المجلة الإلكترونية الشاملة متعددة التخصصات
العدد السادس والثلاثون شهر (٥) ٢٠٢١

Melatonin and it is receptors indirectly induce MCF7 apoptosis and spare MCF10, via clock gene Period 1 up regulation which in turn to upregulate p53.

Corresponding author:
1- Mowafag Hassan Malla Khedir*

King Saud bin Abdulaziz University for Health Sciences

College of medicine

Tlion5610@gmail.com

Lecturer physiology

2- Amal seed

Professor Khartoum university College of medicine

amalsaeed@yahoo.com

Abstract

Introduction Melatonin is antioxidant, anti-ageing, immunomodulation, and anticancer properties. From the epidemiological research, it was postulated that Melatonin has significant apoptotic, angiogenic, oncostatic and anti-proliferative effects on various oncological cells. anti-proliferative effects on various oncological cells but the mechanism of action, how melatonin is anti-cancer still unknown and under study. ***Aim of study.*** In this study we are looking for the relation between melatonin receptors expression clock gene period 1, and p53 expression Conducting such a research regarding this topic is expected to have relation between melatonin hormone and its receptors to clock genes (period 1) and tumor suppresser gene P53 activation to prevent and induce apoptosis in breast cancer cells. **Material and *method*** Cell Culture for MCF-7 and MCF-10 human breast cancer and normal cells respectively were seeded in T75 flasks at 1×10^6 cells/flask in low glucose Dulbecco's modified Eagle's medium (DMEM) according to MCF 7 and MCF10 culture protocol **flow cytometer (muse) ANNEXIN V** to determine the effect of melatonin in MCF7 apoptotic percentage according to protocol. **Reaction** (qRT-PCR) Analysis Total RNA was isolated using the Ambion Aqueous kit (Ambion). All isolated RNA samples were treated with DNase I to remove contaminating genomic DNA. The quality and quantity of the isolated RNA was determined using Agilent Bioanalyzer 2100. One µg of total RNA was reverse-transcribed using first strand cDNA synthesis Kit (Millipore, USA) followed by **real time quantitative PCR (qRT-PCR).** ***Results*** effect of melatonin on MCF7 as melatonin concentration increase the Apoptotic percentage increase at the same time in the same condition there was no significant apoptotic effect of melatonin on MCF10. There is low MTNR1A melatonin receptor expression in patient with breast cancer compared to normal.As melatonin receptors increase the expression of P53 increase as will this action very clear after melatonin receptor transfection. **Discussions**. melatonin prevents breast cancer formation via upregulation period 1 and P53 to induce cell cancer apoptosis. One more thing is the melatonin action in prevention and killing breast cancer is unique action, (is selective action) because under the same experimental conditions of the breast cancer cell MCF7 there was no apoptosis effect on normal cell (cell line MCF10).

Keywords: - Melatonin – Melatonin receptors expression - clock gene Period 1 expression P53 expression

الملخص

المقدمة :- هنالك العدد من وظائف هرمون الميلاتونين منها انه مضاد للاكسدة , مضاد للشيخوخة و يمنع تكوين الخلايا السرطانية و تقدمها كما يعمل على قتل الخلايا السرطانية ذكرا ذالك في العديد من الدراسات السابقة و لاكن ما ينقصنا هو فهم الية عمل الميلاتونين كا هورمون مضاد لسرطان الثدي و هذه المعلومة لاتزال غير واضحة هدف الدراسة :-**هدف الدراسة** تهدف الدراسة لفهم الية عمل الميلاتونين كهرمون مضاد لتكوين سرطان الثدي و محاولة اثبات العلاقة نظرية البحث التى تدور حول دور الميلاتونين في تنظيم واحد من جينات الساعة البيولوجية Period 1 و الذي بدورة يقوم بتنظيم التعبير الجيني للجين المضاد للاورام P53 الذي يعمل علي قتل الخلايا السرطانية . **المواد و طرق القياس** في البدية نعمل علي زراعة الخلايا الثدي السلرطانية و الطبيعية وفق التعلمات المرفقة معها , نستخدم flowcytometer لتقيم اثر هرمون الميلاتونين على عدد الخلايا السرطانية الحية و الميتة بعد يوم من الزراعة نحتاج ايضا الى real time quantitative PCR (qRT-PCR)) لقياس التبير الجيني لكل من مستقبلات هرمون الميلاتونين , التعبير الجيني لجين الساعة البيولوجية period 1 و التعبير الجين لجين المضاد للاورام P53 .**النتائج** كانت النتائج على النحو التالى :-يعمل الميلاتونين على قتل خلايا سرطان الثدي حيث يزداد عدد الخلايا الميتة كلما زاد تركيز هرمون الميلاتونين . ليس للميلاتونية اي تأثير قياسى علي خلايا الثدي الطبيعية . لدي مرضي سرطان الثدي مستقبلات ميلاتونين اقل من الشخاص الطبيعية في نفس اعمارهم . هنالك علاقة خطية بين الجين المضاد للأورام و عدد مستقبلات الميلاتونين و ذالك يتضح جليا بعد عمل زيادة في مستقبلات الميلاتونين transfection plasmid داخل الخلايا السرطانية . **المناقشة** يعمل الميلاتونين على زيادة التعبير الجيني لجين الساعة البيولجية period 1 الذى بدورة يقوم علي زيادة التعبير للجين المضاد للأورم P53 ليعمل علي قتل الخلايا سرطان الثدي , الشيء المهم هو خاصية الانتقاء الختيارة لهرمون الميلاتونين انة يحفز موت الخلايا السرطانية و في نفس الوقت لايؤثر على الخلايا الطبيعة .

الكلمات المفتاحية :- هرمون الميلانتونية , التعبير الجيني لمستقيلات الميلاتونين التعبير الجينى لجين الساعة البيولجية الزمني الاول , التعبير الجينى للجين المضاد للأورام .

Introduction

Melatonin and anti-cancer effect

Melatonin is antioxidant, anti-ageing, immunomodulation and anticancer properties. From the epidemiological research, it was postulated that Melatonin has significant apoptotic, angiogenic, oncostatic and anti-proliferative effects on various oncological cells(Bhattacharya, Patel, Dehari, Agrawal, & Singh, 2019).

Melatonin, a safe endogenous hormone and a natural supplement, has recently been recognized to have antiproliferative effects and the ability to sensitize cells to other anticancer therapies(Zakki et al., 2020). Phenylarsine oxide (PAO) has anticancer potential but it is considered as a toxic agent. Melatonin showed promising potential as a chemotherapeutic agent in combination with Phenylarsine oxide (PAO) to achieve a better anticancer response(Zakki et al., 2020).

Effect of Melatonin in normal cells is inhibitory, inhibits apoptosis.

melatonin inhibits apoptosis in mouse Leydig cells by activating the retinoic acid-related orphan nuclear receptor (ROR) α/p53 signaling pathway. After treatment with 10 ng/mL melatonin for 36 h, RORα mRNA and protein levels were significantly increased ($P < 0.01$). TUNEL and flow cytometry showed that melatonin significantly decreased the TUNEL-positive cell ratio and apoptosis rate ($P < 0.05$). Moreover, melatonin decreased BAX expression and increased BCL-2 expression ($P < 0.05$). However, the RORα inhibitor SR1001 reversed the inhibitory effects of melatonin on apoptosis ($P < 0.05$). Additionally, analysis of p53 expression showed that melatonin inhibited p53 mRNA and protein expression ($P < 0.05$), whereas SR1001 reversed these effects. p53 reversed the anti-apoptotic process involving RORα-mediated melatonin in mouse Leydig cells. Collectively, melatonin inhibited apoptosis via the RORα/p53 pathway in normal cells (Li, Zhao, Liu, Wang, & Lu, 2020).

