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PRINCIPAL INVESTIGATOR: Robert L. Wells, Ph.D.

CONTRACTING ORGANIZATION: Colorado State University
Fort Collins, Colorado 80523-2002

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13. ABSTRACT (Maximum 200 Words)

Melatonin and breast cyst fluids (BCFs) both exert antiproliferative effects on breast cancer cells that may be mediated by growth factors. Our research was the first to identify melatonin in BCF and we hypothesize that it is responsible for BCF's antiproliferative properties. The primary objectives of this study are to establish a clinical BCF sample bank among patients with gross cystic breast disease in order to study of the relationship between this disease, melatonin and related growth agents in BCF, and breast cancer risk. A laboratory effort will use BCF samples to elucidate the contribution of melatonin and related growth agents (EGF, TGF-beta, DHEA-S) to the oncogenic effects of BCFs in the MCF-7 human breast cancer cell model. Our progress to date includes the establishment of informed consent procedures and a BCF sample bank. Patient enrollment and BCF sample collection are on-going. Cell culture experiments have confirmed that physiological melatonin levels inhibit MCF-7 cell growth. We have also performed baseline melatonin, sodium, potassium, and TGF-beta measurements in BCF. Out of 17 subtasks specified in our Statement of Work, 8 have either been completed or were initiated in year 1 and will remain on-going as patient enrollment progresses.

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INTRODUCTION

Long term exposure of breast tissue to estrogen plays a major role in breast tumor formation, although risk factors linked with chronic estrogen exposure only account for a portion of disease occurrence. Other hormones and growth factors are likely to be related to unidentified breast cancer risk factors. The pineal hormone, melatonin, exerts antiproliferative and anticarcinogenic effects via several proposed mechanisms, including the modified secretion of growth factors that are critical for breast tumor development. Prior research suggests that women with high endogenous melatonin levels may have lower breast cancer risks and that melatonin may be an effective therapeutic agent for breast cancer treatment. Women with palpable breast cysts have a 2-4 fold increased risk of developing breast cancer. Despite the identification of several growth agents in breast cyst fluid (BCF) that appear to segregate based on electrolyte (Na,K) composition, it is still unclear what role BCF plays in breast tumor development. BCF exerts an inhibitory effect on the proliferation of breast cancer cells *in vitro*. We hypothesize that this effect is due to the presence of melatonin in BCF, which stimulates production of the growth inhibitory cytokine, transforming growth factor-beta (TGF-beta), in human breast cancer cells. The two major goals of this study encompass clinical and laboratory-based activities. The clinical effort will establish a cyst fluid sample bank to evaluate the relationship between melatonin in BCF and breast cancer risks. The laboratory effort will use the BCF samples to elucidate the contribution of melatonin to the oncostatic effects of cyst fluids in a breast cancer cell model. The relationship between melatonin and specific growth factors in BCF will also be assessed. The specific aims are to: 1) create a BCF sample bank among patients with gross cystic breast disease in order to perform a prospective study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risk; 2) determine melatonin, TGF-beta, epidermal growth factor (EGF), dehydroepiandrosterone-sulfate (DHEA-S), and electrolyte (Na,K) concentrations in BCF samples; 3) perform cell proliferation studies in MCF-7 breast cancer cells treated with BCF and compare growth rates with melatonin and growth factors in BCF; 4) determine whether TGF-beta synthesis is enhanced in BCF-treated MCF-7 cells and whether the enhancement is related to melatonin levels in the BCF; 5) determine the relationship between melatonin and growth agents (EGF, TGF-beta, DHEA-S) in BCF; 6) quantify Na and K concentrations in BCF to determine whether low breast cancer risk BCF (high Na:K ratio) contains elevated levels of melatonin and TGF-beta, and reduced concentrations of EGF and DHEA-S; and 7) determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease.

BODY

The statement of work for this project includes four tasks. The major objectives of each task are as follows:

Task 1: Establish a sample bank for breast cyst fluids in patients with gross cystic breast disease (Specific Aim 1);

Task 2. Determine melatonin, TGF-beta 1 and 2, EGF, DHEA-S, and electrolyte (Na,K) concentrations in breast cyst fluid samples (Specific Aim 2);

Task 3. Perform cell proliferation studies in MCF-7 breast tumor cells treated with breast cyst fluids in order to compare cell growth effects with melatonin and growth factors in BCF (Specific Aims 3 and 4);

Task 4. Perform biostatistical data analyses to: determine the relationship between melatonin and growth-related substances in BCF (Specific Aim 5); determine whether low breast cancer risk (high Na:K ratio) BCFs contain higher levels of melatonin and TGF-beta and lower levels of EGF and DHEA-S (Specific Aim 6); and determine whether melatonin in BCF is associated with known risk factors for breast cancer in patients with gross cystic breast disease (Specific Aim 7).

Each of the four tasks described above were further delineated by a series of subtasks (17 subtasks total). Each of the four subtasks specified under Task 1 have been initiated and two (1C and 1D) have been completed. Each of the three subtasks specified under Task 2 have been initiated and these subtasks will remain on-going until sample collection has been completed. One of three subtasks under Task 3 (3A) has been completed. Task 4 includes seven subtasks that are all directed towards hypothesis testing via statistical data analyses. Since BCF sample collection and data accrual is currently on-going, these subtasks have not yet been initiated. To summarize, out of a total of 17 subtasks specified in the Statement of Work, 8 have either been completed or initiated. A more detailed description of progress towards accomplishment of each project task is provided below.

There were four subtasks established under Task 1 (1A-1D). The objective of Task 1A was to obtain final approval from institutional review boards (IRBs) for modifying proposed human informed consent procedures. Approval to perform human subjects research was required from three separate institutions; Colorado State University, the University of Washington Medical School, and the U.S. Army Medical Research and Materiel Command (USAMRMC). Note that we were precluded from performing any subject recruitment or BCF sample collection until IRB approvals were obtained from each

institution. Final approval for the human subjects informed consent procedures to be used for this study was obtained on from the USAMRMC on October 27, 1999, approximately two months after the project start date. A copy of the approved consent form is presented in Appendix A.

Task 1B required the establishment of protocols for BCF sample collection including sample handling, storage, shipment, and record keeping. In addition to a written description of procedures to be followed concerning sample handling and shipment, the BCF sample collection protocol also includes: a BCF sample data form to be completed by the attending physician (or medical assistant) performing cyst aspirations, sample labeling procedures, a chain of custody form, and a list of contact information for each of the study investigators. A copy of the BCF sample collection protocol is presented in Appendix B. Also included within the scope of Task 1B was the establishment of laboratory safety protocols and standard operating procedures for the research activities included in this project. A Safety Program Plan that describes these protocols is presented in Appendix C.

Once IRB approvals were obtained from the CSU, UW and USAMRMC IRBs, a subcontract was established with Dr. Anderson at the University of Washington Medical School. BCF sample collection protocols were also developed and supplies were purchased within the first quarter of year 1. Thus, procedures were in place for initiation of patient enrollment (Task 1C) by the second quarter of year 1. However, by March 2000, patient enrollment had not yet been initiated. At that time, the research coordinator at the University of Washington (i.e., the assistant to Dr. Ben Anderson) was terminated and a replacement was hired in the same month. Patient enrollment and BCF sample collection commenced within one month of the new research coordinator's start date. To date, a total of 7 patients and their corresponding BCF samples have been enrolled in this study. Because this does not meet our year 1 enrollment goal, we have initiated a collaborative research agreements with two additional medical facilities. One collaboration has been established with Christina Finlayson, MD, who directs the Breast Center at the University of Colorado Health Sciences Center (UCHSC, Denver, CO). Dr. Finlayson has agreed to oversee patient enrollment and BCF sample collection at the UCHSC (see attached letter Appendix D) and we are currently in the process of obtaining IRB approval at that location. We have also established a collaboration with Winfield Craven, MD, at the Breast Diagnostic Center at Poudre Valley Hospital (Fort Collins, CO) (see attached letter Appendix D) and the IRB approval process is currently on-going at this facility as well. Dr. Finlayson expects to enroll about 40 patients and Dr. Craven about 40-60 patients from their respective medical facilities each year. Thus, in year 2 we anticipate that these additional collaborative efforts will more than compensate for our enrollment deficit in year 1 and

that our goal of recruiting 100 subjects by the end of year 2 will be attained. Patient enrollment and BCF sample collection at the UCHSC and Poudre Valley Hospital will be performed at no additional cost to the USAMRMC.

Task 2 specified the determination of melatonin, related growth agents, and electrolyte (Na,K) concentrations in BCF samples. A total of three specific subtasks were defined: 2A) establish baseline assay conditions and quality control criteria for radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs); 2B) perform biochemical assays of melatonin and growth-related agents; and 2C) determine ionic composition (Na,K) of BCF samples by atomic absorption (AA) spectroscopy. Upon grant award, a search was initiated for a research assistant to perform laboratory based analyses and cell culture experimentation. Interviews were performed and a qualified individual was hired within the first quarter of year 1. Work was then initiated to establish baseline cell culture and biochemical assay conditions. To date, biochemical assays have successfully been performed for melatonin, sodium, and potassium in preliminary BCF samples. In addition, we have also completed a baseline assay for TGF-beta. Methods describing each of these assays are presented below.

Melatonin Assay. The quantification of melatonin is performed by the I-125 competitive radioimmunoassay method as described by Rollag and Niswender (1). Assays are performed at the Animal Reproduction and Biotechnology Laboratory (ARBL) at Colorado State University under the direction of Dr. Torrence Nett. Briefly, melatonin is extracted from a 0.5 ml aliquot of BCF sample by addition of 2.5 mL CH_2Cl_2 (dichloromethane). The solvent extract is then treated with 1 ml of 0.1 M sodium bicarbonate, mixed, and centrifuged at 3000 rpm for 15 minutes and the aqueous layer removed. Finally, the extract is treated with 1 ml of de-ionized water, mixed, and centrifuged at 2500 rpm for 10 minutes and the aqueous layer removed. The solvent extract is dried under a nitrogen atmosphere and reconstituted in 1 ml PBS-gel. To perform the RIA, 200 μl of sample is incubated with 100 μl of ^{125}I -melatonin (~40,000 cpm/tube) and 200 μl melatonin antiserum (1:40,000) at 4° C for 4 hours. After 4 hours, 200 μl of anti-rabbit gamma globulin is added to the incubation. At the end of a 24-hour incubation period, 3 ml of cold phosphate buffered saline is added and each sample is centrifuged at 2500 rpm for 30 minutes, the supernatant decanted, and the radioactivity in the pellet is quantified with a gamma counter. Melatonin levels in BCF samples are then determined via a linear equation (standard curve) obtained using similarly assayed known melatonin concentrations in PBS-gel buffer. Results are reported as pg/ml with a method detection limit of 1 pg/ml.

Electrolyte Assays. Samples are analyzed for sodium and potassium content by flame ionization atomic absorption spectroscopy.

Commercial sodium standard solutions (Fisher, Houston TX) are diluted with 1% nitric acid in double de-ionized water to concentrations of 0.125, 0.25, 0.50, 1.0, and 2.0 µg/ml and a standard curve is prepared at an absorbance of 589 nm (sodium lamp). Potassium standards are prepared in similar fashion from a commercial stock standard solution (Fisher, Houston TX) and diluted with 1% nitric acid in double de-ionized water to concentrations of 0.3125, 0.625, 1.25, 2.5, and 5.0 µg/ml and a standard curve is prepared at an absorbance of 766.5 nm (potassium lamp). Data are integrated and recorded using Varian Spectra 300/400 software. Cyst fluid samples are diluted in 1% nitric acid-double de-ionized water with final dilution factors of 1,000X for potassium and 1,000X or 2,000X for sodium. Analytic blanks were prepared from 1% nitric acid in double de-ionized water.

Preliminary data showing breast cyst fluid concentrations of sodium, potassium, and melatonin are presented in Table 1. These

Table 1. Preliminary Data: Breast Cyst Fluid Composition

Patient ID	Cyst Fluid Sodium (mg/ml)	Cyst Fluid Potassium (mg/ml)	Cyst Fluid Na:K Ratio	Cyst Fluid Melatonin (pg/ml)
7	0.76	5.22	0.2	12.4
8	0.42	0.09	4.7	29.5
9	2.95	2.29	1.3	<1.0
11	2.76	0.40	6.9	7.7
12	3.65	0.32	11.4	54.5
13	0.16	2.91	0.1	5.6
14	0.90	5.06	0.2	32.8

analyses allowed for establishment of baseline biochemical assay procedures for several endpoints included in this project. Several patients had BCF melatonin levels comparable to nocturnal serum concentrations in adults (20-60 pg/ml) (2-3). Note that mean melatonin concentrations in BCF samples with Na:K ratios greater than 3 (30.6 ± 23) were nearly three times greater than those with Na:K ratios less than 3 (12.8 ± 14). These results are consistent with our hypothesis that melatonin concentrations in BCF are elevated among low breast cancer risk patients (Na:K ratio > 3). However, these results are based on a limited sample set and should be interpreted with caution. In summary, these preliminary analyses demonstrate that baseline assay conditions have been established for determination of melatonin and electrolytes (Na,K) in BCF.

Transforming Growth Factor (TGF) beta. Baseline TGF-beta assays were performed using an enzyme linked immunosorbent assay (ELISA, Biosource Europe, Belgium). The principle of the method involves the competitive binding of sample TGF-beta and horseradish peroxidase (HRP) labeled TGF-beta to bound antibody in a microtiter plate. After rinsing and adding the HRP substrate-chromogen, TMB, the optical density is measured at 450 nm and is inversely proportional to the sample TGF-beta concentration. The procedure follows the manufacturer's protocol. Briefly, the provided calibration standard (2 ng/ml) is serially diluted in 5-fold increments to produce standards of 400, 80, 16, and 3.2 pg/ml. The wash solution is prepared by diluting 1 ml to 199 ml of DDI water. The positive control is reconstituted with 0.5 ml double de-ionized water, extracted with 2.5 M acetic acid (100 μ l/100 μ l), vortexed, and incubated at room temperature for 15 minutes. It is then diluted 20 μ l to 500 μ l of dilution buffer for a final dilution of 1:52. The sample is extracted with the provided acid containing extraction solution (500 μ l/50 μ l), vortexed, and incubated at room temperature for 10 minutes with continuous shaking. It is then diluted with 0.5 ml dilution buffer to a final dilution factor of 1:2.1. Coated assay wells are secured to the provided frame with positions noted and labeled for standards, samples, and controls. Sample, standard, or control (200 μ l) is pipetted into the appropriate well followed by 50 μ l of TGF-beta-HRP conjugate. The plate is then incubated for two hours at room temperature on a horizontal shaker at approximately 700 RPM. The liquid is aspirated from each well followed by three rinses with wash solution. Within 15 minutes after the final wash, 100 μ l of chromogen is added and the plate is incubated 60 minutes in the dark on a horizontal shaker. Stop solution (100 μ l) is added and the absorbance read at 450 nm on a microtiter plate reader. A standard curve is prepared for each assay and concentrations are calculated from the linear equation derived from the standard curve. Preliminary results from our initial baseline assay show good linearity (regression equation: $y = -30x + 94.45$) with 99.7% of the variance explained (i.e., $R^2 = 0.997$). Thus, baseline assay conditions also been established for TGF-beta determinations. Work on the EGF and DHEA-S assays is in progress.

The subtasks specified by Task 3 included the following: 3A) determine initial dose-range toxicity and proliferation response relationships in MCF-7 cells; 3B) perform cell proliferation and TGF-beta response studies with BCF samples; and 3C) calculate growth indices for each cell culture experiment. A comprehensive literature review was performed in order to compile and evaluate research on the effects of melatonin on MCF-7 breast cancer cell growth that has been published since the preparation of our grant proposal in 1998. A number of studies published within the last 2 years have demonstrated melatonin's oncostatic effects in MCF-7 (4-10) and other cancer and hormone-dependent cell lines (11-13),

although these effects have not been consistently observed (14-15). Thus, the literature review was also required to identify cell culture parameters that have proven effective for melatonin induced growth inhibition in MCF-7 cells (see ref. 4 for a recent review).

