A DISSERTATION ON

Role of Bisphenol A indiuced Lipogenesis in Breast Cancer cells

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DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING
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BY
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UNDER THE SUPERVISION OF
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DECLARATION FORM

I, **Sadiya Zafar**, a student of **B.Tech.-M.Tech. Dual Degree Biotechnology** (5 Year/ X Semester), Integral University have completed my six months dissertation work entitled **"Role of Bisphenol A Induced Lipogenesis in Breast Cancer cells"** successfully from **CSIR-Indian Institute of Toxicology Research,Lucknow-226001** under the able guidance of **Dr. Pradeep Kumar Sharma.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Sadiya Zafar

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हम सभी **सुश्री सदिया जफर** के उज्जवल भविष्य की कामना करते है।

To Whomsoever it May Concern

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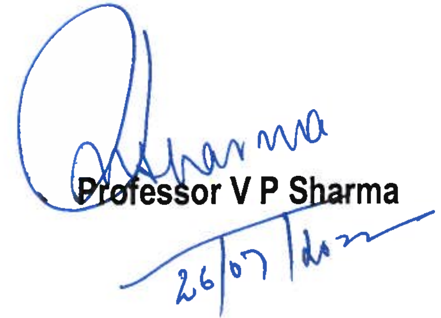
We wish **Ms Sadiya Zafar** success in her future endeavours.



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Certificate that Ms Sadiya Zafar (Enrollment Number 1700100128) has carried out the research work presented in this thesis entitled **"Role of Bisphenol A induced Lipogenesis in Breast cancer cells"** for the award of B.Tech.**-M.Tech. Dual Degree Biotechnology** from Integral University, Lucknow under my supervision. The thesis embodies results of original work and studies carried out by the student himself/herself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **B.Tech.-M.Tech. Dual Degree Biotechnology.**

I wish her good luck and bright future.

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I wish her good luck and bright future.

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I wish her good luck and bright future.

Dr. Alvina Farooqui
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All praise for the Almighty, Who has blessed me with more than I deserve.
My Graduation journey has come to an end, but I was lucky enough for it to end on an extremely happy note with my unforgettable dissertation tenure. I was able to bring this project to completion successfully because of the contribution of many special people.

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LIST OF ABBREVIATIONS

S.No	Abbreviation	Meaning
1	µl	Micro litre
2	µg	Microgram
3	ml	Millilitre
4	µM	Micromole
5	mM	Milli mole
6	nM	Nano mole
7	BPA	Bisphenol A
8	EDC	Endocrine Disrupting Chemical
9	PBS	Phosphate-buffered saline
10	TBS-T	Tris-Buffer Saline Tween 20
11	DMSO	Dimethyl Sulfoxide
12	DMEM	Dulbecco's Modified Eagle Medium
13	°C	Degree Centigrade
14	ERα	Estrogen Receptor α
15	ERβ	Estrogen Receptor β
16	PCNA	Proliferating Cell Nuclear Antigen
17	CERBPα	CCAAT/enhancer-binding proteins
18	ACC	Acetyl-CoA Carboxylase
19	PPARγ	Peroxisome proliferator-activated receptor γ
20	FAS	Fatty Acid Synthase
21	SREBP1	Sterol regulatory element-binding transcription factor 1
22	KLFs	Kruppel like transcriptional factors

23	PREF-1	Preadipocyte factor 1
24	SIRT-1	sirtuin (silent mating type information regulation 2 homolog) 1
25	TAZ	Tafazzin
26	Wnt	Wingless-related integration site".
27	FoxA	Forkhead Box A
28	EMT	Epithelial to Mesenchymal Transition
29	Bcl-2	B-cell lymphoma 2
30	FGF-2	fibroblast growth factor 2
31	ECL	Enhanced chemiluminescence
32	PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
33	RT-PCR	Reverse Transcription–Polymerase chain reaction
34	PFA	Paraformaldehyde
35	ANOVA	Analysis of variance
36	mRNA	Messenger Ribonucleic acid
37	IL-6	Interleukin 6
38	αSMA	Alpha Smooth Muscle Actin
39	HER-2	Human epidermal growth factor receptor 2.
40	PTEN	Phosphatase and TENsin homolog deleted on chromosome 10
41	DAPI	4',6-diamidino-2-phenylindole
42	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
43	SYBR	N, N-dimethyl-N'-[4-[(E)-(3-methyl-1, 3-benzothiazol-2-ylidene) methyl]-1-phenylquinolin-1-ium-2-yl]-N'-propylpropane-1, 3-diamine.
44	cDNA	Complementary Deoxy Ribonucleic acid
45	FBS	Fetal bovine serum

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1. INTRODUCTION:

Cancer is one of the most common disease in the world increasing worldwide. Cancer also attributes around 70% of deaths in the developing countries of the world. Among various cancers, the most prevalent type of cancer in women is Breast cancer. According to the epidemiology data available, about 2.3 million women were diagnosed with breast cancer in 2020 out of which 685,000 women were reported to be dead around the world. By the end of 2020, the total number of alive women with breast cancer were 7.8 million, making it the most common cancer in the world (Sung, H., et *al.,* 2021).

In cancer there is an uncontrolled growth of a particular type body cells which can also spread to other body parts. Likewise in breast cells out of control growth results in the formation of tumour. Cancer of Breasts is of metastatic type so most of the time it spreads to other distant organs like bones, liver, lungs and brain, which why it is often considered as incurable (Sun, Y. S *et al.,* 2017). One important thing to consider is that not all tumours of breast are cancerous, some of them could be non-cancerous or benign and they don't spread to other body parts. Tumour growth can start from any part of the breasts .Humans breasts has the following parts from where cancer can originate:

- Glands that makes milk are the lobules and cancer that occurs here is called as lobular cancer.
- The small tubules that carries milk from the lobules to the nipple are known as ducts. It's the most common site for cancer origination and the cancer is known as ductal cancer.
- Surrounding the ducts and lobes, there is fat and stroma or connective tissue in a breast which helps to hold the ducts and lobes in place. A rare type of cancers can occur here in the stroma of the breasts called Phyllodes.
- Apart from the above mentioned parts, there are also blood and lymph vessels present in a breast and a type of cancer known as Angiosarcoma can start from the linings of these vessels (Sharma G.N. *et al.,* 2010).

The stroma which supports the ducts and lobules in a breast is mainly composed of adipose tissues and lipid molecules. These adipose tissues are the main source of energy in breast cancer cells. As these adipocytes/lipids are a rich source of energy they also help

in the invasion and progression of Breast cancer by releasing different types of proteins which helps the cancer cells to survive (Kothari C *et al.,* 2020).From this it evident that obesity is one of the risk factors of breast cancer. Other factors include lifestyle changes like smoking, alcohol consumption, unbalanced diet, physical inactivity and environmental factors like pollution, hormonal and reproductive factors, different types of drugs, exposure to different radiations, etc. As Breast cancer is a hormone- dependent cancer factors like endocrine disrupting chemicals or EDCs play a major role in its aggressiveness. EDCs includes chemicals like Phthalates, Cadmium and Bisphenols (A, S, F). In various studies, it was found that these chemicals induce different pathways of lipogenesis, thus contributing to the aggressiveness of the tumour cells.

2. REVIEW OF LITERATURE:

When changes in certain genes disrupts the apoptosis process, it starts the development of cancer. The alteration in the normal cell division occurs due to genetic mutation in several groups of genes like oncogenes,tumor suppressor genes ,genes of apoptosis and DNA repairement genes (Williams JL ,2002) .Cancer cells has the power to proliferate and invade to other body cells. This is also the case with breast cancer. Several studies and clinical data suggested several hormones like estrogen, progesterone, and prolactin contributes to the progression and initiation of breast cancer. The reason behind this is that breasts are hormone dependent organs. Their development and growth is mainly controlled by hormones at different stages of life .Thus, these hormones is also linked with the tumour progressions in the breast (Clemons M, Goss P., 2001).

Based on the type of hormone responsible for breast cancer it can be divide into the following type ;Estrogen-receptor positive breast cancer ,Estrogen-receptor negative breast cancer ,Progesterone –receptor positive breast cancer ,Human epidermal growth factor 2 (HER 2) positive breast cancer and triple negative breast cancer cells (Lumachi F. *et al.,*2013)

Table 1: Different cell lines of breast cancer

Breast cancer	**Hormone Involved**	**Cell line**	**References**
ER positive (80%)	Estrogen	ZR-75-1 , BT-474 ,MCF-7	Abubakar M. *et al,*2018
EGFR or HER- 2 positive (20 %)	Human Epidermal growth factor	MDA-MB-468 and MDA-MB-231	Alkabban FM and Ferguson T,2021
PR positive (60 %)	Progesterone	Evsa-T,	Borras, M.,*et al.,1997*

Triple negative (10-20 %)	None	Hs 578T, MDA-MB-157	Chavez, K. J *et al.*,2010

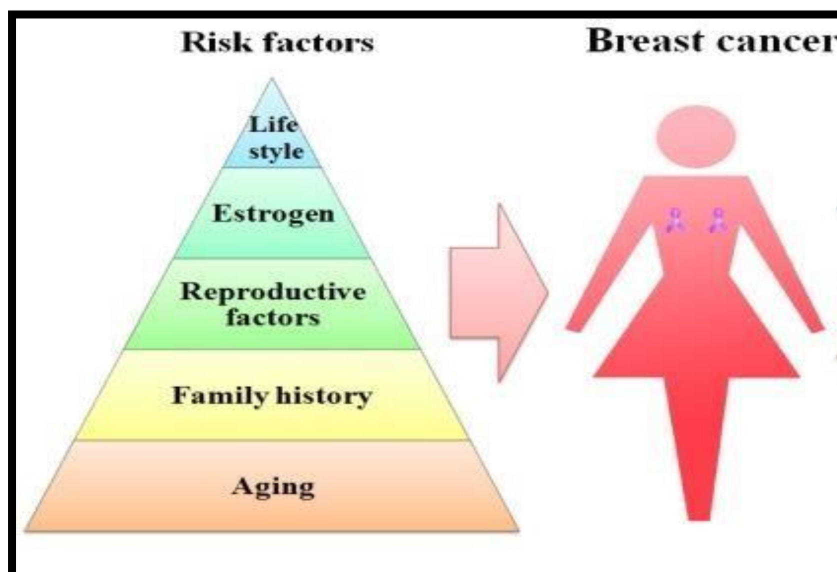


Figure 1: Risk factors of breast cancer.(Adopted from Sun, Y. S *et al.*,2010)

2.1Bisphenol A an Endocrine Disrupting Chemical:

From the above data, it is evident the most common type of breast cancer is ER positive. This is because of the presence of different types of Endocrine disrupting chemicals or EDCs in the environment and gets very easily accumulated in our body which show estrogenic properties and induce progression of breast cancer. One such chemical is Bisphenol A or BPA. It is a synthetic compound which is based on carbon and is used in the production of several plastic products. It shows hormone (estrogenic) properties and can be found almost anywhere in the environment. It interacts with estrogen receptors ERα and ERβ, and brings changes in the proliferation of cells, its apoptosis and also its migration properties, thus increasing the cancer development and progression (Wang, Z. *et al.,* 2016)

