5mM alpha tocopherol solution was added to the well for a final concentration was 50ìM for each well. Next two wells were treated with combination of alpha tocopherol and 17-beta estradiol solution. Again 20ìl of 5mM alpha tocopherol solution was added to the two wells and then 20ìl of the 10-5 M 17-beta estradiol solution was added. For last two wells, Ascorbic acid and alpha tocopherol solutions were added together. 20ìl of 10mM Ascorbic acid solution was added, and then 20ìl of 5mM alpha tocopherol solution was added. These plates were incubated for 24 hours at 39 degrees Celcius.

RNA ISOLATION USING RNEASY(R) PROTECT MINI KIT(250) (QIAGEN)

After 24 hr. incubation, each type of cells was collected into 15ml Falcon tubes using sterile cell scraper. Tubes were then centrifuged for five minutes at 300-x g. After centrifuge was done, supernatant from each tube was discarded, and then 600 ìl of Buffer RLT was added to each tube to disrupt the cells. When Buffer RLT was added to the tube, it was pipetted up and down several times for homogenization. After that, 600ìl of 70 percent ethanol was added to the homogenized lysate of each tube and mixed well by pipetting. 700ìl of each sample was added to RNeasy mini spin column sitting in a 2-ml collection tube (supplied), and centrifuged for 15 seconds at 10,000 rpm. Volume of the each sample exceeded 700ìl, so flow-through of the collection tube was discarded and then rest of the aliquots were added to the same RNeasy column and the each column was centrifuged again. After the centrifuge, flow-through was discarded. 700ìl of Buffer RW1 was pipetted onto each of RNeasy column, and columns were centrifuged for 15 seconds at 10,000 for washing. After that flow-through and collection tubes were discarded. RNeasy column were transferred into a new 2-ml collection tube (supplied). 500ìl of Buffer RPE were pipetted onto the each column, and the columns were centrifuged for two minutes at maximum speed to dry the RNeasy membrane. After the centrifuge was done, RNeasy spin column were placed in a new two-ml collection tube, which were not supplied and old collection tube with filtrate was discarded. RNeasy spin column with new collection tubes were centrifuged at full speed for 1 minute. After this step was completed, each RNeasy column was transferred into a new 1.5-ml collection tube (supplied), and 30ìl of RNase-free water was directly added to the each of RNeasy column. Columns were centrifuged for one minute at 10,000rpm as for final step in collecting mRNA. mRNA from each sample was collected into the tube and the columns were discarded. mRNA sample tubes were immediately placed on ice.

RT (REVERSE TRANSCRIPTION)

Optical Density was first founded for each sample of mRNA, for the calculation for making the concentrations of RNA samples to be equal. RNA samples were diluted in RNase-free water in a new 200ìl tubes. These samples were then denatured at 95 degrees Celcius for one minute. After the denaturation step, to the each tube, following reagents (All from Invitrogen™, Carlsbad, CA) were added: 8ìl of dNTP's, 4ìl of 5X 1st Strand Buffer, 2ìl of 0.1 M DTT, 1ìl of Random Primer, 1ìl Rnase Inhibitor (SIGMA, St Louis, MO). After reagents were added, 1ìl of enzyme,

Super Script™ II RNase H-Reverse Transcriptase (Invitrogen™, Carlsbad, CA) was added to the each sample. Samples were ran in Thermal Cycler (Gene Amp PCR System 9700, Applied Biosystem) for 60 minutes at 40°C, and then 10 minutes at 65°C. After RT was done samples were placed in ice.

PCR (Polymerase Chain Reaction)

To the 200ìl tubes prepared for the cDNA (RT products), following components (Invitrogen™, Carlsbad, CA) were added for PCR: 5ìl of 10X PCR buffer, 1.5ìl of Magnesium Chloride, 2ìl of dNTP's, 1ìl of Estrogen ··receptor forward primer, 1ìl reverse primer, 29ìl of Rnase free water, 10ìl of cDNA. In addition, primers specific for the ,-actin gene was also used as an internal control for the PCR reaction.