Available evidence suggests that there may be several pathways that melatonin acts upon to inhibit cell proliferation. For example, melatonin stimulates transcription of the potent growth-inhibitory cytokine, TGF-beta gene, in MCF-7 cells (16). This effect was recently replicated in MCF-7 cells and the increased TGF-beta transcription was linked with melatonin-induced growth suppression (6). In support of these findings, melatonin-mediated inhibition of benign prostate epithelial cell growth was also shown to be dependent upon increased TGF-beta production (13). Studies in various breast cancer cell lines indicate that melatonin exerts its antiproliferative effects only in estrogen receptor (ER) positive cell lines at physiologically relevant concentrations (17). Although melatonin does not directly interfere with binding of estrogen to the ER (18), it has been shown to alter estrogen-mediated signal transduction (19), inhibit the expression of ER mRNA and protein (20), and destabilize the binding of estrogen-ER complexes to the estrogen response element (7) in MCF-7 breast cancer cells. Other studies suggest that melatonin's growth inhibitory effects may be dependent upon: interactions between melatonin and other hormones or growth factors present in serum (21), on a high plated cell density (4,22), or on an elevated rate of cell proliferation in culture (4,22). A recent study performed using three stocks of MCF-7 cells obtained from different laboratories revealed that differences in the growth inhibitory effects of melatonin among the different MCF-7 stocks were associated with both the level of ER mRNA expression and the degree of estrogen-stimulated cell growth (6). Studies recently performed in our laboratory indicate that under certain conditions melatonin alters MCF-7 cell growth in a manner that is not dependent upon estrogen signaling. After achieving exponential or log phase growth, MCF-7 cells cultured in estrogen depleted, charcoal stripped serum responded to melatonin treatment via uncoupling of oxidative phosphorylation and reduced ATP production (see submitted manuscript, Appendix E). These effects appear to be mediated by the melatonin receptor, since co-treatment of MCF-7 cells with the melatonin receptor antagonist, luzindole, eliminated this effect. Taken together, the results summarized above suggest that melatonin may act through different mechanisms depending upon cell culture conditions. Further research is needed to elucidate the specific molecular mechanisms of melatonin's oncostatic action and the extent to which these processes are modulated by different cell culture conditions.

Cell culture experiments to address Task 3A objectives were initiated in the second quarter of year 1, immediately after our

laboratory research assistant was hired. These experiments have focused on melatonin-mediated growth inhibition using culture conditions (i.e., unstripped serum) that are more likely to mimic BCF treatments in MCF-7 cells. These activities were initiated to ensure that the MCF-7 cells would be sensitive to the effects of melatonin and growth factors present in breast cyst fluid. Physiological concentrations of melatonin were used (1 nM) since previous work has shown this concentration to be effective in inhibiting MCF-7 cell growth (4-10 and Appendix E). Figure 1 shows the results of a cell proliferation experiment using MCF-7 cells obtained from the ATCC, and grown DMEM plus 10% fetal bovine serum. These cells are designated as MCF-7-W. In this experiment a clear growth inhibition was observed in the cultures treated with melatonin for five days, but not for three days. In addition, daily medium changes were found to be more effective than no medium changes during the five day treatment interval. Thus, we used daily medium changes with fresh melatonin added for a total of five days as the treatment protocol in subsequent experiments. Reports in the literature indicated that MCF-7 cells were sensitive to melatonin depending on the cell proliferation rate as modulated by cell seeding densities (22). We therefore kept the cell cultures at a constant, relatively high plating density for each experiment.

Based on the preliminary experiments described above and on discussions with Dr. Steven Hill (Department of Anatomy, Tulane University, New Orleans, LA), we have established the following method for determining growth inhibition by melatonin. Cells are plated in 6-well plates at densities of either 1×10^4 or 3×10^4 cells per well with DMEM medium supplemented with 10% fetal bovine serum. After 24 hours, the medium is aspirated and replaced with serum-free DMEM medium. After 24 hours, the cells become blocked in G₁ phase of the cell cycle, and the medium is changed to DMEM with 10% fetal bovine serum with or without 1 nM melatonin added. After 120 hours, with daily medium changes and fresh additions of melatonin, the cells are trypsinized and counted with a hemacytometer using trypan blue exclusion.

In addition to the experimental methodology described above, we performed parallel experiments using an alternative method of cell counting known as the CyQuant cell proliferation assay (Molecular Probes, Eugene, OR). This method involves the fluorescence measurement of a dye that is incorporated into the DNA of the attached cells. This innovation allows for the measurement of a smaller number of cells growing in the well of the plate and conserves the culture medium (or BCF sample volume) that is required for a given cell culture experiment. We evaluated this alternative method for quantifying cells in multiwell plates because we anticipate the necessity to work with relatively small volumes of BCF from some patients, and this method will allow for a greater overall data yield. Using this method we have obtained

results (data not shown) that are identical to those presented in Figure 1 using hemacytometer and trypan blue exclusion counting.

We undertook further studies to define the melatonin effect in other strains of MCF-7 cells. It has been previously reported that inhibition of MCF-7 cell proliferation by melatonin was cell strain dependent (6). MCF-7-L cells were obtained from Dr. Leia Smith, Colorado State University. These cells are unique in that they have the neomycin resistance (NEO^r) gene inserted into their genome, which results in clonal selection in the presence of the antibiotic, G418. Experiments with these cells were performed under conditions described above. Figure 2 shows that under these strict growth conditions, MCF-7-L cells were inhibited by 1 nM melatonin after five days of treatment at two different plating densities. We have also recently obtained MCF-7-M cells from Dr. Steven Hill at Tulane University that were previously shown to be responsive to the growth inhibitory effect of melatonin (6) and proliferation studies with these cells in response to melatonin treatment are currently underway.

We also characterized the cell strains according to DNA content of actively growing cultures by flow cytometry (Figure 3); DNA content is plotted on the abscissa and cell number is plotted on the ordinate. MCF-7-L cells show a typical DNA histogram for actively growing cells, prominent G_1 peak, a typical S-phase region and G_2/M peak. The MCF-7-W and MCF-7-M cells show atypical DNA content profiles that indicate that these strains are composed of mixtures of at least to sub-populations with differing ploidy. How this genetic diversity came about is unclear, but may involve a degree of genomic instability inherent in the MCF-7 breast cancer cell line. The more typical profile of DNA content present in the MCF-7-L cells is due to the insertion of the NEO^r gene and subsequent clonal selection by G418.

From the foregoing, it is obvious that MCF-7-M and MCF-7-W cells are genetically diverse and not unlike some tumors. This could be advantageous for melatonin inhibition studies, where a tumor cell line is needed. On the other hand, we have observed that MCF-7-L cells show a greater melatonin inhibition response, which may be related to the fact that they are homogeneous. These differences may be important in order to effectively demonstrate growth suppression when the actual cyst fluid samples are tested, and studies are currently planned to examine these potential differences using excess breast cyst fluids that have already been collected (Task 3B). In summary, laboratory efforts to date have established cell culture parameters and treatment protocols for melatonin-induced growth inhibition in the MCF-7 breast cancer cell line. These accomplishments will provide a platform for scheduled work to be completed in year 2.

KEY RESEARCH ACCOMPLISHMENTS

- Approval of informed consent procedures for research in human subjects was obtained from the CSU, UW, and USAMRMC institutional review boards.
- BCF sample collection and handling procedures and laboratory safety protocols have been established.
- Patient enrollment has been initiated and a BCF sample bank has been established.
- Collaborative agreements for patient recruitment and BCF sample collection have been established at two additional medical centers.
- Baseline biochemical assay procedures have been established for melatonin, sodium, potassium, and TGF-beta.
- Cell culture conditions and a treatment protocol that induces melatonin-mediated growth inhibition in MCF-7 human breast cancer cells has been established.
- An alternative cell counting procedure (CyQuant) that minimizes the required BCF volume necessary for cell proliferation assays and optimizes the data yield for each BCF sample has been developed and validated.

REPORTABLE OUTCOMES

- A human breast cyst fluid sample bank has been established as a biological resource for performing studies on the relationship between breast cyst fluid composition, gross cystic breast disease, and breast cancer risk in women.
- We have initiated enrollment of a patient population that will not only allow us to study how cyst fluid composition influences human breast cancer cell growth but we have also incorporated into our study design the possibility for long term follow-up of this population through a consenting procedure that will not require re-contacting any patients, so that the role growth-related agents in breast cyst fluid can be prospectively linked with breast cancer risks.
- We have recently prepared and submitted a manuscript for publication in the peer-reviewed scientific literature that confirms and extends previous reports of melatonin's oncostatic potency in the MCF-7 human breast cancer cell line (Appendix E).

CONCLUSIONS

Breast cancer remains a leading cause of cancer mortality in women worldwide. Because the known risk factors for breast cancer only account for a portion of the disease occurrence, research is needed to determine the role of hormones and related growth agents other than estrogen that are likely to be associated with as yet unidentified breast cancer risk factors. Women with palpable breast cysts have a 2-4 fold increased risk of developing breast cancer, although studies indicate that breast cyst fluid has antiproliferative properties. Clearly, further research is needed to evaluate the role of cyst fluid composition in breast tumor development. Our research has for the first time identified the presence of the oncostatic hormone, melatonin, in breast cyst fluid and we hypothesize that melatonin is responsible for the previously observed antiproliferative effects of breast cyst fluid in vitro.

The primary objectives of this study are to establish a clinical breast cyst fluid sample bank among patients with gross cystic breast disease in order to study of the relationship between gross cystic breast disease, melatonin and related growth agents in BCF, and breast cancer risks in women. A laboratory effort will use the BCF samples from this repository to elucidate the contribution of melatonin and related growth agents to the oncostatic effects of cyst fluids in a human breast cancer cell model. Our progress to date can therefore be divided into clinical and laboratory efforts. On the clinical side, informed consent procedures and a BCF sample bank have been established, and patient enrollment and breast cyst fluid sample collection are on-going. On the laboratory side, cell growth experiments have commenced, resulting in pertinent data confirming that melatonin exerts a growth inhibitory effect on the proliferation of human breast cancer cells in vitro. We have also performed baseline melatonin, sodium, potassium, and TGF-beta measurements in breast cyst fluid. Out of 17 subtasks specified in our Statement of Work, 8 have either been completed or have been initiated and will remain on-going during year 2.

The knowledge gained to date will enable this project to progress to the next step, which encompasses the treatment of MCF-7 breast cancer cells with human BCF to determine how BCF composition relates to its effects breast cancer cell proliferation. Cell culture experiments using BCF samples already collected as well as corresponding biochemical composition assays are scheduled for the first quarter of year 2. Results obtained from this study will add to our understanding of breast cancer biology and help elucidate the mechanism whereby BCF and melatonin inhibit breast cancer cell growth. Information resulting from this project will be valuable for evaluating the potential predictive and therapeutic applications of BCF constituents including melatonin in the development and treatment of breast cancer.

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Figure 1

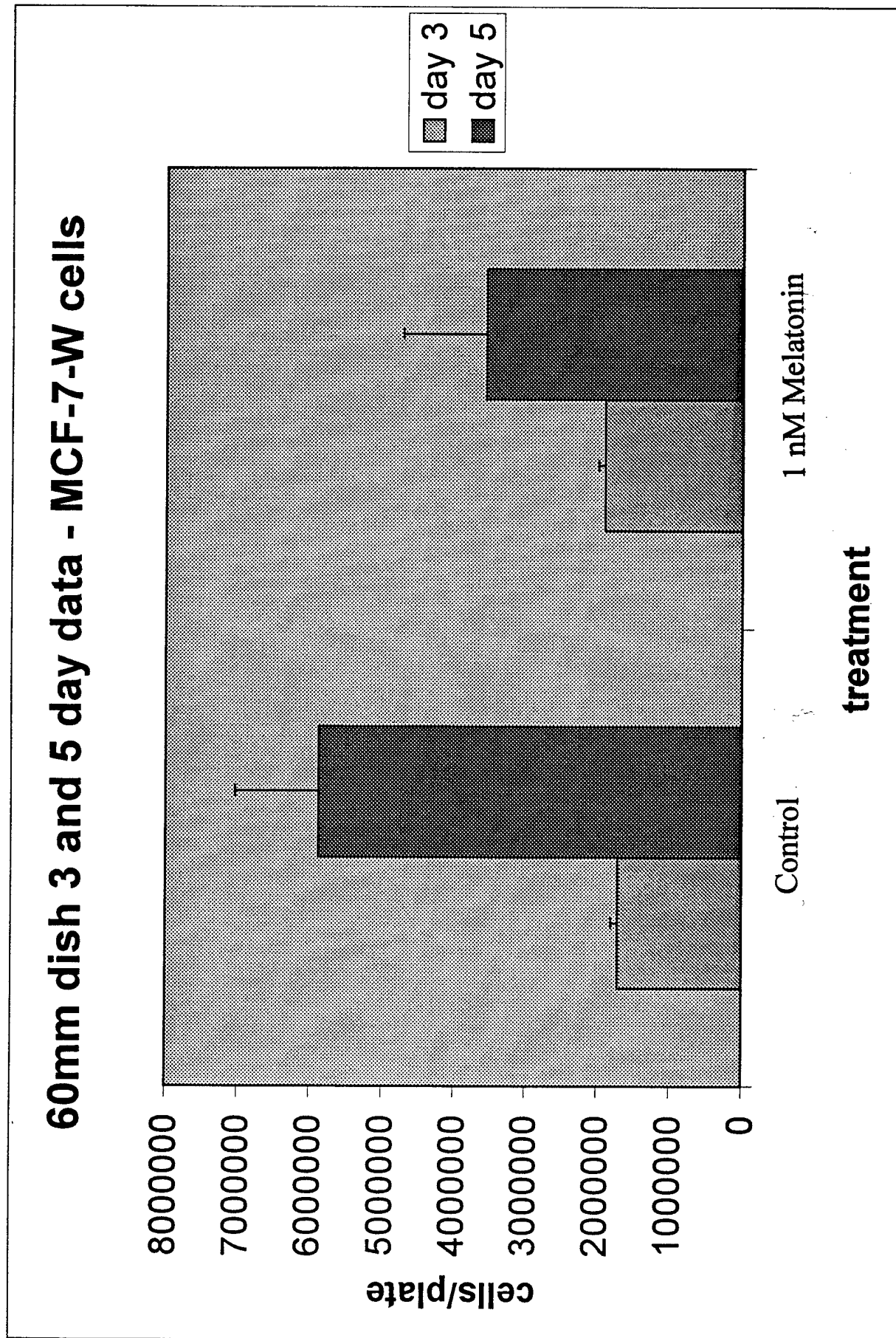


Figure 2.