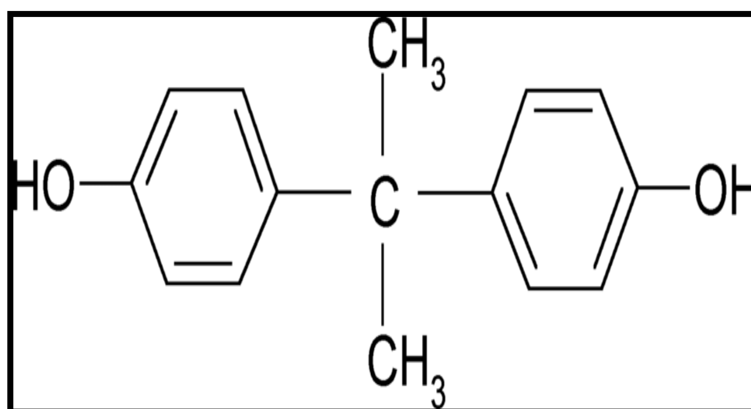


Figure 2: Molecular structure of BPA (Pubchem, CID)

There are various ways by which a human can be exposed to BPA like by ingesting, through mother to foetus, by inhaling it, contact with skin and eyes. Once it enters the human body it causes harm to several tissues and organs like liver,hearth,adipose tissue in breasts ,thyroid etc. It is released by plastics used in foods and drinks either in the form of crystals or powder mostly on heating. Plastics when heated to high temperatures enhances the transfer of BPA to human body .Additionally, polymeric materials when comes in the contact of acidic or basic substances also releases BPA (Gao, H., *et al.,* 2015). BPA also regulates different processes like growth and development of various tissues in the similar fashion of estrogen .For its activation BPA binds to the most common estrogen receptors i.e. ERα and ERβ. In the year 1993 the estrogenic property of BPA was found during a study of finding a estrogen binding protein yeast. The BPA molecule has such a structure that it shows affinity to binds to estrogen receptors (ERα and ERβ). However, the binding ability of BPA is around 1000 to 10000 times less than the estrogen to the receptors ERα and ERβ as BPA has weaker van der Waals attraction towards the estrogen receptors. (Lazúrová, Z., & Lazúrová, I. ,2013).

In another study it was found that BPA shows more estrogenic activity at Nano molar doses than at micro molar. Several in-vitro demonstrations has revealed that like estrogen, BPA also affects the gene expression of several adipogenic and lipogenic markers.It has been seen that it decreases the secreation of adiponectin from adipose cells in humans. As lipogenesisis one the main mechanism behind breast cancer agressivemess, BPA also plays a major role in cancer cells migration and invasion (Desai, M.*et al.,* 2018).

In one study it has been found that BPA imparts chemoresristance to a number of anti-cancer drugs. It is possible as BPA enhances the Bcl-2 expression which is an anti-apoptotic protein. It has also been seen that BPA also effects the ER negative cell line so it possibly also interacts with receptors other than ER (Lapensee, E. W.,*et al.,*2009).

In one study, the effect of BPA on epithelial mesenchymal transition or EMT was examined using a clonal variant MCF-7 CV which also expresses estrogen receptors. It was found that BPA indeed has effects on EMT markers .It up regulates the expression of marker N- cadherin while down regulating E- cadherin protein expressions (Kim, J. Y.,*et al.,*2017).

2.2.Role of Lipogenesis in Breast Cancer:

Adipose tissues ia an endocrine organs and secreate many hornones and growth regulators, as a result it plays a major role in obesity and breast cancer. As we know the stroma of female breasts is consists of these adipocytes and which are mainly lipid molecules and has a great impact in the growth and development of breasts. The process in which the cells loaded with fats known as adipocytes, first develops and then gets accumulated as adipose tissues at various places in a body is called as Adipogenesis. Adipogensis requires the activation of various transcriptional genes (Kothari, C.*et al.,* 2020).

There is a slightly different type of adipocytes present beside the breast cancer cells known as Cancer-associated adipocytes or CAAs which helps in the invasion of cancer cells. It has more aggressive properties than normal adipocytes and helps in the cross talk between adipocytes and breast tumour cells. It releases many chemicals called as adipokines like leptin, adiponectin, several inflammatory factors and many more which has essential roles in the proliferation, angiogenesis, invasion and metastasis of Breast cancer. They all are a part of the breast tumour microenvironment (Rybinska, I.,*et al.,*2021)

Numerous studies have concluded that person with breast cancer have high levels of IL-6 and fibroblast growth factor -2 which results in the resistance to endothelial growth factor therapies.FGF-2 reported to activate ER even when there are no estrogen ligands present which leads to the tomoxifen resistance. Different studies have reported that

leptin, IL-6 and IL-8 factors that are released by adipose tissues assists in the migration and invasion of breast cancer cells (Bernard, J. J., and Wellberg, E. A.,2021).

It has been seen through many studies that adipocytes participates in EMT in many types of cancers. One such study was done on breast cancer cell lines MCF-7, MDA-MB-453, MDA-MB-435S, MDA-MB-231, and MDA-MB-468 to investigate the effect of adipocytes on EMT.They first cultured these cells lines with different adipocytes, then the analysis of changes in morphology, proliferation, EMT markers, migration and invasion was done. They also checked several human cancer specimens for the presence of estrogen and progesterone receptors, HER-2 .They found out that adipocytes induces progression of Breast cancer and enhances its progression. Several EMT markers were found to be up regulated too (Lee, Y., *et al.,*2015).

There are many transcription factors in lipogenesi samong them, PPARγ is the most important one .There is one other transcriptional factor known as C/EBPα ,both these factors works in a positive collaboration and is responsible for increasing expression of several other lipogenesis genes .C/EBPs other than C/EBPα are involved in pre-adipogenesis. SREBP1, KLFs, GATA2/3, PREF-1, SIRT-1, TAZ, Wnt, FoxA are some other adipogenic genes which regulates the PPARγ expressions through various pathways. It has been revealed that PPARγ expression does not initiate cancer in normal breast cells but increases the proliferation rate of tumour cells in Breast cancer (Simeone, P.,*et al.,*2021).

Table 2: Genes/Transcriptional factors involved in Lipogenesis

Gene	**Mechanism**	**Role in Breast cancer**	**Reference**
PPARγ	Master regulater;responsible for terminal differentiation of adipocytes	In normal cells acts as a tumour suppressor while in cancer cells its up regulation will enhance proliferation of cells	Kothari, C.,*et al.,*2020

C/EBPα	Main regulator in pre-lipogenesis; also regulates the expression of PPARγ	In cancer cells targets the hormonal genes and regulates the and takes part in the cell migration and invasion	Nacht, A. S.,*et al.,2019*
Adiponectin	Elevates the differentiation of pre-adipocytes into mature adipocytes; has anti-inflammatory properties	It's up regulation in the cancer cells will reduce cell proliferation ,migration and invasion	Chu, D. T.,*et al.,*2019
Leptin	Has a role in angiogenesis	Promotes cancer cell growth	Chu, D. T.,*et al.,*2019
FAS	Activates the inflammatory pathways by inducing several cytokines like IL-1α, IL-1β, IL-6, and IL-8 leading to obesity; FAS regulates de novo lipogenesis from acetyl-CoA, malonyl-CoA, and NADPH and is expressed at high levels in adipose tissue, liver, and lung.	Up regulation in cancer cells leads to their high proliferation rate	Wueest, S.,*et al.,2010* Ranganathan, G.,*et al.,*2006
ACC	Catalyses the synthesis of Fatty acids in adipogenesis; regulates the rate of	Up regulation to satisfy the high energy demand of tumor cells; leads to decrease sensitivity of	Mentoor, I.,*et al.,*2018

	carboxylation of acetyl-CoA into malonyl-CoA.	tumor cells for chemo and radiation therapy	Fullerton, M. D.,*et al.,*2013

2. 3. Role of BPA in inducing Lipogenesis:

Peroxisome proliferator-activated receptor-γ (PPARγ) activation has been a new approach to cancer therapy. In one of the study, they investigated the effects of two structurally different PPARγ agonists, rosiglitazone and KR-62980 on MCF-7 breast cancer cells. Both agonists inhibited the cell proliferation and colony formation via apoptosis. PTEN expression was increased with decreased Akt phosphorylation by the agonists, whereas agonists actions were abolished in PTEN knockdown cells, indicating the critical role of PTEN in the anti-proliferative effects of PPARγ activation.
Rosiglitazone induced the MCF-7 cell differentiation but KR-62980 did not alter the differentiation pattern with little effects on the lipid accumulation and the expression of lipogenesis markers. These results suggest that PPARγ activation may result in the inhibition of cell proliferation and/or induction of cell differentiation depending on the type of PPARγ agonists, and that KR-62980 may be useful in breast cancer therapy by inducing apoptosis (Kim, K. Y., *et al.,* 2006).

In one study it has been demonstrated that the differentiation of pre-adipocytes is induces by BPA even in the absence of glucocorticoid. It was revealed that despite the lack of GR inhibitor BPA causes increment in the fat disposition, mRNA expression and expression of adipogenic genes like lipoprotein marker and PPARγ. It was also known that BPA activates the C/EBPα which leads to the upregualtion of PPARγ transcriptional factor.Though,estradiol does not have any effect on lipogenesis but estrogen inhibitors decreases the lipogenesis induced by BPA.This suggests that BPA works through non-classical ER pathways (Boucher, J. G.,*et al.,*2014).

In another study 3T3-L1 cells were treated with BPA and the results showed that BPA can activate PPARγ using its response element by around 1.5 fold. It was reveals that BPA enhances differentiation of adipocytes in a dose dependent manner and is required for inducing adipogenesis (Ahmed, S., and Atlas, E., 2016). Similarly BPA found to be

upregualting many of the adipogenic genes like FAS, ACC, IL-6, and IL-8 which are all involved in the cancer progression of breast cells too.

BPA gets inside the adipose tissue because of its lipophilic nature and then it promotes the formation of Tumor micro environment. BPA has been found to start signalling in inflammatory cytokines of adipocytes and also the enhances the tolerance towards glucose. BPA can also take part in the pro-inflammatory and pro-tumorigenic microenvironment as it induces adiposity by doing modifications in epigenetics of some important genes related to adipogenesis and lipogenesis. (Kwon Y.2022).

3. AIM AND OBJECTIVE:

The dissertation entitled "**Role of BPA induced Lipogenesis Breast Cancer Cells"** was designed with the main aim of studying the aggressiveness in Breast cancer due to lipogenesis and how BPA play a role in inducing lipogenesis. My study was divided into two objectives:

1. To evaluate the BPA mediated Lipogenesis in MCF-7 cells
2. To evaluate the molecular mechanism underlying BPA induced lipogenesis in MCF-7 Cells.

4. MATERIALS AND METHODS:

4.1.Materials:

4.1.1.Chemicals:

Table 3: List of Chemicals and Media Used:

Component	Manufacturer	Catalogue No.
Bisphenol A	Sigma-Aldrich	239658
Crystal violet	HiMedia®	TC510
Sodium Bicarbonate	HiMedia®	GRM849
DAPI	Sigma-Aldrich	D9542
MTT	SRL	58945
Nile Red	Sigma-Aldrich	72458
Sodium pyruvate	Gibco™	11360-070
SYBR Green master mix	Sigma-Aldrich	A25742
Trizol	Thermo-Fisher	15596026
High capacity cDNA reverse transcriptase kit	Thermo-Fisher	4368814
DMSO	HiMedia®	67-68-5
Glycine	VWR Life sciences	56-40-6

Tris base	Bio-Rad	1610716
Acrylamide	Bio-Rad	1610101
GW9662	Sigma-Aldrich	M6191
DMEM	Sigma-Aldrich	D9756
Trypsin	Sigma-Aldrich	T4049
Fulvestrant	Sigma-Aldrich	I4409

4.1.2.Cell line:

MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium and positive estrogen receptors (ER) in the cell cytoplasm of breast cancer cell line MCF-7.