Melatonin and vitamin D3 inhibit breast cancer cell growth and induce apoptosis, but they have never been combined as a breast cancer treatment. In MCF-7 breast cancer cells, melatonin together with vitamin D3,

induced a synergistic proliferative inhibition, with an almost complete cell growth arrest at 144 hr. Cell growth blockade is associated to an activation of the TGFβ-1 pathway, leading to increased TGFβ-1, Smad4 and phosphorylated-Smad3 levels. Concomitantly, melatonin and D3, alone or in combination, caused a significant reduction in Akt phosphorylation and MDM2 values, with a consequent increase of p53/MDM2 ratio. These effects were completely suppressed by adding a monoclonal anti-TGFβ-1 antibody to the culture medium. Taken together, these results indicate that cytostatic effects triggered by melatonin and D3 are likely related to a complex TGFβ-1-dependent mechanism, involving down-regulation of both MDM2 and Akt-phosphorylation(Sara Proietti et al., 2011).

Melatonin Down-Regulates MDM2 Gene Expression and Enhances p53 Acetylation in MCF-7 Cells

Compelling evidence demonstrated that melatonin increases p53 activity in cancer cells. p53 undergoes acetylation to be stabilized and activated for driving cells destined for apoptosis/growth inhibition. Over-expression of p300 induces p53 acetylation, leading to cell growth arrest by increasing p21 expression. In turn, p53 activation is mainly regulated in the nucleus by MDM2. MDM2 also acts as E3 ubiquitin ligase, promoting the proteasome-dependent p53 degradation. MDM2 entry into the nucleus is finely tuned by two different modulations: the ribosomal protein L11, acts by sequestering MDM2 in the cytosol, whereas the PI3K-AkT-dependent MDM2 phosphorylation is mandatory for MDM2 translocation across the nuclear membrane. In addition, MDM2-dependent targeting of p53 is regulated in a nonlinear fashion by MDM2/MDMX interplay. Melatonin induces both cell growth inhibition and apoptosis in MCF7 breast cancer cells. We previously reported that this effect is associated with reduced MDM2 levels and increased p53 activity. Herein, we demonstrated that melatonin drastically down-regulates MDM2 gene expression and inhibits MDM2 shuttling into the nucleus, given that melatonin increases L11 and inhibits Akt-PI3K-dependent MDM2 phosphorylation.

Melatonin induces a 3-fold increase in both MDMX and p300 levels, decreasing simultaneously Sirt1, a specific inhibitor of p300 activity. Consequently, melatonin-treated cells display significantly higher values of both p53 and acetylated p53. Thus, a 15-fold increase in p21 levels was observed in melatonin-treated cancer cells. Our results provide evidence that melatonin enhances p53 acetylation by modulating the MDM2/MDMX/p300 pathway, disclosing new insights for understanding its anticancer effect(S. Proietti et al., 2014).

Several epidemiological studies have shown that high levels of melatonin, an indolic hormone secreted mainly by the pineal gland, reduce the risks of developing cancer, thus suggesting that melatonin triggers the activation of tumor-suppressor pathways that lead to the prevention of malignant transformation. Melatonin induces phosphorylation of p53 at Ser-15 inhibiting cell proliferation and preventing DNA damage accumulation of both normal and transformed cells. This activity requires p53 and promyelocytic leukemia (PML) expression and efficient phosphorylation of p53 at Ser-15 residue. Melatonin-induced p53 phosphorylation at Ser-15 residue does not require ataxia telangiectasia-mutated activity, whereas it is severely impaired upon chemical inhibition of p38 mitogen-activated protein kinase activity. By and large, these findings imply that the activation of the p53 tumor-suppressor pathway is a critical mediator of melatonin and its anticancer effects. Therefore, it provides molecular insights into increasing observational evidence for the role that melatonin has in cancer prevention(Santoro, Marani, Blandino, Muti, & Strano, 2012).

melatonin, at physiological concentrations, exerts its antiproliferative effects on MCF-7 human breast cancer cells by inducing the expression of some of the proteins involved in the control of the cell cycle. MCF-7 cells were cultured for 48 h in DMEM media containing either melatonin (1 nM) or the diluent (0.001% ethanol). At this concentration, after 48 hours of incubation, melatonin reduced the number of viable cells in relation to controls. The decreased cell proliferation was coincident with a significant increase in the expression of p53 as well as p21WAF1 proteins.

These results demonstrate that melatonin inhibits MCF-7 cell proliferation by inducing an arrest of cell cycle dependent on an increased expression of p21WAF1 protein, which is mediated by the p53 pathway. (Mediavilla, Cos, & Sánchez-Barceló, 1999)

Clock genes and the tumor suppressor gene p53 It is well recognized that p53 is an important antioncogene that safeguards genomic integrity. When DNA damage or proto-oncogenic mutation occurs, p53 receives upstream signals and triggers the stress response, which promotes the repair or apoptosis of damaged cells(Purvis et al., 2012). The function of the anti-oncogene p53 is tightly connected with the modulation of protein stability, and the P53 protein serves as the most important transcription factor involved in diverse biological processes, including the cell cycle, apoptosis, and DNA-damage response, that are important in maintaining cell homeostasis, preventing gene mutation, and inhibiting tumor formation and development. (Miki, Matsumoto, Zhao, & Lee, 2013; Purvis et al., 2012), The deletion or mutation of the 53 gene is a frequent occurrence in approximately half of all human cancers, including oral cancer.

2. Research Problem

They are several studies confirm the link between melatonin and cancer specially breast cancer. The melatonin act as anti-cancer, the gab of problem is how, what is the physiological action of melatonin that prevent breast cancer. In this study we are looking for the relation between melatonin receptors expression, clock gene period 1, and p53 expression Conducting such a research regarding this topic is expected to have relation between melatonin hormone and its receptors to clock genes (period 1) and tumor suppresser gene P53 activation to prevent and induce apoptosis in breast cancer cells .

3. Research Questions

The main question of this study is: "How to investigate relation between melatonin hormone and it is receptors to clock gene (period1) and tumor suppresser gene P53 activation

This main question is subdivided into the following sub-questions:

1. Will study effect of different melatonin concentration on MCF-7 cell line P53 mRNA expression, and clock gene period 1.
2. Will study effect of different melatonin concentration on MCF-10 cell line P53 mRNA expression, and clock gene period 1.
3. Study melatonin receptors expression M 1 encoding via MTNR1A expression in patient with breast cancer in different stages compare to normal.
4. Study melatonin receptors expression M 2 encoding via MTNR1B expression in patient with breast cancer in different stages compare to normal.
5. Study melatonin receptors expression M 1 encoding via MTNR1A expression in MCF-7 cell line compare to normal MCF-10 cell line
6. Study melatonin receptors expression M 2 encoding via MTNR1B expression in MCF-7 cell line compared to normal MCF-10 cell line .
7. Study Period 1 mRNA expression in patient with breast cancer in different stages compare to normal.
8. Study correlation between period 1 mRNA expression and MTNR1A mRNA expression at the same samples .
9. MCF-7 MTNR1A Transfection (up regulation M1 receptors)
10. Study effect of different melatonin concentration on MCF-7 cell line P53 mRNA expression, and clock gene period 1 **after upregulation** M1 (MTNR1A plasmid transfection)

How to conduct the 10 steps above

1. Tissue cultures for MCF-7 and MCF-10.
2. rt-PCR Sybergreen master mix to identify Melatonin receptors M1 (MTNR1A), M2 (MTNR1B), period 1 and P53 mRNA expression.
3. For upregulation Melatonin receptors M1 in MCF-7 cell line is conducted via plasmid transfection MTNR1A GFP .

Material and Methods

Cell Culture

MCF-7 and MCF-10 human breast cancer and normal cells respectively were seeded in T75 flasks at 1×10^6 cells/flask in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 0.01 mg/ml insulin and 1% penicillin/streptomycin mix and need to be incubated at 37°C in an atmosphere of 5% CO2. The medium renewal has to be performed 2 times per week, while cells should weekly be passaged at a sub-cultivation ratio of 1:3 (11). An alternative cell culture medium would be DMEM supplemented with antibiotics/antimycotics and 10% FBS(12).

Transfections

The day before transfection, we transplanted our cells so that they were confluent 70% to 90% at the time of your experiment. We will be following the 24-well plate format of the Lipofectamine® LTX & Plus™ Reagent protocol. We prepared 4 tubes of 50 µl of Opti-MEM® Medium each and labeled them 1 through 4. We added 2 µl of Lipofectamine® LTX Reagent to Tube 1, 3 µl to Tube 2, 4 µl to Tube 3 and 5 µl to Tube 4. We mixed each tube well by moving or whipping the tube. We prepared a tube containing 250 µl of Opti-MEM® medium and added 5 µg of plasmid DNA. Since our DNA concentration is 1 µg per µl, we will add 5 µl. Then we add 5 µl of Plus ™ reagent and mix well.