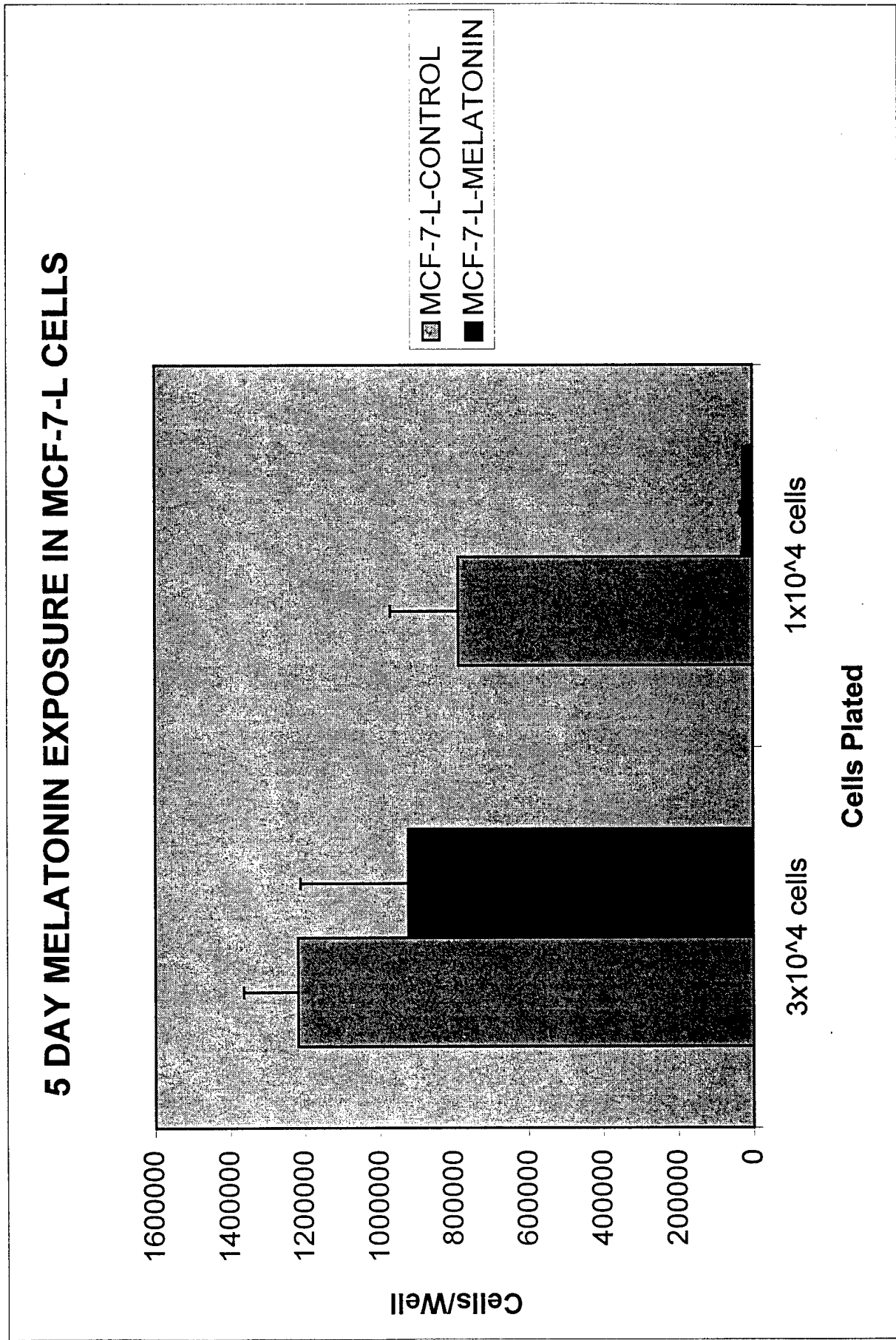


Figure 3

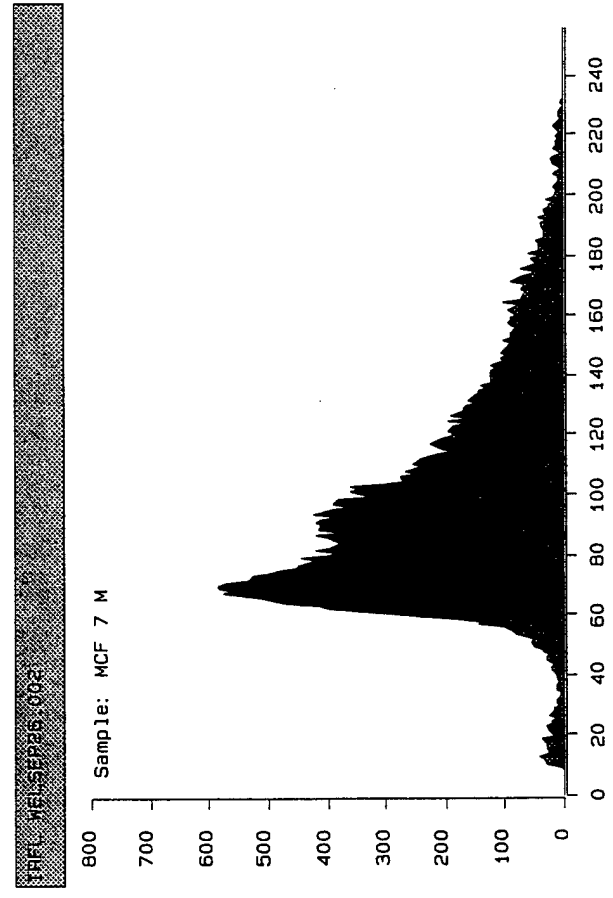
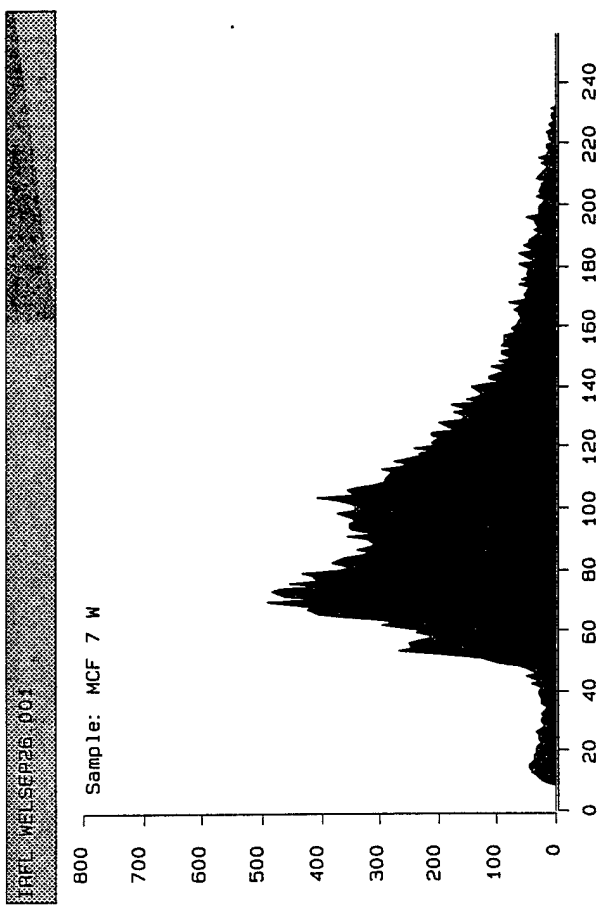
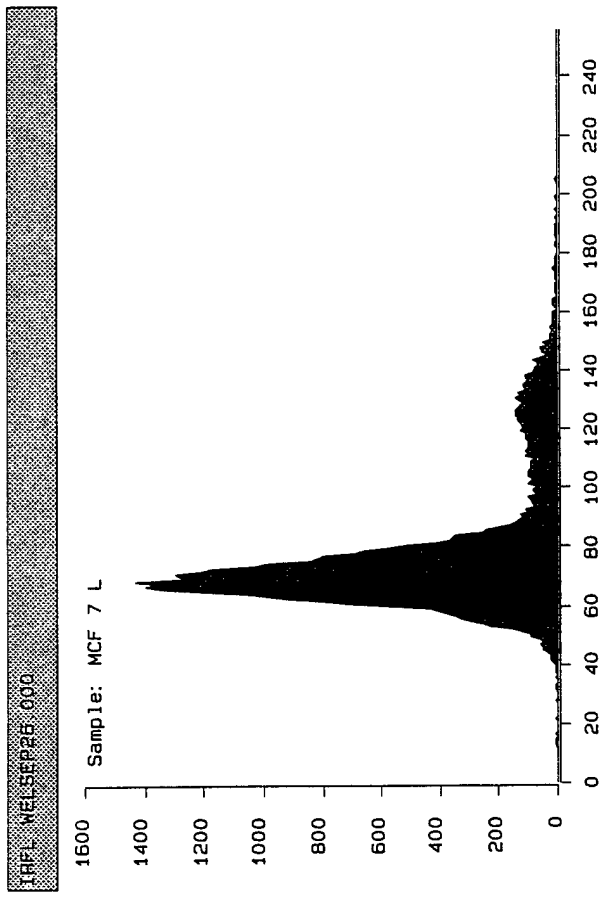


Figure Legends

Figure 1. MCF-7-W cell growth suppression by 1 nM Melatonin. Cells per 60 mm petri dish were counted after 3 and 5 days of melatonin treatment using a hemacytometer and trypan blue exclusion.

Figure 2. Cell plating density effects on Melatonin inhibition. MCF-7-L cells were plated at either 1×10^4 or 3×10^4 cells per well in 6 well plates. After a 5 day exposure to melatonin with daily medium changes the cells were counted by hemacytometer and trypan blue exclusion.

Figure 3. Flow Cytometry histograms of 3 strains on MCF-7 cells in exponential growth. Cell number vs. DNA content is plotted for MCF-7-L (upper left), MCF-7-W (upper right), and MCF-7-M (lower left). See the discussion for a description of the derivation of the strains.

APPENDIX A

MAR 28 2000

CONSENT FORM

UW

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

Investigators:

Benjamin O. Anderson, MD	Associate Professor	UW Department of Surgery	206-543-3680
Julie R. Gralow, MD	Assistant Professor	UW Medicine/Oncology	206-598-4100
James B. Burch, MS, PhD	Dept. of Environ Health	Colorado State Univ.	970-491-6148
Robert Wells, PhD	Associate Professor	CSU Dept. of Radiology	978-491-1087
Beth Aaron, RN	Research Coordinator	UW Department of Surgery	206-543-9322

University of Washington Medical Center Medical Oncology 24-hour emergency phone number: 206-598-6180 Ask that the Oncology Fellow "on-call" be paged.

INVESTIGATORS' STATEMENT

Purpose and Benefits

Melatonin, which is a substance found naturally in the body, has been shown to slow the growth of cancer cells. How melatonin works on cancer cells is not known. This study will look at the relationship between melatonin and cystic breast disease (CBD) to better understand its effect on breast cancer. It will also study other proteins found naturally in the body, called growth hormones and growth factors, which are found in breast fluid and how they effect melatonin. Your doctor has told you that you have CBD. In order to treat your cysts, your doctor will need to remove the fluid inside these cysts with a needle; this is also known as an aspiration. You have consented to this aspiration and are being asked to give part of this breast cyst fluid (BCF) for this study.

You will not receive any direct benefit from participating in this study, however, the results of this study may provide researchers with a better understanding of CBD and its relation to breast cancer.

Procedures

If you agree to be in this study, we will ask you to fill out a questionnaire to provide us with information regarding your breast cancer risks factors. Some of these risk factors include information such as whether or not you have had breast aspirations before, whether or not there is a history of breast cancer in your family, your history of menstruation, abortion, pregnancy and hormone use. The questionnaire is voluntary which means you do not have to fill it out. Should you agree to fill out the questionnaire, it will only take about 5-10 minutes. You may skip any question you do not want to answer. The information that you give us from the questionnaire will be entered into a computer database called the University of Washington Breast Cancer Epidemiologic Register and Neoplastic Index (BERNI). Your identity will be kept confidential which means researchers who use the database to look up information will not be able to identify you by your name. You will only be identified by a number. This data will be used by researchers doing breast cancer studies and who need information about breast cancer

____ Witness's Initials

APPROVED

____ Subject's Initials

03/27/00

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APR 07 2000

risk factors of women who come from many different backgrounds. The information that is entered into the database will stay in the database for an indefinite length of time for researchers to use.

After your doctor collects your BCF, part of the sample you donate for research (which will be no more than approximately 1 tablespoon) will be stored in the University of Washington Breast Cancer Tumor Bank for an indefinite length of time. The other part of your BCF will be shipped to Colorado State University (CSU) where it will be tested for melatonin levels and related growth factors and hormones. Any BCF that is not used at Colorado State University will be destroyed after approximately 3 years. The samples that will be sent to CSU will be linked to information stored about you in the database. However, this link will be coded such that the researchers at CSU will not know your identity unless you give permission as described below.

To follow your health status, the investigators at CSU would like to review the Washington State Cancer Registry or another cancer registry in case you move from Washington State, in the future. In order to do this, we will need your social security number (SSN). Only the investigators will have access to your SSN and the information from the cancer registry. You will not be contacted by any investigators at CSU in regards to this study or any other future studies. Giving us your SSN is voluntary.

Other Information

Your participation in this study is completely voluntary. You can withdraw from this study at any time. You can withdraw your fluid sample from the University of Washington Breast Cancer Tumor Bank at any time, if it has not already been used. You will not receive payment for being in this study. You will not be charged for any study procedures. Your costs for treatment of your cysts are yours and your insurance agency's responsibility. Giving the study team your Social Security Number and date of birth is optional:

In order to access and review the Washington State Cancer Registry, I agree to give the study team my: (please check all of the lines that you agree with)

_____ Social Security Number. _____ Date of Birth

_____ I do not agree to give the study team my SSN for date of birth.

We will keep all data for this study indefinitely. Information you provide for the database will be coded so that only the study team will know your identity and the data will be in locked files indefinitely. If we publish the results of this study, we will not use your name. The sponsor of this study is the US Army Medical Research and Material Command. The sponsor will also have access to your study records as part of their responsibility to protect human subjects in research. You will not be contacted by Colorado State University.

_____ Witness's Initials

03/27/00

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_____ Subject's Initials

APR 07 2000

Investigator's Signature

Name of Physician/Investigator (printed or typed)

Date

Signature of Physician/Investigator

Subject's Statement and Signature

The study described above has been explained to me. I understand that there is a possibility that the cyst fluid sample I am providing for this study may also be used in other research and could potentially have some commercial use in the future. I voluntarily give up all right, title, and interest to any future developments. I am 18 years of age or older and I voluntarily consent to participate in this study. I have had an opportunity to ask questions. I understand that future questions I may have about the research or about my rights as a research subject will be answered by contacting Beth Aaron RN (206-543-9233) or one of the investigators listed above. I will receive a signed copy of this consent form.

Subject Name (printed /typed)

Date

Subject Signature

Subject's permanent mailing address, city, state, zip code

Social Security Number (OPTIONAL)

Date of birth mm/dd/yy (OPTIONAL)

Witness Name (printed/typed)

Date

Witness Signature

Copies to: Subject
 Investigator's File

Witness's Initials

APPROVED

Subject's Initials

03/27/00

APR 07 2000

UNIVERSITY OF WASHINGTON SCHOOL OF MEDICINE
COLORADO STATE UNIVERSITY

SAMPLE DONATION FORM

Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

I voluntarily and freely donate any and all breast cyst fluid samples to the University of Washington and Colorado State University. I understand that there is a possibility that the breast cyst fluid sample(s) that I am providing under this study may also be used for other research studies and could potentially have some commercial applicability in the future. I hereby relinquish all rights, title, and interest in said items.

Signature of Subject

Date

Printed name of Subject

Permanent Address of the Subject

Signature of Witness

Date

Printed Name of Witness

APPROVED

APPENDIX B

PROTOCOL

Role of Melatonin in the Prevention of Breast Cancer in Patients With Cystic Breast Disease

I. Cyst Fluid Sample Collection and Processing

A. As soon as possible after aspiration, breast cyst fluid (BCF) samples should be labeled and placed in a sterile container and refrigerated. The signed consent form and completed questionnaire should be left in a pre-designated secured location (e.g., in a labeled envelope with a secretary or receptionist, or with the sample in the refrigerator). The University of Washington study coordinator (Sharon Bennett 206 616-8384) will be contacted for sample pick-up.

B. The study coordinator will pick up the BCF sample as soon as possible after collection. A Sample Tracking Form will be initiated at this time (see attached). BCF samples will be transported on ice to a laboratory for processing and storage. Samples should be processed under sterile conditions. Fresh samples should be vortexed then centrifuged at 1,500x g for 5 minutes. The supernatant should then be placed in 1ml aliquots in storage tubes, labeled, and stored at -70°C. Label samples on the side and, if possible, on top. Be sure that labels do not fall off after freezing and/or that markers will not rub off. The number of aliquots will vary depending upon the volume BCF collected and should be recorded on the BCF sample data sheet.

Cyst fluid specimen labeling will follow the scheme below:

Example: UBCNN000-xn

U - designates site of collection, U = University of Washington

B - designates sample source, B = Breast

C - designates sample type, C = Cyst fluid

NN - designates year sample was collected, 99 = 1999, 00 = 2000

000 - designates sequential patient collection, 001 = 1st patient collected, 002 = 2nd pt collected

x - designates aliquot samples for each patient, A = 1st aliquot, B = 2nd aliquot. Will also designate the total number of samples collected per patient.

n - designates cyst (if more than one per patient)

Sample of specimen labeling of 1st patient with two different cysts aspirated:

UBC99001-A1, UBC99001-B1, UBC99001-C1, UBC99001-A2, UBC99001-B2

C. Prior to shipping samples, contact a CSU investigator and arrange for an appropriate shipment date. A total of five 1-ml aliquots should be shipped per sample, as available. One aliquot should be stored at the University of Washington. Any remaining aliquots should be shipped to CSU. If 3 aliquots or less are available, they should all be shipped to CSU. Complete a chain-of-custody form (attached) itemizing the sample numbers and number of aliquots included in the package. Place one signed copy of the chain-of-custody form in the package and retain the other copy for project records. For shipment, samples should be double containerized with absorbent material, maintained frozen with dry ice or equivalent, and mailed via courier (overnight express) to Maxine Hennessey (see contact list).

Melatonin in Cystic Breast Fluid Study

THIS FORM MUST BE COMPLETED BY CLINICIAN AT TIME OF PROCEDURE AND ACCOMPANY SPECIMENS TO REFRIGERATOR.

Be sure to number samples if more than one cyst is aspirated and designate location below.

1. Sample Collection Date: _____ Time: _____
(MM/DD/YY) (24 Hour)

Cyst #	Location	Volume (ml)	Breast - Right (R)	Left (L)	Color *
1					
2					
3					
4					
5					

* Include all color codes that apply in table

- (1) clear (3) tan/yellow (5) brown (7) gray/blue
(2) milky/creamy (4) red/pink (6) green (8) black

2. Is this the first time the patient has been seen by a physician for this condition?

- ① Yes ② No

3. If no, how many years has the patient been seeing a physician for this condition? ____

3a. Number of previous aspirations? ____

Clinician name: _____

Comments: _____

TO BE COMPLETED BY STUDY COORDINATOR:

Subject ID Number: _____ Sample ID Number: _____

Sample Freeze Date: _____ Time: _____
(MM/DD/YY) (24 hour)

Sample Shipment Date: _____ Time: _____
(MM/DD/YY) (24 hour)

CSU Person Contacted: _____

APPENDIX C

SAFETY PROGRAM PLAN

**Role of Melatonin in the Prevention of Breast Cancer
in Patients With Cystic Breast Disease**

Prepared for:

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland

Principal Investigator:

Robert L. Wells, Ph.D.
Department of Radiological Health Sciences
Colorado State University
Fort Collins, CO 80523

1.0 AFFIRMATION OF SAFETY PROGRAM

The Affirmation of Safety Program Statement for this project was submitted by telefax to the USAMRMC on March 22, 1999. A copy of this statement is presented in Appendix A.