Sequence of Forward and Reverse Estrogen Alpha Primer:
Forward Primer: 5'-ATCCTGATGATTGGTCTCGTCT-3'
Reverse Primer: 5'-GGATATGGTCCTTCTCTTCCAGA-3'

Sequence of Forward and Reverse Estrogen Beta Primer:
Forward Primer: 5'-GCTCATCTTTGCTCCAGATCTTG-3'
Reverse Primer: 5'-CAATCACCCAAACCAAAGCATC-3'
The samples were denatured at 95°C for 2 minute in a thermal cycler (Gene Amp PCR System 9700, Applied Biosystem), and then added 0.5ìl of Taq DNA polymerase to each tube. The PCR analysis was performed for 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min, then as a final primer extension step, 72°C for 10 minutes. The PCR product was analyzed by gel electrophoresis.

ELECTROPHORESIS

1g of Agarose was added to a flask then 60ml of 1X TBE Buffer solution were added. The flask was then microwaved for about 2 minutes until the solution started boil. After the solution cooled down, 5ìl of Ethidium Bromide was added and mixed well. The gel was then cast onto a horizontal gel electrophoresis apparatus. 45ìl of each PCR product was mix with 9ìl of DNA loading buffer, and 30ìl from the mixture was loaded onto the gel. After electrophoresis, the gel was visualized and analyzed by a Gel Documentation system (UVP) containing an ultraviolet transilluminator.

NITRIC OXIDE DETERMINATION

CELL CULTURING

Same step was performed as for gene expression except that nitric oxide determination was done to the cells in 96 wells plates, so 200ìl from mixture of cells and media were pipetted to the each well. This step was done to 15 wells.

CELL TREATMENT

First five wells were left as control. To next five wells, 2ìl of Ascorbic acid (Vitamin C) solution with concentration of 10mM was added for the final concentration of 100ìl for each well. To last five wells, 2ìl of Alpha tocopherol (Vitamin E) solution with concentration of 5mM was added to each well for the final concentration of 50ìM. Pre-treated cells were incubated for 24 hours at 39 degrees Celcius.

NITRIC OXIDE DETERMINATION

For Nitric Oxide detection, Apollo 4000 Free Radical Analyzer manufactured by World Precision Instrument (Sarasota, FL) was used. NO release was detected with an NO-selective microprobe manufactured also by World Precision Instruments (Sarasota, FL). The redox current was detected by a current-voltage converter circuit and continuously recorded. The dimensions of the probe (30 ìM diameter, 0.5mm length) permitted the use of a micromanipulator (Zeiss-Eppendorff). The sensor was placed approximately 100 ìM above the respective tissue surface. Calibration of the electrochemical sensor was performed by use of different concentrations (10ìl, 20ìl, 40ìl, and 80ìl) of a nitrosothiol donor S-nitroso-N-acetyl-DL-penicillamine (SNAP). Each well was placed under the probe and the graph was observed by the computer interfaced DUO-18 software (World Precision Instruments, Sarasota, FL). When the graph line stabilized, 10ìl of 17-, estradiol solution with concentration of 10-5 M was added to the well to stimulate NO release. Amount of NO release was graphed on the monitor and it was measured.

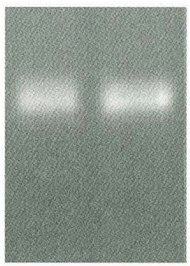


FIGURE 1A

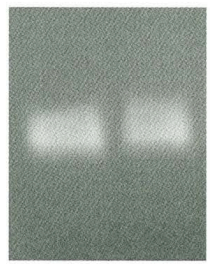


FIGURE 1B

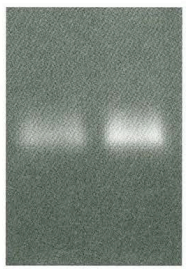


FIGURE 2A

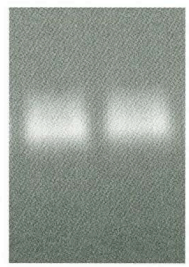


FIGURE 2B

Gene Expression for Estrogen *Alpha* Receptors in MCF-7 Cells

Figure 1A: The band in the left lane represents the amplified cDNA of ER-alpha genes in MCF-7 cells that were treated with 17-*beta* estradiol. The band on the right lane is that of control group of untreated cells. The brightness of the band on the right lane (control group) is slightly brighter than brightness of the band on the left lane (17-*beta*) estradiol, suggesting that 17-*beta*, estradiol slightly down-regulated the gene expression.