Table 4: Background Details of MCF-7 cell line:

Designation	MCF-7
Organism	Homo sapiens
Tissues	Breast
Culture properties	Adherent
Source	laboratory of Dr. Soumya Sinha Roy, CSIR Institute of Genomics and Integrative Biology, India
Organ	Mammary gland

Diseases	Adenocarinoma
Derived from metastatic site	Pleuro effusion
Morphology	Epithelial
Karyotype	Cloning efficiency of the four lines over a 10 fold range, Tumorigenicity.
Culture Medium	The base medium for this cell line is Dulbecco's Modified Eagle's Medium (DMEM) to make the complete growth medium add the following components to the base medium: Fetal Bovine Serum to a final concentration of 10%.
Preservation (Cryopreservation freeze medium)	Complete growth medium supplemented with 5% (v/v) DMSO and 95% FBS. Storage temperature: liquid nitrogen vapour phase
Culture Conditions	Atmosphere : air 95%, carbon dioxide 5% Temperature - 37°C

4.2.Methodology:

4.2.1Cell Viability Assay:

After the MCF-7 cells were exposed to different doses of BPA, its cell viability was investigated to determine its estrogenic properties. At first around 5000 cells were plated in 96-well plate with 100 µl complete DMEM media for 24 hours. Then BPA (range of 1 nM to 1 µM) treatment was given to the cells for the next 24 hours. Then after 24 hours, the cell viability was checked by using MTT assay. BPA was prepared in DMSO and stored in -20 °C.

4.2.2.Colony formation assay:

After the repeated exposures to BPA (50 nM and 100 nM), MCF-7 cells were checked for its colony formation ability. Around 500 cells/well were seeded in a 6-well plate with 2 ml DMEM media. Both control and BPA exposed cells (150 exposures) were seeded in the culture plate. Incubation of 2-3 weeks were given to form colonies. After the incubation period, the media was disarded and the wells were first washed with PBS twice, after which the cells was fixed with 70 % chilled ethanol. Followed by staining with crystal violet solution (0.5 % in methanol) and colonies were counted.

4.2.3.Soft agar assay:

BPA (50 nM and 100 nM) exposed MCF-7 cells were taken around 2500 cells in 1 ml of 0.6 % agar solution(in DMEM) .Each well of culture plate is coated with agar first then cells suspended in 0.6 % agar solution were added to each well. After 20 mins, 500 µl DMEM was added over the agar wells so that colonies could form. After 2-3 weeks ,the imaging of colonies was done with an inverted bright field microscope which has a 20X magnification (Olympus,Japan) .Colony size was measured using software ImageJ 1.48v.

4.2.4.Migration Assay:

Migration potential of MCF-7 cells was evaluated after 150 exposure to 50 nM and 100 nM BPA through a wound healing assay. A scratch was made in a fully confluent flask of both control and BPA exposed MCF-7 cells with a 200 µl sterile tip. Media was changed at different time intervals and images were taken at 0 hour, 24 hours and 48 hours under an inverted bright field microscope with 4X objective (Olympus, Japan).The length of the wound was measured using ImageJ software.

4.2.5.Invasion assay:

Invasion potential of BPA treated MCF-7 cells was investigated using Boyden Chamber. At first 50 µl MAtrigel was placed in a 24 –well plate and allowed to solidify at 37 °C for 10-15 mins. Then 100000 cells in a 100 µl DMEM was put over the matrigel layer. Then inserts were laced on the lower chambers having 500 µl BPA exposed cells in DMEM for the next 24 hours. The cells that were migrated to the lower surface were fixed with 70 % chilled ethanol and then left for drying. After the cells were dried stain them with 0.25 % crystal violet solution for 10 mins. Then wash the inserts and take

images under an inverted bright field microscope with 10X magnification and he cells that invaded were counted.

4.2.6.Western Blotting:

Western blot was done for the analysis of various proteins that were used for targets.20 µl of lysate was taken and electrophoresis was done using 10-12 % SDS Page gels. After that transfer the protein on a PVDF membrane, and incubate with primary antibody followed by secondary antibody. Membranes were then visualized with a Image Quant LAS 500 with ECL reagent. The densitometry of bands was done through Studio™ Lite software. B-Actin and GAPDH was used as loading control.

4.2.7.Quantitative real-time PCR:

First RNA was extracted of both control and treated MCF-7 cells using Trizol .After which cDNA was synthesized using 2 µg RNA in a 20µl Reaction with the help of High density RT cDNA kit. The quantification of gene expression was don using SYBR Green master mix and Quant Studio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific).The formula 2-ΔΔCt was used for calculation of gene expression. B-Actin was used as an internal control.

Table 5: List of Primers used

Gene	Primers
β-actin	Forward 5′ -ATT GGC AAT GAG CGG TTC-3’ Reverse 5′ -CGT GGA TGC CAC AGG ACT-3
FAS	Forward 5’-AAG GAC CTG TCT AGG TTT GAT GC-3’ Reverse 5’-TGG CTT CAT AGG TGA CTT CCA-3’
ACC	Forward 5’-TGA GAC TAG CCA AAC AAT CTC GT-3’ Reverse 5’-AGA AAG TAG AAG CTC CGA TCC T-3’
PPARγ	Forward 5’-GCC CTT TGG TGA CTT TAT GGA-3’ Reverse 5’-GCA GCA GGT TGT CTT GGA TG-3’
ERα	Forward 5′ - CCA CCA ACC AGT GCA CCA TT-3’ Reverse 5′ - GGT CTT TTC GTA TCC CAC CTT TC-3′

4.2.8.Nile Red Assay:

When the flask reached 70% confluency,wash it with PBS twice .Add 4% PFA solution and leave it for 15 minutes followed by fixation with 70% ethanol for 30 mins. Then add Nile Red stain solution (5µl in 10 ml TBS-T buffer) Incubate for 1-2 hours. Visualize the cells under microscope.

4.2.9.Statistical Analysis:

Student's t-test and one-way ANOVA test (with Dunnett's multiple comparisons test) were used to determine the significance level between two or multiple groups, respectively, using GraphPad Prism 8.0.1 software. A P value of <0.05 was considered significant. Data represent the mean ± SD of at least three independent experiments.

5. RESULTS AND DISCUSSION:

5.1.MTT Assay:

By MTT assay we were able to determined the estrogenic effect of BPA by giving different doses to estrogen-positive MCF-7 cells. As seen in the Fig.3, there was a significant increase in the viability of cells at low doses up to 100 nM, while at higher doses i.e. 200 nM to 1 µM a decrease in cell viability can be seen when MCF-7 cells was treated with BPA for 24 hours .50 nM was the minimum dose at which enhancement in the viability could be seen whereas 100 nM was the maximum dose.

From this data the two doses of BPA i.e. 50 nM and 100 nM was decided to treat the MCF-7 cells with BPA with continued exposures once every 24 hours for 200 days to further investigate its effect.

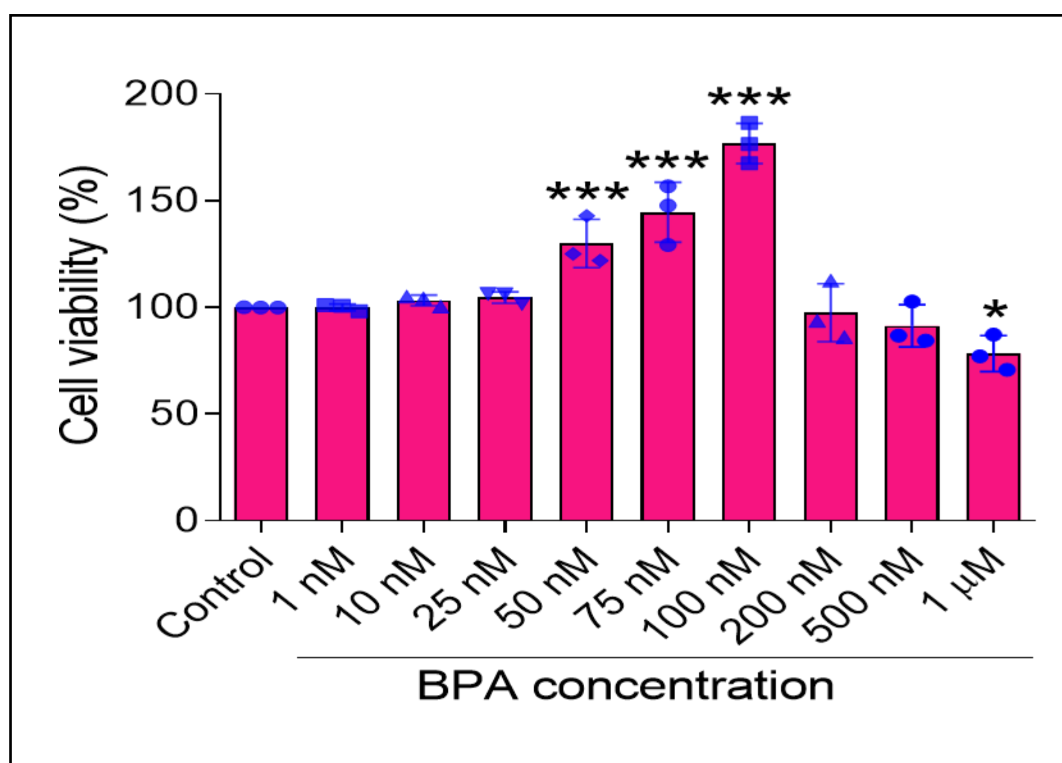


Figure 3: MTT assay in BPA (1 nM-1 µM) MCF- 7 cells for 24 h.

5.2.Effects of repeated exposures of BPA on Estrogen Receptor:

Western blot analysis of ERα gene was done on different number of exposures (50,100,150, 200). The loading control was β- Actin. It has been found that BPA exposure enhances the expression of ERα protein. From Fig 4(a) and 4 (b) it can be seen that there is a notable increase in ERα expression at BPA 50 exposures with a maximum increase at 150 exposures of BPA in both doses i.e 50 nM and 100 nM .In both 50 nM and 100 nM doses the enhancement in ERα expression remain abided after 150 exposures and till 200 exposures without showing any further change.

Then, the mRNA expression of ERα protein was also evaluated in MCF-7 BPA treated cells through RT-PCR technique. As shown in Fig 4(c), an increase of almost 20 fold can be seen in MCF -7 cells exposed with BPA (100 nM) after 150 exposures as compared to control cells.

From both the data of western blot and RT-PCR we can conclude that repeated exposure of BPA enhanced estrogen receptor expression in a time dependent manner.

