University of Reading

Genotoxicity of Cosmetic Chemicals in Human Breast Epithelial Cells

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ABBREVIATIONS

Al	**Aluminium**
AlCl3	**Aluminium chloride**
ATCC	**American Tissue Culture Collection**
BPA	**Bisphenol A**
CO	**Co-activators**
CS	**Cowden syndrome**
D3	**Hexamethylcyclotrisiloxane**
D4	**Octamethylcyclotetrasiloxane**
D5	**Decamethylcyclopentasiloxane**
DMEM	**Dulbecco's Modified Eagle's medium**
DES	**Diethylstilbestrol**
DRC	**DNA repair capacity**
DSB	**Double strand breaks**
E	**Oestrogen**
EDTA	**Ethylenediaminetetraacetic acid**
EGF	**Epidermal growth factor**
ERα	**Oestrogen receptor alpha**
ERß	**Oestrogen receptor beta**
HBSS	**Hank's Balanced Salt Solution**
HRT	**Hormone replacement therapy**
KSA	**Kingdom of Saudi Arabia**
Lilial	**Butylphenylmethylpropional; 2-(4-tertbutylbenzyl) proplonaldehyde**
Mins	**Minutes**
POP	**Persistent organic pollutants**
POL	**Polymerase**
RTPCR	**Real time PCR**
RTPCR	**Reverse-transcriptase polymerase chain reaction**

SCGE **Single-cell gel electrophoresis**

SSB **Single strand breaks**

PRIOR DISSEMINATION OF PARTS OF THIS THESIS

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ABSTRACT

The incidence of female breast cancer is rising globally at unprecedented rates with a near doubling in many countries. Oestrogen is a main risk factor and many environmental chemicals have been shown to possess oestrogenic activity (xenoestrogens) and to enter the human breast from exposure through diet, the domestic environment or personal care products. The aims of this project were to investigate whether xenoestrogens also possess genotoxic activity. The compounds studied were three cyclosiloxanes (D3, D4, and D5), butylphenylmethylpropional (Lilial), triclosan and aluminium salts which are used extensively in personal care products, and bisphenol-A which is used widely in the manufacture of plastics. Genotoxicity was assessed from their ability to enable growth in suspension culture, to damage DNA in a comet assay and to interfere with cellular DNA repair systems in two immortalised non-transformed human breast epithelial cell lines (MCF10A and MCF10F). The ability of non-transformed epithelial cells to grow in suspension culture is an established marker of transformation. All these compounds enabled growth of MCF10A and MCF10F cells in suspension with maximal effects observed at 10^{-10}M D3, 10^{-5}M D4, 10^{-5}M D5, 10^{-5}M bisphenol A, 10^{-7}M triclosan and 10^{-5}M butylphenylmethylpropional (Lilial). The comet assay showed DNA damage after 1 hour exposure to 17β-oestradiol in both cell lines as well as to 10^{-5}M D3, 10^{-5}M D4 or 10^{-5}M butylphenylmethylpropional (Lilial). Reverse-transcriptase polymerase chain reaction (RTPCR) was used to detect effects of these chemicals on mRNA levels in MCF-10A and MCF-10F cells for 14 key DNA repair proteins (BRCA1, BRCA2, ATM, ATR, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111). Increases in mRNA for BRCA1 were detected after both short-term (1 week) and long-term (30 weeks) exposure to 10^{-5}M D3 and 10^{-5}M D4, 10^{-5}M D5 gave an increase only after short-term exposure (1 week). Decreases in BRCA2 mRNA were found after both short-term (1 week) and long-term (30 weeks) exposure to 10^{-5}M D3 and 10^{-7}M triclosan: long-term exposure (30 weeks) resulted in increases after exposure to 10^{-5}M D5, 10^{-5}M bisphenol A and 10^{-5}M butylphenylmethylpropional (Lilial). Western immunoblotting showed that BRCA1 protein was reduced in line with the mRNA results, demonstrating that in this case transcription and translation followed the same pattern. A shorter study using long –term exposure (20 weeks) to aluminium based antiperspirant salts at 10^{-4}M concentrations showed reduced expression also of BRCA1 mRNA and BRCA1 protein together with reduced expression of other mRNAs. In summary, all these compounds showed genotoxic activity in MCF10A and MCF10F cells and points to the potential for reduction in exposure as a strategy for breast cancer prevention.

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DECLARATION

This project is the result of my own investigation except where otherwise stated. Other sources are acknowledged by reference. A reference list is appended.

CONFLICT OF INTEREST STATEMENT

The author has no conflict of interest. The study was funded solely by Jazan University for the academic purposes of postgraduate study.

Chapter 1 Introduction

1.1 The Cell Cycle

The evolution of large multicellular animals with distinct interacting organ systems requires on the one hand that many tissue elements are continually replenished but on the other hand that homoeostatic control of this remodelling is maintained. Individual cells can be directed down paths of proliferation or differentiation. Surplus or abnormal cells can be removed by various forms of cell death, arguably the most important of which is apoptosis, whereby the cell contents are packaged up as apoptotic bodies which can be disposed of by the reticulo-endothelial system without the local milieu being swamped with disorganised enzymes and other bioactive compounds (Houtgraaf et al., 2006).

There are two main apoptotic pathways, receptor mediated and intrinsic. The latter is initiated through mitochondrial caspases as a result of DNA damage or stress to the endoplasmic reticulum and is the mechanism most relevant to the present study. These paths are illustrated in ***Figure 1.1.*** Other forms of programmed cell death exist such as extreme forms of autophagy whereby lysosomal membranes invaginate to engulf the cytoplasmic content.

This introduces the concept of the cell cycle, depicted schematically in **Figure 1.2:**

Cells pass from the resting phase, termed G1, into S phase, where DNA is replicated, on completion of which, after a lag period (G2) the cell divides by mitosis (M), the daughter cells returning to G1. This cycle is broken if cells cease dividing and become quiescent (G0) or terminally differentiate. The cycle can be arrested – primarily at the three points indicated by red lines at the G1/S and G2/M interfaces, or M phase. These are known as cell cycle checkpoints and are important in assessing the integrity of the cell, diverting compromised cells towards destruction by apoptosis.

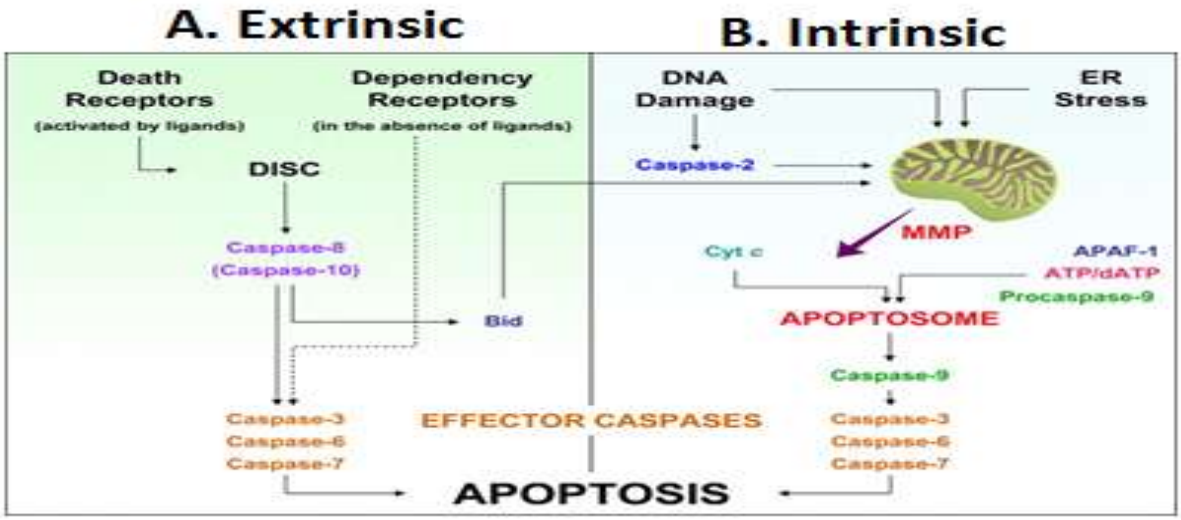


FIGURE 1.1 APOPTOTIC PATHWAYS

Graphic emphasising the two main routes for inducing apoptosis: a. extrinsic receptor –mediated,involving fas/fas ligand interaction, mediated through caspase-8 and bid. b. intrinsic pathways of apoptosis initiated by growth factor withdrawal, chemotherapy or uv irradiation, mediated through bcl2, cytochrome-c and caspase-9. ***Key to abbreviations****: APAF-1: Apoptotic protease activating factor 1, Bid: a pro-apoptotic member of the Bcl-2 family, Cyt c: cytochrome c RE: reticuloendothelium, DISC: Death Induced Signalling Complex, MMP: Matrix Metalloproteinases. Taken from (Kroemer et al., 2007)*

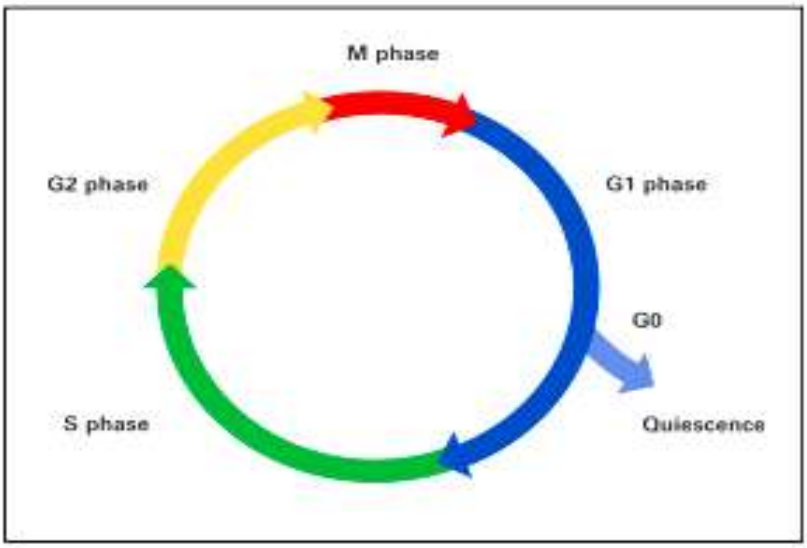


FIGURE 1.2 SCHEMATIC REPRESENTATION OF THE CELL CYCLE

Cells may leave cycle from G1 to temporary quiescence or terminal differentiation. Exit at checkpoints (red lines) normally indicates malfunction and initiates apoptosis. Adapted from Houtgraaf et al 2006.

Controlling the balance of cell proliferation, differentiation and loss is directed by external stimuli activating genetic pathways resulting in the production of appropriate gene products. Accuracy is essential and abnormal products will malfunction. The main cause of abnormal and ineffectual (or counter-productive) products is mutation in relevant genes. It is therefore appropriate here to describe something of the nature of DNA, which codes for amino acids and dictates their sequence in the proteins produced.

1.2 DNA structure and replication

DNA structure is illustrated most simply form in ***Figure 1.3A*** and more three-dimensionally as the double helix, in ***Figure 1.3B***

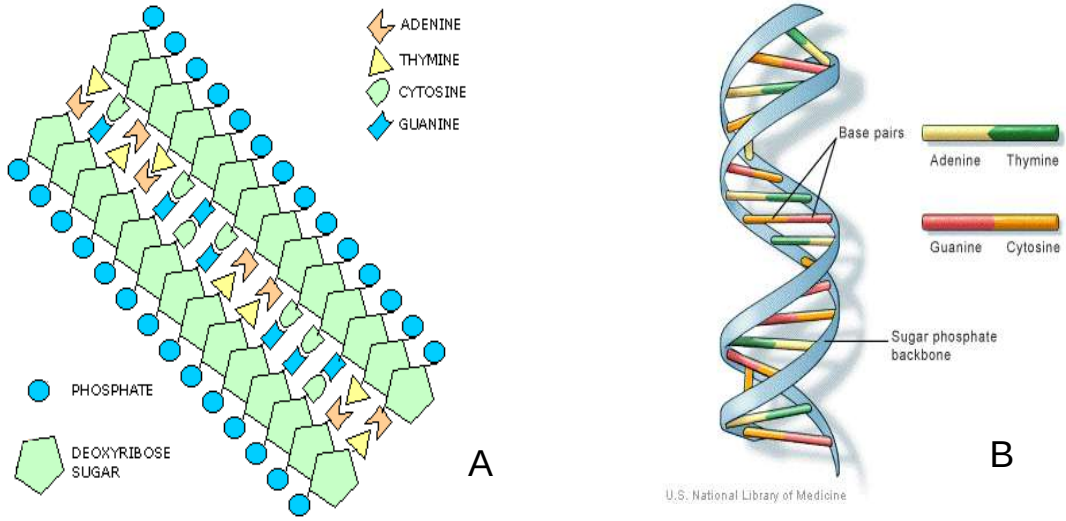


FIGURE 1.3 DNA STRUCTURE

The complementary base pairs are held in place by a sugar-phosphate backbone. In the cell protected by histones. Each three base pairs codes for one amino acid (if that region is for translation. Taken from www.biologyonline.org (A) & National Library of Medicine (USA) (B)

The first step in replication is for a helicase enzyme to break the hydrogen bonds that hold the bases together, splitting the strands. This preferentially starts at an A-T rich region, A-T having only 2 bonds as opposed to the 3 that hold C-G together. This forms a ***replication***

fork into which RNA primers can attach and attract the nucleotides for "extension" of the DNA strands. In the commonest form of replication, each daughter molecule comprises one old and one new strand and is termed "semi-conservative" replication **(Figure 1.4)**

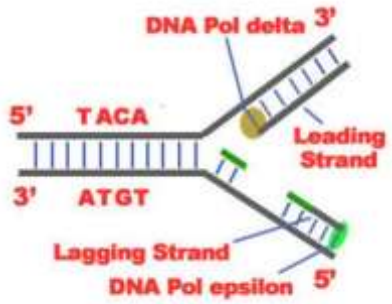


FIGURE 1.4 DNA REPLICATION FORK

Schematic showing DNA replication fork: *Taken from: www.dnareplication.info*

Different polymerase enzymes are required for the 5'-3' "leading" strand and the 3'—5' "lagging" strand, the latter being more complex. The orientation of the complementary strands results from the carbon atom of the sugar molecule to which the phosphate binds (***Figure 1.5***)

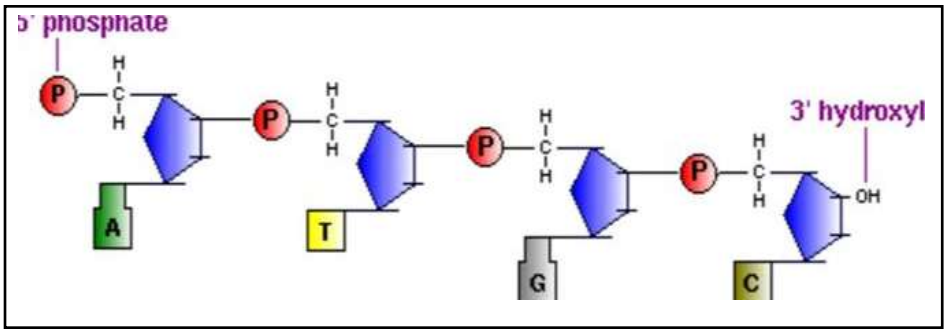


FIGURE 1.5 GRAPHIC ILLUSTRATING THE ORIENTATION OF THE SUGAR-PHOSPHATE BACK BONE OF DNA WITH BASE BOUND.