2.0 RESEARCH OPERATIONS/STANDARD OPERATING PROCEDURES

The scope of this project involves the collection of breast cyst fluid (BCF) samples from patients at the University of Washington School of Medicine Breast Health Center and laboratory-based experimentation and biochemical analysis of these samples by investigators at Colorado State University. The central goal is to determine whether melatonin mediates the antiproliferative effect of BCF on breast cell growth and to define the relationship between melatonin and growth factors in BCF. These goals will be accomplished by performing a series of cell proliferation and biochemical studies with these samples to address the following hypotheses: 1) there is a positive association between inhibition of breast cancer cell growth by BCF and melatonin concentrations in BCF; 2) melatonin levels are positively associated with transforming growth factor (TGF) beta in BCF; 3) melatonin levels are inversely associated with epidermal growth factor (EGF) and dehydroepiandrosterone-sulfate (DHEA-S) in BCF; and 4) low breast cancer risk BCF (high sodium:potassium [Na:K] ratio) contains higher melatonin levels than high risk (low Na:K ratio) fluid. Breast cyst fluid aspirations will be performed by licenced physicians at the University of Washington School of Medicine Breast Health Center using standard surgical procedures. The laboratory-based activities of this project are described below.

MCF-7 Cell Proliferation Studies. The growth inhibitory effects of cyst fluids will be measured in MCF-7 cells using previously established methods. Cells will be plated into 24-well culture plates at a density of 1×10^5 cells/well. Serial dilutions of cyst fluids will be made in standard cell culture growth medium (DMEM plus 10% FBS, 1x non-essential amino acids, $0.01 \mu\text{g/ml}$ insulin, $0.05 \mu\text{g/ml}$ hydrocortisone), and added directly to cell cultures. Following trypsinization, cell enumeration will be performed by Coulter counting (Particle Data Inc. Model E1 zone 180) on days 1, 2, and 3. A growth index will be calculated for each assay by dividing the mean cell number in the treated cultures by that in the control replicates. Acutely toxic or hazardous chemicals will not be used in this phase of the project. Standard laboratory safety protocols will be used to perform these experiments.

Biochemical Analyses of Breast Cyst Fluids. The measurement of melatonin will be performed using a radioimmunoassay (RIA).

originally developed at Colorado State University (1). DHEA-S will be performed using a validated, commercially available RIA kit (Diagnostic Products Corp., Los Angeles, CA). The RIAs to be performed will use ^{125}I -labeled reagents and will therefore incorporate standard precautions for working with radioactivity. Assays for melatonin and DHEA-S will be performed at the Animal Research and Biotechnology Laboratories (ARBL) at Colorado State University.

Electrolytes (Na and K) will be analyzed using atomic absorption spectrophotometry. Determination of EGF, and TGF beta 1 and 2 in BCF (also in cell cultures for TGF-beta) will be made using validated enzyme-linked immunosorbent assays (ELISA) (R&D Systems, Inc., Minneapolis, MN). These analyses will be performed by strict adherence to the supplied protocols, including the establishment of standard curves on day of assay with supplied standard reagents. ELISA's will be performed in a 96-well format, using a Elx800 Bio-Tek plate reader. These assays do not require the use of acutely toxic or hazardous chemicals. Thus, standard laboratory safety protocols will employed. Dr. Wells will oversee the measurement of TGF-beta, EGF, and electrolytes.

The laboratory-based procedures for this project will be performed in accordance with standard laboratory safety protocols. Experimental and analytical work using BCF samples will be performed using human biosafety level 1 (BL1) practices and procedures. A BL1 lab is suitable for work involving agents of minimal potential hazard to laboratory personnel and the environment. Biosafety Level 1 represents a basic level of containment which relies on standard microbiological practices. Standard precautions for biological fluids will be implemented for BCF sample shipment. Samples will be double-containerized with adsorbent material inside the second container, and shipped frozen (on dry ice) via express (overnight) mail. Laboratory personnel will have specific training in the procedures to be conducted in the laboratory and will be supervised by a scientist with general training in laboratory safety.

Standard Practices:

1. Access to the laboratory is limited or restricted at the discretion of the laboratory director when experiments or work with cultures and specimens are in progress.
2. Persons wash their hands after they handle viable materials and animals, after removing gloves, and before leaving the laboratory.
3. Eating, drinking, smoking, handling contact lenses, and applying cosmetics are not permitted in the work areas

where there is reasonable likelihood of exposure to potentially infectious materials. Persons who wear contact lenses in laboratories should also wear goggles or a face shield. Food is stored outside the work area in cabinets or refrigerators designated and used for this purpose only.

4. Mouth pipetting prohibited; mechanical pipetting devices are used.
5. All procedures are performed carefully to minimize the creation of splashes or aerosols.
6. Work surfaces are decontaminated at least once a day and after any spill.
7. All cultures, stocks and other regulated wastes are decontaminated before disposal by an approved decontamination method, such as autoclaving. Materials to be decontaminated outside of the immediate laboratory are to be placed in a durable, leakproof container and closed for transport from the laboratory. Materials to be decontaminated at off-site from the laboratory are packaged in accordance with applicable local, state and federal regulations, before removal from the facility.
8. An insect and rodent control program is in effect.

Special Practices

None required.

Safety Equipment:

1. Special containment devices or equipment such as a biological safety cabinet are generally not required for manipulations of agents assigned to Biosafety Level 1. It is recommended that laboratory coats, gowns, or uniforms be worn to prevent contamination or soiling of street clothes.
2. Gloves should be worn if the skin on the hands is broken or if a rash exists.
3. Protective eyewear should be worn for anticipated splashes of microorganisms or other hazardous materials to the face.

Laboratory Facilities:

1. Each laboratory contains a sink for handwashing.
2. The laboratory is designed so that it can be easily cleaned. Rugs in laboratories are not appropriate, and should not be used because proper decontamination following a spill is extremely difficult to achieve.

3. Bench tops are impervious to water and resistant to acids, alkalis, organic solvents, and moderate heat.
4. Laboratory furniture is sturdy. Spaces between benches, cabinets, and equipment are accessible for cleaning. If the laboratory has windows that open, they are fitted with fly screens.

The amount of BCF that will be handled during the laboratory phase of this project is not likely to exceed 1 milliliter per sample. In case of a spill involving BCF, the area should be cleaned using a bleach solution. Dr. Bob Ellis, University Biosafety Officer, should be contacted in the event of an extensive spill. He can be reached at the following numbers: 970 491-6729 (am phone), 970 491-5740 (pm phone), or 970 490-4981 (pager). He will consult with the principal investigator concerning the need for additional clean-up and recommendations concerning the need for a Hepatitis B vaccination.

Basic Procedures for Working with Hazardous Chemicals (Radionuclides):

1. In case of eye contact, promptly flush eyes with water for a prolonged period (15 minutes) as a minimum and seek medical attention.
2. In case of ingestion, encourage the victim to drink large amounts of water, unless the MSDS or label suggests otherwise, and seek medical attention.
3. In case of skin contact, remove contamination by appropriate procedures and seek medical attention. Do not wear contaminated clothing until it has been washed.
4. Promptly clean up spills, using appropriate protective apparel and equipment and proper disposal. If cleanup is beyond laboratory's capability, call CSU Police (CSUPD) (911) for assistance.
5. Avoid "routine" exposures. Develop and encourage safe habits. Avoid unnecessary exposure to chemical agents by any route.
6. Do not smell or taste chemicals. Vent apparatus which may discharge toxic chemicals (vacuum pumps, distillation columns, centrifuge buckets, etc.) into approved local exhaust devices.
7. Inspect gloves and glove boxes before use.
8. Insure that laboratory hoods are in proper working order before use.
9. Do not allow release of toxic substances in cold rooms or warm rooms, since these have contained atmospheres.
10. Only those chemicals for which the quality of the laboratory, its security and ventilation systems are

- appropriate should be used.
11. Avoid eating, drinking, smoking, chewing gum or other substances, or application of cosmetics in areas where laboratory chemicals are present. Wash hands before conducting these activities or using toilet.
 12. Avoid storage, handling or consumption of food or beverages in storage areas, refrigerators, glassware or utensils which are also used for laboratory operations.
 13. Handle and store laboratory glassware with care to avoid damage; do not use damaged glassware. Use extra care with Dewar flasks and other evacuated glass apparatus; shield or wrap them to contain chemical hazards and fragments should implosion occur. Use equipment only for its designed purpose.
 14. Wash all areas of exposed skin (shower if appropriate or required by laboratory practices) before leaving laboratory area. Remove possibly contaminated personal protective equipment and/or clothing before washing prior to leaving.
 15. Avoid practical jokes or other behavior, which might confuse, startle or distract another worker.
 16. Never use mouth suction for pipetting or starting a siphon.
 17. Confine long hair (including beards) and loose clothing. Wear shoes at all times while in the laboratory but do not wear sandals, perforated shoes or sneakers (cloth shoes).
 18. Keep the work area clean and uncluttered, with chemicals or equipment being properly labeled and stored. Clean up the work area on completion of any operation and at the end of each day.

Personal Protection:

1. Appropriate eye protection must be worn by all persons, including visitors, where chemical hazards are present in a form that could cause eye problems. Avoid use of contact lenses unless medically necessary; if they are used inform supervisor and co-workers so that they can take additional eye protection precautions suggested by physician.
2. Wear appropriate gloves when a potential for contact with corrosive or toxic materials exists, wash them before removal and replace them periodically.
3. Use appropriate respirators when air contaminants are not sufficiently restricted by engineering controls, inspecting the respirator and its fit before each use. Unless concentration is known and another type respirator

- is permitted by OSHA and/or the CHO, a supplied air respirator is suggested for work with chemicals. Routine work in areas where air containments are not sufficiently restricted by engineering controls must not be done unless there is assurance that individuals involved are capable of wearing respirators.
4. Laboratory coats or other protective dress used in the laboratory should be immediately removed in case of overt contamination. Coats should then be decontaminated and appropriately cleaned. Person involved may need to wash or shower.
 5. Use other protective and emergency apparel and equipment appropriate to the work and as approved for use by the CSC.
 6. Seek information and advice from Environmental Health Services (EHS) about hazards (e.g., MSDS, etc.), plan appropriate protective procedures and practices and plan for positioning of equipment before beginning any new operation.
 7. If operation will be left unattended: leave lights on; place an appropriate sign on the door; and provide for containment of toxic or flammable substances in case of failure of a utility service (such as cooling water, or a hood) to the unattended operation.
 8. Use a laboratory hood for operations, which might result in the release of toxic or flammable vapors or dust.
 9. As a rule of thumb, a hood or other local ventilation device should be used when working with any appreciable volatile substance with a TLV of 50 ppm or less, and for any substance with a flash point of less than 200° F.
 10. Confirm adequate hood performance before use. Keep hood closed (to at least the marked level showing maximum opening at which a satisfactory velocity can be maintained) at all times except when adjustments or set ups are being made within the hood. Keep materials stored within hoods to a minimum and do not block vents or air flow.
 11. Leave the hood "on" when it is not in use if toxic or flammable substances are stored therein or if it is uncertain whether adequate general laboratory ventilation will be maintained when it is "off".
 12. Be alert to unsafe conditions and see that they are corrected.
 13. Each laboratory activity/operation must plan for waste disposal. Deposit chemical wastes in appropriately labeled receptacles and follow all other waste disposal procedures of the University.
 14. Avoid working alone in a building. Never work alone in a

laboratory using any procedure which could be hazardous.

Work with Chemicals of High Chronic Toxicity (Radionuclides):

1. Examples of these chemicals are dimethylmercury, nickel carbonyl, benzo-a-pyrene, N-nitrosodiethyl amine, those that cause sexual dysfunctions and human carcinogens and/or substances with high carcinogenicity potency in animals.
2. In addition to the procedures above, the following additional precautions are required:
3. Conduct all transfers and work with these substances in a "controlled area", a restricted access hood, glove box, or portion of a lab designated for use of highly toxic substances, for which all people with access are aware of the substances being used and necessary precautions.
4. Prepare a plan for use and disposal of these materials and obtain the approval of the laboratory supervisor or PI.
5. Protect vacuum pumps against contamination by scrubbers or HEPA filters and vent them into the hood. Decontaminate vacuum pumps or other contaminated equipment, including glassware, in the hood before removing them from the controlled area. House vacuum should not be used for these operations. Vacuum pump oil should be considered as hazardous until tested. Decontaminate the controlled area before normal work is resumed therein.
6. On leaving the controlled area, remove any protective apparel (placing it in an appropriate, labeled container) and thoroughly wash hands, forearms, face and neck.
7. During housekeeping, use a 2 bucket system of wet mopping or a vacuum cleaner equipped with a HEPA filter instead of dry sweeping.
8. If using toxicologically significant quantities of such a substance on a regular basis (e.g., 3 times per week), consult a qualified physician concerning the desirability of regular medical surveillance.
9. Assure that the "controlled area" is conspicuously marked with warning and restricted access signs.
10. Assure that contingency plans, equipment and materials to minimize exposures of people and property in case of accidents are available.
11. Store containers of these materials only in a ventilated, limited access area in appropriately labeled, unbreakable, chemically resistant, secondary containers.
12. For a negative pressure glove box, ventilation rate must be at least 2 volume changes/hour and pressure at least

- 0.5 inches of water. For a positive pressure glovebox, thoroughly check for leaks before each use. A log of these checks must be available for inspections by the CHO or alternate. In either case, trap the exit gases or filter them through a HEPA filter and then release them into a laboratory hood or other dedicated exhaust system.
13. Use chemical neutralization for decontamination whenever possible. Ensure that containers of contaminated waste (including washings from contaminated flasks) are transferred from the controlled area in a secondary container under the supervision of a person authorized to generate hazardous waste.

3.0 FACILITY EQUIPMENT AND DESCRIPTION

Cell culture experiments using BCF samples and assays for EGF, TGF-beta, Na and K will be conducted in the laboratory of Dr. Robert Wells (Rooms 421 and 427, Molecular and Radiological BioSciences Building, CSU). This lab is equipped with fume hoods and fire extinguishers, as well as three Class IIB and two Class IIA HEPA filtered biological safety cabinets. An emergency shower and eyewash station is located in the hallway outside the entrance.

Assays for melatonin and DHEA-S will be performed in the RIA Lab (Rooms W175 and W177) of the Animal Research and Biotechnology Laboratories (ARBL) at Colorado State University. This lab is equipped with a fume hood and fire extinguisher. An emergency shower is located in the hallway outside the entrance. The lab is also equipped with an approved radio-iodination hood and the necessary refrigerated storage areas for working with radioactive iodine.

4.0 HAZARD ANALYSIS

This project will employ routine biochemical and cell culture experimentation in a laboratory setting. Each experimental procedure has published methods and can be performed without significant risks to safety given appropriate compliance with the established experimental and laboratory safety protocols. The cell culture studies will not include any toxic or hazardous chemicals. Because human biological fluids will be used, an adverse event could involve exposure to an infectious agent. However, BCF is not known to be infectious and the subjects in this study are not known carriers of pathogenic microorganisms. Although there is some possibility of infection via exposure to BCF (assuming a significant breach of established safety protocols), the probability is considered low. Although this project will not involve work with recombinant DNA, an institutional biosafety

approval is required at CSU for any work with human biological fluids. The approval number is 99-002B (Appendix B).

The RIAs to be performed as part of this project will use ^{125}I -labeled reagents and will therefore incorporate standard precautions for working with minute amounts of radioactivity. ^{125}I is a gamma emitter and is a human carcinogen at sufficient dose. An adverse event could involve exposure to radioactive agents. However, such an event is also considered unlikely given the facilities and protocols that are currently in place. The RIA Laboratory at CSU's ARBL conducts RIAs on thousands of samples each year and has maintained an exemplary safety record. See section 3.0 and 8.0.

Colorado State University currently maintains chemical hygiene, radiation safety, bloodborne pathogen, hazard communication, and related programs that are administered by the Environmental Health Services Department. A description of these programs and copies of CSU's Chemical Safety Handbook, Biosafety Handbook, and Hazardous Chemical Waste System Manual can be accessed on the Internet at <http://www.colostate.edu/depts/ehs>.

5.0 RADIOACTIVE MATERIALS

The radioimmunoassays for melatonin and DHEA-S in BCF samples will use ^{125}I -melatonin and ^{125}I -DHEA-S, respectively, in trace amounts. Disposal of radioactive reagents will be performed according to standard university protocols approved by the university Radiation Safety Committee and Radiation Control Office. Radioactive waste will be stored for decay in approved storage areas and/or disposed of at a licenced offsite repository. Colorado State University operates its Radiation Safety Program under a broad scope license, granted by the State of Colorado, Department of Public Health and Environment. A copy of the NRC license for CSU is presented in Appendix C.

6.0 RECOMBINANT DNA

This project will not involve work with recombinant DNA.

10.0 BIOLOGICAL DEFENSE PROGRAM REQUIREMENTS

Not Applicable


11.0 LITERATURE CITED

1. Rollag MD, Niswender GD. Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimens. *Endocrinology*. 98:482-9. 1976.

Affirmation of Safety Program Statement

The organization affirms that there is an existing safety program that is in accordance with appropriate Federal, State, and local regulations, as required by the Occupational Safety and Health Act. Also, affirms that hazards have been identified, eliminated, and/or controlled and that research may be performed safely under the laboratory conditions. The organization shall be held responsible and liable for inaccuracies of the information provided, failure to implement an effective safety and occupational health program, and/or adverse conditions that may result from the failure of the recipient to identify hazard information.