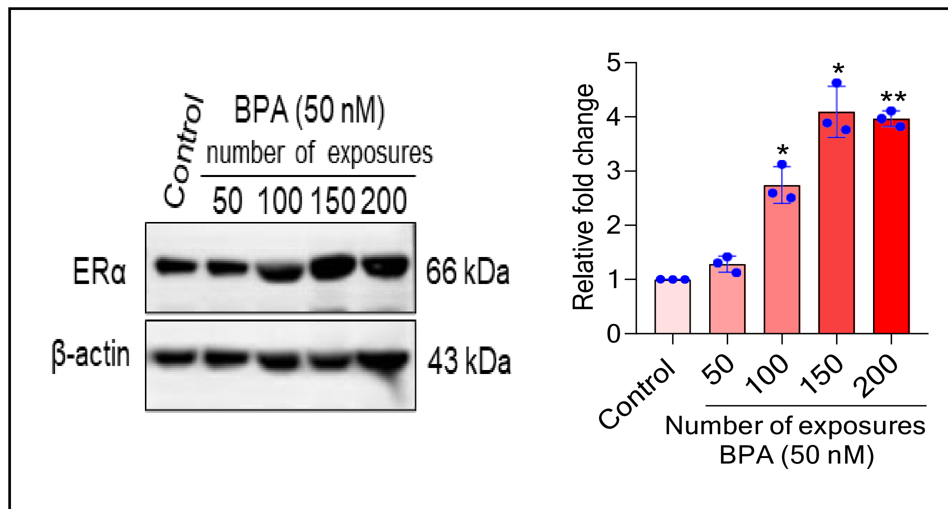


Figure 4 (a): Western blot analysis of MCF-7 cells for ERα in BPA 50 nM

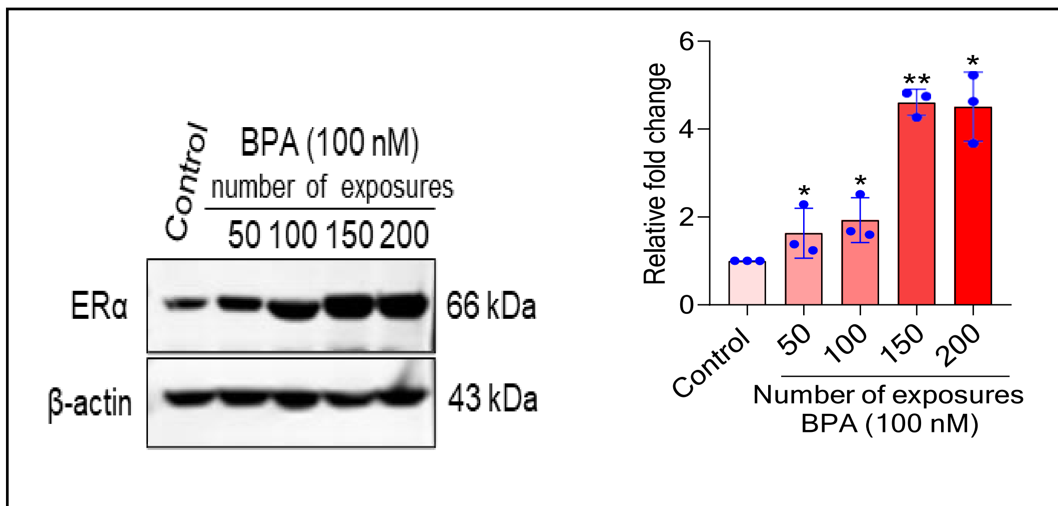


Figure 4 (b): Western blot analysis of MCF-7 cells for ERα in BPA 100 nM

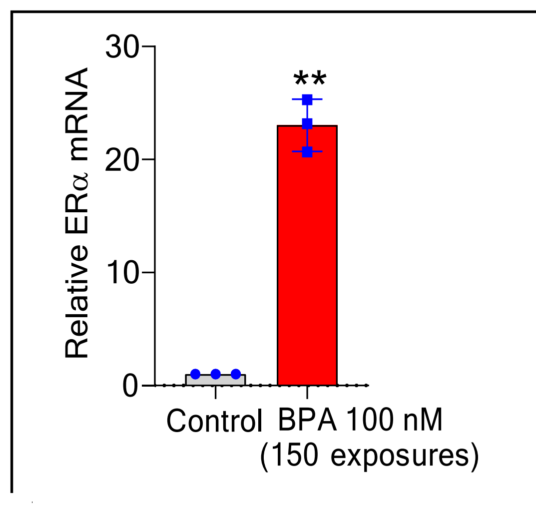


Figure 4 (c): Relative mRNA expression of ERα in MCF-7 wild type and BPA (100 nM) treated cells.

5.3.Effect of BPA on Proliferative markers:

When Immunoblotting analysis for Proliferative markers was performed using β-actin as a loading control .It was found that because of the repeated exposure to BPA (50 nM and 100 nM) there was an elevation in the expression of PCNA i.e. proliferating cell nuclear antigen with time in MCF-7 cells as seen in Fig . 5a and 5b respectively.

Also from Fig. 5 (c) it can be noted that there was also an increase in the expression of survivin protein which is also a cell proliferation marker with the increasing exposure in the BPA (100 nM) exposed MCF-7 cells.

From both the analysis of PCNA and survivin it is fair to say that BPA exposure enhances the expression of proliferative markers in a time dependent manner in MCF-7 cells.

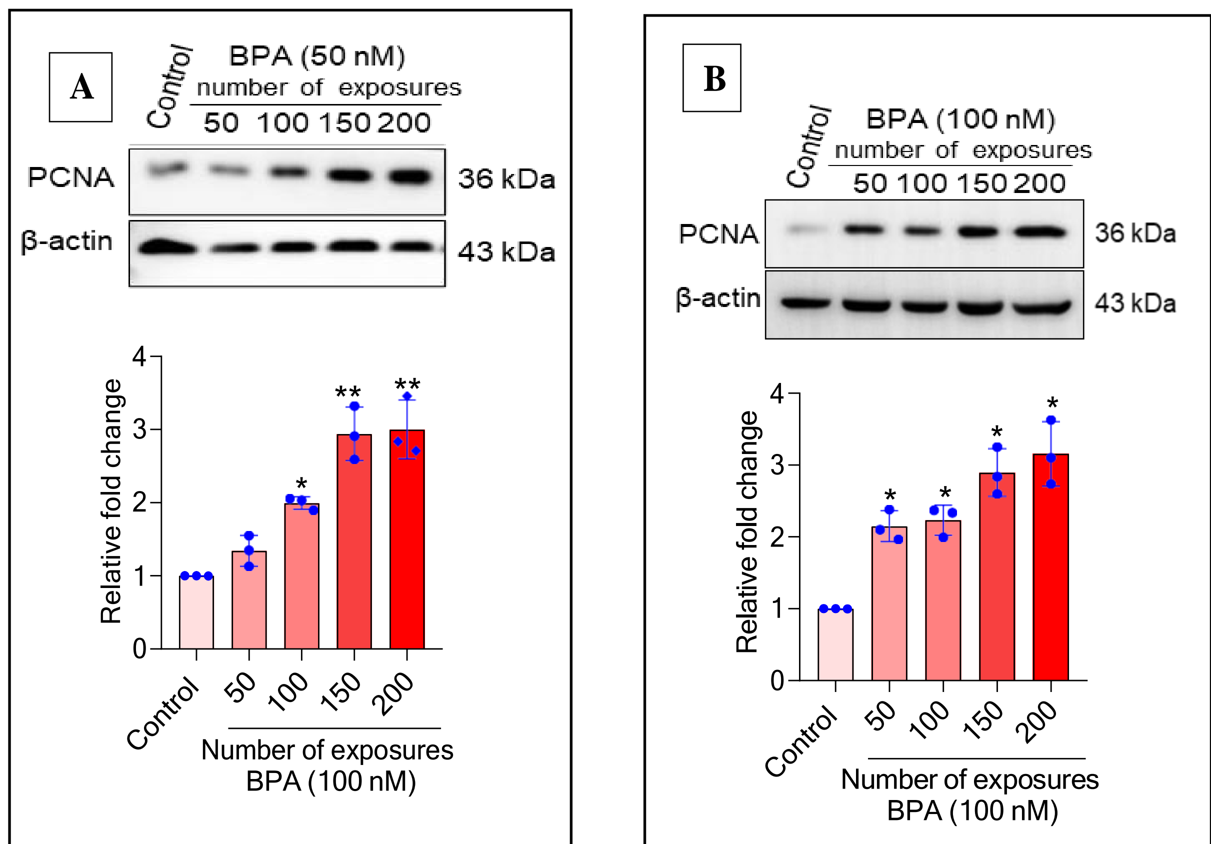


Figure 5: Western blot analysis of MCF-7 cells for PCNA in BPA **(A)** 50 nM dose **(B)** 100 nM

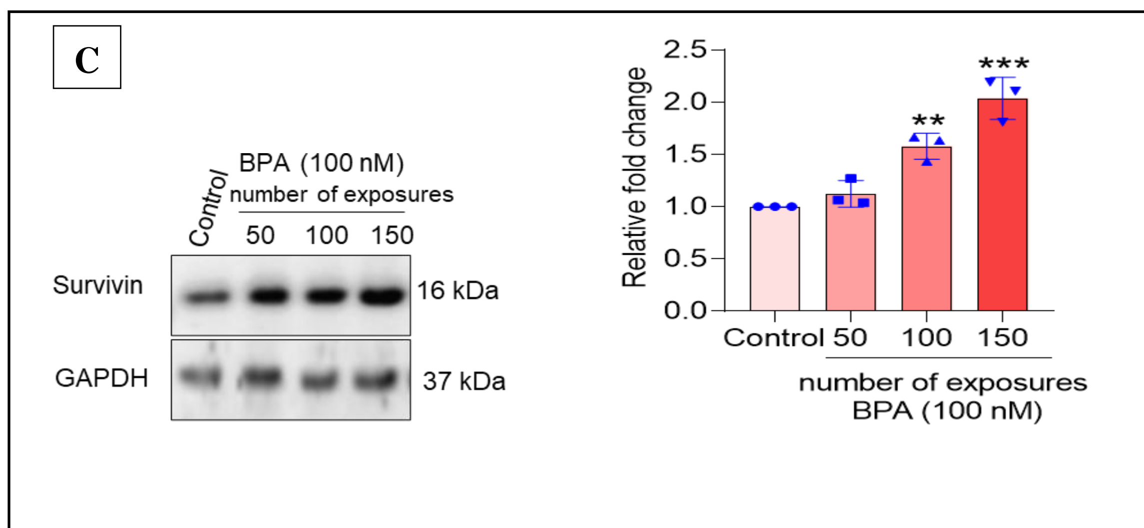


Figure 5 (c): Western blot analysis of MCF-7 cells for Survivin in BPA 100 nM

5.4.Protein expression of ERα and PCNA following inhibition of ERα by Fulvestrant:

Then next to check whether the enhancement in proliferation due to BPA is caused by its estrogenic property, we used the inhibitor fulvestrant on BPA (100 nM) exposed MCF-7 cells.

After the treatment we did western blot analysis of Fulvestrant inhibited BPA exposed MCF-7 cells to check the expression of ERα and PCNA. We found that fulvestrant which is a inhibitor of ERα decreases the expression of both ERα and PCNA in BPA (100 nM) exposed MCF-7 cells with a 2 fold change in each case. (Fig.6)

Hence, we can conclude that BPA has estrogenic properties and that is why there is an enhancement of proliferation in MCF-7 cells after repeated long term exposures with BPA.