Single strand illustrated: sugar blue, phosphate red. *Taken from: www.vivo.colostate.edu*

This complementary sequencing is important in the design of molecular probes and primers for polymerase-chain-reaction based protocols.

1.3 DNA damage

DNA is under constant attack from various destructive agents including endogenous, as well as exogenous sources. Endogenous damage may result from spontaneous base loss or various types of base modification (for instance; cytosine deamination, converting it to uracil) caused by exposure to metabolic products such as reactive oxygen species, or mispairing errors introduced during replication (Lindahl, 1993, Gates, 2009). Exogenous damage has many sources including; UV light, X-rays or gamma radiation, thermal disruption or chemical exposure. DNA damage types can be broadly subdivided into base damage and backbone damage (Hoeijmakers, 2001).

1.3.1 Base damage

1.3.1.1. Deamination/Methylation

Epigenetic changes to DNA (i.e. not involving changes in base sequence) can regulate gene expression – often by silencing the gene (Keedy et al., 2009) However, they can lead to DNA mutation.This is notably the case with tumour suppressor genes. Cytosine residues in DNA can undergo hydrolytic deamination converting them to uracil residues. Up to 500 deamination of cytosine events take place in human cells per day. Other deamination reactions include conversion of adenine to hypoxanthine, 5-methylcytosine to thymine and guanine to xanthine. An example is shown in **Figure 1.6.**

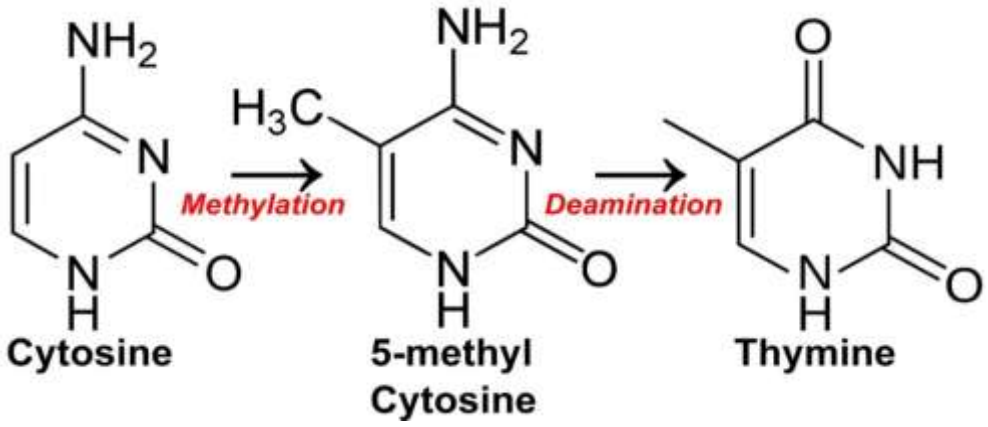


FIGURE 1.6 SCHEMATIC EXAMPLE OF METHYLATION/DEAMINATION
Source: (Bandarian et al., 2003)

Specific DNA damage is categorized by possible endogenous or exogenous sources and the corresponding DNA repair pathway for each type of damage. Hydrolysis of the glycosidic bonds holding a base to the DNA backbone is also common (Gates, 2009).

1.3.1.2. Oxidation

One of the causes of DNA base damage is exposure to reactive oxygen species generated during either normal cellular oxygen metabolism, from exposure to UV light or a wide range of other exogenous sources. A frequent oxidative lesion is 8-hydroxyguanine (8-OHdG); this mutagenic lesion shows preference to pair with adenine rather than cytosine during replication. This lesion is estimated to occur at a rate of up to 500 events per day, the same rate as cytosine deamination (Tudek et al., 2003), Gates, 2009). This is the most commonly studied DNA base oxidation product ***(Figure 1.7)***; in another response to oxidative stress, thymine converts to thymidine glycol.

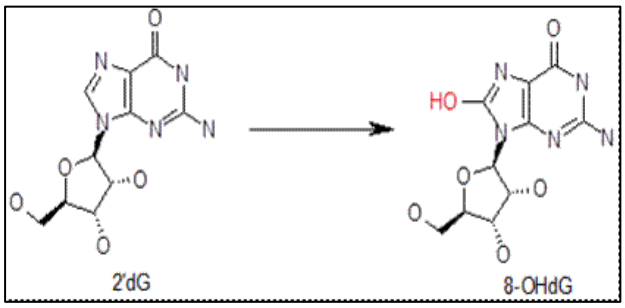


FIGURE 1.7 OXIDATION OF GUANINE

1.3.1.3. Alkylation

Another source of DNA base damage is where an alkyl group attaches to the DNA base giving alkylation products such as O2-alkylthymine, O4-alkylthymine, O6-methylguanine and O6- ethylguanine. This binding may prevent DNA replication causing mutation or cell death. Alkylation can be generated by both endogenous sources (for instance; oxidative by-product or cellular methyl donors such as S-adenosylmethionine) and exogenous sources (fuel combustion, tobacco exposure or anticancer therapies e.g. cisplatin) (Engelbergs et al., 2000) Fu et al., 2012).

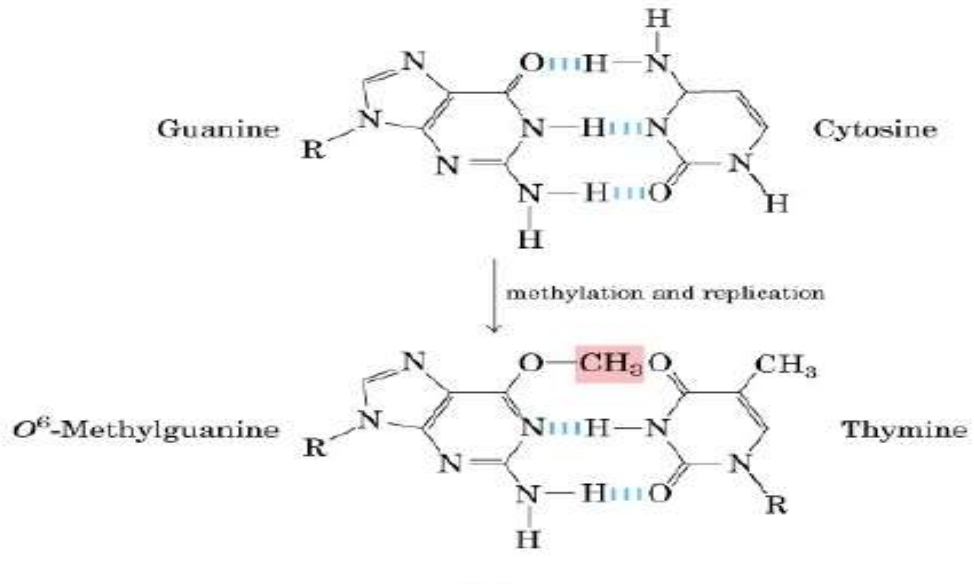


FIGURE 1.8 METHYLATION OF DNA BASES
TAKEN FROM HTTP://WWW.SLIDESHARE.NET/BENLAUFER/LONGLASTING-ALTERATIONS-TO-DNA-METHYLATION

1.3.2. Backbone damage

The DNA backbone is under constant exposure to environmental and endogenous agents that create thousands of lesions per cell each day (Lindahl, 1993). While some of these lesions like abasic sites or single strand breaks (SSB) are considered to be toxic, double strand breaks (DSB) are considered to be the most harmful. There are three major classes of DSB structures that can be toxic if not repaired.

(1) Two-ended DNA double-strand break, created by direct fracture of a DNA duplex.

(2) One-ended DNA double-strand break, created when a replication fork encounters a DNA single-strand break.

(3) Daughter strand gap, created when lagging or leading strand progression is inhibited by a DNA lesion (Helleday et al., 2007).

1.3.2.1. Double strand breaks

DSB are commonly the result of radiation or radiomimetic chemicals, but can also occur from physical stresses when polymerase enzymes encounter single strand lesions and the replication system fails. Mis-repair of these can cause major rearrangements of genetic material.

1.4. DNA repair

1.4.1. Relationship of damage to repair pathways

8 Methylation of DNA Bases DNA damage can be as simple as single altered or substituted DNA bases, or be more extensive, as is evident with lesions in both strands (double strand breaks). ***Figure 1.9***

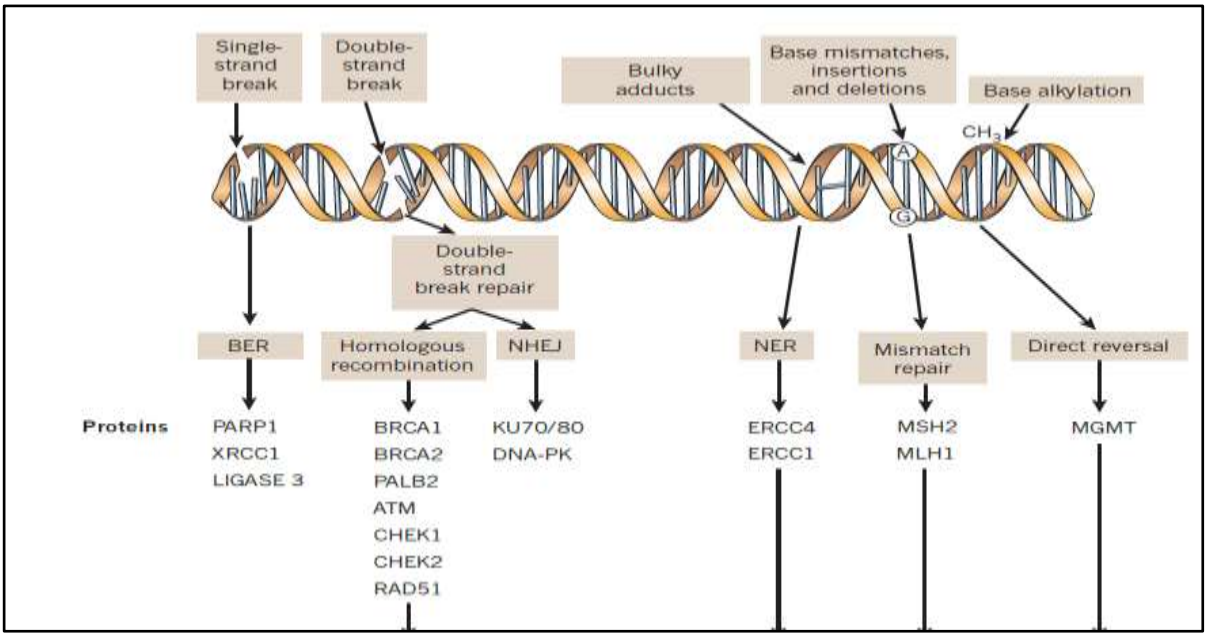


FIGURE 1.9 DNA DAMAGE, ITS REPAIR & THE PROTEINS EFFECTING THE REPAIR
Adapted from (Lord and Ashworth, 2012)

As implied in ***Figure 1.9,*** Repair mechanisms can be classified by the type of damage they are adapted to repair:-

The **Nucleotide Excision Repair** pathway deals with "bulky" base adducts resulting from external agents such as drugs or UV radiation as well modifications resulting from oxidative stress. They disrupt transcription or cause it to proceed in an error-prone fashion. The mechanism falls into two categories, a *global* system that deals with replicating genomic DNA (Fagbemi et al., 2011) and a *transcription coupled* variation that deals with lesions affecting RNA polymerase (Diderich et al., 2011). ***Base Excision Repair*** corrects non-"bulky" base modifications that occur frequently and as a result of normal mitochondrial production of reactive oxygen species. For example,180 guanines are estimated to be oxidized to 8-oxo-dG per mammalian genome per day (Lindahl, 1993). Base excision repair is initiated by a lesion- and site (nuclear or mitochondrial) specific DNA glycosylase. It is active in resting cells as well as dividing ones.

Mismatch Repair is an evolutionarily well conserved process going back to bacterial organisation. It both recognizes and repairs base-base mismatches and insertion-deletion loops derived from errors in DNA replication or homologous recombination (in which nucleotide sequences are exchanged between DNA molecules, a process designed for controlled genetic diversity driving evolution) (Jiricny, 2006, Modrich, 2006). ***Double strand break repair*** is designed to cope with serious DNA damage such as translocations. Unrepaired, these activate an apoptotic cell death response (Jackson and Bartek, 2009).

If repair is satisfactorily completed, any cell cycle arrest may be cancelled and the cell progresses normally to mitosis. If the damage cannot be fixed, then cells will senesce or move into apoptosis, with chromatin fragmentation and the formation of apoptotic bodies, mediated by caspases from the mitochondria. These bodies are relatively non-toxic, compared to the products of necrosis and are mopped up by cells of the reticulo-endothelial system. (Houtgraaf et al., 2006)

It is a third possibility, where the damage is not repaired but the cell persists and can divide, which potentially leads to carcinogenesis. Cancer formation relies on cell proliferation, so it is the compartment in tissues (such as in most epithelia) with a relatively high cell turnover – not so much the stem- or terminally differentiated cells, but the transit-amplifying region or population – that is most susceptible to tumourigenic effects from DNA damage. These alternative pathways are represented in ***Figure 1.10***

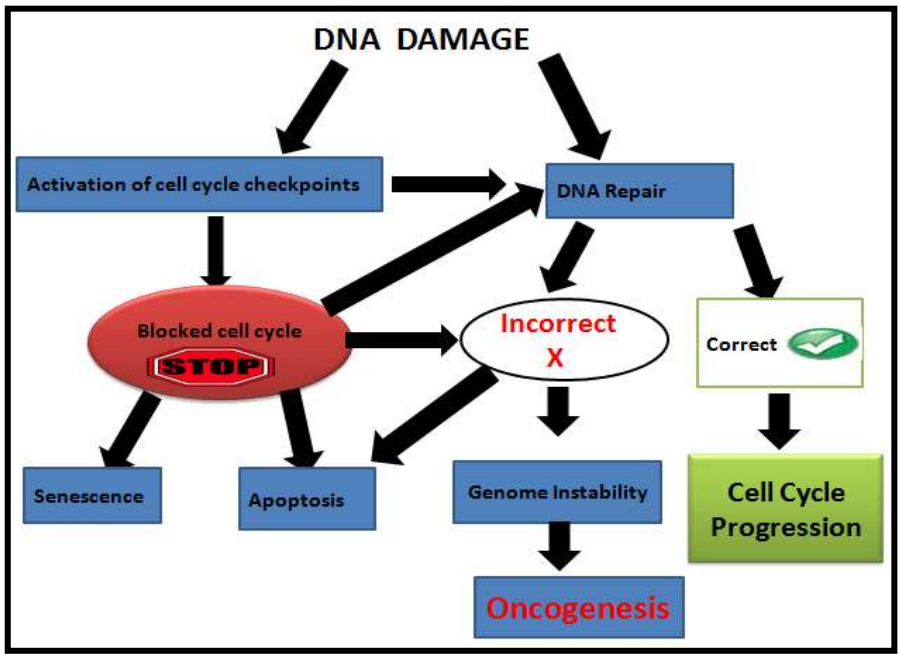


FIGURE 1.10 FLOW-CHART OF THE CELLULAR RESPONSE TO DNA DAMAGE (ADAPTED FROM HOUTGRAAF ET AL., 2006).