Add 50 µl of the diluted DNA to each of Lipofectamine® LTX dilutions in tubes 1 through 4. We incubate the compound for 5 minutes at room temperature. After the 5-minute incubation period, we removed the 24-well plate containing our cells from the incubator and brought them to the workspace in the lid. We added 50 µl of the DNA reagent complex from tubes 1 to 4 to wells 1 to 4 of a 24-well plate, respectively. Then we put our 24-well plate back into the incubator and grew cells for one day at 37 ° C. After incubating our cells, assess the transfection efficiency in each well by displaying GFP fluorescence.

Quantitative Real Time Polymerase Chain

Reaction (qRT-PCR) Analysis Total RNA was isolated using the Ambion Aqueous kit (Ambion). All isolated RNA samples were treated with DNase I to remove contaminating genomic DNA. The quality and quantity of the isolated RNA was determined using Agilent Bioanalyzer 2100. One µg of total RNA was reverse-transcribed using first strand cDNA synthesis Kit (Millipore, USA) followed by real time quantitative PCR (qRT-PCR). qRT-PCR was performed with a 7900HT Fast Real-Time PCR System(Applied Biosystems, USA), using 2 µL cDNA, 10 µL 2X Sybergreen Master mix (150mM Tris, pH 9.2, 40mM(NH4)2SO4, 5mMMgCl2, 0.02% Tween-20, 0.4mM dNTPs, 1.25 Units Taq Polymerase, 1X Sybergreen) and 0.5 µL of 20µM gene-specific primers (Table 1). Primers were designed based on theoretical optimal conditions, which included primer melting temperature, primer annealing temperature, GC content, cross homology and primer secondary structures. All primers were purchased from Bio-Basic Canada Inc. (Ontario, Canada). The specificity and size of the PCR products were tested by adding a melt curve at the end of the amplifications, analysis on a 2% agarose gel of the bands. Amplicon Bands were isolated and sequenced.

The reaction protocol consisted of one activation cycle of 50◦C for 2min followed by 95◦C for 15 s. Thereafter, 40 cycles of denaturation at 95◦C for 15 s, and at 60◦C annealing/extension for 2min were performed. Although normalization to RPL13 and Ubiquitin C showed similar trends, all values were normalized to Ubiquitin C. The $2^{-\Delta\Delta CT}$ method was used for relative quantification for qRT-PCR experiments.

Primers used in the Methods.

Primers	Forward	Reverse	Accession number
MTNR1A	CTGTCGGTGTATCGGAACAAG	CCAACGGGTACGGATAAATGG	NM_005958
Period 1	GCCAACCAGGAATACTACCAGC	GTGTGTACTCAGACGTGATGTG	NM_002616
P53	GTTGAGTGGAAAGTACGGAACG	TGTGGGTGCTTGTGTAACCAG	NM_001126049

Statistical Analysis

Statistical analysis was carried out using Spss software ver.20. Fold change in mRNA expression was calculated for qRT-PCR results and analysis was carried out using One Way ANOVA followed by (t test) for pairwise comparisons and comparisons against the Normal group.

Ethical approval

This Research is part of PhD thesis which posted to Al Jazeera university /Sudan postgraduate 2018.

Results

Effect of different melatonin concentration from 1 nm up to 3 nm on estrogen positive cell line MCF7 cell

flowcytometry analysis for estrogen positive MCF 7 cell line without melatonin after 24 hours

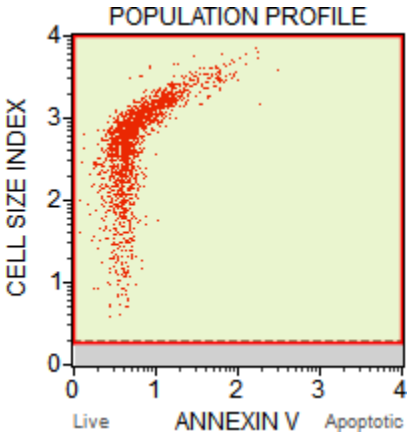


Figure 1 population profile MCF 7 controle without melatonin

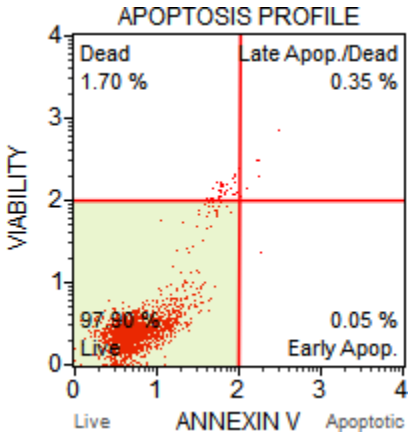


Figure 2 apoptosis profile MCF 7 controle without melatonin

Table 1:- Cytometer analysis for estrogen positive MCF 7 cell line without melatonin after 24 hours

Cell's status	Cell Conc. (Cells /mL)	% Gated
Live (LL):	5.35E+06	97.90 %
Early Apoptotic (LR):	2.73E+03	0.05 %
Late Apop. / Dead (UR):	1.91E+04	0.35 %
Debris (UL):	9.28E+04	1.70 %
Total Apoptotic:	2.18E+04	0.40 %

Flow cytometer analysis for estrogen positive MCF 7 cell line after 1nm melatonin induction for 24 hours

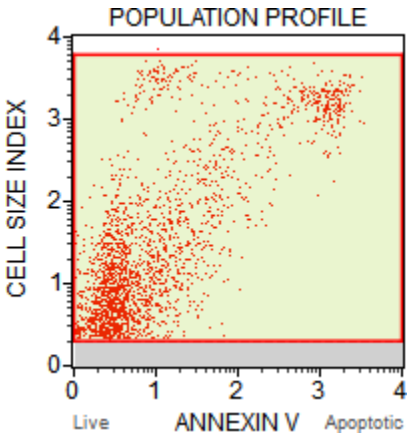


Figure 3 population profile MCF 7 after 1nm melatonin induction for 24 hours

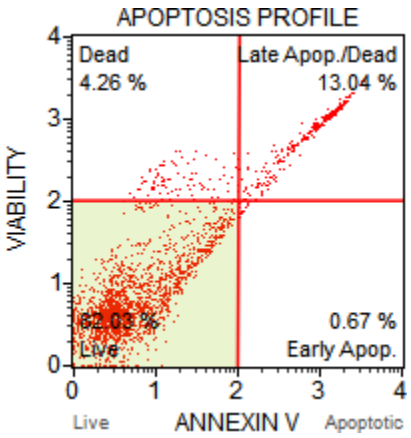


Figure 4 apoptosis profile MCF 7 after 1nm melatonin induction for 24 hours

Table 2 flowcytometer analysis for estrogen positive MCF 7 cell line after 1nm melatonin induction for 24 hours

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL):	3.45E+06	82.03 %
Early Apoptotic (LR):	2.80E+04	0.67 %
Late Apop. / Dead (UR):	5.48E+05	13.04 %
Debris (UL):	1.79E+05	4.26 %
Total Apoptotic:	5.76E+05	13.71 %

Flow cytometer analysis for estrogen positive MCF 7 cell line after 2 nm melatonin induction for 24 hours

Figure 6 population profile MCF 7 after 2 nm melatonin induction for 24 hours

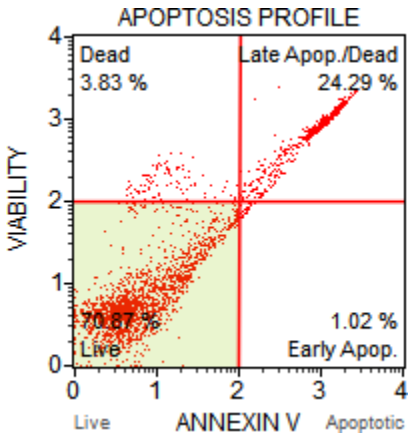


Figure 5 apoptosis profile MCF 7 after 1nm melatonin induction for 24 hours

Table 3 effect of 2 nm melatonin concentration on estrogen positive cell line

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	3.06E+06	70.87 %
Early Apoptotic (LR) :	4.38E+04	1.02 %
Late Apop./ Dead (UR) :	1.05E+06	24.29 %
Debris (UL) :	1.65E+05	3.83 %
Total Apoptotic :	1.09E+06	25.30 %