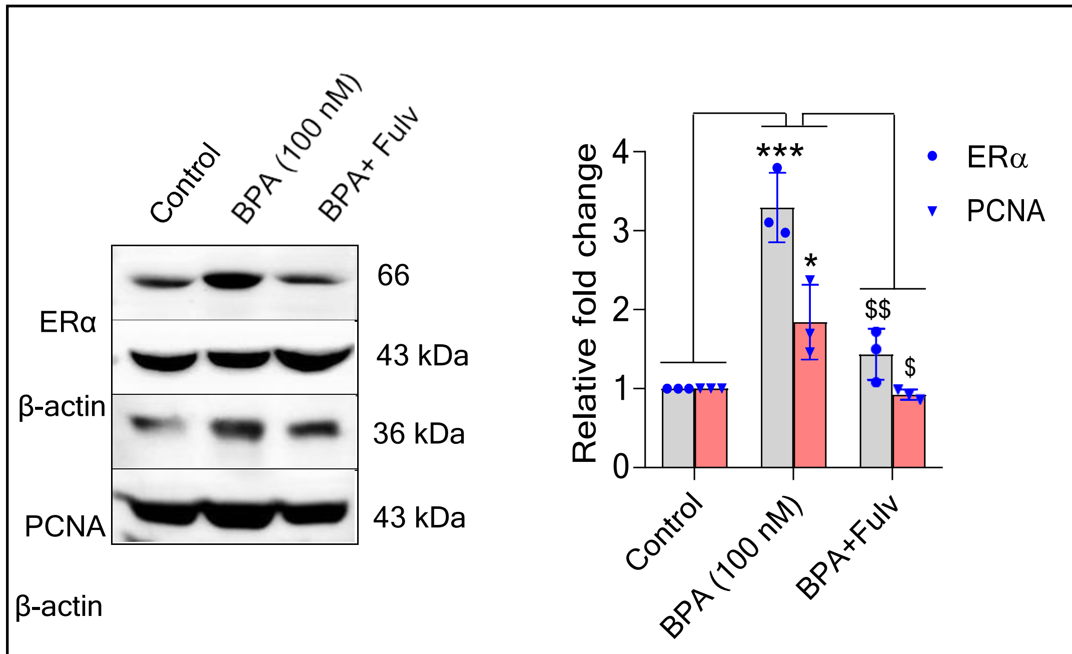


Figure 6: Western blot analysis to check the effect of Fulvestrant on the expression of ERα and PCNA in BPA exposed MCF-7 cells.

5.5.Effect of BPA exposure on lipid content:

To study the effect of BPA on lipid content in MCF-7 cells, immunofluorescence analysis was performed .The Nile red was used to stain the lipids in the cells with DAPI as a counter-stain.

It was observed that the relative intensity of fluorescence is significantly much more in BPA (100 nM) treated MCF-7 cells as compared to control. Thus it was concluded that BPA increases the lipid content in breast cancer cells.

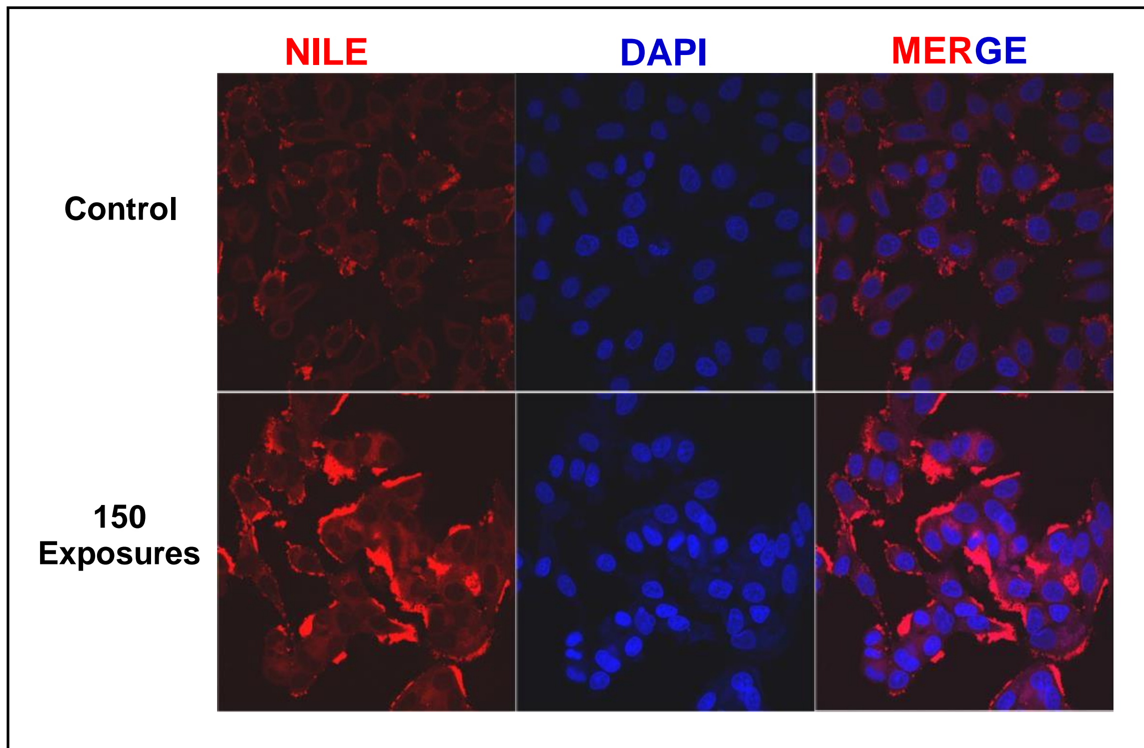


Figure 7 (a): Immunofluorescence analysis using Nile red to stain lipid content in MCF-7 cells and the counter stain is DAPI

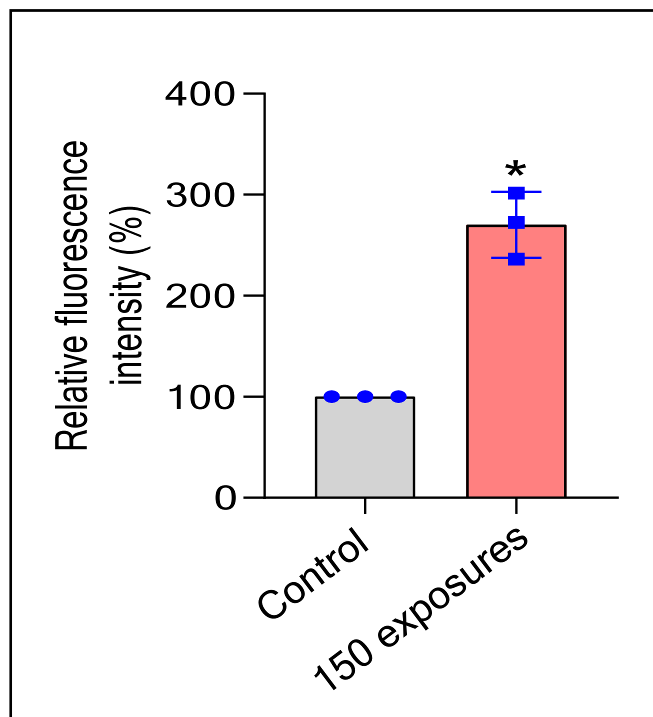


Figure 7 (b) : Relative change in the fluorescence intensity in the BPA(100 nM) treated MCF-7 cells as compared to control.

5.6.Effects of BPA on Lipogenic Markers:

To check the effect of BPA on lipogenesiswestern blot analysis was done followed by qRT-PCR analysis.From Fig.8(a) and 8 (b) we can see that adipogenic markers FAS,ACC and PPARγ upregulates in the BPA 150 exposure cells as compared to control cells in MCF-7 cell line. FAS increases with 2 fold change, ACC with 1.5 fold change while PPARγ has a 0.6 fold change.

Also in Figure 8 (c) and 8 (d) we can see that all three markers has enhaced expression in BPA (100 nM) treated MCF-7 cells than the wild type cells.

It is evident that BPA induces lipogenesis by upregulating its various markers.

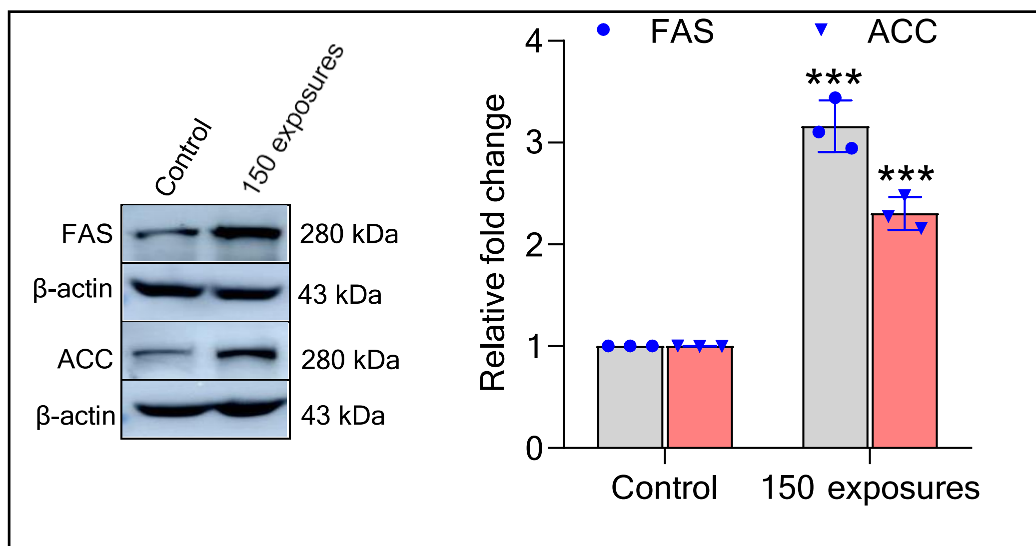


Figure 8 (a): Western blot analysis for FAS and ACC in MCF-7 wild type And BPA (100 nM) treated cells.

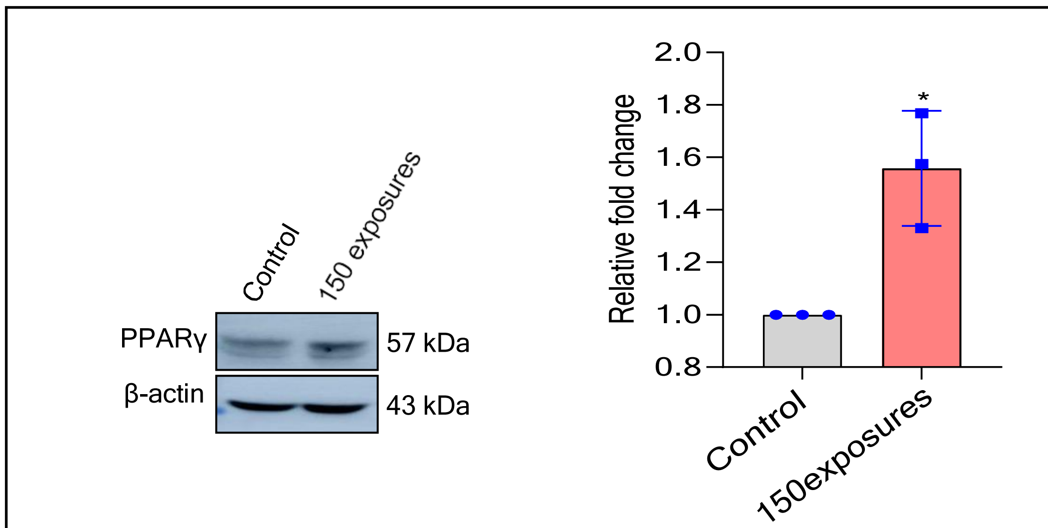


Figure 8 (b) : Western blot analysis for PPARγ in both MCF -7 Wild type and BPA treated cells.