In proliferating cells, cell cycle checkpoints will be activated, leading to cell cycle arrest allowing activated DNA damage repair machinery to work. When repair is complete, the cell may proceed through the cell cycle. If the damage proves irreparable, the cell cycle can be blocked permanently, leading to senescence or apoptosis. If unrepaired damage is not detected and persists, this constitutes mutation; genomic instability ensues, potentially leading to oncogenesis. (***Figure 1.11)*** depicts pathways and gives examples of checkpoint regulation in eukaryotic cells

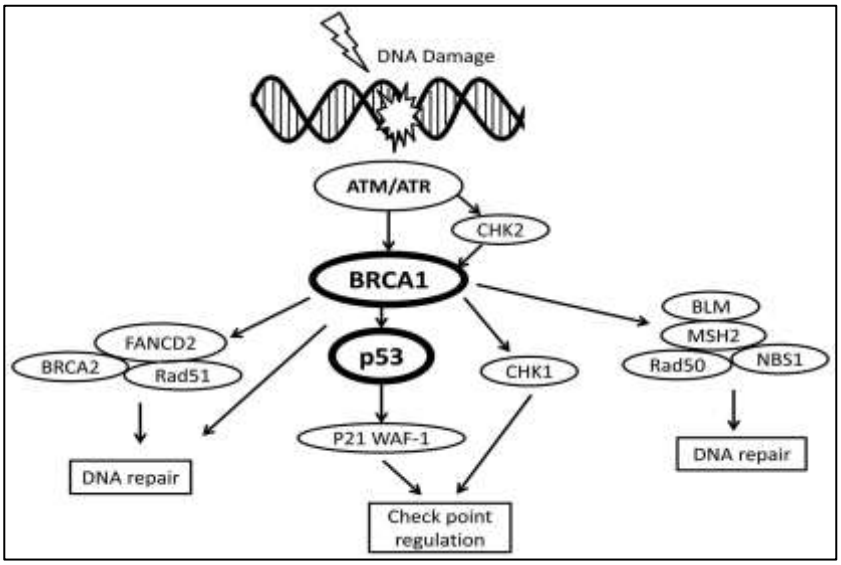


FIGURE 1.11 SCHEMATIC REPRESENTATION & OVERVIEW OF DNA REPAIR & CHECKPOINT REGULATION IN CELL CYCLE SIGNALLING PATHWAYS
ADAPTED FROM (KITAGISHI ET AL., 2013)

These and other molecules whose production is modulated from that associated with normal cell cycling in response to DNA damage or repair activity are described in detail below. All have been analysed in the present study as potential markers of ongoing genotoxicity.

1.4.2 Fourteen repair DNA genes analysed in this study

BRCA1, P53, ATR and ATM products are essentially concerned with damage detection and initiation of cell cycle checkpoints ***(see Figure 1.11)***. The remaining nine genes and products are directly related to repair mechanisms

1.4.2.1 p53

The P53 gene is located on the short arm of chromosome 17 (17p13.1)..(McBride et al., 1986)The gene spans 20 kb, with a non-coding exon 1 and a very long first intron of 10 kb. P53 was originally described in 1979, was pronounced the "guardian of the genome" (Lane, 1992) and the first gene to be given the label "tumour suppressor". These genes exist in the germline and if a germline mutation is passed on the host is congenitally one step further along the path towards carcinogenesis, requiring just a mutation of the second copy to precipitate complete p53 dysfunction. This is loss of heterozygosity.

P53 mutation is the most common genetic change associated with many cancers and lies at the centre of a cascade of enzymes, with regulatory kinases (including ATM) upstream of it detecting DNA damage and activating the P53 gene. Lysine acetylation and/or serine phosphorylation in the C-terminus of the protein promotes DNA binding to strand ends and catalyse renaturation. Published work regarding familial syndromes such as Li-Fraumeni, in which there is an association between sarcomas, brain tumours and breast cancer at a young age shows a frequent link to abnormalities in the p53 gene (Benson et al., 2009).
Many human tumour –associated p53 mutants have properties not seen in the normal protein. Virally induced cancers are frequently associated viral proteins that degrade p53 or inhibit its action (Vogelstein and Kinzler, 2001). Among the actions of P53 are inhibition of the cell cycle at the G2 arrest point, inducing a number of pro-apoptotic proteins, the best known of which is Bax. Specific down-stream targets of P53 that negatively influence invasion and metastasis include the *mammary serine protease inhibitor and* KAI1.There is a correlation between some types of p53 mutation and clinical outcome in breast cancer. Mutations impairing DNA binding were found in the more aggressive cancers (Alsner et al., 2000).

1.4.2.2 BRCA1

The ***BRCA1 gene***, located on chromosome 17 at q21 codes for the product named **breast cancer protein-1**. It is a multi-domain protein with an amino-terminal zinc-finger ring

domain that has been shown to alter caspase activity in a pro-apoptotic manner (Johnson 2002). It is responsible for repairing damaged DNA and involved in the control of cell proliferation in mature tissues (Hall et al., 1990) and in embryogenesis (Durant and Nickoloff, 2005). BRCA1 mutations are strongly associated with breast cancer (Miki et al., 1994).

BRCA1 was first cloned in 1994 (Miki et al., 1994). The gene product is directly involved in repairing damage (Boulton, 2006). The BRCA1 protein interacts with other molecules – including RAD51 and BARD1 gene products, *inter alia,* to mend breaks in double DNA strands. These breaks can occur as chromosomes exchange genetic material during normal mitosis. BRCA1 is therefore crucial in maintaining genetic integrity (Durant and Nickoloff, 2005, Paull et al., 2001). The BRCA2 gene (coding for breast cancer type 2 susceptibility protein) was cloned in 1994 by Wooster (Wooster et al., 1994). Its product is also involved in the repair of damaged DNA in the nucleus.

1.4.2.3 BRCA2

BRCA2 (location 13q12-13) also acts as a tumour suppressor gene. i.e. it behaves recessively and requires loss of heterozygosity to exert a deleterious effect, which is in fact an aberration in controlling the survival and proliferation of genetically compromised cells (Abdulrahman and Rahman, 2012b). BRCA 2 does not appear to interact with BRCA1 and complexes between the two proteins has not been demonstrated, BRCA2 interacts directly with 70 amino-acid BRC motifs on RAD 51 recombinase (Liu & West, 2002).

The BRCA2 protein interacts with, inter alia, RAD51 and PALB2 products in repairing DNA (Buisson et al., 2010, Wooster et al., 1995, Xia et al., 2006). As a so-called *tumour suppressor* gene it behaves in recessive fashion, so mutations are only effective where there is loss of heterozygosity. Around 50% to 60% of women who inherit BRCA1 (or indeed BRCA2) gene mutations will develop breast cancer by the age of 70. (Benson et al., 2009). All *BRCA2* mutations found so far appear to have been inherited, suggesting that there is a “founder” effect in play, where the mutation is common in certain relatively closed populations and can theoretically be ascribed to a single common ancestor (e.g. Neuhausen, 1996)

1.4.2.4 ATR

The cytogenetic location of the ATR gene is 3q23.The ATR (ataxia-telangiectasia- and Rad3-related) kinase is, collaboratively with the ATM gene product, a critical component of the system for maintaining genomic integrity. It is related to the gene in *Saccharomyces sp* which is responsible for cell cycle arrest by phosphorylating the checkpoint kinase CCHK1

(source: genecards.org). The product is a serine/threonine kinase acting in response to genotoxic stresses.

It functions both in parallel and cooperatively with ATM (joining the ATM process further downstream), but in respect of a more diverse range of DNA damage including ionising and non-ionising radiation, hypoxia and stalled replication forks (Abraham, 2001) Activated ATR phosphorylates *inter alia*, p53, BRCA1 and CHK1. Based on its central function in DNA damage repair, abnormal *ATR* is considered likely to cause susceptibility to cancer.

1.4.2.5 ATM

The ATM gene is located at 11q22.3, comprises 66 exons, 62 encoding a 3056 amino acid protein (Savitsky et al., 1995). The disease Ataxia-telangiectasia is a progressive disease affecting cerebellar function, microvascular lesions (telangiectasia), radiation sensitivity and immunodeficiency. ATM is a PI3K-related protein kinase, with the kinase activity associated with a highly conserved C-terminal region. It has functions to do with various aspects of normal organ development, but in the cancer context its role in double strand DNA break repair is crucial. A protein complex (named MRN) recruits the ATM product to sites of damage. Cascades of activities are initiated that involve the cell cycle checkpoint enzyme CHK2 as well as P53. It is involved in both a rapid and delayed response to damaged DNA (Ahmed and Rahman, 2006). Although the disease is associated with an increased cancer risk(Taylor and Byrd, 2005), the converse was not true in a study by FitzGerald (FitzGerald et al., 1997)so that breast cancer cases did not hold an increased rate of ATM mutation.

1.4.2.6 BRIP 1

This acronym derives from the gene product function, viz. BRCA1 Interacting Protein C-terminal helicase 1. The gene is located at 17q22.2 and comprises 184,751 base pairs. It is also known as FANCJ or BACH1. It is commonly a germ-line mutation, suspected of being the product of a founder effect it belongs to a family of DNA helicases that include XPD and as such, along with its BRCA association, should in principle act as a tumour suppressor. However, cancer patients do not always show loss of heterozygosity and BRIP-1 appears to possess *only low-to-moderate penetrance linked to no more than a two-to threefold increase in breast cancer risk (Cantor and Guillemette, 2011). Germ-line mutations show a much reduced incidence of childhood cancers than occur with BRCA1*

1.4.2.7 CHK1

Human Chk1 is located on chromosome 11 on the cytogenic band 11q22-23. Chk1 is a highly conserved protein kinase relatively unchanged through evolution covering all eukaryotic organisms. Gene expression analysis indicates an important role for this gene in "triple negative breast cancer" i.e. they do not express the oestrogen receptor, progesterone receptor or Her2 (human epidermal growth factor) gene products. It is therefore an important potential target in this hard-to-treat group of patients (Cantor and Guillemette, 2011). Its deletion causes catastrophic events during mitosis. Chk1 is essential to the mammalian DNA damage checkpoint, is important in embryogenesis and, as ATR regulates Chk1, hence its significance in cancer, both mechanistically and as a therapeutic target (Liu et al., 2000). Phosphorylation of CD25A by CHK1 product is required for delaying cell cycle progression in response to double strand breaks. It also integrates signals from ATM and ATR.

1.4.2.8 CHK2

The CHEK2 gene is located on chromosome 22q12.1. The gene contains 14 exons, and encodes a protein of 543 amino acids. The protein Chk2, a serine/threonine kinase, CHK2 serves as a tumor suppressor by playing an important role in DSB responses leading to cell cycle checkpoint arrest, apoptosis and DNA repair. Heterozygous germline mutations in *CHK2* are associated with a p53-independent variant form of the Li-Fraumeni syndrome (Bell et al., 1999) also, CHK2 mutations are also found in sporadic cancers (Dong et al., 2003) and down regulation of CHK2 protein has been reported in several cancers (Bartkova et al., 2004). Of interest, despite this reported importance of Chk2 for G1 and G2 cell cycle arrest, no gross effect on cell cycle arrest after DNA damage is observed in *CHK2*-deficient mice, suggesting that this role of Chk2 is not essential (Takai et al, 2002). Multiple pathways causing redundancy in certain genes is not uncommon (Lynce 2016)

1.4.2.9 PALB2

The PALB2 gene is found in the human genome on the chromosome 16 p (short) arm, at location 12.2. Interacting with the BRCA2 product, the PALB gene product causes *Fanconi anaemia* which is an inherited condition characterised primarily by bone marrow failure. Its involvement with DNA repair confers cancer predisposition status on it, with an estimated 2.3x elevated risk in the presence of a monoallelic mutation (Rahman et al., 2007). It may

act independently of the BRCA genes although it also collaborates with them in homologous recombination and double-strand break repair.

1.4.2.10 PARP1

Located at 1q42.12, spanning 47,412 base pairs, Parp1 codes for the Poly ADP-Ribose Polymerase 1 enzyme. It is primary involvement is with the repair of single-strand DNA breaks through the base excision repair pathway, although it also interacts with the BRCA genes in double strand break repair. Among its activities aside from DNA repair, it is thought to play a role in the origins of Fanconi anaemia and type-I diabetes. It tends to be upregulated some twofold in 30% of infiltrating ductal carcinomas of the breast (Ossovskaya et al., 2010). It is another target molecule of specific interest in "triple negative" breast cancer.

1.4.2.11 RAD50

The archetype RAD50 gene is characterised in yeasts of the Saccharomyces genus where it is involved in double-strand DNA break repair, but a similar gene is found in the human genome, with a cytogenetic location 5q31, coding for a 153kD zinc-binding protein It forms part of the 'MRN' complex with MRE11 and NBS1. Mutations in RAD50 are thought to cause an inherited DNA breakage-prone disease similar to Nijmegen breakage syndrome, which displays a stunted microcephalic phenotype, with a propensity for cancers of the lymphoid system. Too few Human RAD50 mutations have been described to make firm conclusions about their impact, but mice with mutations either die young or, in 20% of survivors, succumb to lymphoma or leukaemia (Sheikh et al., 2015)

1.4.2.12 RAD51

The RAD51 gene, located on the q arm of chromosome 15; it is essential in homologous DNA recombination. Mutations may have a greater association with ovarian than breast cancer with one study finding no pathogenic mutations in breast cancer only families (Pennington and Swisher, 2012). There is also a lack of incidence in particular demographic populations.

1.4.2.13 PTEN

Germline mutations are rare but can cause PTEN Hamartoma Tumour Syndromes the best known of which is Cowden syndrome (CS). ZHANG et al., 2013 reported CS to be associated with a high risk of breast cancer. In families with CS, ~80% have *PTEN* germline mutations; female CS patients have a 25–50% lifetime risk of breast cancer.

Over 90% of affected individuals will develop some clinical manifestation – usually hyperplastic and including muco-cutaneous lesions, during the third decade of life. Conversely some 40% of invasive breast cancers exhibit loss of heterozygosity at the PTEN locus (10q 23.3) . Functionally the normal PTEN product is a negative regulator of the PI3K/Akt/mTOR pathway which is overactive in many cancers (Pradella et al., 2014). In the cytoplasm PTEN product antagonises the phosphotidylinositol kinase pathway. It also accumulates in the nucleus, where it interacts with a small ubiquitin modifier to stabilise DNA. Cells lacking nuclear PTEN are hypersensitive to oxidative stresses (nih.gov/genes).

1.4.2.14 STK11 /LKB1

STK11 (also known as LKB1) protein is a serine/threonine kinase that acts effectively as a tumour suppressor. Mutation in this gene leads to disruption of cell polarity and is responsible for Peutz-Jeghers syndrome, an intestinal polyposis disease, that also carries an increased risk of breast cancer. (Bardeesy et al., 2002)Up to 8% and 31% of Peutz-Jeghers syndrome, at ages 40 and 60 years of age develop breast cancer.

1.5 Hallmarks of Cancer

The clinical result from serial DNA replication damage or mutagenesis from external sources as described above is uncontrolled proliferation and tumour formation. In some circumstances this may be spatially confined and lacking in invasive capacity giving risecto a benign tumour. More commonly a tumour progresses and metastasises, becoming malignant and therefore appropriately designated a cancer with attendant activated oncogenes, loss of suppressor gene heterozygosity and recruitment of host elements to support the continued growth.

Six important alterations in cell physiology required to transform normal cells into malignant cancer cells have been proposed firstly self-sufficiency in growth signals, secondly insensitivity to antigrowth signals, thirdly limitless theoretical potential for growth, fourth evasion of apoptosis, fifth sustained angiogenesis and finally, tissue invasion and

metastasis (Hanahan and Weinberg, 2000). These are represented schematically in ***figure 1.12*** and the basic mechanisms by which they operate are listed below.

(1) **Enabling replicate immortality**: the self-sufficiency hallmark is characterised mechanistically by autocrine stimulation, i.e. the ability of cells to manufacture their own growth factors, freeing them from exogenous homoeostatic control. There is also a positive paracrine feedback between cells in a single local milieu.