Figure 1B: Expression levels for *beta*-actin primer remained constant for two samples in Figure 1A, which verified equivalent sample loading.

Figure 2A: The band on the left represents the amplified cDNA of ER-*alpha* genes in a group of MCF-7 cells that were treated only with ascorbic acid, and the band on the right lane represents amplified cDNA(ER-*alpha*) of the group treated in a combination of 17-*beta* estradiol and ascorbic acid. The brightness of the band on the right lane (a group treated with the combination) is shown to be brighter than the brightness of the band in the left lane (a group treated with ascorbic acid), which means that more cDNA was amplified in the group treated with the combination. Also when the two bands in Figure 2A are compared to the control band in Figure 1A, the control group of Figure 1 is brighter than the left lane of Figure 2 (ascorbic acid) but dimmer than the right lane of Figure 2A(17-*beta* estradiol and ascorbic acid). This indicates that ascorbic acid alone resulted in a down-regulation (less cDNA were amplified) of ER-*alpha* gene expression and the combination of ascorbic acid and 17-*beta* estradiol resulted in an up-regulation.

Figure 2B: Expression levels for *beta*-actin primer remained constant for two samples in Figure 2A, which verified equivalent sample loading.

GENE EXPRESSION

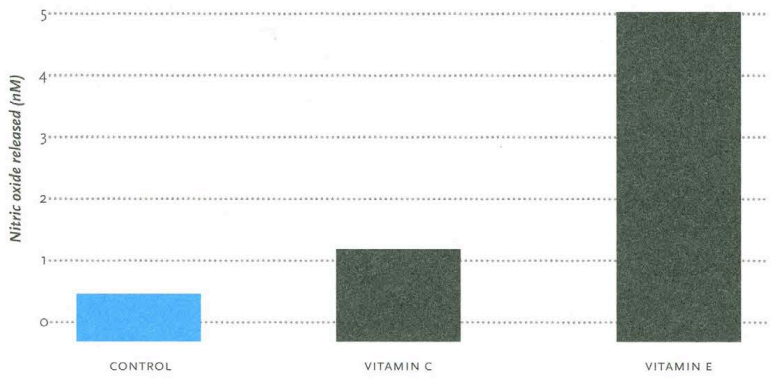
PCR products were analyzed on a two percent Agarose gel and a gel documentation system containing a UV transilluminator (UVP) (Figure 1-4). Brightness of the bands from the results of *beta*-actin gene expressions (Figure 1B, 2B, 5B) verified equivalent sample loading. Figures 1A and 2A below show bands that represent the amplified amount of cDNA for ER-*alpha* genes. The brighter the band is, the more amplified cDNA is present. Since the cDNA was generated by a Reverse Transcription of mRNA, the brighter the band, the greater level of the expression of the ER-*alpha* gene that produced the mRNA. The right lane in Figure 1A shows the bands of amplified cDNA of a control group of untreated MCF-7 cells (right lane), and the experimental group treated with 17-*beta* estradiol (left lane). A slight difference in the brightness of the two bands can be noticed. It appears that the control group band is brighter than the 17-*beta* estradiol treated group, which means that the ER-*alpha* gene had diminished expression in the group treated with estradiol. In other words 17-*beta* estradiol appears to have down-regulated the gene. Figure 2A shows the band of the group treated with ascorbic acid (left lane), and the band of the group treated with the combination of ascorbic acid and 17-*beta* estradiol (right lane). When the left lane in Figure 2A (ascorbic acid treatment) is compared to the right lane of Figure 1A (control), the control again appears brighter, suggesting that ascorbic acid also down-regulates the expression of the ER-*alpha* gene. The right lane (ascorbic acid and 17-*beta* estradiol treated) is brighter than both the left lane (ascorbic acid treatment) of Figure 2A and the right lane (control) of Figure 1A, suggesting that when ascorbic acid is in a combination with estrogen, it can up-regulate the ER-*alpha* gene.