EARLIE Thomas
Printed or Typed Name


Signature

DIRECTOR
Title

3/19/99
Date

ENVIRONMENTAL HEALTH SERVICES
Name of Organization



Office of Regulatory Compliance
Office of Vice President for Research
and Information Technology
Fort Collins, CO 80523
(970) 491-1563
FAX (970) 491-2293

Dr. Robert Wells
Radiological Health Sciences

Re: Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease

January 22, 1999

You have biosafety approval from our office for the above-referenced project. The approval number is 99-002B.

Sincerely,

A handwritten signature in cursive script that reads "Celia Walker".

Celia Walker
Director,
Office of Regulatory Compliance

STATE OF COLORADO
DEPARTMENT OF PUBLIC HEALTH AND ENVIRONMENT

RADIOACTIVE MATERIALS LICENSE

Pursuant to the Radiation Control Act Title 25, Article 11, CRS 1989, Replacement Volume, as amended, and the State of Colorado Rules and Regulations Pertaining to Radiation Control, Part 3, and in reliance on statements and representations heretofore made by the licensee designated below; a license is hereby issued authorizing such licensee to transfer, receive, possess and use the radioactive material(s) designated below; and to use such radioactive material(s) for the purpose(s) and at the place(s) designated below. This license is subject to all applicable rules, regulations, and orders now or hereafter in effect of the Colorado Department of Public Health and Environment and to any conditions specified below.

Licensee

1. Name: Colorado State University
Environmental Health Services

3. In accordance with the application dated October 13, 1997, License Number Colo. 002-19 is amended in its entirety.

4. Expiration date: October 31, 2002

2. Address: Fort Collins, CO 80523

5. Reference number:

6. Radioactive materials
(element and mass no.)

7. Chemical and/or
physical form

8. Maximum quantity licensee may
possess at any one time

A. Any radioactive material with atomic numbers 1-96 inclusive, except as noted below

A. Any

A. 370 GBq (10 Ci) total

B. Any radioactive material with atomic numbers 1-96 inclusive, except as noted below

B. Fixed on foils or electrodeposited onto metal

B. 925 GBq (25 Ci) total

C. Any radioactive material with atomic numbers 1-96 inclusive, except as noted below

C. Sealed sources

C. 185 GBq (5 Ci) total

D. Any radioactive material with atomic numbers 1-96 inclusive, except as noted below

D. Sealed sources

D. 370 GBq (10 Ci) total

E. Any radioactive material with atomic numbers 1-96 inclusive

E. Any

E. As necessary

F. Source material

F. Any

F. 500 pounds

G. Source material

G. Any

G. 1,500 pounds

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6. Radioactive materials (element and mass no.)	7. Chemical and/or physical form	8. Maximum quantity licensee may possess at any one time
H. Cesium 137	H. Sealed sources	H. 1.85 GBq (50 mCi)
I. Americium 241:Be	I. Sealed sources	I. 11.1 GBq (300 mCi)
J. Americium 241:Be	J. Sealed sources	J. 55.5 GBq (1.5 Ci)
K. Any radioactive material with atomic numbers 1-96	K. Any	K. 37 GBq (1 Ci) total

CONDITIONS

- 9.A. Radioactive materials authorized in Item 6.A. to be used for research and development as defined in RH 1.4 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*; for purposes of teaching; calibration; veterinary nuclear medicine; or non-human, In-vitro clinical and laboratory testing.
- B. Radioactive materials authorized in Item 6.B. to be used for research and development as defined in RH 1.4 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*; for purposes of teaching; or quality control.
- C. Radioactive materials authorized in Item 6.C. to be used for research and development as defined in RH 1.4 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*; for purposes of teaching; calibration; or veterinary nuclear medicine.
- D. Radioactive materials authorized in Item 6.D. and 6.F. to be used for research and development as defined in RH 1.4 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*; for purposes of teaching; or calibration.
- E. Radioactive materials authorized in Item 6.E. to be STORED ONLY as waste which was buried prior to December 30, 1985.
- F. Radioactive materials authorized in Item 6.G. and 6.K. to be STORED ONLY awaiting lawful disposal.
- G. Radioactive materials authorized in Items 6.H. and 6.I. to be used in CPN model MC Series moisture/density gauges to determine the moisture content and density of soils and construction materials.
- H. Radioactive material authorized in Item 6.H. may also be used in a CPN model 500 Series depth probe for the measurement of moisture content of soils and construction materials.
- I. Radioactive material authorized in Item 6.J. to be STORED ONLY.

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- 10.A. Radioactive material may be used and stored at Colorado State University Campuses, Research Centers, and on property owned or controlled by Colorado State University within the State of Colorado.
- B. Radioactive materials authorized in Items 6.H. and 6.I. may be used on the Colorado State University Campus and Research Centers, and at temporary job sites of the licensee anywhere in the State of Colorado where the State of Colorado maintains jurisdiction for regulating the use of radioactive materials.
11. The licensee shall comply with the provisions of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*, Part 4, "Standards for Protection Against Radiation", and Part 10, "Notices, Instructions and Reports to Workers: Inspections".
- 12.A. Radioactive material shall be used by, or under the supervision of individuals designated as users by the Colorado State University Radiation Safety Committee.
- B. Radioactive materials authorized in Items 6.H. and 6.I. shall be used by individuals, designated as users by the Colorado State University Radiation Safety Committee, who have successfully completed a training course in the safe use and handling of portable nuclear gauges which has been accepted by the U.S. Nuclear Regulatory Commission or an Agreement State.
- C. The Colorado State University Radiation Safety Committee Chairperson shall maintain written records indicating the date and basis of approval of designated users.
- 13.A. The Radiation Safety Officer is James C. Graham, M.S.
- B. The Alternate Radiation Safety Officer is James P. Abraham, M.S.
14. Each sealed source authorized in Item 6 of this license shall be tested for leakage in accordance with the requirements of RH 4.16 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*, at intervals not to exceed six months, except as stated below:
- A. Each sealed source designed for the purpose of emitting alpha particles shall be tested at intervals not to exceed three (3) months.
- B. Each sealed source authorized in Items 6.H. and 6.I. shall be tested for leakage and/or contamination at intervals not to exceed one (1) year.
- C. Each sealed source fabricated by the licensee shall be inspected and tested for construction defects and leakage and/or contamination prior to use or transfer as a sealed source. If the inspection or test reveals any construction defects or 185 Bq (0.005 μ Ci) or greater of contamination, the source shall not be used or transferred as a sealed source until it has been repaired, decontaminated, and retested.
15. Sealed sources containing radioactive material, not fabricated by the licensee, shall not be opened.
16. Experimental animals administered radioactive materials or their products shall not be used for human consumption.

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17. The licensee may not dispose of experimental animals containing radioactive material by burial in soil except as specified in RH 4.37 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*.
- 18.A. Detector cells containing titanium tritide foil shall only be used in conjunction with a properly operating temperature control mechanism which prevents foil temperatures from exceeding 225 degrees Centigrade.
- B. Detector cells containing scandium tritide foil shall only be used in conjunction with a properly operating temperature control mechanism which prevents foil temperatures from exceeding 325 degrees Centigrade.
- 19.A. Individuals involved in operations which utilize, at any one time, more than 3.7 GBq (100 mCi) of Hydrogen 3 in a non-contained form, other than metallic foil, shall have bioassays performed within one week following a single operation and at weekly intervals for continuing operations. Records of the bioassays shall be maintained for inspection by the Department and the action points listed below shall be observed.
- B. (1) Tritium shall not be used in such a manner as to cause any individual to receive a radiation exposure such that urinary excretion rates exceed 1.04 MBq (28 μ Ci) of tritium per liter when averaged over a calendar quarter.
- (2) Urinalysis shall be performed at weekly intervals on all individuals who work in the restricted areas of facilities in which tritium is used. If the average concentration of tritium in urine for any single individual during a calendar quarter is less than 370 kBq (10 μ Ci) per liter, urinalysis may be performed on that individual at monthly intervals for the following calendar quarter and may continue at monthly intervals so long as the average concentration in the calendar quarter remains below 370 kBq (10 μ Ci) per liter. The urine specimen shall be collected on the same day of the week insofar as possible.
- (3) A report of an average concentration in excess of the limit specified in B. (1) above for any individual shall be filed, in writing, within thirty (30) days of the end of the calendar quarter with the Director, Laboratory and Radiation Services Division, Colorado Department of Public Health and Environment, 8100 Lowry Boulevard, Denver, Colorado 80220-6928. The report shall contain the results of all urinalyses for the individual during the calendar quarter, the cause of the excessive concentrations, and the corrective steps taken or planned to assure against a recurrence.
- (4) Any single urinalysis which discloses a concentration of greater than 1.85 MBq (50 μ Ci) per liter shall be reported, in writing, within seven (7) days of the licensee's receipt of the results, to the Director, Laboratory and Radiation Services Division, Colorado Department of Public Health and Environment, 8100 Lowry Boulevard, Denver, Colorado 80220-6928.
20. Individuals involved in operations which utilize, at any one time, more than 50 millicuries of I-125 and/or I-131 or unvented laboratory operations involving 10 mCi of I-125 and/or I-131 in a noncontained form shall have bioassays performed within one week following a single operation. Records of the bioassays shall be maintained for inspection by the Department and the action points listed below shall be observed.

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- 20.A. Whenever the thyroid burden at the time of measurement exceeds 4.44 kBq (0.12 μ Ci) of I-125 or 1.48 kBq (0.04 μ Ci) of I-131, the following actions shall be taken:
- (1) An investigation of the operations involved, including ventilation surveys shall be carried out to determine the causes of exposure and to evaluate the potential for further exposures.
 - (2) If the investigation indicates that further work in the area might result in exposure of a worker to concentrations that are excessive, the licensee shall restrict the worker from further exposure until the source of exposure is discovered and corrected.
 - (3) Corrective actions that will eliminate or lower the potential for further exposures shall be implemented.
 - (4) A repeat bioassay shall be taken within 1 week of the previous measurement in order to confirm the effectiveness of the corrective action taken or to verify internal radioiodines present.
 - (5) Reports or notification shall be provided as required by RH 4.52 and 4.53 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*.
- B. If the thyroid burden at any time exceeds 18.5 kBq (0.5 μ Ci) of I-125 or 5.18 kBq (0.14 μ Ci) of I-131, the following actions shall be taken:
- (1) Prevent the individual from any further handling of I-125 or I-131 until the thyroid burden is below the above limits.
 - (2) Carry out all steps described above.
 - (3) As soon as possible, refer the case to appropriate medical consultation for recommendations regarding therapeutic procedures that may be carried out to accelerate removal of radioactive iodine from the body. This should be done within 2-3 hours after exposure when the time of exposure is known so that any prescribed thyroid blocking agent would be effective.
 - (4) Carry out repeated measurements at approximately 1-week intervals at least until the thyroid burden is less than 4.44 kBq (0.12 μ Ci) of I-125 or 1.48 kBq (0.04 μ Ci) of I-131.
21. Pursuant to RH 11.3.10 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control* and this condition, the licensee is authorized to possess for **STORAGE ONLY** radioactive material identified in Item 6.G. and contained in uranium mill tailings, provided that the ultimate disposition of this material is in accordance with prior written approval of the Department.
- 22.A. The licensee may transport radioactive material or deliver radioactive material to a carrier for transport, in accordance with the provisions of Part 17 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*, "Transportation of Licensed Material".

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- 22.B. The transportation of Colorado radioactive materials shall be subject to all applicable regulations of the Colorado Public Utilities Commission, Colorado Department of Transportation, Colorado Department of Public Safety, Colorado Department of Revenue (Port of Entry), U.S. Department of Transportation, and other agencies of the United States having jurisdiction. When the U.S. Department of Transportation Regulations (Title 49, Chapter I, Code of Federal Regulations) are not applicable to shipments by land of Colorado radioactive material by reason of the fact that the transportation does not occur in interstate or foreign commerce, the licensee must be in compliance with the requirements relating to packaging of the radioactive material, marking and labeling of the package, placarding of the transport vehicle, and accident reporting set forth in the regulations of the U.S. Department of Transportation.
- C. Except for plutonium contained in a medical device designed for individual human application, no plutonium, regardless of form, shall be delivered to a carrier for shipment by air transport or transported in an aircraft by the licensee.
- 23.A. All users of radioactive material authorized in Items 6.H. and 6.I. who are likely to receive an occupational dose exceeding 10% of any applicable limit specified in Part 4 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control* must be equipped with personnel monitoring devices capable of detecting both gamma and neutron radiation.
- B. If personnel monitoring devices will not be used, the Radiation Safety Officer must maintain written records demonstrating that personnel dose monitoring was not required pursuant to RH 4.18 of the Regulations.
24. The licensee shall maintain a use log indicating gauge number, user, date, and location of use for radioactive materials authorized in Items 6.H. and 6.I.
25. For use of radioactive materials authorized in Items 6.H. and 6.I. the licensee shall list the telephone number(s) of the Acting Radiation Safety Officer in the procedures manual for emergency notification.
26. Prior to the use of licensed materials outside the State of Colorado, or at any facility under exclusive Federal jurisdiction including a facility within the State of Colorado, the Licensee shall comply with the applicable provisions of 10 CFR 150.20 or if the use shall take place in an Agreement State the licensee shall comply with the applicable provisions of that State's reciprocity requirements.
27. The licensee shall not transfer possession and/or control of materials or products containing radioactive material as a contaminant except:
- A. by transfer of waste to an authorized recipient;
 - B. by transfer to a specifically licensed recipient; or,
 - C. as provided otherwise by specific condition of this license pursuant to the requirements of RH 3.22 of the State of Colorado *Rules and Regulations Pertaining to Radiation Control*.
28. Radioactive material shall not be used in or on human beings or in products distributed to the public.

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29. The State of Colorado Rules and Regulations Pertaining to Radiation Control shall govern the licensee's statements in applications or letters, unless the licensee's statements are more restrictive than the regulations. Except as specifically provided otherwise by this license, the licensee shall possess and use radioactive material described in Items 6, 7 and 8 of this license in accordance with statements, representations, and procedures contained in:
- A. the application and attachments dated October 13, 1997; and
 - B. the Colorado State University RADIATION CONTROL MANUAL of the University's Radiation Safety Program, Revised 1997; and
 - C. the correspondence dated October 29, 1997 (received March 30, 1998).

FOR THE COLORADO DEPARTMENT OF PUBLIC HEALTH AND ENVIRONMENT

Date APRIL 27, 1998

By Charles E. Mattson

APPENDIX D

August 25, 2000

Jim Burch, M.S., Ph.D.
Assistant Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

Dear Dr. Burch:

We are pleased to write a letter of support for your grant, "The Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease". After we receive IRB approval for study, we look forward to participating as an ancillary site. We expect to enroll 40 patients per year.

If you have any questions, please call me at (303) 315-8671.



Christina A. Finlayson, MD
Assistant Professor
GI, Tumor and Endocrine Surgery



POUDRE VALLEY HOSPITAL

1024 South Lemay Avenue
Fort Collins, Colorado 80524-3998
(970) 495-7000
www.pvhs.org

September 27, 2000

James Burch, M.S., Ph.D
Assistant Professor
Department of Environmental Health
Colorado State University
Fort Collins, CO 80523

Dear Dr. Burch:

I am pleased to have the opportunity to collaborate with you on your project: "The Role of Melatonin in the Prevention of Breast Cancer in Patients with Cystic Breast Disease". After Institutional Review Board approval, the Breast Diagnostic Center anticipates providing patient data and breast cyst fluid for biochemical analysis. I will also be available for consultation regarding data analysis and manuscript preparation. We expect to enroll between 40 and 60 patients per year.

If you have any questions, please contact me at 970-495-8600.

Sincerely,

Winfield M. Craven, M.D.
Co-Director
Breast Diagnostic Center
Poudre Valley Health Systems

WMC/lm

APPENDIX E

Mitochondrial perturbations and cytotoxicity following melatonin treatment of MCF-7 human breast tumor cells.

Scott A., Cosma G., Frank A., Wells R., and Gardner H., Dept. Environmental Health, Dept. Pathology, and Dept. Radiological Health Sciences, Colorado State University, Fort Collins, CO, US Army Ctr Environ Health Research, Ft. Detrick, MD

ABSTRACT:

Clinical and laboratory studies have provided evidence of oncostatic activity by the pineal neurohormone, melatonin. However, these studies have not elucidated its mechanism of action. The following series of MCF-7 breast tumor cell studies, conducted in the absence of exogenous steroid hormones, provide evidence for a novel mechanism of oncostatic activity by this endogenous hormone. We observed a 40-60% loss of MCF-7 cells after 20 hr treatment with 100 nM melatonin, which confirmed and extended previous reports of its oncostatic potency. Interestingly, there were no observed changes in tritiated thymidine uptake, suggesting a lack of effect on cell cycle/nascent DNA synthesis. Further evidence of a cytotoxic effect came from morphologic observations of acute necrosis and autophagocytosis accompanied by degenerative changes in mitochondria. Studies of mitochondrial function via standard polarography revealed a significant increase in oxygen consumption in melatonin treated MCF-7 cells. Enzyme-substrate studies of electron transport chain (complex IV) activity in detergent permeabilized cells demonstrated a concomitant 53% increase ($p < 0.01$) in cytochrome c oxidase activity. Additional studies of succinate dehydrogenase activity (complex II) as determined by reduction of (3-4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (M.T.T.) demonstrated a significant increase ($p < 0.01$) in melatonin treated cells and further confirmed accelerated electron transport chain activity. Finally, there was a 64% decrease ($p < 0.05$) in cellular ATP levels in melatonin treated cells, as measured by chemiluminescence. The G-protein coupled melatonin receptor blocker, Luzindole, abrogated the cytotoxic and mitochondrial effects of

melatonin. These studies demonstrate a receptor modulated pathway of cytotoxicity in melatonin treated MCF-7 tumor cells with apparent uncoupling of oxidative phosphorylation which may represent a novel mechanism of tumor prevention.