(2) **Evading growth suppressors:** a complementary hallmark is a loss of sensitivity to anti-proliferative signals. Transforming growth factor-β (TGF-β) is one such signal and disruption of its associated pathway is conducive to unregulated growth.

(3) **Sustaining proliferative signalling**: the number of daughter generations of somatic non-germline) cells are governed by the erosion of telomeres during repeated replication. It is indeed the function of telomeres to act as a buffer zone allowing for errors in the start of chromosome replication. The result is the so-called Hayflick limit – generally around 40 divisions, after which cells senesce. The upregulation of the enzyme telomerase reverse transcriptase promotes regeneration of telomeres, effectively immortalizing the cell.

(4) **Resisting cell death**: apoptosis is a method for arranging the death of effete or compromised cells in an organised, packaged fashion capable of removal by the reticulo-endothelial system without the microenvironment becoming awash with free enzymatic cell content. It is regulated by extrinsic or intrinsic pathways. In relation to carcinogenesis it is compromise in the latter that causes cells with defective DNA to persist. Later in tumour progression, resistance to external “death factors” such as FAS become important.

(5) **Inducing angiogenesis** : no structure can gain size without nutrition and oxygen. Neovasculature is therefore required in the growing tumour, by the process of angiogenesis, stimulated invasion of the cancer by vessels of host origin. Tumour cells commonly secrete high levels of vascular endothelial growth factor to this end.

(6) **Activating invasion and metastasis**: the final hallmark is tissue invasion and metastasis. The normal agents of tissue remodelling such as E-cadherin and the matrix metalloproteinases are deployed aberrantly by the tumour to effect expansion of the primary tumour, replacing the original tissue architecture (invasion) and detachment of cells or aggregates of cells, which can migrate into the lymphatics or bloodstream, trap in a downstream capillary bed and cause distant foci of malignancy ((metastasis).

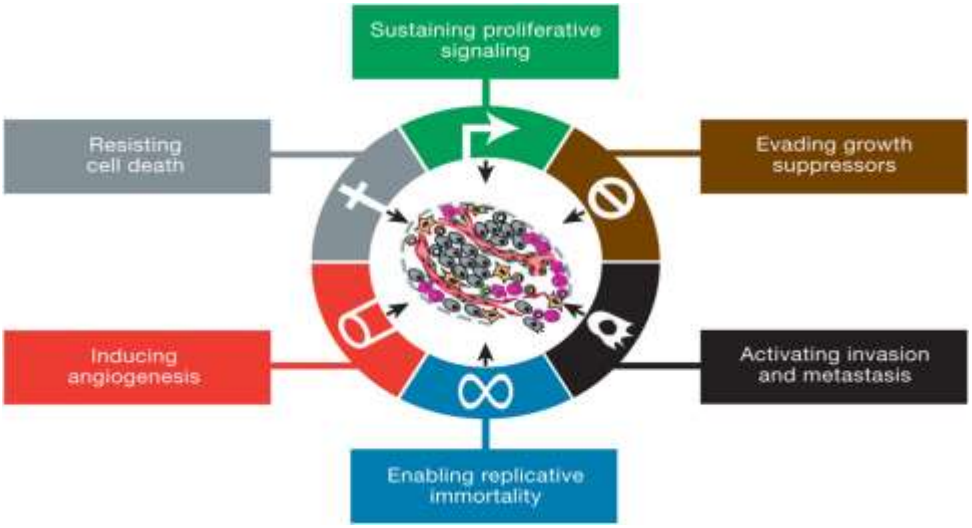


FIGURE 1.12 THE HALLMARKS OF CANCER

The six hallmark capabilities as originally proposed in 2000. (Graphic taken from Hanahan and Weinberg, 2000)

The scheme represented above has been further developed and alternatively expressed as shown in ***figure1. 13.*** This figure shows additionally two emerging hallmarks and two enabling characteristics. Firstly the ability of cancer cells to modify the normal metabolism of their cell-type of origin in order to support neoplastic proliferation. In the changed milieu within the cancer – particularly relating to oxygen tension - utilising manipulation of the inflammatory cytokine system. This, along with the requirement for neovascularisation and tissue remodelling led to the concept of cancer as “wounds that do not heal “(Dvorak, 1986), or perhaps equally “wounds that will not stop trying to heal”

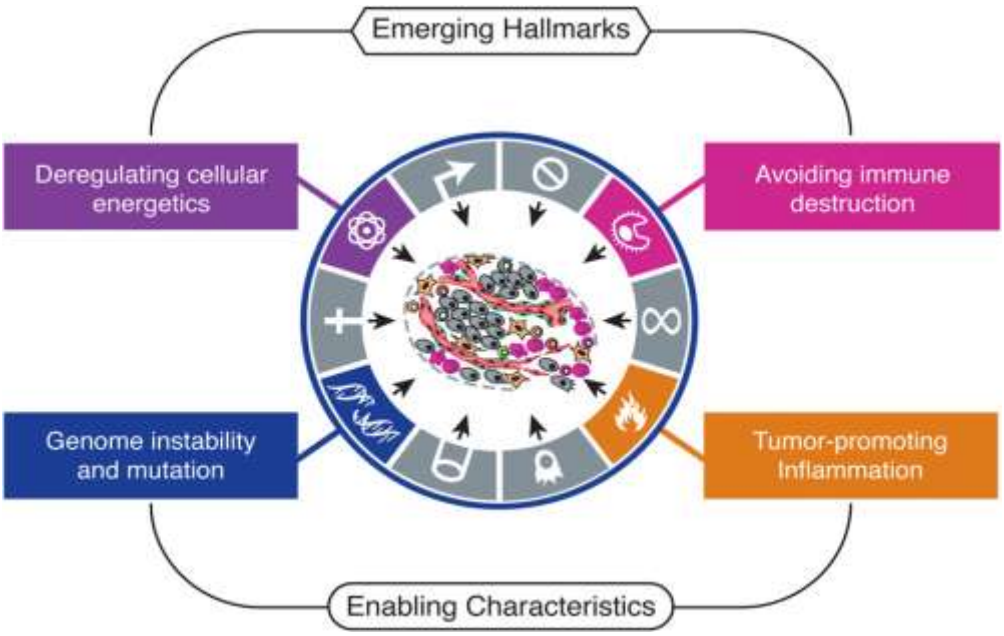


FIGURE 1.13 EMERGING HALLMARKS AND ENABLING CHARACTERISTIC. TAKEN FROM (HANAHAN AND WEINBERG, 2011).

The overall picture that emerges, then, is of interacting genomic, epigenetic and non-genomic alterations, driven by intrinsic and environmental factors often involving excess free radical formation or the inability to clear these molecular species effectively, producing a multistep cascade of chance events enabling a mutant cellular phenotype to emerge that does not recognise normal homoeostatic regulators. The underlying influence is an abrogation of the cell's ability to monitor its genomic integrity and rectify errors in replication. The compounds studied in this project, are constituents of cosmetics and which have been proposed to exert their influence on breast cancer through stimulation proliferation (Darbre, 2006a) but their ability to drive DNA mutation and genomic instability were previously unstudied in breast cells.

1.6. Breast cancer

1.6.1. Overview

Breast cancer is the formation of a malignant tumour that has developed from cells, normally epithelial cells, in the breast. The mammary gland exhibits complex cell kinetics; cycles of epithelial proliferation and regression accompany pregnancy and lactation (Pai et al., 2015). This dynamic makes the organ particularly susceptible to diseases dependent for their pathology on cell proliferation. It has been argued that the involution phase of the mammary cycle, accompanied by much apoptosis and tissue remodelling is a particularly vulnerable period (Schedin, 2006, Lyons et al., 2009).

1.6.2. Incidence and mortality

Breast cancer is heterogeneous in its presentation and diverse in its prevalence, stratification, morphology and prognosis (Al Tamimi et al., 2010). Breast cancer currently occurs throughout the world, and is the most common reason for death by cancer in women (Murray and Lopez, 1997). It has high incidence rates in more developed countries; rates in the most underdeveloped countries are low but increasing over time (Key et al., 2001).

The incidence is lowest in Eastern Asia (Ferlay, 2013) appears relatively low in East Africa with about 18,000 new cases and 10,000 cancer related deaths per year. In Western Europe, the incidence is five times higher with about 40,000 deaths recorded due to breast cancer in 2008 (Abdulrahman and Rahman, 2012a). The incidence is similar in Central and Eastern Europe with roughly 115000 new cases and more than 47000 deaths in 2008.

Many papers report breast cancer as the most common cancer in women in the UK since 1997; in 2009, there were 48,788 new cases, 48,417 women and 371 men. In 2010 there were 11,633 deaths from breast cancer in the UK (Marmot et al., 2012, Moss et al., 2012).

In the Kingdom of Saudi Arabia (KSA), despite the prevalence of breast cancer being lower than in Western countries (Al-Kuraya et al., 2005) it is still the most frequent malignant tumour in Arabian females, at 21.8%. Breast cancer in Saudi women exhibits differences from the disease manifestations in Western countries. According to Ezzat (Ezzat et al., 1999) breast cancers presenting in Saudi women are normally advanced at the time of diagnosis, and affect mostly females of 46-50 years of age. This differs markedly from the characteristic 60-65 years seen in developed countries where also advanced disease is relatively infrequent at diagnosis (Al Tamimi et al., 2010, Ezzat et al., 1999).

1.6.3 Risk factors.

Loss of function of the BRCA1 or BRCA2 genes is a main cause of inherited susceptibility to breast cancer (Roy et al., 2012). This has demonstrated the central importance of these genes in repair of DNA in breast cells, and the consequences of loss of their function to the breast cells. More recently, inherited loss of other components of DNA repair have also been suggested to contribute to breast cancer susceptibility (Kitagishi et al., 2013). The question still remains, however, as to whether the source of the DNA damage is natural replication errors or exposure to exogenous chemicals or radiation. Agents which can stimulate excess proliferation in breast cells have greater potential to generate errors at replication which if not repaired could result in DNA mutations and genomic instability. Such agents may be endogenous hormones, most notably oestrogens, or exogenous chemicals which can mimic oestrogen action. However, exposure to environmental radiation could

also provide sources of DNA damage which these women cannot repair, as has been shown from studies of breast cancer incidence in survivors of the atomic bomb at Hiroshima (Tokuoka et al., 1984) Alternatively, the DNA damage might result from exposure to environmental chemicals or their metabolites. Even the main endogenous hormone, oestradiol, is known to produce metabolites which can damage DNA (Sancar et al. 2004).

Although diet and alcohol consumption have been implicated in breast cancer, the main risk factor for breast cancer remains hormonal and in particular lifetime exposure to oestrogen. However, many pollutant chemicals enter the human body via oral, respiratory, or dermal routes which have endocrine-disrupting properties and can mimic or interfere with the action of oestrogen (Key et al., 2001, Lipworth, 1995). Increased exposure to endogenous oestrogens can result from early onset of menarche, late onset of menopause, null parity, late age of first pregnancy, lack of breastfeeding, and use of exogenous oestrogens derived from personal choices such as using the contraceptive pill or hormone replacement therapy (HRT) (Lipworth, 1995, Key et al., 2001).

The source of such chemicals may be phytoestrogens (from plants), pharmacological oestrogens, or man-made oestrogen-mimicking chemicals (xeno-oestrogens). They are sufficiently common that Darbre has reported that, with oestrogen playing such a prominent role in breast cancer, serious consideration needs to be given to the potential ability of exogenous oestrogen-like substances to drive the development and growth of breast tumours. In addition to the plant environmental oestrogens are hormones taken medicinally, as oral contraceptives or hormone replacement therapy (HRT). Oral contraceptives and HRT are confirmed as influencing breast cancer risk; so other xeno-oestrogenic compounds might also act similarly, either alone or in combination, if they enter the human breast (Darbre, 2010)

Environmental oestrogenic chemicals may enter the human body in food contaminated with persistent organic pollutants (POPs) as residues in dietary animal fat; and also through skin, air and oral exposure in the home to chemical components of plastics, cleaning materials, air fresheners, pesticides/herbicides, and soft furnishings. Also, Darbre suggests that alternative mechanisms of exposure may be through protracted application of cosmetics that contain compounds with oestrogenic activity (Darbre 2006, 2010).

These compounds have been studied for their ability to drive proliferation of breast epithelial cells but less attention have been given to other properties of these chemicals

which could enable other hallmarks of cancer, such as DNA mutation and genomic instability,

1.7 Components of Cosmetics

Many personal care products, including cosmetics, are applied to the skin on a regular basis as lotions, creams or sprays. Underarm cosmetics are applied to the local area of the breast directly, and dermal absorption of component chemicals has been suggested as an explanation of why more than half of all breast cancers in the UK start in the upper outer quadrant of the breast (Darbre 2005, Darbre and Charles 2010). The component chemicals are included in the products for a variety of reasons. The chemicals studied in this thesis were added for the purposes of conditioning and spreading (cyclosiloxanes), fragrance/ fragrance fixing (Lilial), antimicrobial deodorant / preservative (triclosan) and antiperspirant (aluminium salts). Use of plastic containers for the products may allow leaching of bisphenol A into the products.

1.7.1 Cyclosiloxanes

The siloxanes are a large group of compounds with a backbone of alternating silicon and oxygen atoms and with hydrocarbon groups attached to silicon side chains. The silicon-oxygen atoms in the cyclosiloxanes are singly bonded and form a ring. Hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) are common examples(Horii and Kannan, 2008). The general chemical structures of cyclosiloxanes are shown in ***figure 1.14.***

Cyclosiloxanes are used in the manufacture of silicone products. Also, they are used in cosmetics as conditioning and spreading agents. (Luu and Hutter, 2001) D5 is the most frequent siloxane found in all environmental matrices sampled except for air, where D4 predominates(Flassbeck et al., 2003). Also, Lu et al pointed out that D4 is permitted in personal care products at 60% by weight(Luu and Hutter, 2001).

D4 is considered toxic in many studies and interferes with the female reproductive system (Lieberman et al., 1999a, Hayden and Barlow, 1972).McKim et al (2001) report D4 to possesses intrinsic oestrogenic activity in *in vitro* models and *in vivo* uterotrophic assays(McKim et al., 2001). Studies have not shown D5 to be oestrogenic, however D5 has

been demonstrated to cause uterine endometrial adenocarcinomas in some animal studies. Also, there are potential dangers related to the possible impact of D5 on the neurotransmitter dopamine and the hormone prolactin (Ben-Jonathan, 2001, Besser et al., 2005).

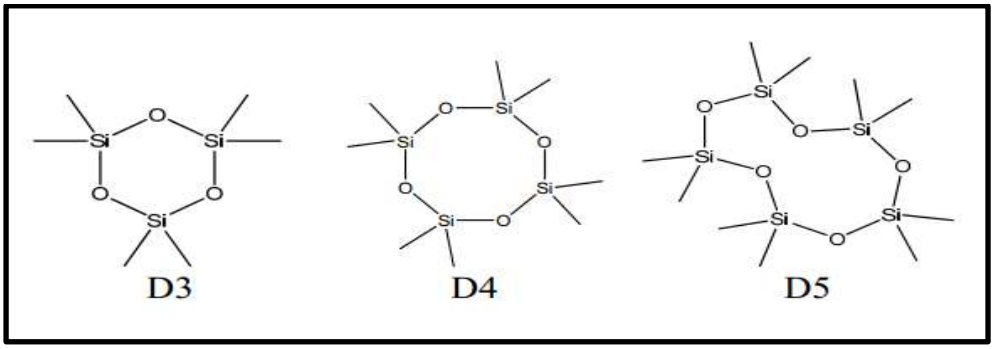


FIGURE 1.14 GENERAL STRUCTURE OF CYCLOSILOXANES.