Flow cytometer analysis for estrogen positive MCF 7 cell line after 3 nm melatonin induction for 24 hours

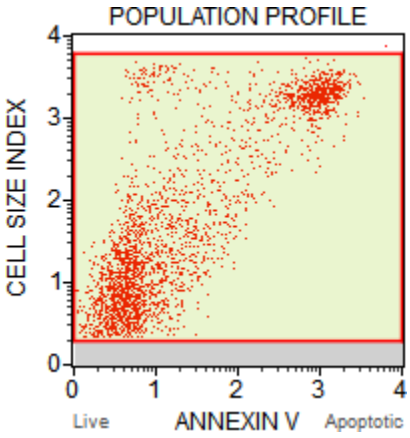


Figure 7

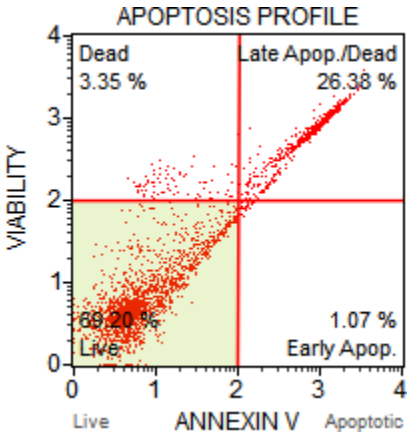


Figure 8

Table 4 effect of 3 nm melatonin concentration on estrogen positive cell line

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL):	2.83E+06	69.20 %
Early Apoptotic (LR):	4.36E+04	1.07 %
Late Apop. / Dead (UR):	1.08E+06	26.38 %
Debris (UL):	1.37E+05	3.35 %
Total Apoptotic:	1.12E+06	27.45 %

Effect of different melatonin concentration from 1 nm up to 3 nm on cell line MCF10 normal cell line

Flow cytometer analysis for MCF 10 cell line normal cell line without melatonin after 24 hours

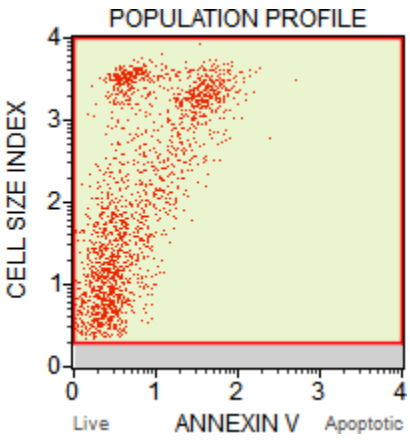


Figure 9 population profile MCF 10 controle without melatonin

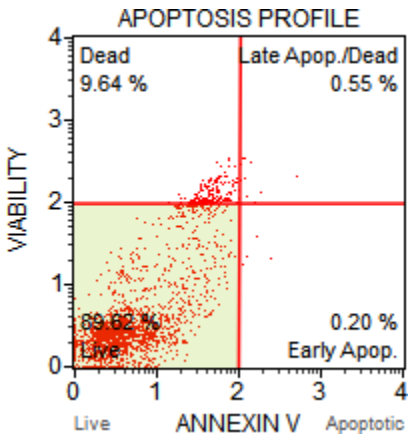


Figure 10 apoptosis profile MCF 10 controle without melatonin

Table 5 flowcytometer analysis for MCF 10 cell line normal cell line without melatonin after 24 hours

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL):	4.12E+06	89.62 %
Early Apoptotic (LR):	9.18E+03	0.20 %
Late Apop. / Dead (UR):	2.52E+04	0.55 %
Debris (UL):	4.43E+05	9.64 %
Total Apoptotic:	3.44E+04	0.75 %

Flow cytometer analysis for MCF 10 cell line after 1nm melatonin induction for 24 hours

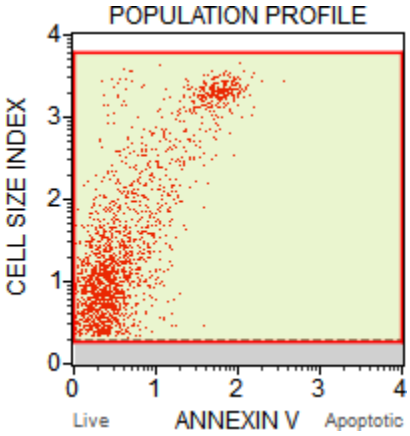


Figure 11 population profile MCF 10 after 1nm melatonin induction for 24 hours

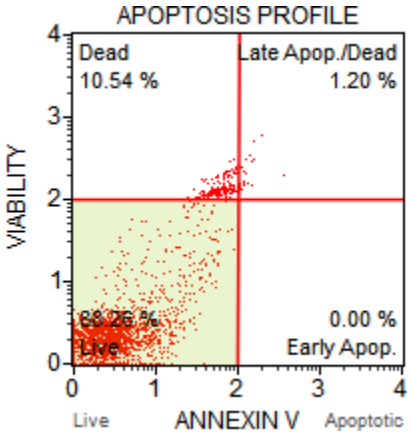


Figure 12 apoptosis profile MCF 10 after 1nm melatonin induction for 24 hours

Table 6 effect of 1 nm melatonin concentration on MCF 10 normal cell line

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL)	4.29E+06	88.26 %
Early Apoptotic (LR)	0.00E+00	0.00 %
Late Apop./ Dead (UR)	5.82E+04	1.20 %
Debris (UL)	5.12E+05	10.54 %
Total Apoptotic	5.82E+04	1.20 %

flowcytometer analysis for MCF 10 cell line after 2 nm melatonin induction for 24 hours

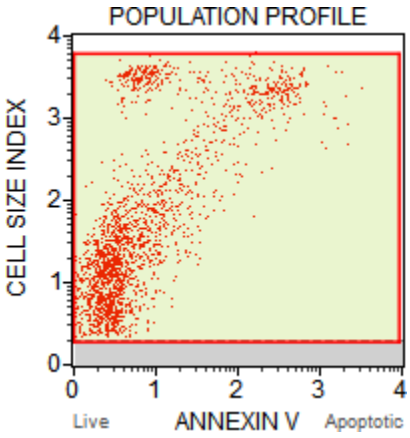


Figure 13 Figure 14 population profile MCF 10 after 2 nm melatonin induction for 24 hours

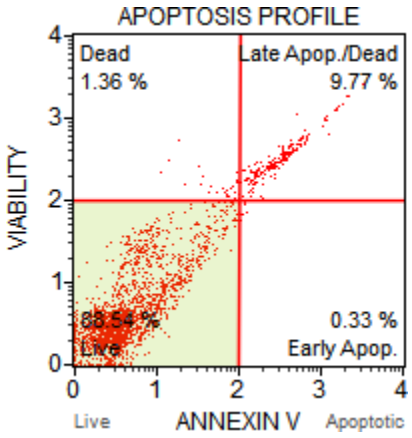


Figure 15 apoptosis profile MCF 10 after 2nm melatonin induction for 24 hours

Table 7 effect of 1 nm melatonin concentration on MCF 10 normal cell line

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL):	5.08E+06	88.54 %
Early Apoptotic (LR):	1.88E+04	0.33 %
Late Apop. / Dead (UR) :	5.60E+05	9.77 %
Debris (UL):	7.81E+04	1.36 %
Total Apoptotic:	5.79E+05	10.10 %

flowcytometer analysis for MCF 10 cell line after 3 nm melatonin induction for 24 hours