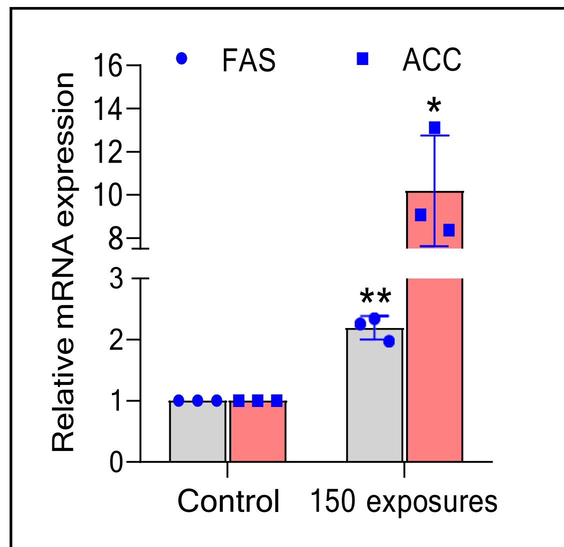


Figure 8 (c) : Relative mRNA expression of FAS and ACC in MCF-7 cells exposed with BPA(100 nM) compared to wild type.

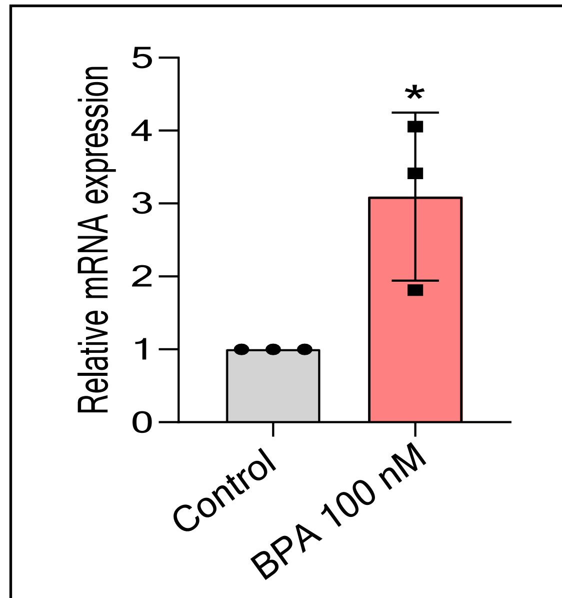
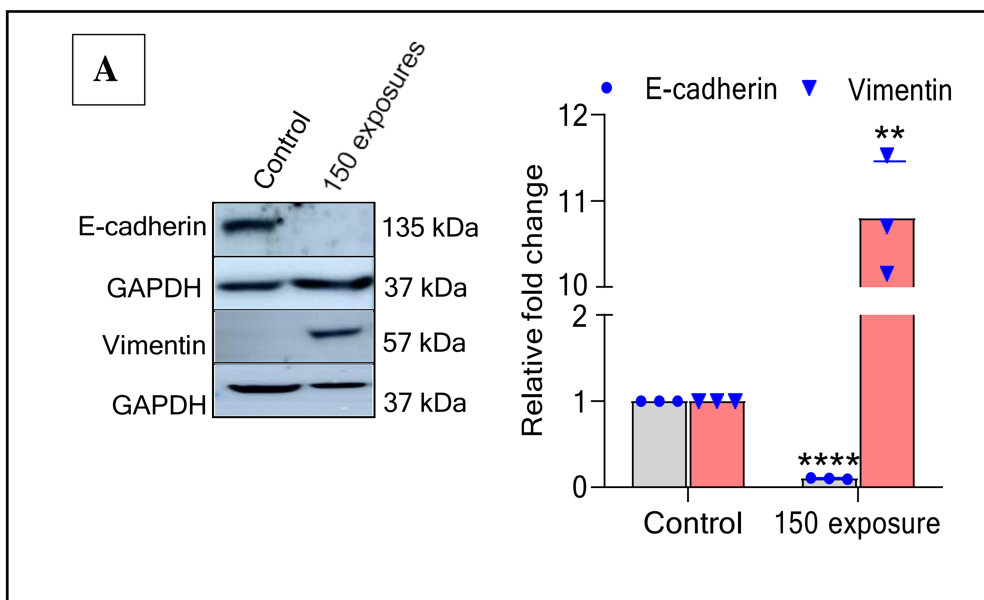


Figure 8 (d): Relative mRNA expression of PPARg in both MCF-7 wild Type and BPA(100 nM) exposed cells

5.7.Effect of BPA on EMT markers:

To investigate the changes in EMT markers E-cadherin, vimentin, snail, slug and αSMA in MCF-7 treated cells immunoblotting analysis was performed. From Fig 9 (a) and (b) it is evident that BPA up regulates the expression of EMT markers in breast cancer cells.



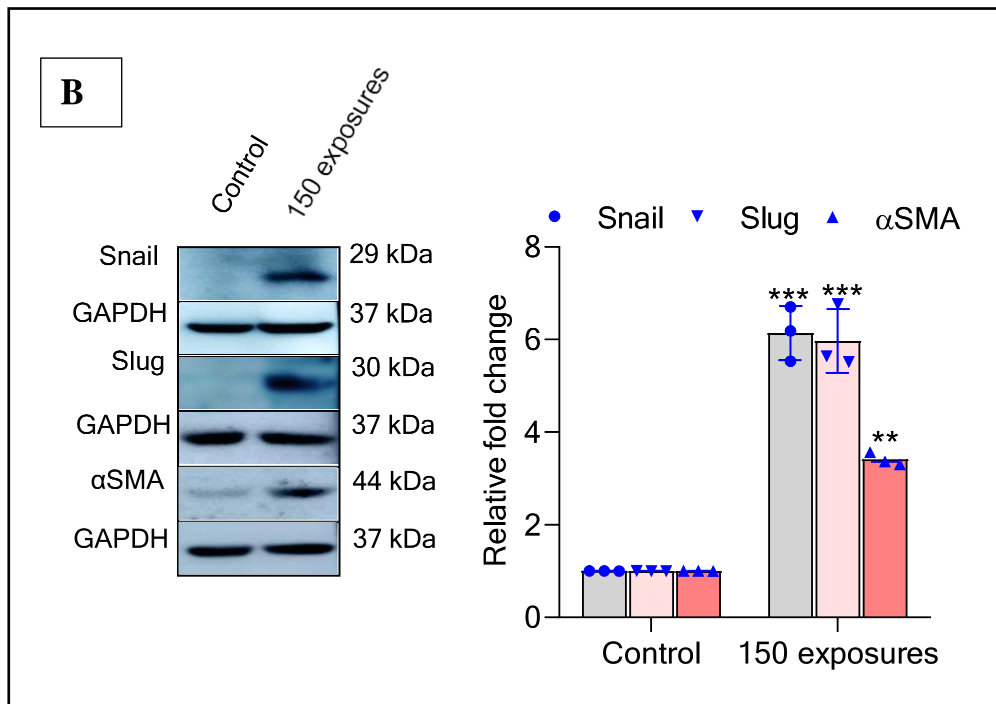


Figure 9: Immunoblotting analysis of **(a)** E-cadherin and vimentin **(b)** snail, slug and αSMA in MCF-7 cells treated with 100 nanomole BPA

5.8.Effect of PPARγ Inhibition in MCF-7 cells:

PPARγ inhibitor GW9662 (μM) was added into MCF-7 cells and analysis was done through western blotting .It has been found that on increasing the dose of GW9662 ,expression of PPARγ decreases .Various doses of 10,20,40 μM was administered and compared against the untreated cells.

Various other parameters were also analysed as described in the sections followed .It has been found out that Inhibition of PPARγ suppresses the adipogenic signaling pathways in MCF-7 cells

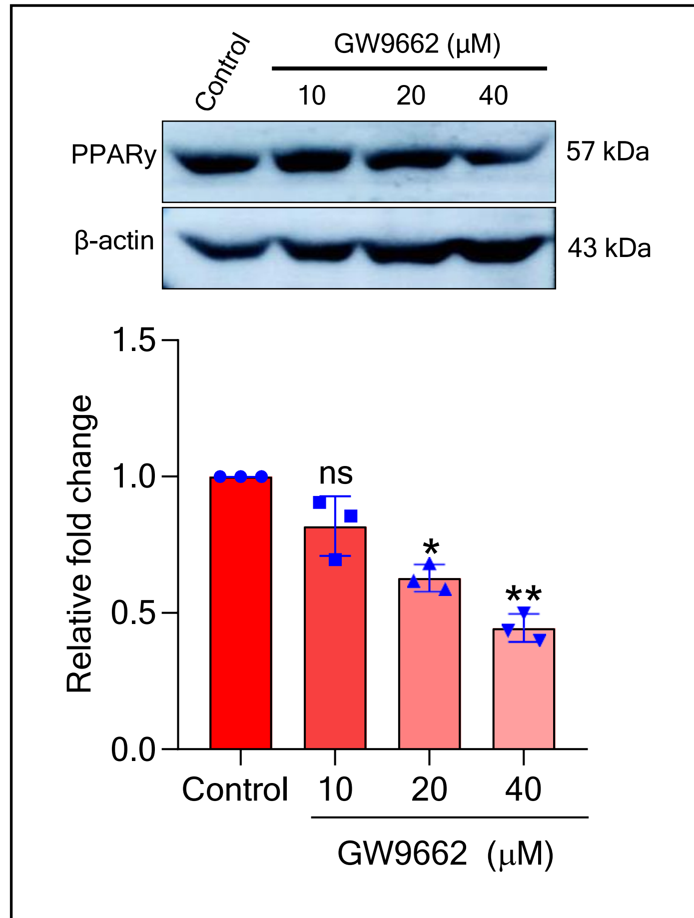


Figure 10: Western blot analysis for PPARγ when inhibited by GW9662 in MCF-7 cells

5.9.Effect of Inhibition of PPARγ on lipid content in MCF-7 cells:

Nile red analysis was performed for the evaluation of lipid content in MCF-7 cells treated with BPA(100 nM).Based on the Figure 11 (a) and (b) we can see that lipid content in 150 exposure BPA MCF-7 cells increases and then on inhibition of PPARγ by GW9662 it decreases as compared.

The relative fluorescence intensity in MCF-7 Treated cells is 100 times more than in wild type but then again in PPARγ inhibited cells it comes down by 80 % as compared to treated cells.