Defined as any compound having a ring of alternating silicon and oxygen atoms, bonded together. Adapted from(Zheng et al., 2012, Rayeza et al., 1999)

1.7.2. Fragrances

Many synthetic musk fragrances are manufactured and used in perfumes, skin creams, deodorants, soaps, and detergents. Both polycyclic musks(Gomez et al., 2005, Bitsch et al., 2002) and nitromusks(Bitsch et al., 2002) have been shown to have oestrogenic activity as have benzyl salicylate, benzyl benzoate and butylphenylmethylpropional (Lilial) (Charles and Darbre, 2009a). Also, Benzyl salicylate, benzyl benzoate and butylphenylmethylpropional
(Lilial ^{TM}) are further compounds used currently in a wide range of cosmetics applied around the human breast, including underarm cosmetics. They are added primarily as fragrances and/or fragrance fixers. Additionally, in a recent survey, butylphenylmethylpropional and benzyl salicylate were found as labelled on 43% and benzyl benzoate on 28% of the cosmetic products surveyed (Buckley, 2007) Some of these musks have been measured in human milk (Kuklenyik et al., 2007, Reiner et al., 2007, Darbre and Charles, 2010). The general chemical structure of Lilial is shown in ***figure 1.15.***

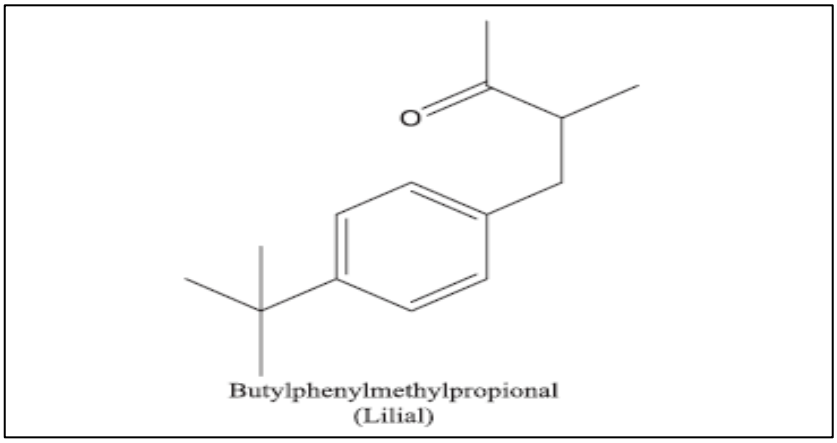


FIGURE 1.15 CHEMICAL STRUCTURES OF BUTYLPHENYLMETHYLPROPIONAL [LILIAL]
CAS no.80-54-6; 2-methyl-3-(4-tert-butylphenyl)propanal; 2-(4-tert-butylbenzyl)propionaldehyde; p-tert-butyl-α-methylhydrocinnamic aldehyde].(adapted from Charles and Darbre, 2009)

1.7.3. Triclosan

Triclosan (5-chloro-2(2,4-dichlorophenoxy)-phenol) is also known as Irgasan. The chemical structure of triclosan is shown in ***figure 1.16.*** It is an antibacterial agent incorporated into soaps, toothpastes, first-aid products, fabrics, plastic goods and in cosmetics to improve storage properties (James et al., 2010). Also, it is incorporated into deodorants to stop microbial growth on the skin surface, which metabolises sweat producing body odour (Liu B, 2002). Triclosan has been shown to inhibit enoylreductase enzymes involved in type II fatty acid syntheses in certain bacteria. Set against these useful properties, Triclosan is also cytotoxic to human breast cancer cells (Foran et al., 2000, Darbre, 2006a). Triclosan can be detected in aquatic environments including river and ground water. It therefore also gets into sediments and biomaterial including fish and human breast milk (Adolfsson et al., 2002, Kim et al., 2011a). In recent times, Gee et al have demonstrated that triclosan exerts both oestrogenic and androgenic effects on human breast cancer cells (Gee et al., 2008b).

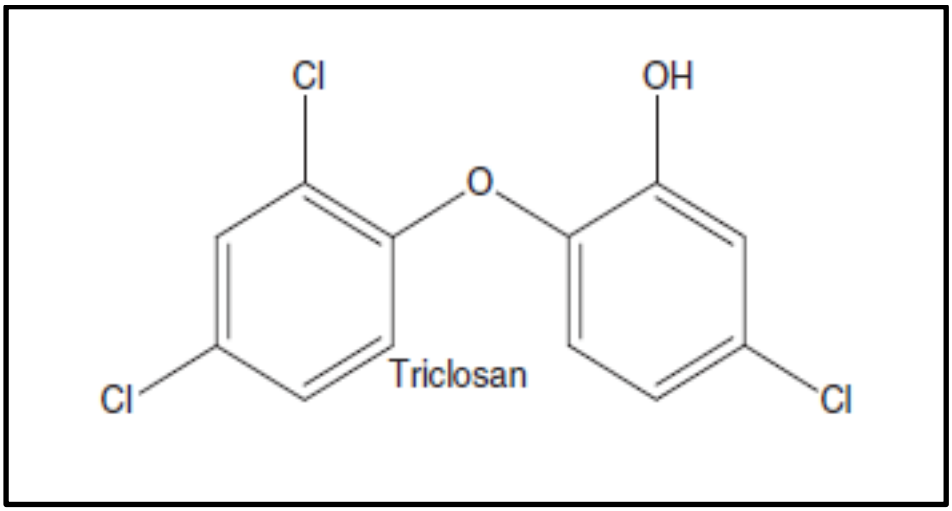


FIGURE 1.16 GENERAL STRUCTURE OF TRICLOSAN
CHEMICALLY IT IS 2,4,4'- TRICHLORO-2'-HYDROXYPHENYL ETHER). FUNCTIONAL GROUPS INCLUDE BOTH PHENOLS AND CHLORIDES(DARBRE, 2011)

1.7.4. Aluminium

Aluminium (Al) is the third most abundant element after (oxygen and silicon), and its compounds make up about 8% of the Earth's surface (Exley 1998). The human breast is exposed to aluminium from many sources including diet and antiperspirants (Krewski,et al. 2007; Mannello, et al. 2011). The action of Al salts in antiperspirants arises from their ability to prevent sweat reaching the skin surface, this perhaps through the formation of a physical plug of precipitated salts and desquamated cells at the superficial orifice of the sweat duct (Laden and Hunter 1998; Darbre 2005). One published paper has demonstrated Al at higher levels in breast tissue from outer compared to inner breast quadrants, which may result from antiperspirant use in the underarm area (Exley, Charles et al. 2007). Darbre has suggested that the large number of breast cancers developing in the upper outer quadrant of the breast might be related to antiperspirant application patterns. (Darbre, 2005, Darbre and Charles, 2010).

Al is, moreover, identified to be capable of causing DNA damage, epigenetic effects and many other biochemical aberrations within cells. Al exposure has been reported to be neurotoxic and contributes to neurodegenerative diseases such as Parkinson!s (Flaten, 1990) and Alzheimers disease (Zatta et al., 2009; Mannello et al., 2011). Al is classed as a metalloestrogen because in MCF-7 human breast cancer cells it has compromised the binding of oestradiol to estrogen receptors and enhanced transcription from an estrogen-responsive gene in (Darbre, 2006). Recently, Sappino et al have suggested that aluminium chloride ($AlCl_3$) is not generically mutagenic, but functions similarly to an activated

oncogene by inducing proliferation stress, DNA double strand breaks (DSBs) and senescence in the normal mammary epithelial cell line MCF10 A (Sappino et al., 2012)

1.7.5. Bisphenol A

Bisphenol A (BPA) (4,4-isopropylidenediphenol) is not directly applied to the skin as a lifestyle choice. It is relevant here because it is likely to leach from plastic containers. It is involved in the manufacture of polycarbonate plastics and epoxy resins (Vandenberg et al., 2007a). In the home BPA is commonplace, found in plastic food containers, baby bottles, and lining metal food cans(Welshons et al., 2006).BPA is a diphenyl compound that has two hydroxyl groups in the para position; it thus has similarities (see ***Figure 1.17***) to the physiological oestrogen 17β- oestradiol and the synthetic oestrogen diethylstilboestrol(Soto et al., 2008), mainly in the presence of hydroxyl residues at either end of ring-containing molecules of similar dimension.. Kang et al (Kang et al., 2006) have reported leaching of BPA into food or water from plastic containers. This migration is influenced by the manufacturing process, storage environments and heating by end-users(Kang et al., 2006). BPA has oestrogenic properties (Bcnefeld-largensen and Long, 2007); in animals BPA has been demonstrated to induce mammary hyperplasia (Durande et al., 2007). It is detectable in human breast milk., (Kuruto-Niwa et al., 2007).

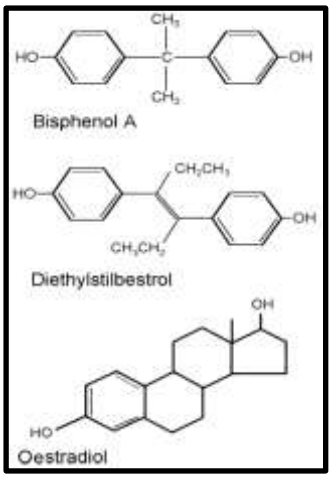


FIGURE 1.17 CHEMICAL STRUCTURE OF BISPHENOL A (BPA), DIETHYLSTILBESTROL (DES) AND 17B-OESTRADIOL

The structures of BPA and DES show greater similarities to each other than either are to the native hormone 17β oestradiol. This illustrates the range of structures capable of binding to the oestrogen receptor.(Soto et al., 2008).

AIMS OF THIS THESIS

The aims of this project were:

- To investigate possible genotoxic actions of the environmental chemicals bisphenol A, triclosan, three cyclosiloxanes (D3, D4, D5), butylphenylmethylpropional (Lilial) and aluminium using MCF10A and MCF10F immortalised non transformed human breast epithelial cells in culture, The effect of each chemical was studied:-
    - On the ability of MCF10A and MCF10F cells to form colonies in suspension culture, a change which constitutes a strong indicator of transformation in these cells (Soule,1990).
    - On DNA damage assessed before and after exposure to each chemical using the comet assay (Collins, 2004).
    - On expression of mRNAs for DNA repair enzymes in MCF10A & MCF10F cells. This was carried out using real time RTPCR for BRCA1,BRCA2,p53,ATM,ATR, Rad50&51, CHK1&2, PARP1, BRIP1, PALB2, PTEN and STK111 Short term (7days) and long term (30 weeks) effects of the chemicals were compared.

    - Western immunoblotting was used to assess whether changes in BRCA1 mRNA are reflected by similar changes in protein production.

Chapter 2 Materials and Methods

2.1 Cell proliferation experiments

Cells were suspended from stock dishes by treatment with trypsin- EDTA solution (as described in section 2.2) and added to an equal volume of phenol red free DMEM including 5 % (v/v) dextran-charcoal-stripped-FCS (DCFCS), penicillin (100 U/ml) streptomycin (100µg/ml) and 2mM glutamine (Darbre et al., 2003). Cells were counted on a haemocytometer and added to the required volume of phenol red free DMEM as above at a concentration of 0.2×10^5 cells/ml. Cells were plated in 0.5-ml aliquots into 4-well plastic tissue culture dishes (Nunc, Roskilde, Denmark). The medium was replenished after 24 h to contain the required concentration of treatment or an ethanol vehicle control and cell counts completed by counting released nuclei on a ZBI Coulter Counter as described in section 2.6, below. Test medium was changed routinely every 3 days and cells were counted at time zero after 24 hours of plating and after 7 or 14 days.

2.2 Cell culture models for studying DNA repair

Breast cancer is a classic case of hormone dependency in cancer. The sub-clone of the cell line MCF10 (MCF10F), as used in this study was instrumental in establishing that oestrogens are indeed themselves fully carcinogenic in that natural 17beta oestradiol and its metabolites exert as much neoplastic transformation as chemical carcinogens such as benzopyrene (Russo and Russo, 2006). This concept has been reinforced by Darbre (Darbre, 2012), contrasting the use of normal cell lines with models derived from metastatic tumour although she also points out that supraphysiological concentrations of hormone are required. This background is, however more than adequate to justify the use of the immortalised but non-neoplastically transformed MCF10A and MCF10F cell lines as the in vitro models of breast cancer here.

The MCF10 cell line was derived from fibrocystic breast tissue by long-term culture in low-calcium, serum free medium and characterized by Soule (Soule et al., 1990b). The MCF10A cells were derived from an adherent population in those cultures, while the MCF10F line was cloned from floating cells but is now grown on substrates, with anchorage dependency. The cells are non-tumourigenic *in vivo*, have epithelial morphology and stain for epithelial sialomucins, cytokeratins and milk fat globule antigen. On plastic substrates the MCF10A cells are adherent but not fully contact inhibited, tending to pile up to form domes. The use of both cell lines in this study is justified as the 10F clone has some anchorage independence in its past history and the colony-forming assay described below is predicated on assessing growth potential in suspension,

2.3 Culture of stock MCF10A and MCF10F cells

MCF10A and MCF10F immortalized, non-transformed human breast epithelial cells (Soule et al., 1990a) were obtained from the American Tissue Culture Collection (ATCC). Cells were grown as monolayer cultures in Ham'sF-12and Dulbecco's modified Eagle's medium (DMEM) containing phenol red(1:1 v:v ratio) (Invitrogen, Paisley, UK) supplemented with 5% (v/v) horse serum (Invitrogen), 10µgml–1 insulin (Sigma), epidermal growth factor (EGF) (Sigma) and 500ng/ml hydrocortisone (Sigma). Media were supplemented with 5ml Penicillin/ streptomycin solution containing 10,000 units of penicillin and 10mg/ml streptomycin. Cell lines were kept at37°C and in a humidified atmosphere of 10% carbon dioxide in air. Cell stocks were sub cultured with 0.0 6% trypsin/ 0.02% Ethylenediaminetetraacetic acid, (EDTA) (pH7.3) at weekly intervals.

2.4 Sub culturing of cells

All stock cells were sub- cultured at weekly intervals. MCF10A cells were taken from one 9cm diameter dish and medium was sucked off. The cells were washed with 2 ml Hank's Balanced Salt Solution (HBSS) (Invitogen, UK) to remove the serum which would inhibit the action of trypsin. After that, cells were incubated at 37°C with HBSS containing 0.06% trypsin (w/v), 0.02% EDTA, pH 7.3 (Invitrogen, UK) for 15minutes (mins) for MCF10A. The cells suspensions were then supplemented with to 2 ml of relevant stock medium and re plated at a 1/10 dilution into the relevant culture medium in 9-cm tissue culture dishes (Nunc, Denmark).