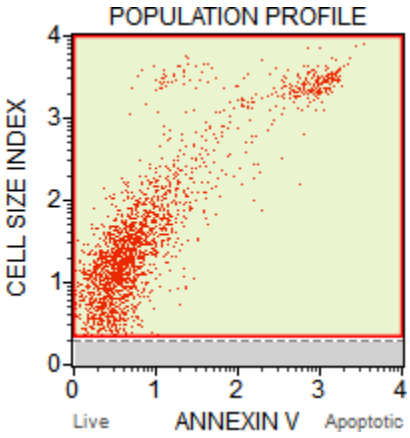


Figure 16 Figure 17 population profile MCF 10 after 3 nm melatonin induction for 24 hours

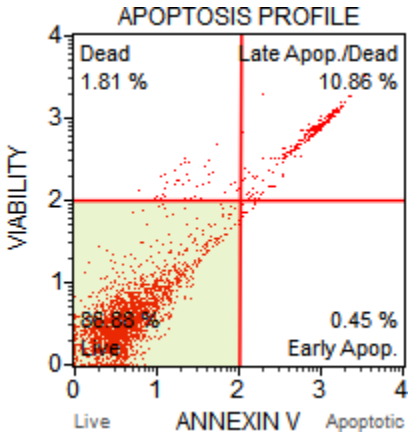
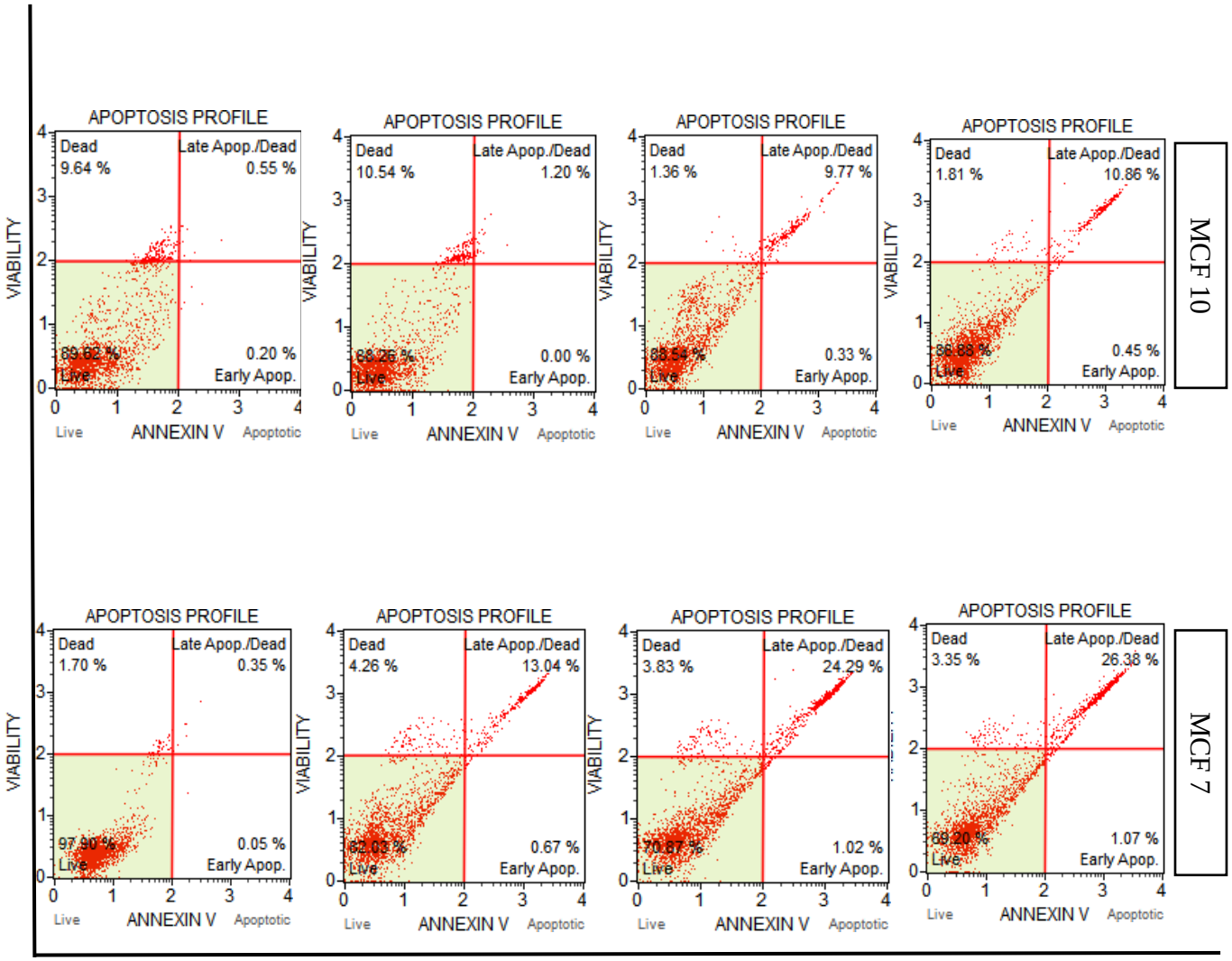


Figure 18 apoptosis profile MCF 10 after 3nm melatonin induction for 24 hours

Table 8 effect of 1 nm melatonin concentration on MCF 10 normal cell line

Cell's status	Cell Conc. (Cells / mL)	% Gated
Live (LL):	6.22E+06	86.88 %
Early Apoptotic (LR):	3.24E+04	0.45 %
Late Apop. / Dead (UR):	7.78E+05	10.86 %
Debris (UL):	1.30E+05	1.81 %
Total Apoptotic:	8.10E+05	11.31 %



Control without melatonin	1 nm Melatonin concentration	2 nm melatonin concentration	3 nm melatonin concentration

Figure 19 explain the melatonin induce MCF7 apoptosis but at the same time there is no significant effect on normal cell MCF10.

Table 9 Model Summary [b] effect of different melatonin concentration on MCF7 apoptosis

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.963[a]	.926	.890	4.13015

a. Predictors: (Constant), Melatonin concentration

b. Dependent Variable: MCF7 Apoptotic percentage

Table 10 ANOVA [b] effect of different melatonin concentration on MCF7 apoptosis

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	430.035	1	430.035	25.210	.037[a]
	Residual	34.116	2	17.058		
	Total	464.152	3			

a. Predictors: (Constant), Melatonin concentration

b. Dependent Variable: MCF7 Apoptotic percentage

Table 11 Coefficients[a] effect of different melatonin concentration on MCF7 apoptosis

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	2.804	3.456		.811	.502
	Melatonin concentration	9.274	1.847	.963	5.021	.037

a. Dependent Variable: MCF7 Apoptotic percentage

There was a significant effect of different melatonin concentration from 1 nm up to 3 nm on the live MCF 7 cell line estrogen positive ($p < 0.037$)