INTRODUCTION

Melatonin is a neurohormone, synthesized and secreted by the pineal gland in humans and other vertebrate species [review[1;2]]. It appears to play a number of homeostatic physiologic roles in the immunology and neuroendocrinology of most mammalian species [review[1;2]]. In addition to its interactions with these physiologic systems, melatonin also displays several other biological properties, including a direct quenching effect on oxygen free radicals, which we have previously described [3]. In addition, several studies have demonstrated an oncostatic activity by melatonin against breast cancer cell proliferation *in vitro* [4-7]. These reports have demonstrated a growth inhibitory effect by melatonin in the estrogen responsive human breast carcinoma cell line, MCF-7, at nanomolar concentrations that approach circulating physiologic levels of the hormone [5;8-10].

Despite these confirmatory reports of oncostatic activity by melatonin, the mechanism of action by which this endogenous agent inhibits tumor cell proliferation remains unresolved [for recent review, see][11-14]. For example, a number of diverse morphologic changes have been described in MCF-7 cells following treatments with melatonin, but no distinct or insightful pattern of subcellular degenerative changes have emerged from these prior investigations [4;15]. In addition, studies have focused on changes in cell cycle kinetics following MCF-7 cell treatments with melatonin, including slight delays in the G₁-S transition and accumulation of G₁ phase cells, however, there has been no elucidation of underlying mechanism of potential cell cycle arrest or block [16-18]. Interestingly, an "estrogen-rescue" by melatonin on cell cycle effects has been describe wherein the cell cycle arrest was reversed by the addition of estradiol [18]. Several other biochemical changes have been demonstrated in melatonin-treated MCF-7 cells, including changes in the secretion of estrogen-regulated

growth factors (e.g., *c-myc*, TGF α , *c-fos*, TGF- β) [19] and downregulation of the estrogen receptor [8;20] [review][14]. Clinical studies have also provided generally supportive evidence of oncostatic activity by melatonin, both in cohort studies of melatonin levels in breast cancer/non-cancer patients, as well as in studies of breast cancer survival in patients enrolled in melatonin supplementation regimens [11;21-23]. However, a recent review of these clinical studies has pointed out discrepancies and conflicting evidence for the support of melatonin as an alternative oncostatic agent [24].

Perhaps what is even more unclear are the previously described effects of melatonin on the estrogen receptor positive MCF-7 cell line grown with and without estrogen in the medium. Separate studies with varying concentrations of estrogen in culture medium have provided evidence of cell cycle arrest [16;17], proliferation [9;25;26], induction of the estrogen early responsive gene, *c-myc* [19], and morphologic aberrations [4;15]. Because of these reports suggestive of interactions between melatonin and estrogen signaling pathways, the studies described herein were performed in estrogen free culture conditions, so that analysis of melatonin effects on cell growth and respiration could be explored in a direct fashion. In addition, since melatonin has been identified as a ligand for a G-coupled receptor as well as being a potent antioxidant, we investigated whether the receptor antagonist, Luzindole, would block the mitochondrial and cytotoxic effects that we observed in the MCF-7 cells.

MATERIALS AND METHODS:

Cell Culture. MCF-7 human breast adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Rockville MD) at passage 154. The MCF-7 cell line was initially isolated from the pleural effusion of a metastatic breast cancer, and expresses both estrogen and progesterone receptors. Cell cultures were maintained in a humidified incubator at 5% CO₂ and 37⁰C. All reagents and supplies were purchased from Sigma, St. Louis, MO, unless otherwise specified. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (FBS 10% v/v), MEM non-essential amino acids (1% v/v), penicillin-streptomycin (1% v/v), hydrocortisone (200 nM), sodium pyruvate (1 mM), and insulin (0.003 unit/ml). Prior to experiments, cells were grown in phenol red free medium supplemented with charcoal-dextran treated FBS to minimize exogenous hormone levels (≤ 0.114 pM). Melatonin concentration in the treated serum was below the detection limit of 1.9 picomole per liter as measured by radioimmunoassay [27]. Cells were maintained in the estrogen-depleted medium for three to four days and were in mid to late log phase growth at the time of experimental treatments. Cells were enumerated by hemocytometer count using Trypan Blue dye viability assay or by Coulter cell counter (Particle Data Inc., model EL Zone 180).

Tritiated thymidine uptake. MCF-7 cells were seeded at a density of 8×10^5 cells per 60 mm culture dish and maintained in experimental medium until late log phase growth. After 16 hours of exposure to melatonin, vehicle, or estradiol, cultures were pulsed with [³H]thymidine (0.1 mCi/ml) for 30 minutes. After rinsing with 4⁰C phosphate buffered saline, ice cold trichloroacetic acid was added for 10 minutes, and followed with ice cold 70% ethanol. Cells were lysed with Toplayer II (18.15g NaOH, 29.3g NaCl, 3.81g tetra-sodium

EDTA, 5.23g Brij-58, in one liter double deionized water). A scintillation cocktail was prepared with 75 ul HCl, 300 ul H₂O, and 10 ml of Ready Safe (Beckman Coulter, Miami, FL) and cell lysates were analyzed on a Beckman LS 5801 liquid scintillation system using counts per minute (CPM) obtained from the tritium window. CPMs were standardized to mean cell counts from cultures grown in parallel.

Electron Microscopy. Cells were prepared by routine glutaraldehyde fixation, osmium tetroxide post-fixation, sequential dehydration, infiltration, and plastic imbedding prior to staining and examination.

Polarography/oxygen consumption: MCF-7 cells were harvested and re-suspended in medium to provide a concentration of 1×10^6 cell per analysis. Samples were incubated for 30 minutes at 37°C and 5% CO₂ immediately prior to polarographic measurements. Oxygen uptake was measured by an Instech Laboratories (Horsham, PA) micro-chamber instrument with a Clark type oxygen electrode and circulating water jacket maintained at 35°C. Oxygen levels were quantified using a Yellow Springs Instruments (Yellow Springs, OH) model 53 oxygen monitor calibrated from 100 to 0% oxygen saturation by the addition of sodium sulfite to air saturated deionized water. Data measurements were taken over a linear range of oxygen consumption ($R^2 > 0.99$) for 20 minutes.

Cytochrome c oxidase assays: The cytochrome c oxidase microtiter plate assay was performed with minimal modification using the method of Chrzanowska-Lightowlers [28]. Briefly, MCF-7 cell membranes were permeabilized with 0.25% saponin for ten minutes prior to adding substrate mix containing cytochrome c and 3,3'-diaminobenzidine-tetrachloride (DAB). The oxidation of DAB maintains a constant substrate pool of reduced cytochrome c and forms a polymer that absorbs visible light at a wavelength of 450 nm. Enzyme assays

were standardized to cell number in 96 well microtiter plates with approximately 1.5×10^6 cells required per assay well for optimal detection of enzyme activity. Kinetic readings taken on a Bio-tek EL_x 800 plate reader were linear for at least fifteen minutes in all samples.

Background absorption was measured in wells with medium but no cells (assay blank) and subtracted from cell samples. The addition of 3 mM potassium cyanide to control wells inhibited 100% of enzyme activity, thus verifying that the reaction was catalyzed by cytochrome c oxidase. Data is reported as mean velocity of enzyme activity (linear slope of DAB oxidation= Δ absorbance/minute) per 10^6 cells.

In addition, we used the AlamarBlue™ dye assay (AccuMed International, Inc. Westlake OH) to detect changes in electron transport complex IV activity. AlamarBlue dye is reported by the manufacturer to be a final electron acceptor between the reduction of oxygen and cytochrome c oxidase (cyt.a₃). The dye is reduced by the removal of oxygen and its replacement by hydrogen with the amount of reduced dye (emitting at 590 nm) being proportional to the activity of cyt.a₃. Cells were incubated with AlamarBlue for four hours at 37°C as described by the manufacturer's protocol. The amount of dye reduced during the incubation was quantified by fluorometric measurements using a Cytofluor 2300 instrument with excitation filter at 530 nm and emission at 590 nm. Data is reported as fluorescent units of reduced dye per cell per four-hour incubation.

Analysis of succinate dehydrogenase activity.

Mitochondrial complex II activity was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with minor modification of the original Mosmann method [29]. Briefly, 5×10^4 MCF-7 cells were seeded in 48 well microtiter plates in 500 ul DMEM supplemented with penicillin streptomycin, insulin, sodium pyruvate, non-essential

amino acids, hydrocortisone and charcoal dextran treated FBS as described previously. After three days growth, the cells were re-fed, then treated two days later with fresh medium containing treatments. MTT was dissolved in PBS at 5 mg/ml and filter sterilized. After 17 hours of treatment, each well received 75 μ l of MTT dye and was incubated for three additional hours. Medium was aspirated with care not to disturb formazan crystals formed by the cell metabolism. DMSO was then added to each well (200 μ l) and placed on a plate shaker at low speed. After 5 minutes, 150 μ l of the DMSO solubilized dye solution was transferred to a 96 well microtiter plate and 550 nm absorbance read on a Bio-tek EL_x 800 microtiter plate reader. Cells from parallel treatments were enumerated by hemocytometer and Trypan Blue dye exclusion to standardize MTT results by cell number.

Luciferin-Luciferase Chemiluminescent ATP Assay: Quantification of cellular ATP by chemiluminescent measurement was performed as described in Sigma Chemical technical bulletin number BL-100 using luciferase-luciferin in glycine buffer (cat. no. L0633).

Luminometry measurements were recorded using an EG&G Berthold Lumat model LB9501/16 luminometer. Cell samples were extracted with 0.1% triton X and diluted five-fold in assay buffer containing 100 mg BSA and 10 ml each of 200 mM Tris HCL, 20 mM EDTA, and 100 mM MgSO₄ at pH 7.8. Results were standardized to cell protein using the BCA protein assay (Pierce, Rockford, IL.) and reported as moles of ATP per mg protein.

Statistical analysis. Where applicable, results were analyzed by one way analysis of variance (ANOVA) using Minitab^R software. Comparison of sample means to control means was determined by Dunnett's multiple means test or Student's T tests with the assumption of equality of variance. Two tailed critical values were used in all experiments with level of significance as indicated. Significance of oxygen consumption curves was done with

Minitab^R software by statistical comparison of slopes within the context of multiple linear regression [see for example, ch 14, Kleinbaum et al. [30]]. All experiments were conducted in triplicate or quadruplicate fashion.

RESULTS. Our initial proliferation studies of melatonin treated MCF-7 cells confirmed and extended the previous reports of its oncostatic activity in breast tumor cells. In our studies, we performed 17-20 hr melatonin treatments, after which time the cells were enumerated by directly counting the cells (via coulter and/or hemocytometer). As shown in Figure 1, we observed a 40% to 60% dose-responsive loss of cells following these short-term treatments with melatonin, at concentrations as low as 1 nM melatonin. Interestingly, cell numbers were not different from control in 100 nM melatonin cultures simultaneously treated with 1 μ M luzindole. Considering the abbreviated time-course of these effects, the results suggested a cyto-lethal mechanism of oncostatic action, rather than an inhibition of cell proliferation. Further evidence for this cyto-lethality came from our tritiated thymidine uptake/nascent DNA synthesis studies. As depicted in Figure 2, following the same melatonin treatments described above, we found no significant inhibition of tritiated thymidine uptake in treated vs. control MCF-7 cell cultures. On the other hand, cells treated with 1 nM 17 beta-estradiol demonstrated a significant increase in tritiated thymidine uptake, thus confirming a positive proliferative response to estrogen in the MCF-7 cells. These results suggested a lack of inhibitory effect by melatonin on the cell cycle/DNA synthesis, and provided further evidence for a cyto-lethal mechanism of action.

Acute cytotoxicity in the form of necrotic cell death was confirmed by light and electron microscopy. Figure 3 displays one of many pale staining "ghost" like bodies in the melatonin treated cells. In addition to these obviously degenerating cells, a number of inclusion bodies were present within the cytoplasm of otherwise normal cells. A 15,000 x transmission electron micrograph (Figure 4) of the inclusion body in Figure 3, identified it as a phagocytized cell in advanced stages of degeneration. The results of these experiments

demonstrated that melatonin treatment of the MCF-7 carcinoma cells resulted in acute necrotic cell death accompanied by autophagocytosis.

A further finding of electron microscopy experiments was morphologic alterations of mitochondrial ultrastructure in the melatonin treated cells. The electron micrograph depicted in Figure 5 shows an MCF-7 cell 18 hours after melatonin treatment and demonstrates effects ranging from normal mitochondrial ultrastructure to swollen cristae, degenerate cristae, and dissolution of the outer mitochondrial membrane.

Because of the acute toxicity that melatonin appeared to inflict in the MCF-7 cells, as well as our morphologic evidence for specific organellar degradation, we proceeded to explore mitochondrial activity/respiration in melatonin treated breast tumor cells. Using standard polarography, we first measured oxygen consumption in MCF-7 cells treated with melatonin in similar fashion to the previously described proliferation studies. As shown in Figure 6, 100 nM melatonin treatment resulted in an increase (185% of control respiration $p < 0.05$) in oxygen consumption in MCF-7 cells by 17 hours but no significant change was present when luzindole was added concurrently with the melatonin.

Our next analyses of mitochondrial respiration explored specific electron transport chain enzyme complex activities by directly performing enzyme-substrate assays in detergent permeabilized MCF-7 cells. These studies demonstrated a 53% increase in cytochrome c oxidase activity in melatonin treated MCF-7 cells (Figures 7). We further investigated this apparent change in electron transport chain activity by employing the use of a mitochondrial vital dye, AlamarBlue™, which undergoes a detectable change in fluorescence following reduction by complex IV. Eighteen hours following melatonin treatment, a dose-responsive 70% to 125% increase in AlamarBlue dye fluorescence was present compared to vehicle

treated control cultures (Figure 8). Once again, the addition of 1 μ M luzindole to the 100 nM melatonin cultures returned values to the level of controls.

To confirm that the increase in electron transport activity was not restricted to complex IV, we evaluated succinate dehydrogenase activity (complex II). Results from the MTT assay (Figure 9) demonstrated a 15% ($p < 0.05$) increase in formazon dye reduction in the MCF-7 cells following nineteen hours of treatment with melatonin and suggested increased activity of complex II of the electron transport chain. Complex II activity in cultures treated with 1 μ M luzindole concurrently with 100 nM melatonin were not significantly different from control values.

The increases in mitochondrial oxygen consumption and electron transport activity suggested that melatonin may act to uncouple oxidative phosphorylation from electron transport in breast tumor MCF-7 cells. To determine whether this effect was exerted by melatonin in the cells, we measured cellular ATP levels by chemiluminescence, and, as shown in Figure 10, observed that 100 nM melatonin treatment resulted in a significant loss of ATP in MCF-7 cells. Thus, melatonin appears to act as an uncoupler of oxidative phosphorylation, and may exert its cyto-lethality in breast tumor cells via this proximal toxic event.

DISCUSSION. Acute toxicity as well as perturbation of mitochondrial respiration was clearly apparent in the MCF-7 cells cultured under estrogen-free conditions following 16-20 hour melatonin treatments (Figures 3 and 4). These cytotoxic and respiratory effects were not observed when the melatonin G-protein coupled receptor antagonist, luzindole, was present nor were the effects observed in parallel studies with estrogen containing medium (data not shown). It is unlikely that luzindole prevented entry of highly lipophilic melatonin molecules into the MCF-7 cells, and it is equally improbable that estrogen directly inhibited either melatonin receptor binding or melatonin entry into the cells. These findings clearly suggest that a receptor mediated pathway modulates melatonin cytotoxicity and mitochondrial effects in MCF-7 cells and that estrogen inhibits these effects.

Previous studies have shown that melatonin oncostatic effects in MCF-7 cells are in part mediated through the estrogen response pathway through as yet incompletely defined mechanisms [review [11;13;14]]. However, one of the proposed mechanisms, that of estrogen receptor down-regulation [20], is questionable in light of the current studies in which the medium was essentially depleted of estrogen (≤ 0.114 pM). A second receptor-mediated melatonin pathway reported in MCF-7 cells is the induction of the *c-myc* gene [19], which is also induced by estrogen [31] and upregulates the transcription of several key glycolytic enzymes [32;33]. Although anaerobic glycolysis is well established as a primary means of tumor cell energy production, recent evidence strongly implicates an important role of mitochondrial ATP in priming glycolysis through the activity of hexokinase II, a key glycolytic regulatory enzyme [34-38]. Since hexokinase II, is reportedly induced by *c-myc* [33], it is likely that both melatonin and estrogen mediated upregulation of *c-myc* may increase the consumption of mitochondrial ATP by hexokinase II activity. Furthermore,

receptor inhibition by luzindole would abolish downstream effects of melatonin, such as those resulting from *c-myc* induction.