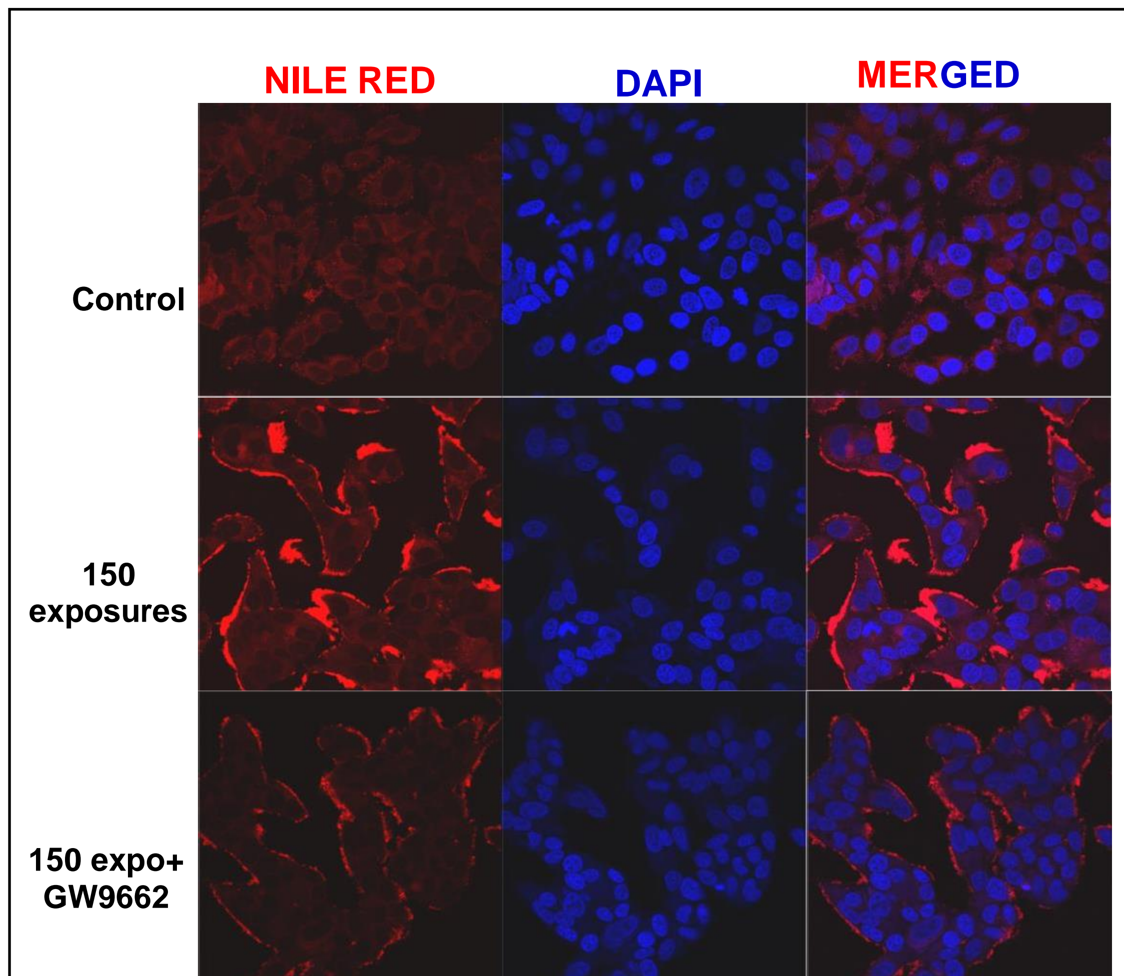


Figure 11 (a): Immunofluorescence analysis of MCF-7 cells to check lipid content after PPARγ inhibition using Nile red stain

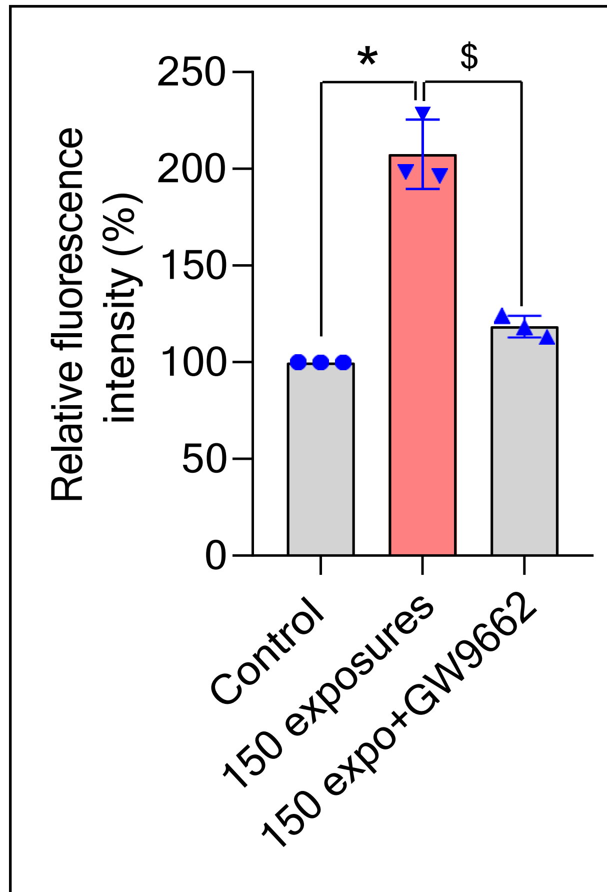


Figure 11(b); Relative fluorescence intensity in MCF-7 wild type, BPA (100 nM) treated and PPARγ inhibited cells

5.10.Effect of PPARγ inhibition on proteins of Adipogenesis:

Western blot analysis for the expression of PPARγ, FAS and ACC was done to check the changes that PPARγ inhibition might have brought in MCF-7 cells. B-actin was used as a loading control.

In figure 13 we can see all three lipogenesis proteins were less expressed in PPARγ inhibited cells as compared to BPA treated MCF-7 cells. PPARγ has seen reduced fold change by 2, FAS by 3 and ACC by 1.5 compared to 150 exposure BPA cells.

Hence we can conclude that inhibition of PPARγ leads to the suppression of lipogenesis proteins expression and thus leading to deactivation of various signalling pathways involved in Adipogenesis.

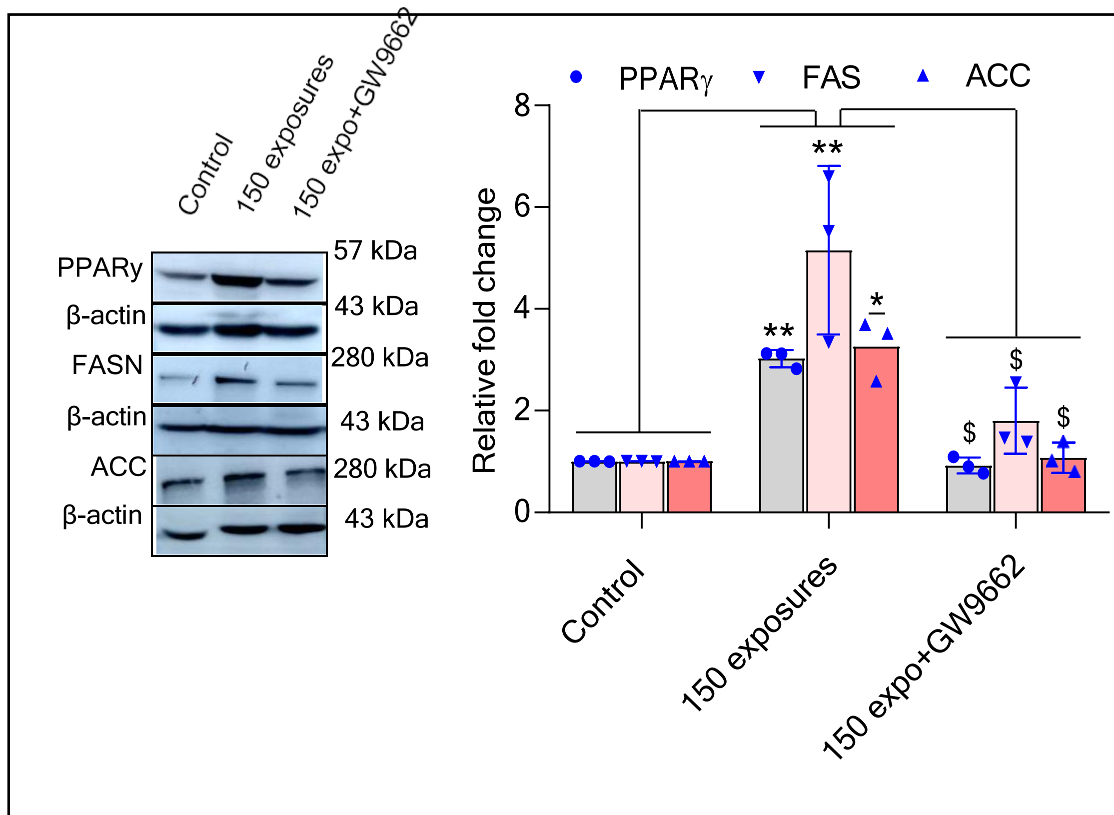


Figure 12: Immunoblotting analysis for PPARγ,FASN,ACC in PPARγ inhibited MCF-7 cells exposed to 150 times BPA(100 nM)

5.11.Effect of PPARγ inhibition on colony formation and anchorage independent growth of MCF-7 cells:

The colony formation ability and anchorage independent growth in MCF-7 cells is induced by BPA .After the inhibition of PPARγ the effects on both these properties were evaluated through Cologenic assay and soft agar assay respectively.

In figure 13 (a) colonies induced by BPA was reduced due to the inhibition of PPARγ by GW9662.Likewise in figure 13 (b) the colony size in PPARγ inhibited cells is smaller than the BPA treated cells.

Both these data suggests that PPARγ inhibition suppresses both colony formation and anchorage independent growth in mCF-7 cells.

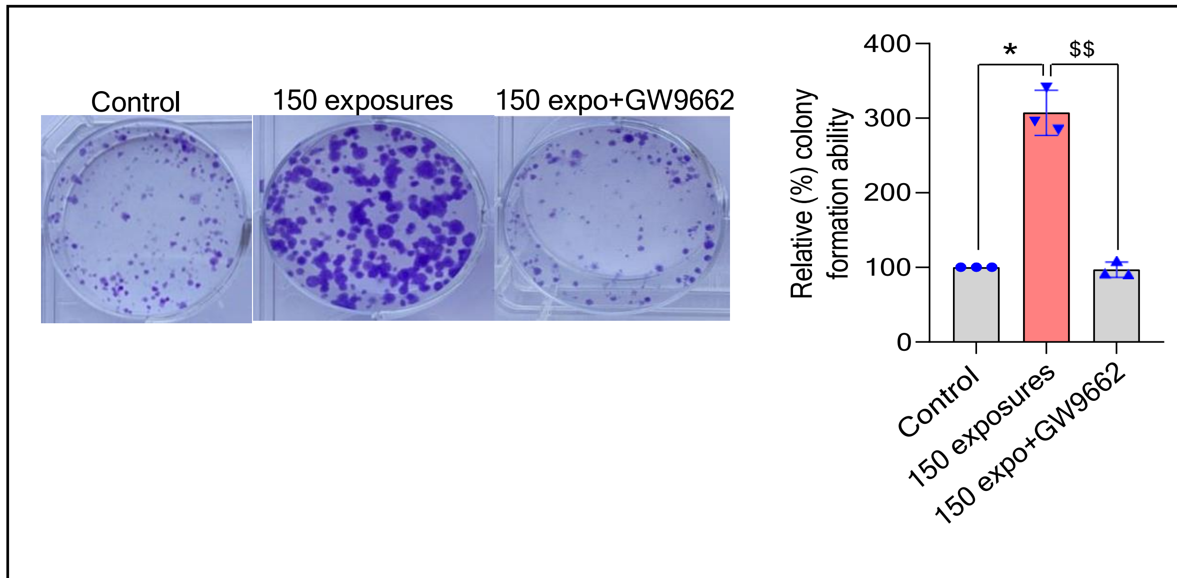


Figure 13 (a): Clonogenic assay analysis in MCF-7 cells treated PPARγ inhibitor against BPA treated cells