In the RT-PCR for estrogen-*beta* receptors, it was difficult to compare the expression levels because the bands (shown in Figures 3, and 4) were so faint. A possible explanation for such a low expression level might be because in MCF-7 cells, the *alpha*-receptor is the prevalent form and the *beta*-receptor is hardly detectable.[xviii]

It appears that the anti-proliferative effect of ascorbic acid on MCF-7 cells reported in the literature[xix] is related to the down-regulation of estrogen receptor gene expression in MCF-7 cells treated with ascorbic acid. There was also a slight down-regulation in the gene expression level in the group treated with 17-*beta* estradiol and it seems that this down regulation was caused by a negative feedback due to the addition of 17-*beta* estradiol.

Previous work found that when estrogen is added to the melatonin, an antioxidant, the anti-proliferative effect of melatonin was reversed. Similarly in this research, it appears that the down-regulating effect on the ER *alpha*-re-

FIGURE 6



Nitric Oxide Determination

Average amount of nitric oxide released for the control group was 0.4 nM. Two groups treated with vitamin C and E released more NO than the control group but overall vitamin E caused more increase in NO release than vitamin C

FIGURE 3

FIGURE 4

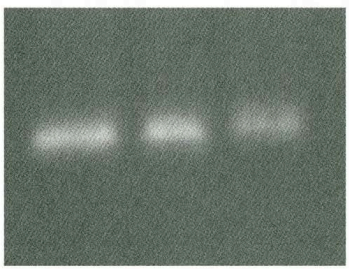


FIGURE 5B

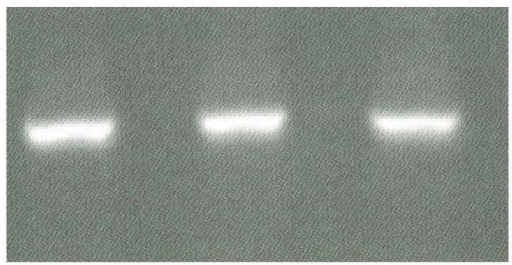


FIGURE 5B

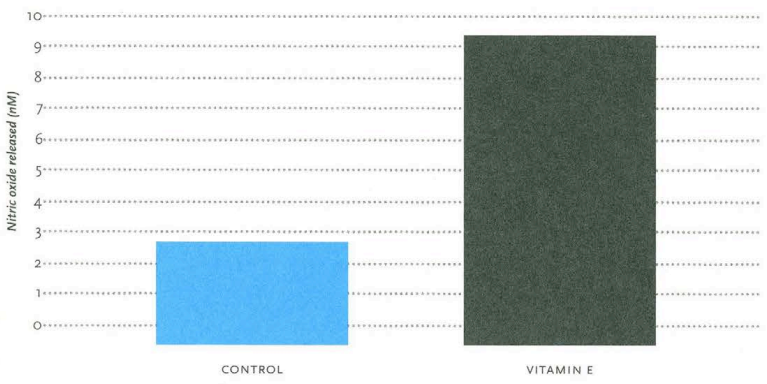
Gene Expression for Estrogen *Beta* Receptors in MCF-7 Cells

Figures 3 and 4: Possibly due to the fact that estrogen *alpha*-receptors are the prevalent form in MCF-7 cells and that beta-receptors are hardly detectable, a minimal amount of cDNA for beta receptors were amplified. This caused the brightness of the bands visualized on the gel to be very faint. No conclusions could be drawn.

Gene Expression for Estrogen *Alpha* Receptors in MCF-7 Cells

Figure 5A: From left: control group (untreated), *alpha* tocopheral (vitamin E) treated group, and the group treated in a combination of 17-*beta* estradiol and *alpha* tocopheral. No significant difference in the brightness of the bands of control group and vitamin E treated group could be found, but when third band (vitamin E and 17-*beta* estradiol) is compared to other two bands, the third band is less bright. The combination of *alpha* tocopheral and estrogen appears to have down-regulated ER-*alpha* gene expression level.
Figure 5B: Expression levels for *beta*-actin primer remained constant for all three samples, which verified equivalent sample loading.