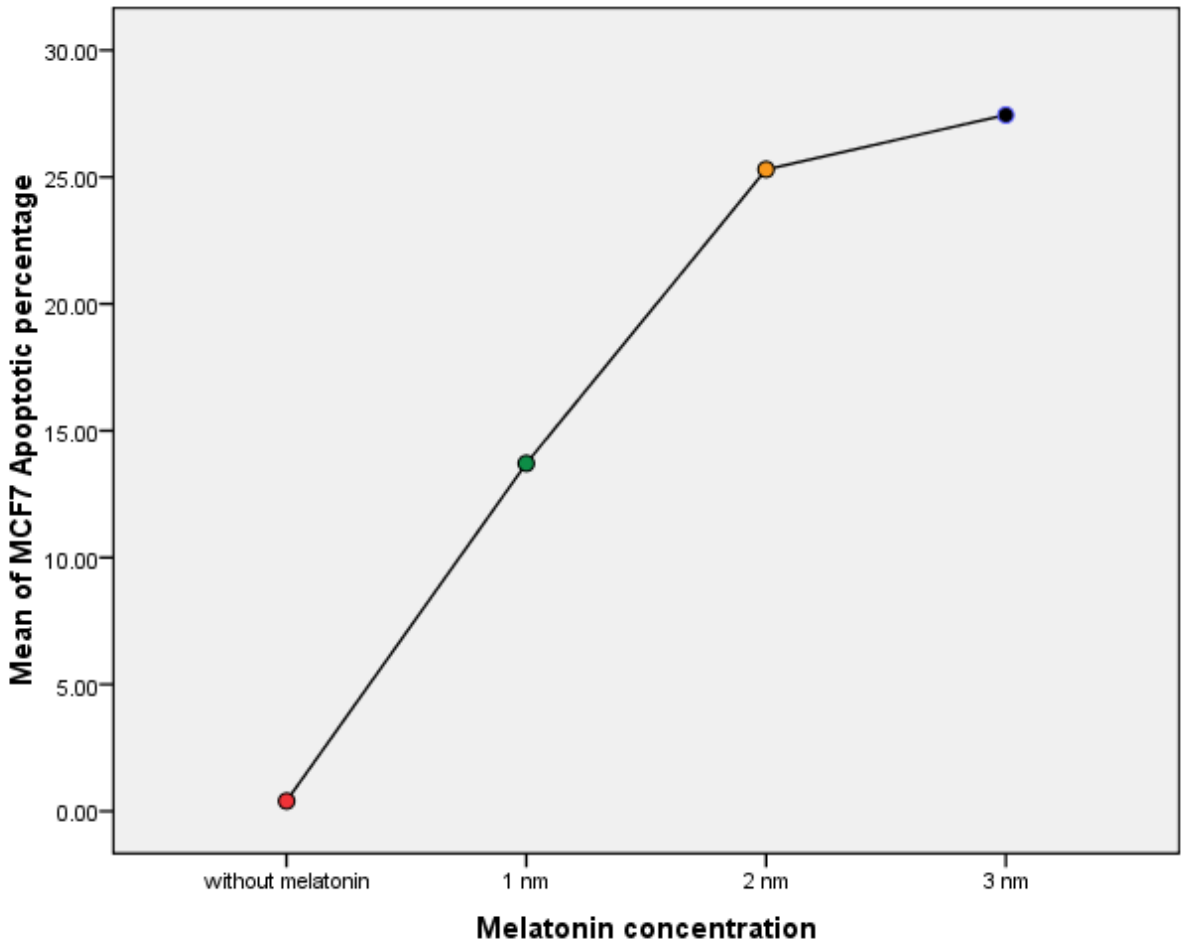


Figure 20 effect of different melatonin concentration on estrogen positive MCF7 cell line

Table 12 ANOVA[b] effect of melatonin on MCF10 normal cell line

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	82.337	1	82.337	12.504	.072[a]
	Residual	13.169	2	6.585		
	Total	95.506	3			

a. Predictors: (Constant), Melatonin concentration

b. Dependent Variable: MCF10 Apoptotic percentage

Table 13 Coefficients[a] effect of melatonin on MCF10 normal cell line

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	-.247	2.147		-.115	.919
	Melatonin concentration	4.058	1.148	.928	3.536	.072

a. Dependent Variable: MCF10 Apoptotic percentage

MCF 10 non-significant effect of melatonin on MCF10 normal cell line

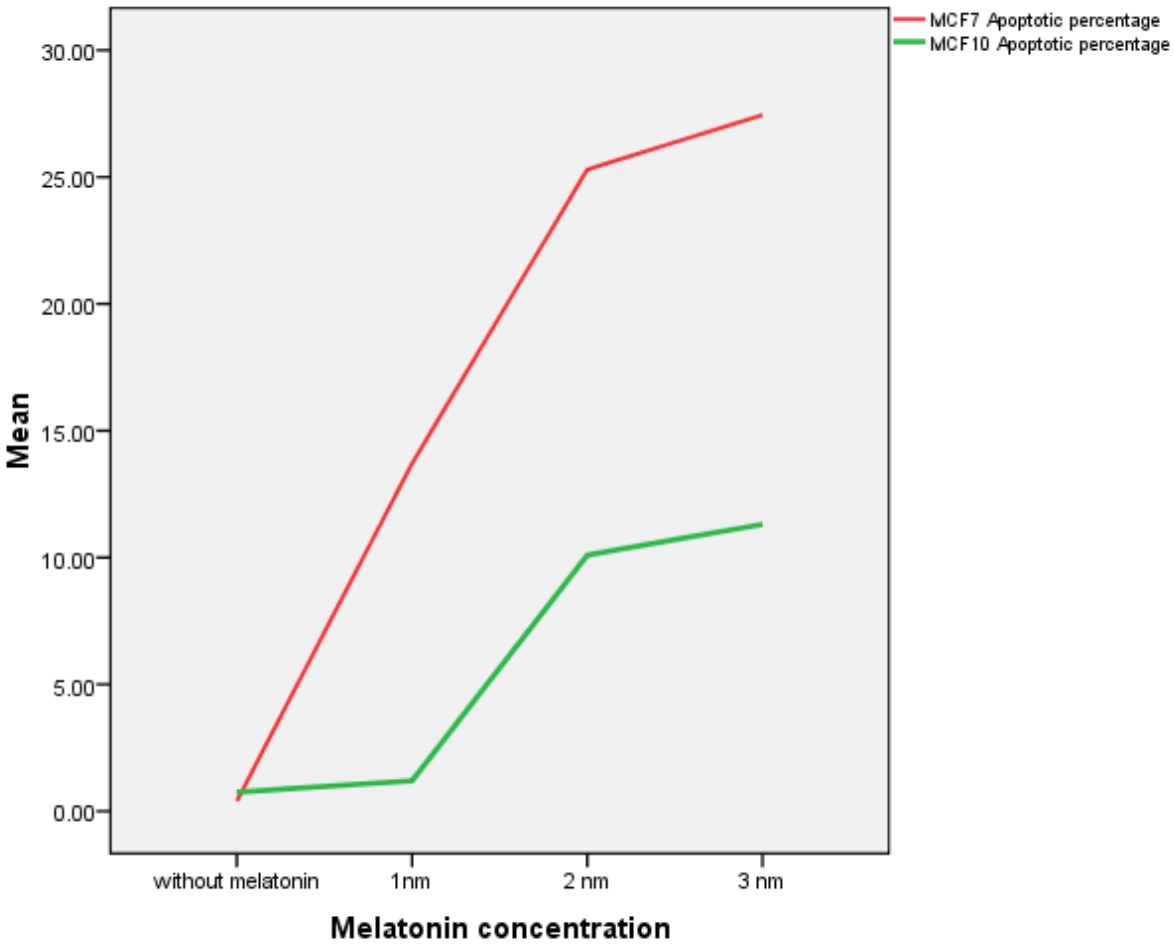


Figure 21 explain effect of melatonin on MCF7 as melatonin concentration increase the Apoptotic percentage increase at the same time in the same condition there was no significant apoptotic effect of melatonin on MCF10.

Effect of different melatonin concentration on MCF10 normal cell line P53 expression (Control)

Table 14 Descriptive different melatonin concentration on MCF10 normal cell line P53 expression (Control)

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
Control		3	-.7700	1.01158	.58404	-3.2829	1.7429	-1.88	.10	
1nm		3	-1.4867	2.37068	1.36871	-7.3758	4.4024	-4.16	.36	
2nm		3	.7300	1.18655	.68505	-2.2176	3.6776	.03	2.10	
3nm		3	5.6467	1.00059 E1	5.77690	-19.2093	30.5027	-.22	17.20	
4nm		3	-.5667	1.98165	1.14411	-5.4894	4.3560	-2.74	1.14	
Total		15	.7107	4.80507	1.24066	-1.9503	3.3716	-4.16	17.20	
Model	Fixed Effects			4.73489	1.22254	-2.0133	3.4347			
	Random Effects				1.28485	-2.8566	4.2780			.78111

Table 15 ANOVA different melatonin concentration on MCF10 normal cell line P53 expression (Control)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	99.050	4	24.763	1.105	0.406
Within Groups	224.192	10	22.419		
Total	323.242	14			

Study effect of different melatonin concentration on MCF7 apoptosis markers, tumor suppressor gene (P53) before and after MTNR1A plasmid transfection