Our data demonstrated increased electron transport activity along with reduced ATP levels in MCF-7 cells cultured in the absence of estrogen, thereby suggesting that oxidative phosphorylation was uncoupled. Interestingly, it has been previously suggested that melatonin was associated with increased levels of the mitochondrial uncoupling protein, thermogenin [review [39]]. This protein or a similar functioning protein would readily orchestrate the controlled uncoupling of oxidative phosphorylation in such manner as our data indicated. Thus, with increased consumption of mitochondrial ATP through enhanced glycolytic activity, the uncoupling of oxidative phosphorylation demonstrated in our studies would be expected to rapidly result in MCF-7 cytotoxicity.

The striking inhibition of melatonin induced MCF-7 cytotoxicity by estrogen may also result from the hormonal modulation of electron transport subunit proteins which undergo as much as a sixteen fold upregulation by estrogen in some cell types [40]. In this manner, MCF-7 cells would overcome the uncoupling effect of melatonin and provide sufficient mitochondrial ATP for continued growth. Conversely, in the absence of estrogen, the limited synthesis of cytochrome C oxidase subunit proteins would impede electron flow and thereby increase cell susceptibility to cytotoxicity by uncoupling of oxidative phosphorylation.

The relationships between melatonin and estrogen receptor pathways clearly require further study to elucidate hormonal crosstalk in breast tumor cells. Our studies may suggest a mechanism through which the hormone melatonin can block aberrant cell growth during periods of low physiologic estrogen levels. Further studies using *in vivo* models will be

required to address the relevance of these MCF-7 cell studies to melatonin inhibition and prevention of breast cancer.

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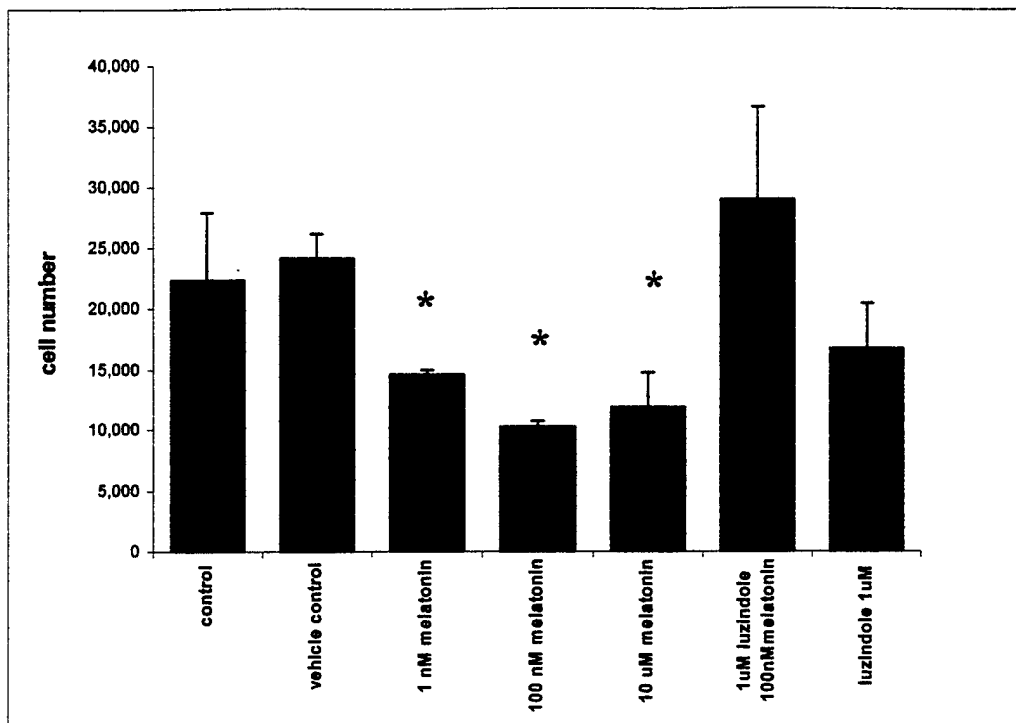


Figure 1. MCF-7 cells treated for 18 hours with vehicle control, melatonin, and Luzindole.

Cells were enumerated by Trypan Blue dye exclusion assay and hemocytometer count. Treatments were performed in triplicate in 48 well microtiter plates. Statistically significant differences from control are indicated by * ($p < 0.05$ by one way ANOVA and Dunnet's comparison of means to control test).

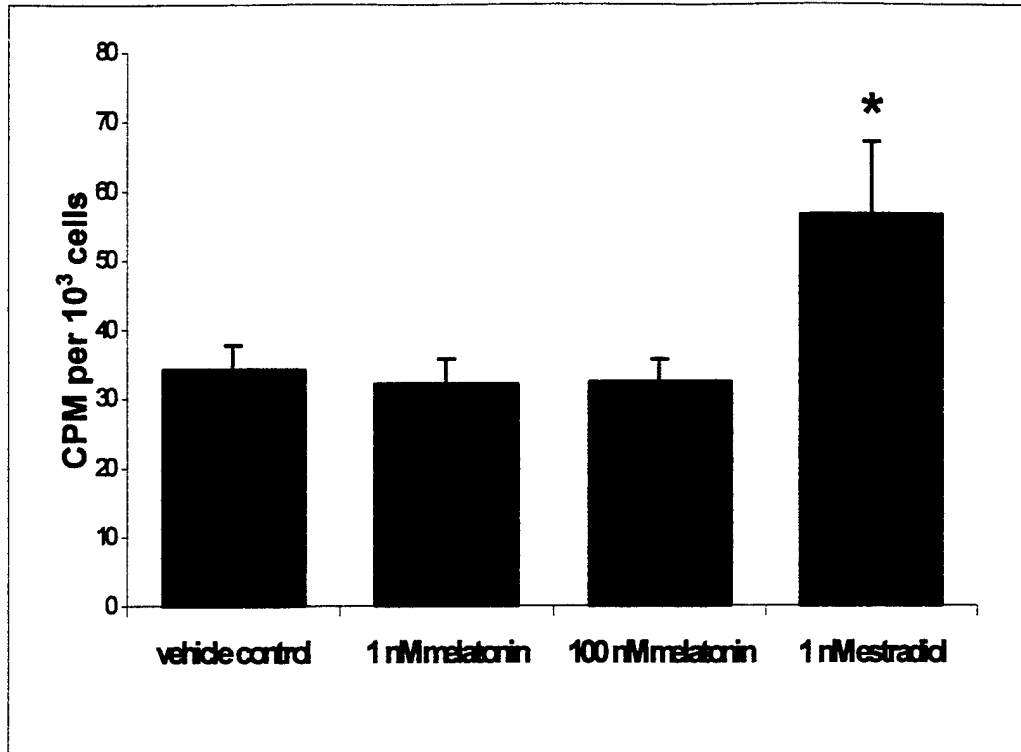


Figure 2. Tritiated thymidine uptake by MCF-7 cells 16 hours following melatonin and 17 β -estradiol treatment.

Tritiated thymidine studies were conducted as described in Methods. No difference in DNA synthesis was evident after either 1 nM or 100 nM melatonin treatment. Significant difference (* $p < 0.05$) between estradiol and control groups by Student's T test is indicated by asterisk. All treatments were performed in triplicate.

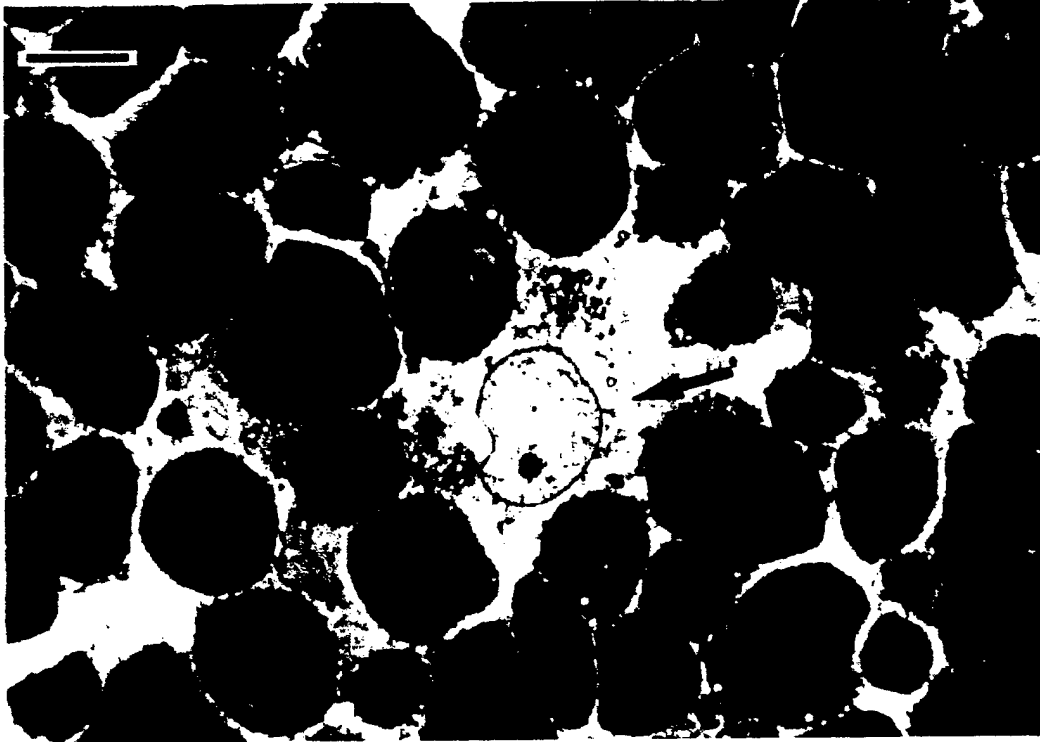


Figure 3. Light micrograph of MCF-7 cells 18 hours after treatment with 100 nM melatonin.

(H and E stain 1000x magnification) Multiple pale staining cells (arrow) were present in slides from melatonin treated cells but absent in both the vehicle control (0.00005% ethanol) and estrogen (1 nM) treated cultures. Many of the treated cells contained cytoplasmic inclusion bodies (arrowhead) that appeared to be degenerating cell bodies.

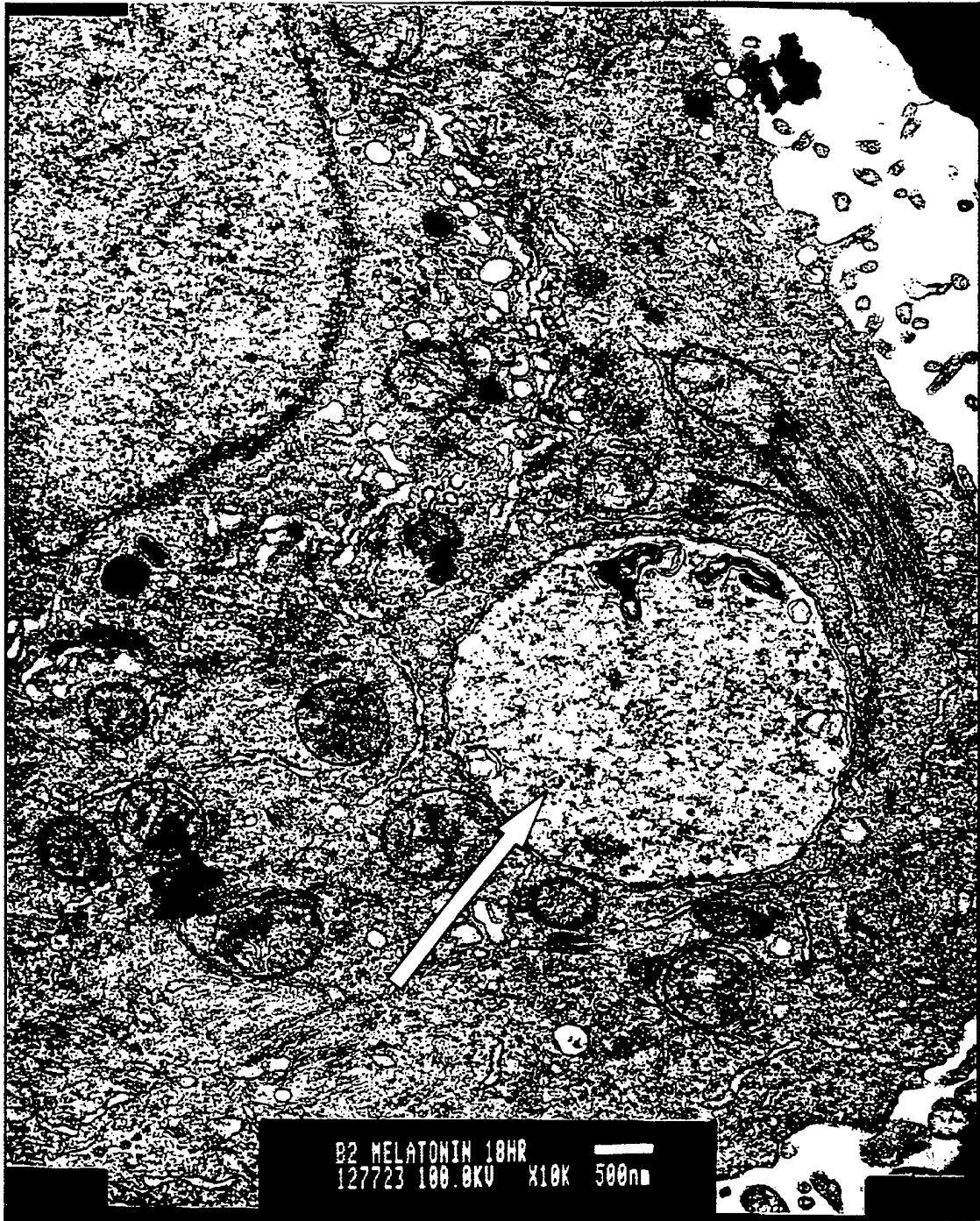


Figure 4. Transmission electron micrograph of MCF-7 cell 18 hours after treatment with 100 nM melatonin. (magnification 10,000x)

The inclusion body (phagolysosome) shown in figure 3 is indicated by the arrow. Note the presence of nuclear remnants and degenerating organelles within the phagolysosome.

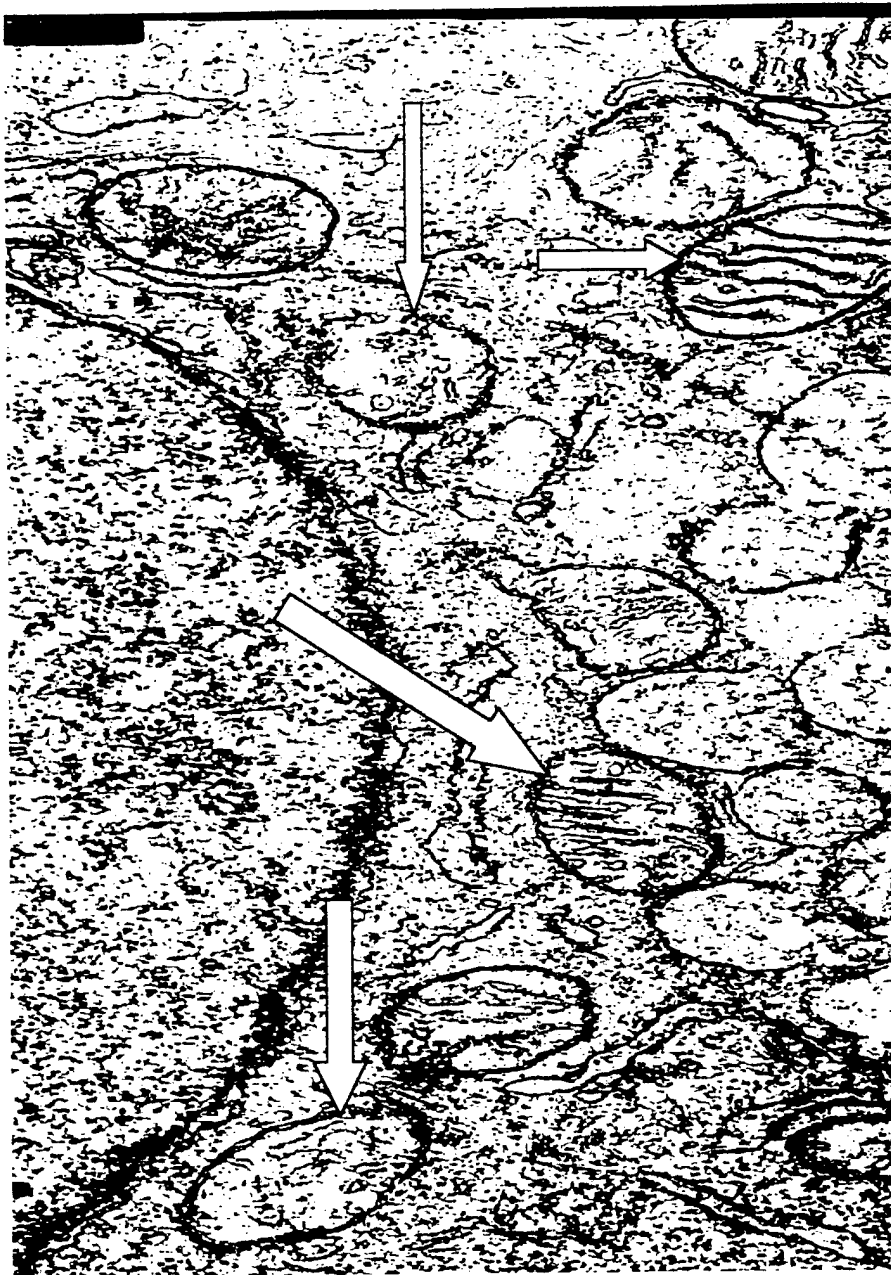


Figure 5. Transmission electron micrograph of MCF-7 cell 18 hours after treatment with 100 nM melatonin. (magnification 15,000 x)

Mitochondria (arrows) demonstrate varying states of degeneration. A normal mitochondrion (upper right) had distinct morphologic ultrastructure and clearly defined cristae. Other mitochondria demonstrated changes that included swelling of cristae, degeneration of cristae, and degeneration of mitochondrial membrane.