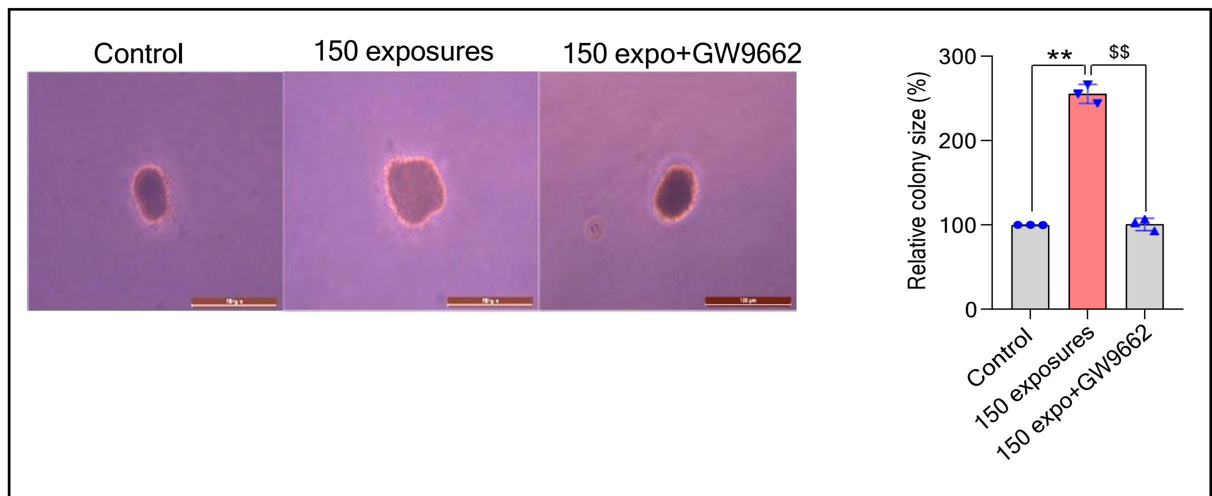


Figure 13 (b): Non-Anchorage growth in MC7- 7 cells treated with PPARγ inhibitor with GW9662.Scale bar of 100 µm

5.12.Effect of Inhibition of PPARγ on EMT markers expression:

To check the effect of PPARγ inhibition by GW9662 on EMT Marker in MCF-7 cells immunoblotting analysis for Slug, snail, vimentin was performed.

Expression of all three EMT markers slug, snail, and vimentin were inhibited in the presence of PPARγ antagonist GW9662. A decrease of Fold change was observed in the PPARγ inhibited MCF-7 cells when compared to BPA (100 nM) treated MCF-7 cells (Fig. 14)

From this observation it is safe to say that PPARγ inhibition MCF-7 cells down regulates the EMT marker proteins.

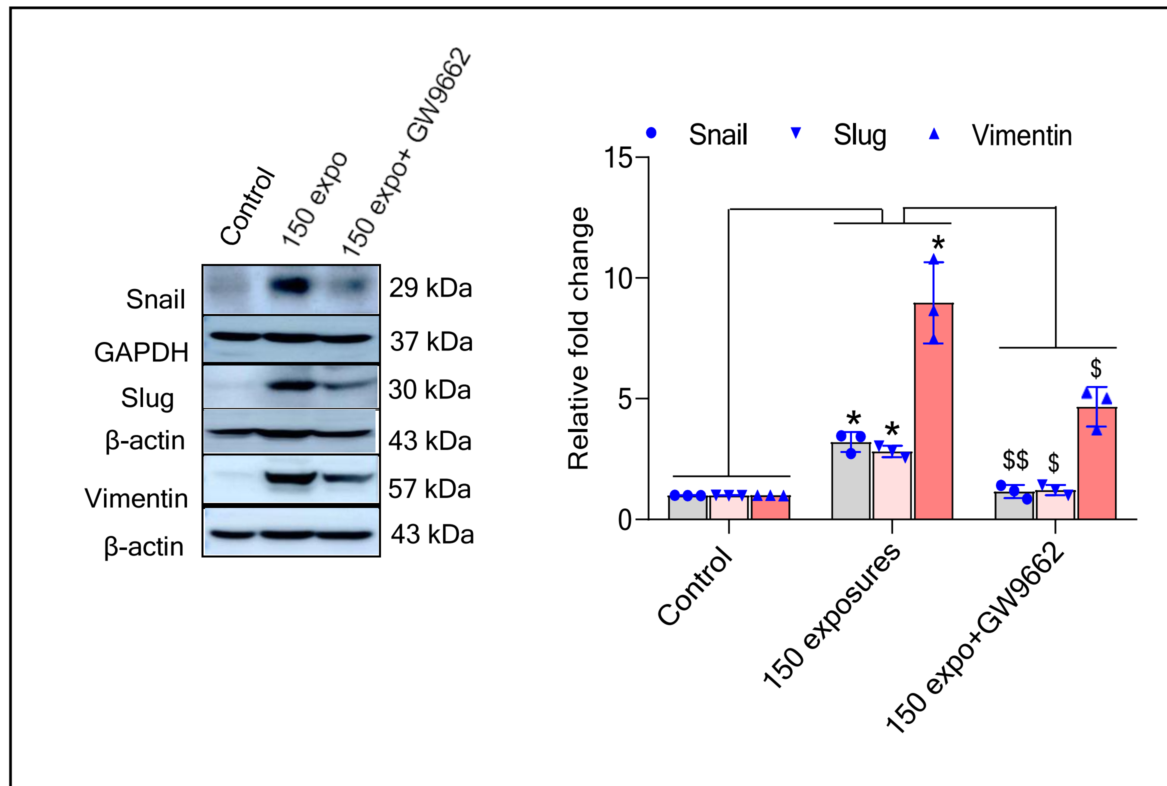


Figure 14: Western blot analysis for snail, slug, and vimentin in PPARγ inhibited MCF-7 cells against BPA (100 nM) treated cells.

5.13.Effect of PPARγ inhibition on migration and invasion of MCF-7 cells:

To investigate the role of PPARγ inhibition on migration and invasion of MCF-7 cells, scratch assay and invasion assay were carried out respectively. It was found out that in BPA(100 nM) exposed MCF-7 cells migration and invasion potential of cells increases as compared to the wild type indicating enhancement of aggressiveness of tumour cells.

It has been observed that when PPARγ inhibitor GW9662 was added to these BPA exposed cells it reduces the migration potential by 2 folds (fig 15 a) and invasive potential by 1.5 folds (fig 15 b).From this observation we can conclude that inhibition of PPARγ also inhibits the migration and invasive potential of MCF-7 cells

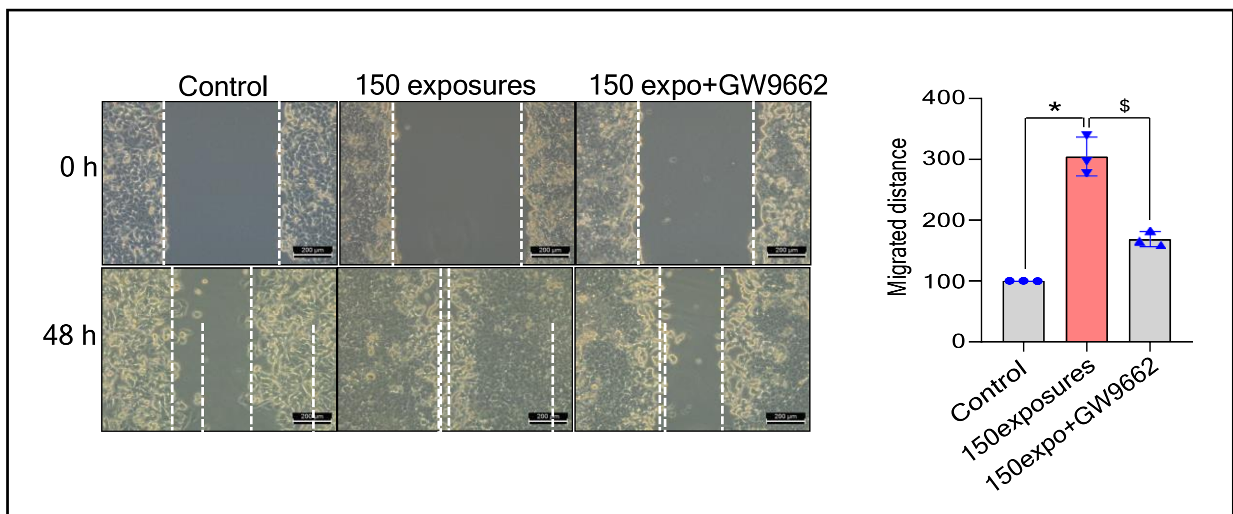


Figure 15 (a): Wound healing assay in PPARγ inhibited MCF-7 cells

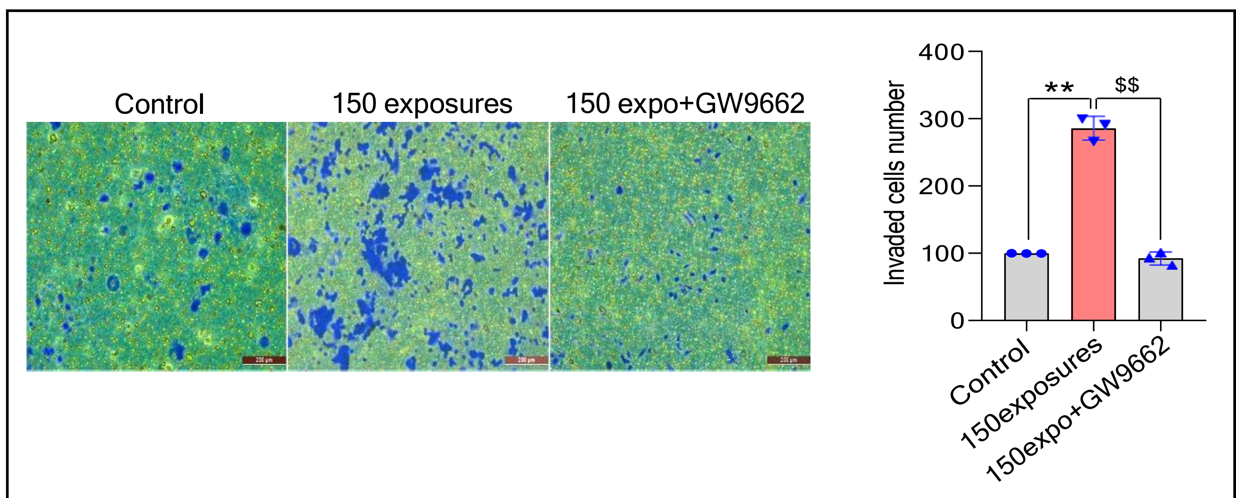


Figure 15 (b): Invasion assay (Boyden chamber) in BPA exposed MCF-7 cells treated with GW9662 inhibitor

6. CONCLUSIONS:

The study undertaken illustrates the Effect of BPA induced lipogenesis in the breast cancer cells. The cells MCF7 were exposed to BPA (100 nM) up to 150 exposures were used to determine the various parameters. It was found that BPA leads to the aggressiveness of breast cancer cells by increasing the expression of adipogeneic markers .It was further confirmed by PPARγ inhibition that Lipogenesis induced by BPA leads to the aggressiveness of cancer cells in MCF-7 cells. As on PPARγ inhibition the expression of EMT markers ,adipogenic markers was decreased along with reduction in , colony formation ability, Anchorage independent growth ,migration and invasive potential indicating the role of Adipogensis in Tumour migration and invasion .

However, its still unknown that how BPA regulates different signalling pathways in lipogenesis and alter tumorigenesis .The mechanism behind the affects that BPA brings in adipocytes is yet to be investigated. This could prove beneficial in the treatment of breast cancer as adipocytes poses as a great therapeutic agent for breast cancer.

7. REFERENCES:

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