Table 16 Descriptive (P53) before and after MTNR1A plasmid transfection

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
mRNA P53 expression MCF7 Before MTNR1A transfection	control	3	-1.2106E2	1.8347E2	1.05928E2	-5.7684E2	3.3471E2	-3.32E2	-1.10E0
	1nm	3	-9.2000E0	6.9950E0	4.03859E0	-2.6577E1	8.1767E0	-1.68E1	-3.07E0
	2nm	3	-7.4667E-1	9.3554E-1	.54013	-3.0707E0	1.5773E0	-1.81E0	-.05
	3nm	3	1.4900E0	3.4395E-1	.19858	6.3559E-1	2.3444E0	1.18	1.86
	4nm	3	3.3097E1	1.6510E1	9.53221E0	-7.9171E0	7.4110E1	1.74E1	5.03E1
	Total	15	-1.9285E1	8.8604E1	2.28774E1	-6.8352E1	2.9782E1	-3.32E2	5.03E1
mRNA P53 expression MCF7 After MTNR1A transfection	control	3	-2.2873E1	1.0176E1	5.87533E0	-4.8153E1	2.4062E0	-3.46E1	-1.64E1
	1nm	3	-2.6433E0	1.1213E0	.64736	-5.4287E0	1.4204E-1	-3.69E0	-1.46E0
	2nm	3	4.6667E-1	5.6862E-2	.03283	3.2541E-1	6.0792E-1	.42	.53
	3nm	3	4.9567E0	6.0623E0	3.50007E0	-1.0103E1	2.0016E1	.94	1.19E1
	4nm	3	1.2767E1	6.4147E0	3.70352E0	-3.1683E0	2.8702E1	7.84	2.00E1
	Total	15	-1.4653E0	1.3328E1	3.44140E0	-8.8464E0	5.9157E0	-3.46E1	2.00E1

Table 17 ANOVA(P53) before and after MTNR1A plasmid transfection

		Sum of Squares	df	Mean Square	F	Sig.
mRNA P53 expression MCF7 Before MTNR1A transfection	Between Groups	41938.937	4	10484.734	1.543	.263
	Within Groups	67969.830	10	6796.983		
	Total	109908.768	14			
mRNA P53 expression MCF7 After MTNR1A transfection	Between Groups	2121.644	4	530.411	14.514	.000
	Within Groups	365.437	10	36.544		
	Total	2487.081	14			

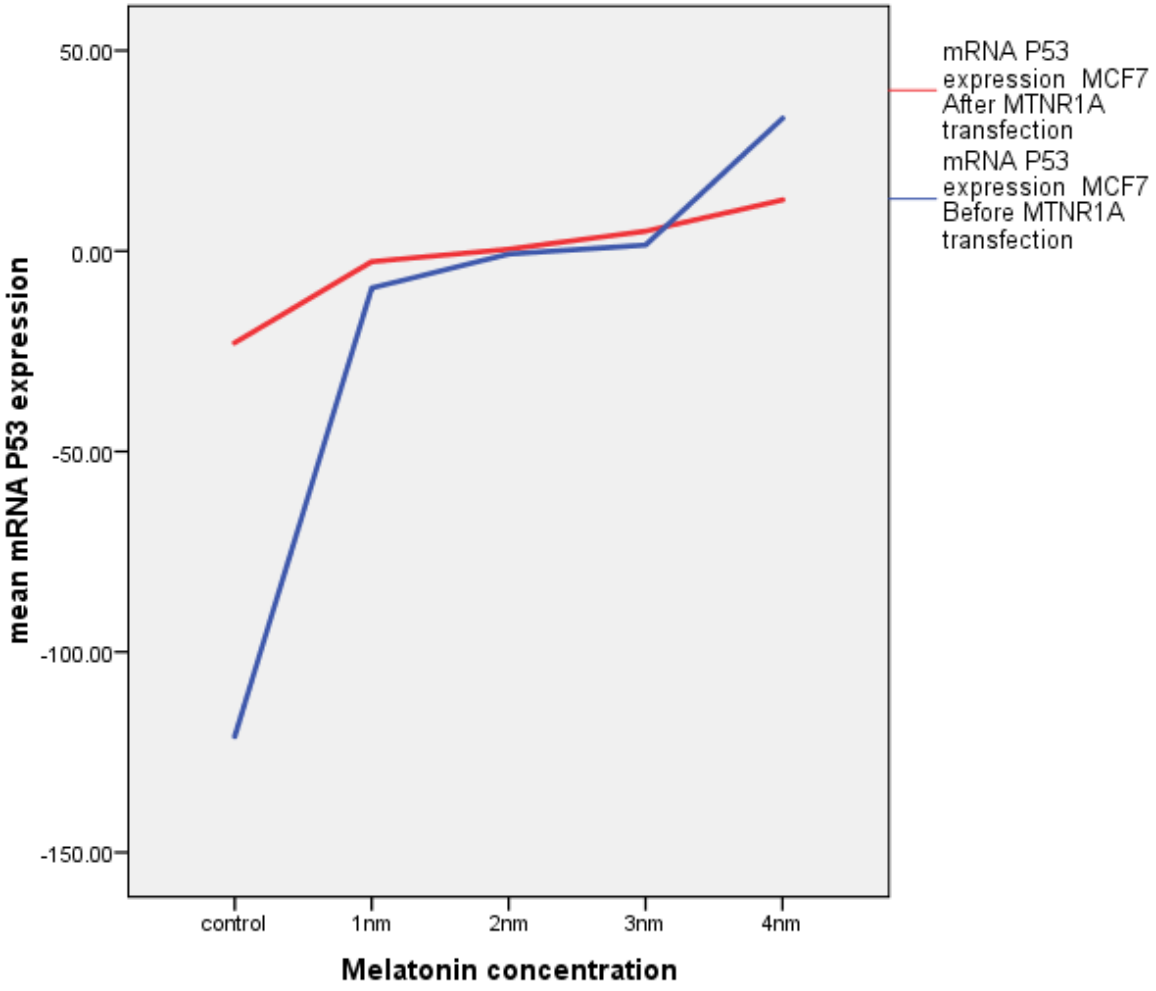


Figure 22 ***The blue one explains the effect of melatonin concentration on cell line MCF7 P53 expression before melatonin receptor M1 upregulation was only significant at melatonin concentration 4 nm. the red one explain the effect of melatonin concentration on cell line MCF7 P53 expression after melatonin receptor M1 upregulation was significant at all melatonin concentration from 1 nm up to 4 nm because melatonin signals induce by both melatonin concentration and melatonin receptors upregulation.***

Table 18 Independent Samples Test comparison between mRNA MTNR1A expression Normal cell line MCF10 VS cancer cell line MCF7 estrogen positive

Group Statistics

	MCF7 VS MCF10	N	Mean	Std. Deviation	Std. Error Mean
mRNA MTNR1A Expression	MCF7 Cell line	3	-6.5133	5.77448	3.33390
	Normal MCF10 Cell line	3	6.1367	5.89542	3.40373

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
mRNA MTNR1A Expression	Equal variances assumed	.058	.821	-2.655	4	.050	-1.26500E1	4.76448E0	-2.58783E1	.57830
	Equal variances not assumed			-2.655	3.998	.050	-1.26500E1	4.76448E0	-2.58805E1	.58054

There was a significant difference in the melatonin receptor MTNR1A expression (M=-6.5133, SD=5.77448) and patient with breast cancer (M=6.1367, SD=5.89542) conditions; t (4) = -2.655, p = 0.050"

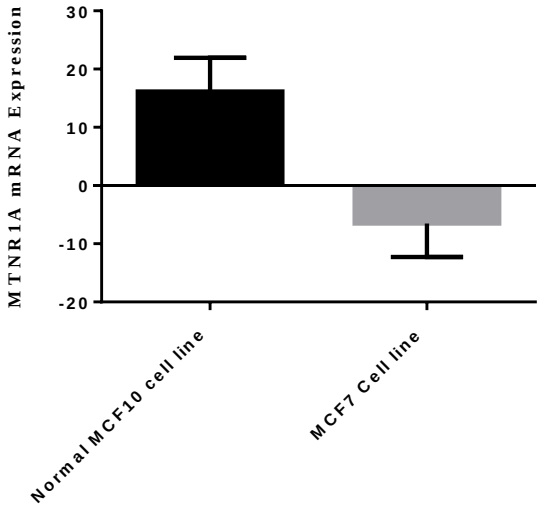


Figure 23 Independent Samples Test comparison between mRNA MTNR1A expression Normal cell line MCF10 VS cancer cell line MCF7 estrogen positive

MTNR1A expression in normal subject compared to patient with breast cancer.