2.5 Long term incubation of cells with cosmetic chemicals

A vial of stock cells (MCF10A) was taken from liquid nitrogen and thawed in order to start of the long –term exposure of cells to agents of interest. This ensured a similar starting passage number as used in previous experiments (Daly et al., 1990) and enabled comparisons with starting control cells at any point of time. Cells were grown for 3 weeks as stock cells before any experiment commenced. After this period of maintenance, cells were washed twice with HBSS (Invitogen, UK) and disaggregated with trypsin/EDTA (section 2.2). The cells were seeded into 6-well tissue culture plates (Nunc, Denmark) and grown in a humidified atmosphere of 10% carbon dioxide in air at 37°C for up 30 weeks with media changed every 3 days. Stock cells were passaged every 7 days. Cells were maintained with or without test compound **(See Table 2-1).**

Table 2-1 The cosmetic –related chemical tested for the study

Chemical	MW	Source	CAS No	Purity
Bisphenol A	228.29	Aldrich 239658-50G	80-05-7	99.95%
Triclosan	289.5	CIBA Irgasan DP300	3380-34-5	97-103.0%
Butylphenylmethylpropional; 2-(4-tert-butylbenzyl)propionaldehyde (Lilial)	204.31	Sigma 95338-10MG-F	80-54-6	90.0%
Hexamethylcyclotrisiloxane (D3)	222.47	Aldrich 235687-25G	541-05-9	98.5%
Octamethylcyclotetrasiloxane (D4)	296.62	Aldrich 23,569-5	556-67-2	95-99.8%
Decamethylcyclopentasiloxane D5)	370.78	Aldrich 444278-50ML	541-02-6	90%
Al chloride	133.34	563919 Aldrich	7446-70-0	99.99%
Al Chlorohydrate	36.46	258148 Sigma-Aldrich	7647-01-0	

Compounds were diluted in ethanol to give stock solutions. Stocks were then diluted 1:10,000 into culture medium. Controls contained equivalent amounts of ethanol.

2.6 Assay of colony growth in methocel suspension culture

A solution of 2% methylcellulose (methocel), made from 1gm of methyl cellulose in autoclaved 100ml glass bottles with magnetic stirrers, plus 50ml of stock medium MCF10A stirred for 2 days in the cold (4^0C room.) For experiments, this was diluted with an equal volume of the same stock medium without methylcellulose but containing 2×10^5cells/ml together with either ethanol vehicle (control) or treatment 17β-oestradiol, hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5), bisphenol A (BPA), triclosan or butylphenylmethylpropional (Lilial). Subsequently, 2.5ml aliquots were seeded into six-well suspension culture plates (Costar) and grown for 21 days. Cells were fed every 7 days by the addition of 0.5 ml fresh MCF10A media. The number of colonies in each dish was counted manually every 7 days under the microscope. The mean size of colonies was measured using a 10× objective on an inverted Nikon EclipseTE200 microscope using software NISElementsAR-2.10 with the micron marker facility switched on. The average colony size was determined from 10 colonies for each field of view and results were calculated as the average colony size ± SEM for 15 fields of view. Results were compared statistically by One-way analysis of variance (ANOVA): Dunnett's Multiple Comparison Test, using Graph Pad Prism 5™.

2.7 Cell counting using a Coulter counter

Cells were counted using a Coulter counter after 24 hours (hrs) to provide the plating density or after (7, 14 and 21 days) to measure cell growth. In this method, cells were counted as nuclei them rather than whole cells. This avoids the problem of cells clumping as nuclei appear to have no attraction for one another. Cells were washed twice with 0.25 ml saline solution (0.9% w/v NaCl in water) and lysed in 0.5 ml HEPES/MgCl solution (made from 10ml from 1M HEPES and 0.305 gm MgCl2.6H2O per litre in distilled water) were added plus 2 drops of Zapolobin (Beckman Coulter). They were left for 20-30 min at room temperature (RM). The released nuclei were checked under the microscope and counted in Isoton on ZBI Coulter counter (Beckman Coulter).

2.8 Counting cells from methocel culture by Coulter counter

Five ml of saline (0.9% w/v NaCl in water) was added to each well. The resulting cell suspensions were harvested into 50ml tubes and centrifuged for 20 min at 4000 rpm in an Eppendorf centrifuge 5810. The supernatants were removed and the cell pellets washed twice with1 ml saline and lysed in 1 ml HEPES/MgCl solution - 10ml of 1M HEPES and 0.305 gm MgCl2.6H2O per litre in distilled water plus 2 drops of Zapoglobin (Beckman Coulter). They were left rocking for one hour at room temperature. The released nuclei were checked under the microscope and counted in 9 ml Isoton™ on a ZBI Coulter counter (Beckman Coulter).

2.9 Measurement of DNA damage

Single cell gel electrophoresis, also known as the 'comet' assay, is a rapid and sensitive method of assessing, semi-quantitatively, DNA damage *in vitro* and *in vivo*, identifying individual cells so affected. It addresses factors which modify mutagenesis and carcinogenesis (Collins, 2004, Pandey et al., 2006) and as such has particular relevance to the present study. The comet assay works on the principle that strand breakages in DNA lead to the relaxation of the super-coiled duplex molecule with the production of smaller strands which can be stretched and separated by electrophoresis under alkaline conditions, where the breaks are more labile (Singh et al., 1988). Negatively charged free DNA then moves towards the anode. (Pandey et al., 2006) DNA migration is a function of both the size and the number of broken DNA strands and tail length is directly related to damage. The standard comet assay protocol was designed for use with lymphocytes and hepatocytes (Singh et al., 1988,Van Dyk and Pretorius, 2005) the method described here was adjusted for the current usage with anchorage-dependent epithelial cells such as

MCF10A. Comet assays run at different pH measure damage differently. At neutral pH it measures only single strand breaks; at the alkaline pH used here it detects both single and double strand breaks.

2.10 Alkaline single-cell gel electrophoresis (SCGE) Comet assay

The alkaline single gel electrophoresis (SCGE) or comet assay was employed to detect single strand and double strand breaks in DNA and alkali-labile DNA adducts in the immortalised human breast epithelial cell line MCF10A. It is best not to use transformed cells where there is genomic instability and considerable endogenous DNA damage and polyploidy. The MCF10A cells are appropriate as they are not transformed and have a relatively diploid genome.

The MCF10A cells were plated out at a concentration of $0.4x10^{-5}$ cells/ml, using the same stock cell culture medium as described previously in section (2.1.). Cells were seeded into 9cm dishes containing 10 ml of medium. After 24 hours the medium was changed to the same medium containing the required concentration of the test compound, positive control or vehicle control for one hour exposure. After one hour, the culture medium was sucked off and plate was washed twice with phosphate buffered saline (PBS). The cells were scraped from the well into a 15 ml tube in PBS. The cells were counted on the haemocytometer. Cells were centrifuged, the supernatant was removed and the cell pellet embedded in 0.5% low- melting point agarose (Sigma) at a final concentration of about 10^4 cells/ml. Slides were prepared with 0.5% standard melting point agarose in PBS (0.05g in 10ml PBS) heated in a microwave to dissolve and 110 µl pipetted onto a poly-L-lysine-coated frosted microscope slide. A coverslip was placed carefully over the agarose and chilled at 4^oC for 10-15 minutes to allow the agarose to solidify. The coverslips were removed carefully. After that, 0.5% low melting point agarose was prepared in PBS (0.05g in 10ml PBS) by heating in a microwave to dissolve. 65µl of low melting point agarose was mixed at 37^oC with 10µl of the cell suspension.

The agarose/cell mixture was placed on top of the previous layer of agarose on the slide. Slides were covered with coverslips and chilled at 4^oC until solid for 10-15 min. All steps were done with minimal exposure to light to reduce background DNA damage (eg in a dark cold room or wrapped in foil on the bench).Then coverslips were removed and slides were placed in a chilled lysis solution at 4^oC in the dark, for one hour. The slides were removed from lysis solution (10mM Tris pH 10 (pH adjusted with NaOH), 2.5M NaCl, 100mM EDTA (di-Na salt), 1% triton X-100 added fresh) and placed in the Comet assay tank at 4^oC. The tank was filled with just enough electrophoresis buffer (0.3M NaOH, 1mM EDTA, pH> 10) to completely cover the slides. The slides were left in the electrophoresis buffer for 20-30

minutes at 4oC in the dark to allow the DNA to unwind. The slides were subjected to electrophoresis at 4^{o}C in the dark at 25volts for 20 minutes (or 15V for 30 min). The current was restricted to <300milliamps. Following electrophoresis, the slides were removed from tank and placed in tray of neutralisation buffer (Tris 0.4M pH7.5) 3 times for 5 minutes each time and fixed by placing in cold methanol for 5 minutes. The DNA on the slides was visualised by one drop 4',6-diamidino-2-phenylindole (DAPI) per slide and viewed under a fluorescence microscope.

An Axia fluorescence microscope (Carl Zeiss-Axioimage. A1) fitted with anaxiocam microscope camera was used to visualise the comets, using a x20-25 objective. The extent of the DNA damage is in proportion to the migration of DNA from the head into the comet tail. Fifty cells per treatment were scored and tail length was measured in micrometers (µm). The average tail length was determined from 50cells for each field of view and results were calculated as the average tail length ± SEM. Statistical analysis constituted using One-way ANOVA (analysis of variance) from the SPSS software package.

N.B. (DES) was used as a control for the comet assay because previous work has confirmed that exposure to DES produces DNA damage visible as comet in the assay. (Anderson et al 1998).

2.11 PCR based techniques

As an overview, a schematic of the steps required to generate real-time RT-PCR results is depicted in ***figure 2.1.***

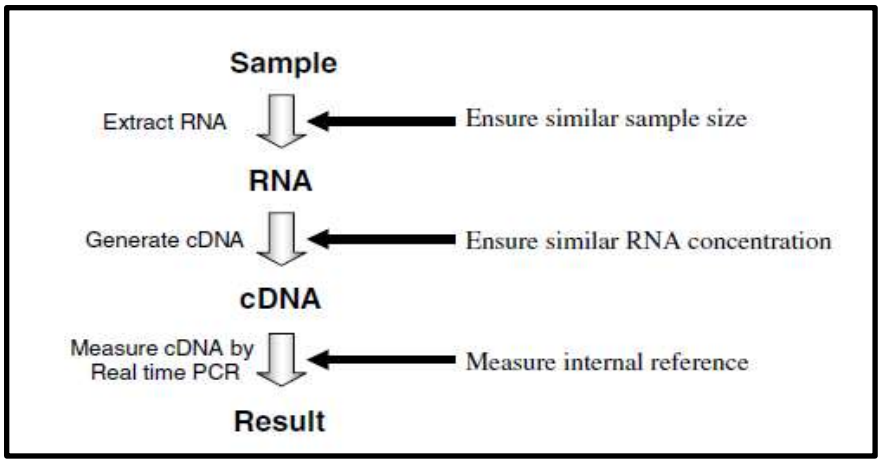


FIGURE 2.1 PROCESS REQUIRED TO GENERATE A REAL TIME RT-PCR RESULT.
Black arrows indicate points, which should be considered for a good normalization strategy (Huggett et al., 2005).

2.11.1 Total cellular RNA extraction

MCF10A cells were added to the required volume of Ham'sF-12 and Dulbecco'smodified Eagle's medium (DMEM) containing phenol red mixed in a 1: 1 ratio by volume, to achieve a concentration of 0.8×10^5 cells/ml, and plated in 16 ml aliquots in 9 cm plastic tissue culture dishes to achieve an adherent monolayer. Cells were left for 7 days, when the medium was changed to stock medium supplemented with the required concentration of test compound, ethanol vehicle or 17β- oestradiol positive control. The cells in the 4-well dishes were counted using the Coulter counter as described in section 2.6.

After 7 days the cells in the 9 cm dishes were washed with isotonic saline and harvested into ice- cold isotonic saline using a rubber policeman, pelleted by centrifugation and cell pellets were stored at -80°C. Whole cell RNA was produced using the RNeasy™ Kit with on-column DNase treatment as described by the manufacturer (Qiagen) as follows:-

Firstly, 600 µl RTL buffer and 6 µl β -mercaptoethanol (β-ME) was added to the cell pellet and vortexed thoroughly. Next, the lysate was pipetted onto a QIA shredder spin column placed in a 2 ml collection tube and centrifuged for 2 min at maximum speed in a microcentrifuge. Then, one volume (600µl) of 70% ethanol was added to the homogenized lysate and mixed well by pipetting cleared lysate to precipitate DNA but not RNA. Subsequently, 700 µl of the sample was applied, including any precipitate, to an RNeasy mini column placed in a 2 ml collection tube. The tube was closed gently, and centrifuged for 15s at 8000x g (≥10,000 rpm). The flow-through was discarded. Then the procedure was repeated with remaining lysate. The flow through was discarded again.

After that, 700µl Buffer RW1 was added to the RNeasy column. The tube was closed gently, and centrifuged for 15 s at ≥8000xg (≥10,000 rpm) to wash the column. The flow-through was discarded with collection tube. Consequently, the RNeasy column was transferred into a new 2 ml collection tube. 500 µl Buffer RPE was pipetted onto the column. The tube was closed gently, and centrifuged for 15 s at ≥8000xg (≥10,000 rpm) to wash the column. The flow-through was discarded. Next step, another 500 µl buffer RPE was supplemented to the RNeasy column. The tube closed gently and centrifuged for 2 min at ≥8000xg (≥10,000 rpm) to dry the RNeasy Silica-gel membrane. The RNeasy column was placed in a new 2 ml collection tube and the old collection tube with the flow-through was discarded. It was spun in a microcentrifuge at full speed for 1 min. Finally, the RNeasy column was placed in to a new 1.5 ml collection tube and 50 µl of RNase–free water was pipetted directly onto the RNeasy silica gel membrane. The tube was closed gently and left for 1-2 mins, then centrifuged for 1 min at ≥8000xg (≥10,000 rpm) to elute the RNA. The concentration of RNA was assessed as OD 260nm using a BioMate^{TM}3 spectrophotometer.

2.11.2 RNA Quantification using Agilent Bioanalyzer

The Agilent 2100 bioanalyzer was used for checking the quality and the integrity of extracted RNA samples. For examination with the Agilent 2100 bioanalyzer, the RNA 6000 LabChip® kit (Agilent Technologies, USA) was used. The guidelines of the assay protocol were strictly followed. Firstly, the gel was prepared: 550µl of RNA 6000 Nano gel matrix was added into a spin filter then centrifuged at 1500 g for 10 minutes at ambient temperature. Secondly, the Gel-Dye Mix was prepared: RNA 6000 Nano dye concentrate was vortexed for 10 seconds, and spun down briefly. 1µl of dye was added into a 65µl aliquot of filtered gel. After that, the solution was vortexed well and spun at 13000 g for 10 min at room temperature. Thirdly, the Gel-Dye Mix was loaded onto the chip priming station. Gel-dye mix (9.0 µl) was pipetted into the wells marked. Subsequently, the plunger was set to 1 ml and then the chip priming station closed. The plunger was pressed until held by the clip. The clip was released after waiting for exactly 30 sec; the plunger was pulled back slowly to the 1ml position after waiting for 5 sec. In the next step, the chip priming station was opened and 9.0 µl of gel-dye mix was pipetted into the wells marked. The remaining gel-dye mix was discarded. After that, 5µl Agilent RNA 6000 Nano Marker was loaded in all 12 sample wells and the wells marked. Finally, 1µl ladder or 1µl Sample was loaded into appropriate wells. 1 µl of RNA 6000 Nano Marker was pipetted in each unused sample well. The chip was placed horizontally in the adapter of the IKA vortexer and vortexed for 1 min at 2400 rpm. The chip was run in the Agilent 2100 bioanalyzer for 5 min.

2.11.3 Analysis of RNA quality and quantity

The main aim of the Agilent 2100 bioanalyzer experiments was to check the quality and the integrity of extracted RNA samples. The example shown relates to the cyclosiloxane series of experiments, but applies to all other series.