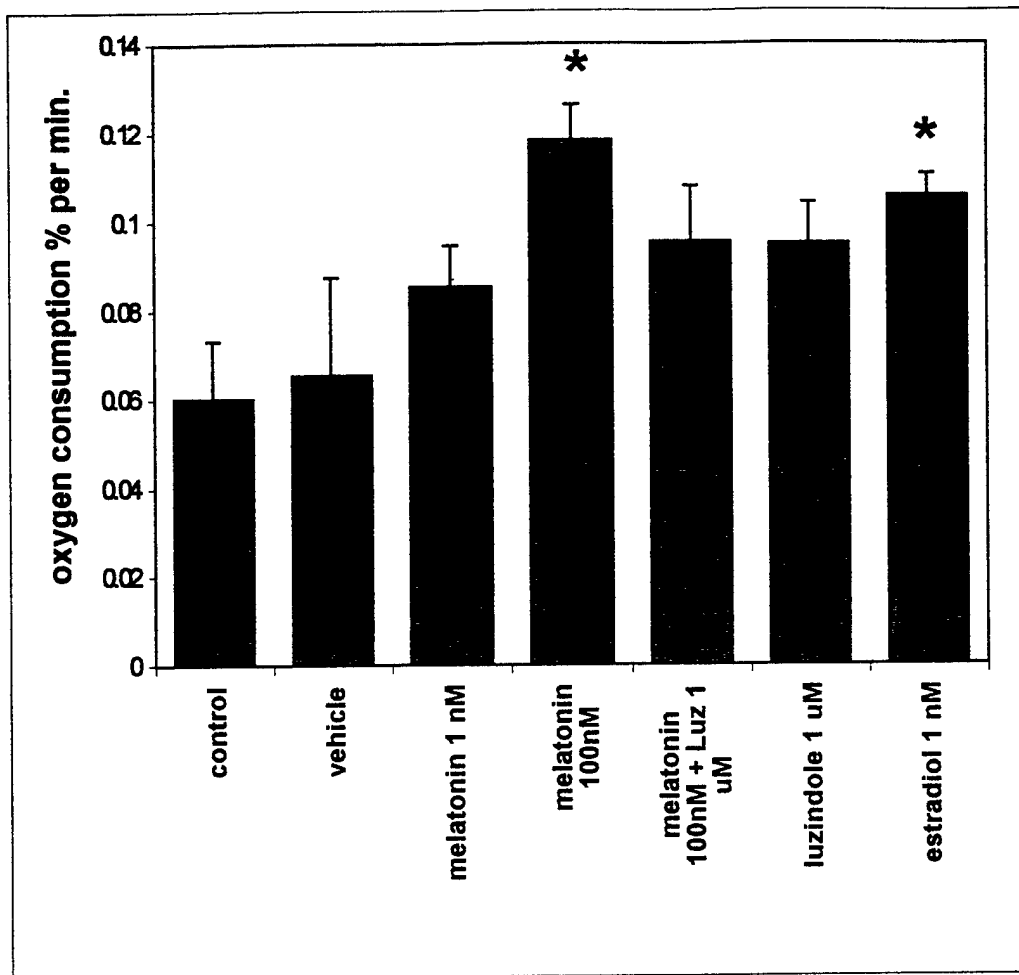


Figure 6. Oxygen uptake by MCF-7 cells 18 hours after treatments with vehicle (0.00005% ethanol), melatonin, Luzindole, and estrogen.

Slopes for oxygen consumption curves were derived from a linear range ($R^2 \geq 0.99$) over 20 minutes. Bars represent mean slope of triplicate samples with error bars representing one standard deviation of each set mean. The slope of both 1nM estradiol and 100 nM melatonin treated groups were significantly higher than vehicle control by one-way ANOVA and Tukeys comparison of means test (* $p < 0.05$).

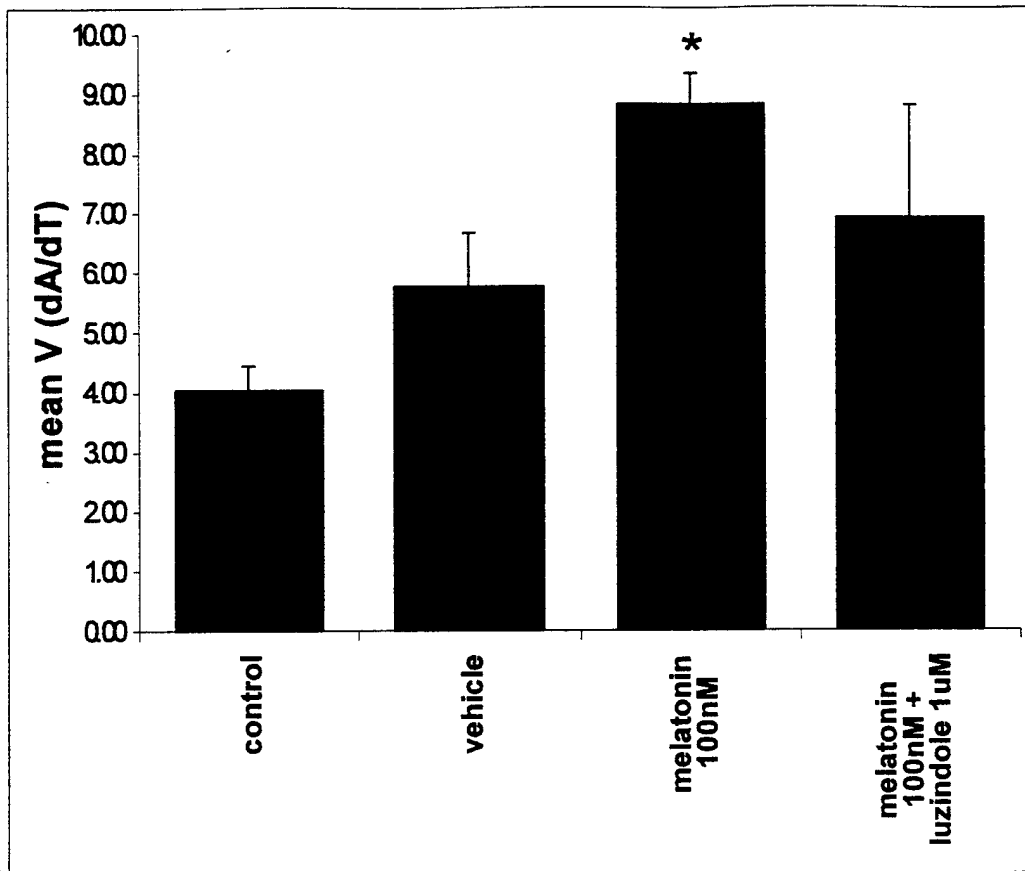


Figure 7. Cytochrome C oxidase activity by microtiter plate method (colorimetric measurement of oxidized DAB) in MCF-7 cells 18 hours after treatment with vehicle, melatonin, Luzindole, or 17 β -estradiol.

The enzyme reaction rate (mean V/minute) was standardized to cell number. Only 100 nM melatonin was significantly different from control (* $P < 0.01$ by one way ANOVA with Fishers comparison of means test). The addition of 1 μ M luzindole to the 100 nM melatonin treatment group returned cytochrome C oxidase activity to control values.

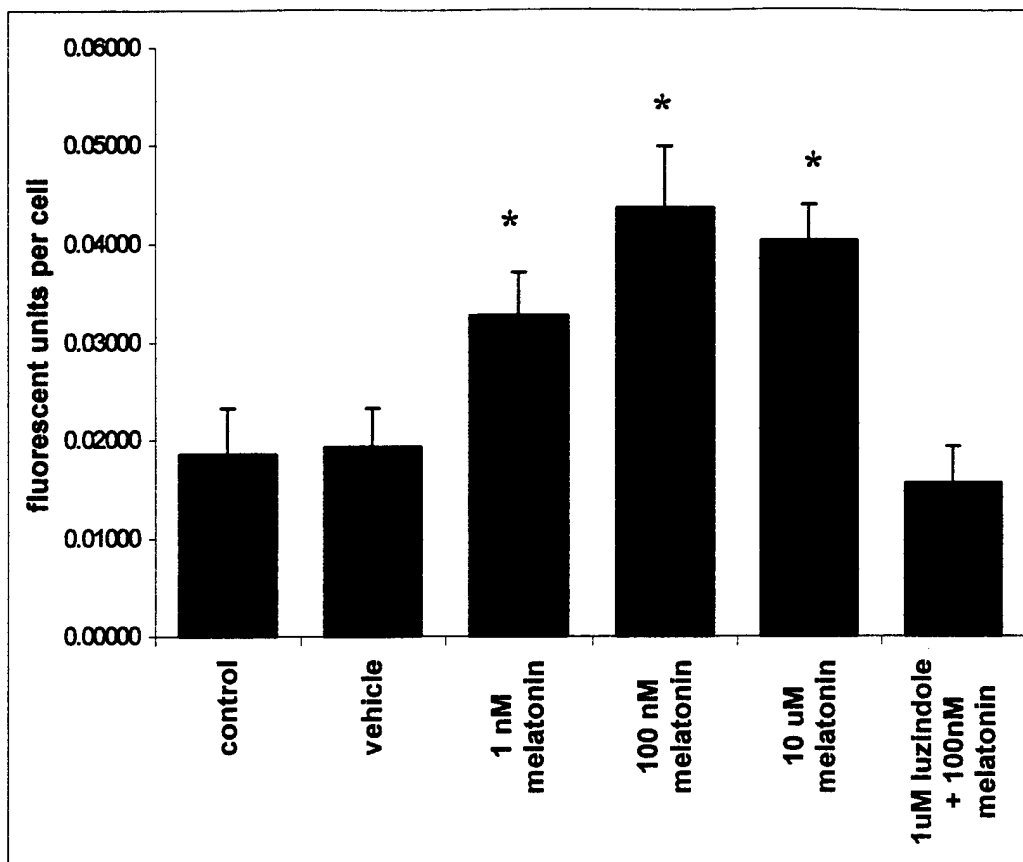


Figure 8. Cytochrome C oxidase activity (reduction of AlamarBlue) in MCF-7 cells 18 hours after treatment.

Data is reported as fluorescent units of AlamarBlue dye reduction during a four-hour incubation period per million cells. Fluorescence after 1 nM, 100 nM, and 10 uM melatonin treatment was significantly higher than control fluorescence by one way ANOVA and Dunnett's comparison of means to control test. The addition of 1 uM Luzindole to 100 nM melatonin returned the fluorescence units (activity of the enzyme) to control values.

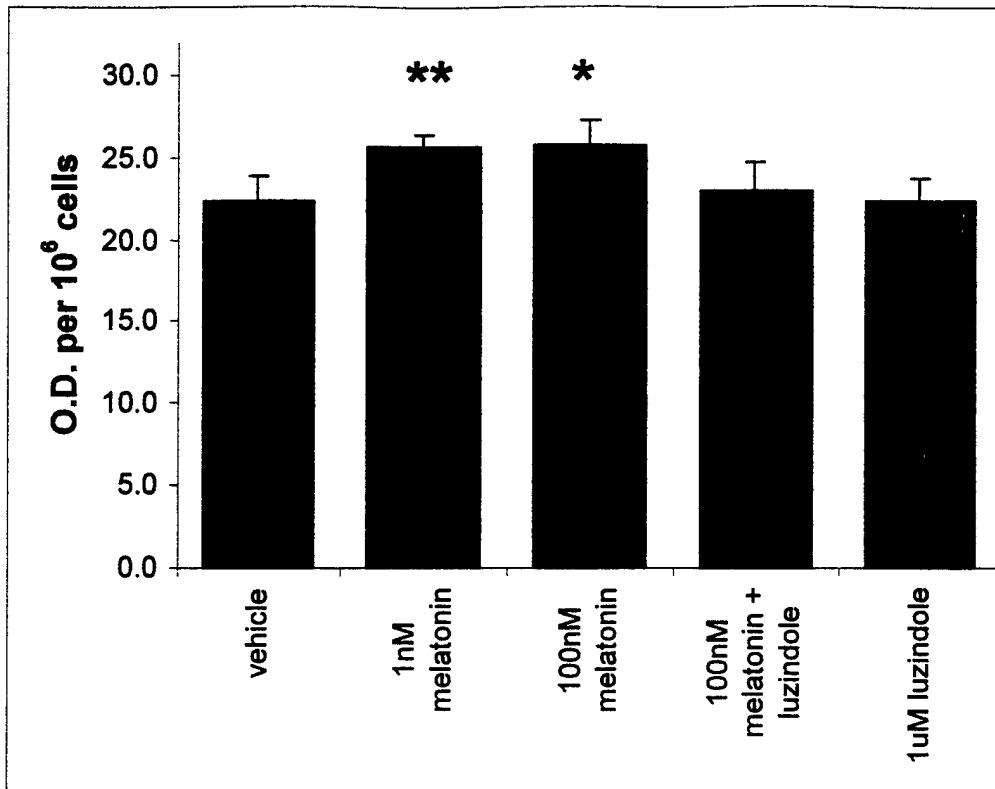


Figure 9. Activity of succinate dehydrogenase by MTT assay.

MCF-7 cells were treated for 19 hours with vehicle (0.003% ethanol), 1 nM melatonin, 100 nM melatonin, 1 uM luzindole, or combination of 100 nM melatonin plus 1 uM luzindole. Optical density of the microtiter plate assay was standardized to cell number and results represented as absorbance per million cells. O.D. following 1 nM and 100 nM melatonin treatments were significantly higher than vehicle control O.D. ($p < 0.01$ and $p < 0.05$) by two tailed Students T test.

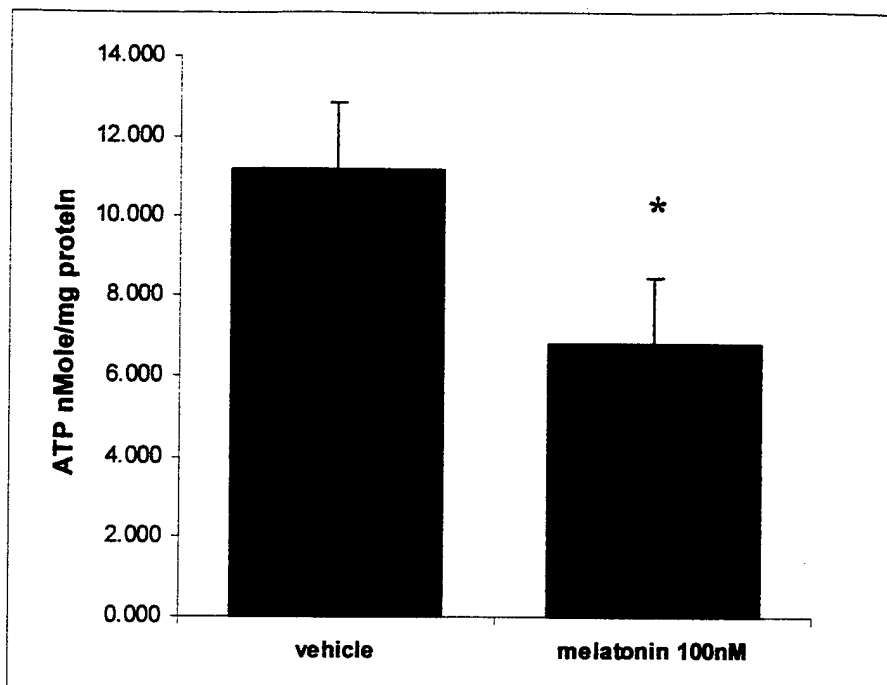


Figure 10. ATP concentration in MCF-7 cells 21 hours after treatment with 100nM melatonin or vehicle (0.00005% ethanol).

ATP was measured by luciferin-luciferase chemiluminescent assay. Crude data (relative light units) were converted to nMole of ATP from a standard curve and reported as nMole of ATP per mg protein. Melatonin treated cells had significantly lower total ATP than vehicle controls ($p < 0.05$ by Student's T test)