FIGURE



Nitric Oxide Determination

A direct comparison of the contol group and vitamin E group.

ceptor gene caused by ascorbic acid was counteracted by the up-regulating addition of 17-*beta* estradiol.

Figure 5A, shows by contrast that in the *alpha* tocopheral (vitamin E) treatment, *alpha* tocopheral alone (middle lane) did not cause much change in the gene expression level of estrogen *alpha*-receptors when compared to the control group (first lane from left). However, the band from the combination of *alpha* tocopheral and 17-*beta* estradiol (third lane from left) is dimmer and therefore the combination of *alpha* tocopheral and 17-*beta* estradiol caused down-regulation in the gene expression level of ER-*alpha*. It appears that *alpha* tocopheral and ascorbic acid have opposite effects when combined with the addition of 17-*beta* estradiol.

NITRIC OXIDE DETERMINATION

A total of 15 trials were performed for nitric oxide determination. Figure 6 shows the bar graph of the average amounts of NO release for three groups of MCF-7 cells with a different treatment for each group.

Overall, compared to the control group, both vitamin C and E increased the level of nitric oxide release. However, it appears that vitamin E was more effective in boosting NO release. Figure 7 shows that this is true in a direct comparison of the control and vitamine E. As mentioned in the introduction, an excessive amount of NO may have negative effects, but the amount of NO increased by vitamin E was not in a reach to be considered as an excessive level. Since NO is a scavenger for other free radicals that may promote a cancer, it appears that vitamin E may has positive effects in limiting cancer development by increasing NO release.

FUTHER RESEARCH

More studies regarding antioxidants and estrogen receptors will have to be done. In the future, Real Time PCR may be conducted, which is the type of PCR that gives the exact quantity of amplified cDNAs. By getting an exact quantity through conducting Real Time PCR, it would be possible to get clearer data. More trials can be conducted in addition. Also in the future, a variety of other antioxidants other than vitamin C and E will be used for similar types of experiments.

CONCLUSION

Although further studies will have to be done, this study demonstrated the overall effect of ascorbic acid (vitamin C), *alpha* tocopheral (vitamin E) and their combination with 17-*beta* estradiol on ER-*alpha* gene expression. This study

demonstrated that ascorbic acid might have positive effects in limiting the spread of breast cancer by decreasing the estrogen receptor level. However, this study also revealed that the combination of estrogen and ascorbic acid could cause up-regulation of ER-*alpha* gene expression, which may be a possible explanation for the phenomena of greater proliferation that occurred in the previous studies. It can be suspected that the combination of estrogen and ascorbic acid can make cells more vulnerable and may accelerate the MCF-7 cell growth due to the increased level of estrogen receptors.

In contrast to ascorbic acid (vitamin C), *alpha* tocopheral (vitamin E) caused a down-regulation of ER-*alpha* gene expression level when the cells were treated in combination with 17-*beta* estradiol. Acting alone, *alpha* tocopheral showed no effect on regulation of this gene.

This study also demonstrated the effect of antioxidants on the release of nitric oxide on the surface estrogen receptor of MCF-7 cells. This study showed that the both antioxidants increased NO level but vitamin E was more effective than vitamin C, suggesting that vitamin E may have positive effects in preventing cancer. Vitamin C and Vitamin E appears to be working by different mechanisms since vitamin E cause a significant release of NO, whereas vitamin C caused a significant decrease of ER-*alpha* gene expression in the MCF-7 human breast cancer cells. Overall, the data from this study will help further understand how antioxidants work in the prevention of cancers and other type of diseases.

ENDNOTES

i. Refernce 1
ii. Refernce 2
iii. Refernce 2
iv. Refernce 2
v. Refernce 3
vi. Refernces 4-8
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viii. Refernces 9-10
ix. Refernces 11- 12
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xiii. Refernce 23
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xvi. Refernces 32-33
xvii. Refernce 34
xviii. Refernces 11-12
xix. Refernce 35

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