Representative samples of mRNA prepared from MCF10A cells grown with or without cyclosiloxanes D3, D4 and D5 for 7 days are shown in ***Figures 2.2 and 2.3***. ***Figure 2.2*** shows RNA preparations gave high quality RNA that contain two bands at 42 and 48 kb equivalent to 28S and 18S rRNA. RNA quantitation can be achieved using capillary electrophoresis (microfluidics) on the Agilent 2100. It requires only 25 ng of input RNA. A 2:1 ratio in the area under the peaks for 28S and 18S rRNA indicates intact total RNA. Degradation is indicated by less pronounced peaks for 28S and 18S rRNA and a 28S:18S rRNA ratio significantly less than 2:1. ***Figure 2.3*** shows a quantitative analysis of the same experiment. Just the two peaks show at the correct location in all samples.

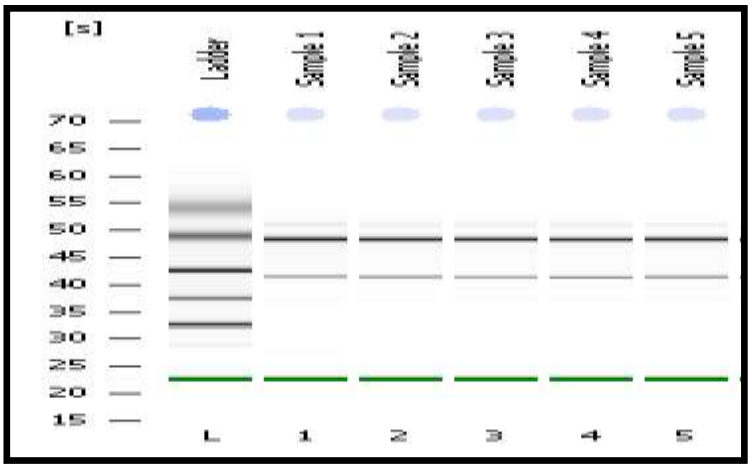


FIGURE 2.2 IMAGE OF GEL FROM AN RNA NANO CHIP RUN ON THE AGILENT 2100 BIOANALYZER
Shows the RNA quality in 5µl RNA samples from MCF10A human breast epithelial cells.

The RNA ladder shows bands of 15-70 KB markers. Cells were grown in stock medium with no addition (1), with 10^{-8} 17β- oestradiol (2), with 10^{-5}M Hexamethylcyclotrisiloxane (D3), with 10^{-5}M octamethylcyclotetrasiloxane (D4) or with 10^{-5} decamethylcyclopentasiloxane.

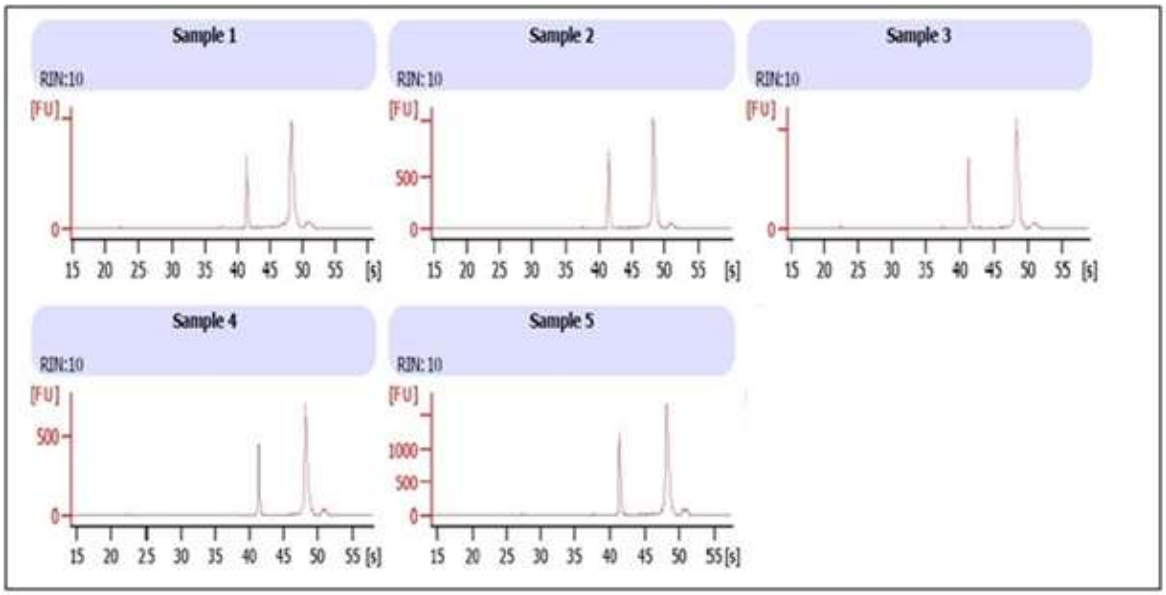


FIGURE 2.3 ANALYSIS OF GEL IMAGE FROM FIGURE 2.5. AGILENT 2100® BIOANALYSER
Scans of High Integrity Total RNA are shown by the 18S and 28S peaks at 39 and 46 seconds, respectively. MCF10A Cells were grown in stock medium with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2), 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4), or10^{-5}M decamethylcyclopentasiloxane (D5) for 7 days.

2.11.4 Selection of β-actin mRNA loading control for normalisation of PCR

Figure 2.4 shows the Ct values from real-time RT- PCR for expression of the housekeeping gene β-actin in MCF10A and MCF10F immortalised non-transformed human breast epithelial cells which were untreated or treated with the cyclosiloxanes (D3, D4 and D5), Lilial, Triclosan and BPA. The strong expression of β-actin with similar Ct values throughout demonstrates that use of β-actin for normalisation as a loading control is technically sound.

Figures 2.5 **and** ***2.6*** show Ct values for β actin mRNA in cells after one week and 30 weeks incubation respectively. There is no significant difference in the observed levels of this putative normalization control between the agents tested, irrespective of the incubation time (1 or 30 weeks).

MCF10A cells MCF10F cells

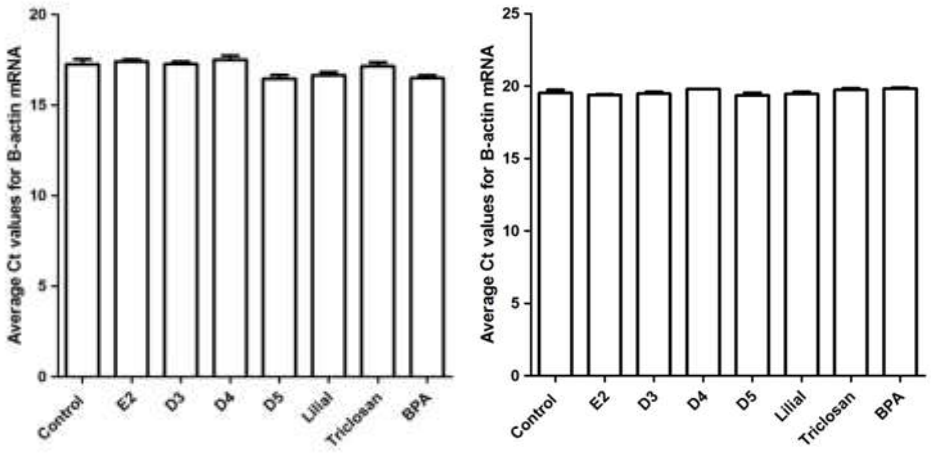


FIGURE 2.4 RT- PCR ANALYSIS OF B-ACTIN MRNA IN MCF10A AND MCF10F CELLS FOR CYCLOSILOXANES (D3, D4 AND D5) LILIAL, TRICLOSAN AND BPA

Cells were grown in stock medium with no addition (control) or in the presence of 10^{-8} 17β-oestradiol (E2), 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4) or 10^{-5}M decamethylcyclopentasiloxane (D5) 10^{-5}M Lilial, 10^{-7}M Triclosan and 10^{-5}M Bisphenol A (BPA) , .Average ± SE of 3 technical replicates for RT PCR of β- mRNA.

MCF10A cells MCF10F cells

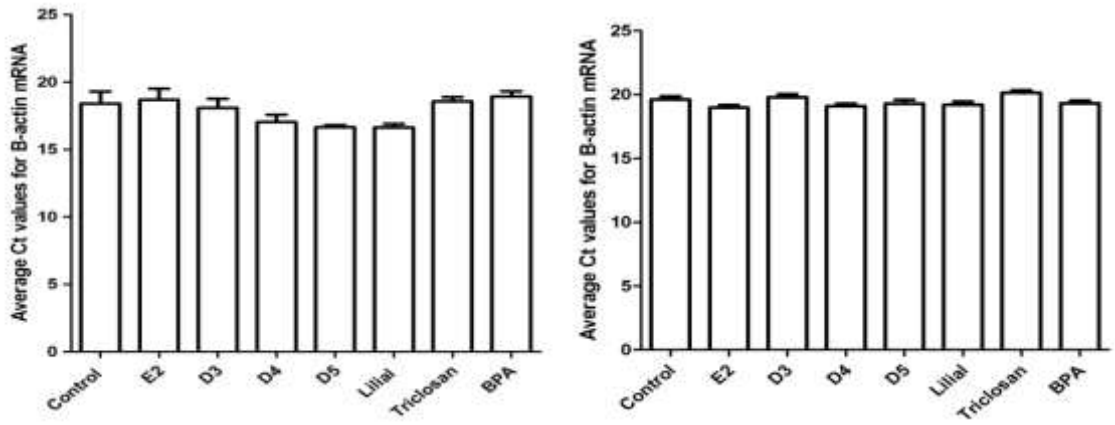


FIGURE 2.5 RT- PCR ANALYSIS OF B-ACTIN MRNA IN MCF10A AND MCF10F CELLS: SHORT TERM CULTURE FOR CYCLOSILOXANES (D3, D4 AND D5), LILIAL, TRICLOSAN AND BPA

Cells were grown in stock medium with no addition (control) or in the present of 10^{-8} 17β-oestradiol (E2), 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4) or 10^{-5}M decamethylcyclopentasiloxane (D5) 10^{-5}M Lilial, 10^{-7}M Triclosan and 10^{-5}M Bisphenol A (BPA). Average ± SE of 3 biological replicates for RT PCR of β- mRNA for short term (1week).

MCF10A cells MCF10F cells

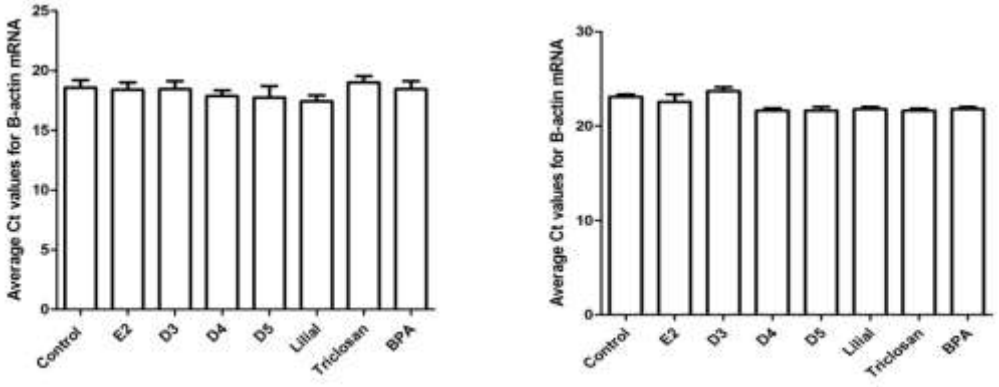


FIGURE 2.6 RT- PCR ANALYSIS OF B-ACTIN MRNA IN MCF10A AND MCF10F CELLS: LONG TERM CULTURE FOR CYCLOSILOXANES (D3, D4 AND D5), LILIAL, TRICLOSAN AND BPA

Cells were grown in stock medium with no addition (control) or in the present of 10^{-8} 17β-oestradiol (E2), 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4) or 10^{-5}M decamethylcyclopentasiloxane (D5) 10^{-5}M Lilial, 10^{-7}M Triclosan and 10^{-5}M Bisphenol A (BPA).Average ± SE of 3 biological replicates for RT PCR of β- mRNA for long term (30week).

Figure 2.7 shows the Ct values from real-time RT- PCR for expression of the housekeeping gene β-actin in MCF10A immortalised non-transformed human breast epithelial cells which were untreated or treated with the Alcl3 and Al hydrochloride The strong expression of β-actin with similar Ct values throughout demonstrates that use of β-actin for normalisation as a loading control is technically sound

In ***Figure 2.7*** Ct value for β actin mRNA after 20 weeks. There is no significant difference in the observed levels of this putative normalization control between the agents tested

Biologically

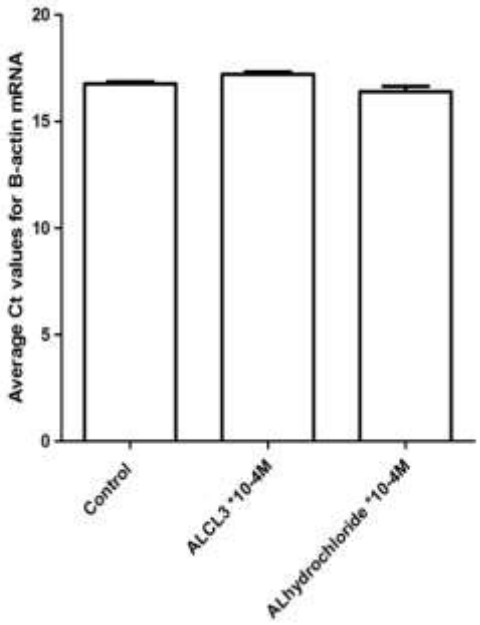


FIGURE 2.7 RT- PCR ANALYSIS OF B-ACTIN MRNA IN MCF10A : LONG TERM CULTURE FOR ALCL3 AND AL HYDROCHOLRIDE .

Cells were grown in stock medium with no addition (control) or in the present of 10^{-4} Alcl3, 10^{-4}M Al hydrochloride, Average ± SE of 3 biological replicates for RT PCR of β- mRNA for long term (20 week).

2.11.5 c-DNA synthesis for real time (RT)-PCR

First strand cDNA was synthesized using the first strand synthesis Qiagen kit according to the manufacturer's instructions. First, 2 µl DNA buffer, 1µgRNAand RNase – free water to a volume of 11 µl were mixed in a small microcentrifuge tube by pipette and incubated 2 min at 42 °C, then placed on ice. Secondly, 1 µl reverse- transcription master mix, 4µl

Quanti Script RT buffer and 1µl RT Primer Mix were mixed in another microcentrifuge tube. In the next step, the 14 µ template RNA from Step 1 was added to step 2 (total vol 20µl), then incubated for15 min at 42°C, then incubated 3 min at 95°C and stored at -20°C/ -80°C.The product was diluted 1:20 for RTPCR.

2.11.6 Real- time RT- PCR analysis

For each reaction of RT-PCR, 2x 7µl QuantiTectSyber Green PCR master mix, QuantiTect primers (1.4µl) (Table 2.2) and RNase free water (0.6 µl) were mixed in a 96 well plate, (Thermo Scientific UK, 1900 High speed low profile PCR plate). A total of 9 µl of master mix as described above was pipetted into each well and 5 µl of cDNA sample was added. The 96 well dish was vortexed. The AB Applied Biosystems method was used for RT-PCR. The thermal profile for all reaction was 95°C for 15 min, followed by 40cycles of 94°C for 15s and 60°C for 1 min. All reactions were performed in triplicate. The β-actin gene has been conventionally used as an internal control or housekeeping gene to normalize the expression of the target gene(s) or mRNA levels (Wong and Medrano, 2005) and was used here as a reference to normalize the expression value of BRCA1,BRCA2,p53,ATM,ATR, Rad50&51, CHK1&2, PARP1, BRIP1, PALB2,PTEN and STK111.