Table 19 ANOVA mRNA MTNR1A Expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1337.034	7	191.005	3.393	.006
Within Groups	2251.806	40	56.295		
Total	3588.841	47			

*There is low MTNR1A melatonin receptor expression in patient with breast cancer compared to normal P < than 0.05 = 0.006**

At the same sample in table 15 we are going to measure clock gene period 1 expression in normal subject compared to patient with breast cancer.

Table 20 ANOVA mRNA Period1 Clock gene Expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1653.349	7	236.193	2.506	.031
Within Groups	3770.705	40	94.268		
Total	5424.054	47			

*There is low clock gene period 1 melatonin receptor expression in patient with breast cancer compared to normal P < than 0.05 = 0.031**

Melatonin receptor M1 encoding via MTNR1A clock gene period 1 expression in normal and patient with breast cancer in different stages correlation.

Table 21 ***Liner regression Model Summary*** *MTNR1A expression and clock gene period1 expression*

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.598[a]	.358	.344	8.70277

a. Predictors: (Constant), mRNA MTNR1A Expression

Table 22 Liner regression ANOVA[b]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1940.094	1	1940.094	25.616	.000[a]
	Residual	3483.960	46	75.738		
	Total	5424.054	47			

a. Predictors: (Constant), mRNA MTNR1A Expression

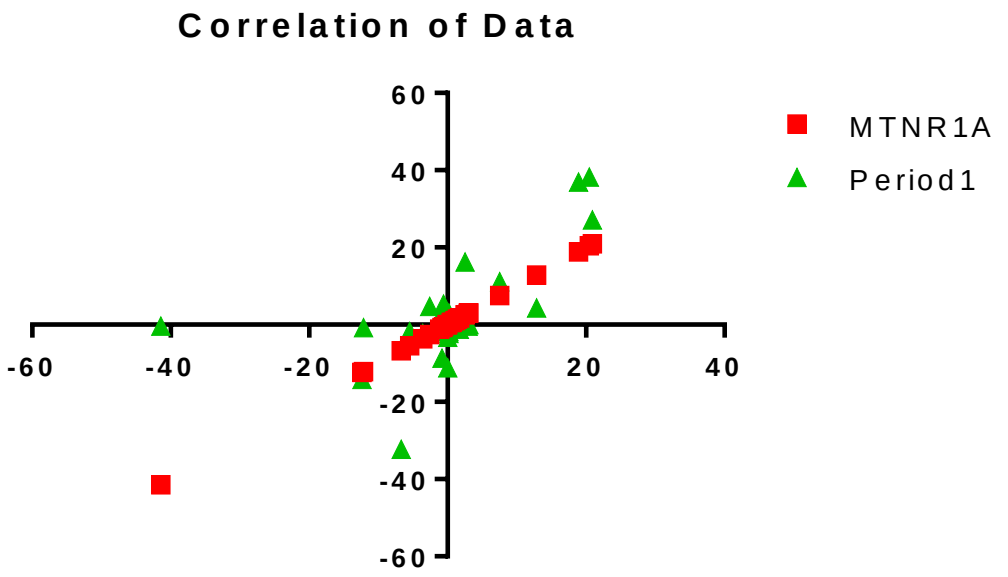
b. Dependent Variable: mRNA Period1 Clock gene Expression

Table 23 Liner regression Coefficients[a] MTNR1A expression and clock gene period1 expression

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	1.339	1.257		1.066	.292
	mRNA MTNR1A Expression	.735	.145	.598	5.061	.000

a. Dependent Variable: mRNA Period1 Clock gene Expression

There is strong correlation between melatonin receptor MTNR1A expression and clock gene period1 expression P < than 0.05 = 0.000 expression of clock gene period 1 depend on MTNR1A expression*



Discussion

From the epidemiological research, it was postulated that Melatonin has significant apoptotic, angiogenic, oncostatic and anti-proliferative effects on various oncological cells but the mechanism of action, how melatonin is anti-cancer still unknown and under study.

Since the physiological role of melatonin is to provide cell information about circadian rhythm, we are going to study relation between melatonin, clock gene (period1) and P53, mRNA expression in order to understand pathogeneses of breast cancer related to melatonin dysfunction. according to current study we found that there is linear relation between melatonin, P53 and period 1 expression. That means the melatonin prevents breast cancer formation via upregulation period 1 and P53 to induce cell cancer apoptosis. One more thing is the melatonin action in prevention and killing breast cancer is unique action, (is selective action) because under the same experimental conditions of the breast cancer cell MCF7 there was no apoptosis effect on normal cell (cell line MCF10). Conclusion Any condition decreases the Melatonin receptors or melatonin hormone is risk for breast cancer formation development.

References

Barabutis, N., Tsellou, E., Schally, A. V., Kouloheri, S., Kalofoutis, A., & Kiaris, H. (2007). Stimulation of proliferation of MCF-7 breast cancer cells by a transfected splice variant of growth hormone-releasing hormone receptor. *Proceedings of the National Academy of Sciences, 104*(13), 5575. doi: 10.1073/pnas.0700407104

Bhattacharya, S., Patel, K. K., Dehari, D., Agrawal, A. K., & Singh, S. (2019). Melatonin and its ubiquitous anticancer effects. *Molecular and cellular biochemistry, 462*(1-2), 133-155.

Comşa, Ş., Ciuculescu, F., & Raica, M. (2012). Mesenchymal stem cell-tumor cell cooperation in breast cancer vasculogenesis. *Molecular medicine reports*, *5*(5), 1175-1180.

Li, Z., Zhao, J., Liu, H., Wang, J., & Lu, W. (2020). Melatonin inhibits apoptosis in mouse Leydig cells via the retinoic acid-related orphan nuclear receptor α/p53 pathway. *Life sciences, 246*, 117431. doi: https://doi.org/10.1016/j.lfs.2020.117431

Mediavilla, M. D., Cos, S., & Sánchez-Barceló, E. J. (1999). Melatonin increases p53 and p21WAF1 expression in MCF-7 human breast cancer cells in vitro. *Life Sci, 65*(4), 415-420. doi: 10.1016/s0024-3205(99)00262-3

Miki, T., Matsumoto, T., Zhao, Z., & Lee, C. C. (2013). p53 regulates Period2 expression and the circadian clock. *Nature communications*, *4*, 2444.

Proietti, S., Cucina, A., D'Anselmi, F., Dinicola, S., Pasqualato, A., Lisi, E., & Bizzarri, M. (2011). Melatonin and vitamin D3 synergistically down-regulate Akt and MDM2 leading to TGFβ-1-dependent growth inhibition of breast cancer cells. *Journal of pineal research, 50*, 150-158. doi: 10.1111/j.1600-079X.2010.00824.x

Proietti, S., Cucina, A., Dobrowolny, G., D'Anselmi, F., Dinicola, S., Masiello, M. G., . . . Bizzarri, M. (2014). Melatonin down-regulates MDM2 gene expression and enhances p53 acetylation in MCF-7 cells. *J Pineal Res, 57*(1), 120-129. doi: 10.1111/jpi.12150

Purvis, J. E., Karhohs, K. W., Mock, C., Batchelor, E., Loewer, A., & Lahav, G. (2012). p53 dynamics control cell fate. *Science, 336*(6087), 1440-1444.

Ryan, K. M., Phillips, A. C., & Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. *Current opinion in cell biology, 13*(3), 332-337.

Santoro, R., Marani, M., Blandino, G., Muti, P., & Strano, S. (2012). Melatonin triggers p53Ser phosphorylation and prevents DNA damage accumulation. *Oncogene, 31*(24), 2931-2942. doi: 10.1038/onc.2011.469

Zakki, S. A., Muhammad, J. S., Li, J.-L., Sun, L., Li, M.-L., Feng, Q., . . . Inadera, H. (2020). Melatonin triggers the anticancer potential of Phenylarsine Oxide via induction of apoptosis through ROS generation and JNK activation. *Metallomics*.