2.11.7 Statistical analysis of real- time RT- PCR

The comparative threshold cycle (Ct) method was used to calculate the amplification factor. Triple replicates were performed for each gene and average expression values were computed for subsequent analysis. The relative expression level of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method. This was then repeated for each of the three biological replicates generated from independent cell cultures after 30 weeks of culture, and results presented show the average ± SE (n = 3) of the three biological replicates. According to the $2^{-\Delta\Delta CT}$ method, results are presented relative to the control value of 1.0 for cells grown in the absence of Al. Statistically significant differences were determined using ANOVA Dunnett in Graph Pad Prism 5™.

TABLE 2-2 Primers for RT-PCR

QuantiTect Primers	Species	Gene ID	Amplicon length	Detected transcript	Product code
ATR	**Human (Homo sapiens)**	**545**	**125bp (NM_001184)**	NM_001184 (8258 bp)	**QT00030779**
ATM	**Human(Homo sapiens)**	**472**	**134bp (NM_000051)**	NM_000051 (13147 bp)	**QT-00061593**
β-Actin	**Human(Homo sapiens)**	**60**	**104bp (NM_001101)**	NM_001101 (1852 bp)	**QT-01680476**
BRCA1	**Human (Homo sapiens)**	**672**	**60bp (NM_007294)**	NM_007294 (7224 bp)	**QT-00039305**
BRCA2	**Human(Homo sapiens)**	**675**	**93bp (NM_000059)**	NM_000059 (11386 bp)	**QT-00008449**
BRIP1	**Human(Homo sapiens)**	**83990**	**97bp (NM_032043)**	NM_032043 (8166 bp)	**QT00086548**
CHK1	**Human(Homo sapiens)**	**1111**	**123bp (NM_001114121)**	NM_001114121 (2699 bp)	**QT00006734**
CHK2	**Human(Homo sapiens)**	**11200**	**134bp (NM_001005735)**	NM_001005735 (1991 bp)	**QT01016155**
p53	**Human(Homo sapiens)**	**7157**	**112bp (NM_000546)**	NM_000546 (2591 bp)	**QT-00060235**
PALB2	**Human(Homo sapiens)**	**79728**	**100bp (NM_024675)**	NM_024675 (4069 bp)	**QT00068523**
PARP1	**Human(Homo sapiens)**	**142**	**86bp (NM_001618)**	NM_001618 (4001 bp)	**QT00032690**
PTEN	**Human(Homo sapiens)**	**5728**	**108 bp(NM_000314)**	NM_000314 (5572 bp)	**QT00086933**
Rad50	**Human(Homo sapiens)**	**10111**	**131bp (NM_005732)**	NM_005732 (6597 bp)	**QT00037170**
Rad51	**Human(Homo sapiens)**	**5888**	**108 bp(NM_001164270)**	NM_001164270 (2177 bp)	**QT00072688**
STK111	**Human(Homo sapiens)**	**6794**	**91 bp (NM_000455)**	NM_000455 (3286 bp)	**QT01008980**

2.12 Western Immunoblotting

2.12.1 Preparation of whole cell lysates

Cells were seeded at a density of 0.8x 10^5 cells/ ml in growth medium as described in section 2.9. These cultures were incubated in a humidified atmosphere of 10 % carbon dioxide in air at 37°C for 7 days in 9 cm and 4 well tissue culture dishes (Nunc, Denmark). 7 days later 4 well dishes were counted in order to calculate cell density at the time of harvest for protein preparation.

2.12.2 Protein Extraction

Cultured cells were washed twice *in situ* with ice cold phosphate buffered saline (1M, PBS, sigma, UK). Cells were removed from the culture dish using a rubber cell scraper and pelleted in 15 ml tubes by centrifugation. The appropriate amount of lysis buffer [50nM Tris-HCL pH7.4, 250mM NaCl, 5mM EDTA, 0.3 % triton x-100,0.3 mM 4-(2- Aminoethly) benzenesulfonly fluoride hydrochloride (AEBSF), 10 µg/ml leupeptin and 2 µg/ml aprotonin] was added to the cell pellet to give a final concentration of 1x105 cells/µl. These samples were kept on ice for 30 minutes and then passed through needles of decreasing size from 19G to25 G in order to break down cellular organelles and DNA. These lysates then were centrifuged at 13000 rpm for 5 minutes at 4°C in a microcentrifuge. The supernatants were transferred to fresh tubes and kept at -80 °C.

2.12.3 Protein Quantification

Protein in each cell lysates was quantified using the Pierce BCA reagent (Thermo Scientific, USA). Protein standards were prepared by mixing different amount of bovine serum albumin (BSA) (1mg/ml) with lysis buffer and distilled water to obtain a total volume of 10 µl in each tube. Cell lysates samples were prepared by mixing 2 µl of cell lysates and 8 µl of distilled water in order to have total 10 µl volumes. Protein standards and cell lysate samples were prepared in triplicate. Pierce BCA reagent was prepared according to the manufacturer's instructions and 200 µl was added into each tube. These tubes were incubated at 60 °C for 30 min. These samples were downloaded into a 96 well plate to be read at OD 560 nm in an EMAX plate reader. Protein in each well was calculated from a standard curve of the BSA protein standards.

2.12.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

In order to separate the protein in samples, treated ethanol vehicle or 17β- oestradiol positive control sodium dodecyl sulphate- polyacrylmide gel electrophoresis (SDS- PAGE)

was performed. Protein samples were diluted 1:1 v:v with Lamelli buffer (Bio-Rad, UK) and incubated at 100 °C for 3 min before loading into the wells of the gel. Mini protean TGX stain free precast gel (7.5%, BioRad, UK) were used for western blot analysis. Precision plus Protein ™ Unstained Standard (BioRad, UK) was used as a protein marker. Depending on the protein amount obtained from the assay, 30 or 50 µg of protein samples were loaded into the wells. The gel was then run at 200 volts until the bromophenol blue dye reached the bottom of the gel.

2.12.5 Gel Activation

The gel was placed on the stain free tray of the Gel Doc ™EZ System in order to activate for 5 minutes for best sensitivity by Gel Doc ™ EZ System (Bio- Rad, UK) using Image Lab software.

2.12.6 Transfer of protein to PVDF membrane

Protein samples were transferred onto polyvinylidene difluoride (PVDF) membrane using the Trans Blot Turbo Transfer pack mini format containing 0.2 µm PVDF membrane (Bio-Rad).

Firstly, the bottom of the prepared papers from the pack were placed onto the Trans-Blot Turbo system cassette. Then the activated gel was placed onto the PVDF membrane followed by replacing papers taken from the top part of the pack. Any bubbles were removed with by rolling. The transfer was run for 3 minutes according to the manufacturer's protocol for TGX gels of the Trans-Blot Turbo system.

2.12.7 Taking PVDF membrane image for total protein calculation

The transferred PVDF membrane was quickly placed on the stain free tray and imaged using the Gel Doc ™ EZ System (Bio-Rad, UK) with Image Lab software. If the PVDF membrane became dyed, it was washed with methanol for few seconds before placing in the Gel Doc ™ EZ System. The total protein in each lane was quantified in *Image Lab* and this value used for normalisation of immunoblots.

2.12.8 Immunostaining of protein

After transfer, the PVDF membrane was washed in 20 ml TRIS-Buffered Saline (TBS) (50 mM Tris PH 7.6, 150 mM Nacl, 2mM KCL) for 5 minutes. The membrane was incubated at room temperature in 25 ml of blocking buffer (2.5 ml 10x TBS, 22.5 ml double distilled water, 1.25 g non- fat dried milk, 25 µl Tween 20). In order to remove blocking buffer the membrane was washed with 15 ml of TBS-T (100 ml 10xTBS, 900 ml double distilled water, 1 ml Tween 20) for three times for 5 minutes each. The membrane then was incubated with primary antibody solution at a concentration as given in **Table2.3**. It was diluted by TBS-T with 5% w/v BSA. This antibody solution and the membrane were put into a heat sealable

bag and incubated at 4 °C overnight with gentle rocking. Next day the membrane was washed with TBS-T three times for 5 minutes each. Then the membrane was incubated with appropriate HRP- linked secondary antibody solution containing blocking buffer (***Table 2.3***). The membrane was put inside a heat sealable bag with this solution for 1 hour at room temperature with gentle shaking. The membrane was washed x3 with TBS-T for 5 minutes each time.

The Amersham ECL prime Western Blotting Detection Reagent (GE Healthcare, UK) was prepared according to the manufacturer's instructions. The membrane was incubated in the reagent for 5 min and the membrane was placed, protein side down, on SARAN wrap. Images of the membrane were taken using the imager Image Quant LAS 400 Mini (GE Healthcare, UK) with different exposure times.

TABLE 2-3: The concentrations of primary and secondary antibodies used in Western Blotting

	Manufacturer	**Source**	**Dilution**
Primary antibodies			
BRCA1	Cell signalling #9010	Rabbit	1:1000
Secondary antibodies			
Anti-Rabbit HRP-linked	Cell signalling #7074	Goat	1:3000
Precision Protein StrpTactin-HRP conjugate	BioRad #1610381		1:5000
B-actin	Cell signalling #8457		1:1000

2.12.9 Quantification of protein & statistical treatment of results

Quantification of protein was performed by measuring relative intensities of appropriate bands as imaged by Image Quant LAS 400 Mini (GE Healthcare, UK). The numbers obtained for each band were divided by the total protein amounts in the lane as obtained from the Gel Doc ™ EZ System by Image Lab software. Results were plotted and analysed using GraphPad Prism™.

Band signals were normalised relative to digitally quantified total protein using the Bio-Rad stain-free system according to manufacturer instructions. All results show the average ± SE (n = 3) of biological replicates generated from three independent cell cultures and were analysed for statistical significance using ANOVA post-hoc Dunnett test B

2.12.10 Molecular sizing of BRCA1 protein (His- tag protein)

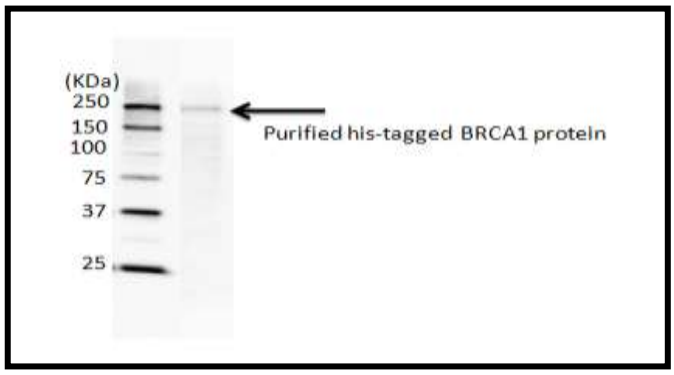


FIGURE 2.8 WESTERN IMMUNOBLOTTING GEL OF HIS-TAGGED BRCA PROTEIN WITH MW MARKER LADDER

The molecular weight markers were biotinylated unstained precision plus from BioRad. Gel shows coincidence of 250KDa standard & the purified his-tagged BRCA1 protein.

2.13 Statistical analyses

Statistical analyses for suspension growth and RTPCR were performed as one way ANOVA with *post-hoc* Dunnett test. The Dunnett test compares a set of means of treatment groups against the mean of a single control group (Upton and Cook, 2008). Statistical analysis for western immunoblotting was performed using either one way ANOVA with *post-hoc* Dunnett test or a student t-test, comparing two independent groups. (David and Gunnink, 1997)

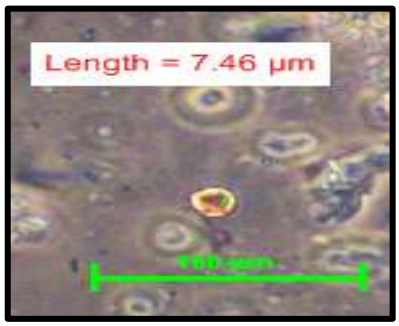
Chapter 3: Results

3.1 Cyclosiloxanes

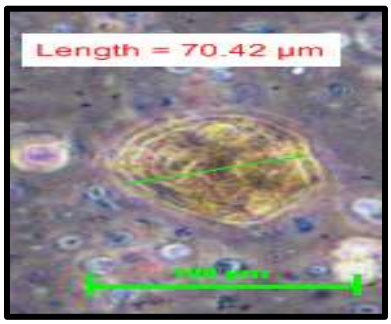
Cyclosiloxanes are widely used as conditioning and spreading agents in personal care products which are applied around the region of the human breast. In this study, we used MCF10A and MCF10F immortalised non-transformed human breast epithelial cells to investigate the ability of the cyclosiloxanes hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) to enable growth in suspension culture, to damage DNA, and to interfere with DNA repair systems.

3.1.1 Effect of cyclosiloxanes D3, D4 & D5 on morphology of MCF10A& MCF10F colonies in suspension culture:

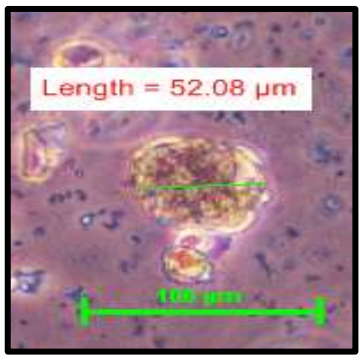
The ability of anchorage-dependent epithelial cells to grow in suspension culture has long been acknowledged as the property of cells in *vitro* to be most closely correlated with transformation in *vivo* (Shin et al, 1975). MCF10A and MCF10F cells are spontaneously immortalised normal human breast epithelial cells which are normally dependent on anchorage to a substratum for their growth. In their stock medium, they grow in monolayer culture but not to any great extent in suspension culture unless 70 nM 17β-oestradiol is added (Pugazhendhi and Darbre, 2010). In these experiments, MCF10A cells were grown in semi-solid suspension culture with a range of concentrations of 17β- oestradiol, D3, D4 and D5 for up to 21 days. Photomicrographs of colonies after21 days are shown in ***Figure 3.1.*** In the absence of treatment, only small colonies were found ***(Figure 3.1A)*** but larger colonies were observed with 70nM 17β-oestradiol (***Figure 3.1B***) or with 10^{-5}M concentrations of D3, D4 or D5 ***(Figure 3.1C-E)***. Similar results were found with MCF10F cells (data not shown)

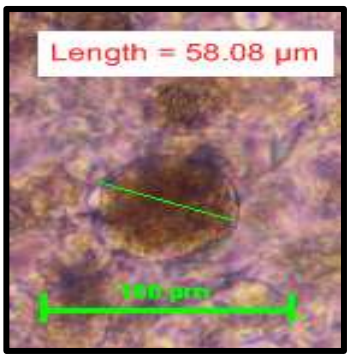


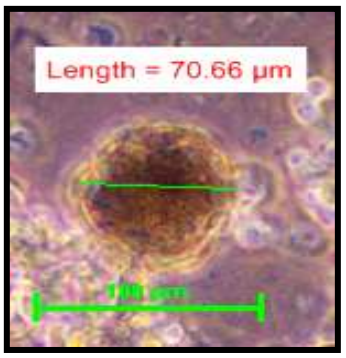
(A) No treatment



(B) 17β- oestradiol (E2)







(**C**) D3 (**D**) (D4) (**E**) (D5)

Figure 3.1 Colonies of MCF10A cells growing in semi-solid methocel suspension culture after 21 days exposure to cyclosiloxane D3, D4 and D5

Cells were grown with:- (A) no treatment (B), 70 nM 17β- oestradiol (C), 10^{-5}M D3 (D), 10^{-5}M D4 (E), 10^{-5}MD5. Phase contrast images.

3.1.2 Dose response effects of D3 on the size and number of MCF10A and MCF10F colonies in suspension culture

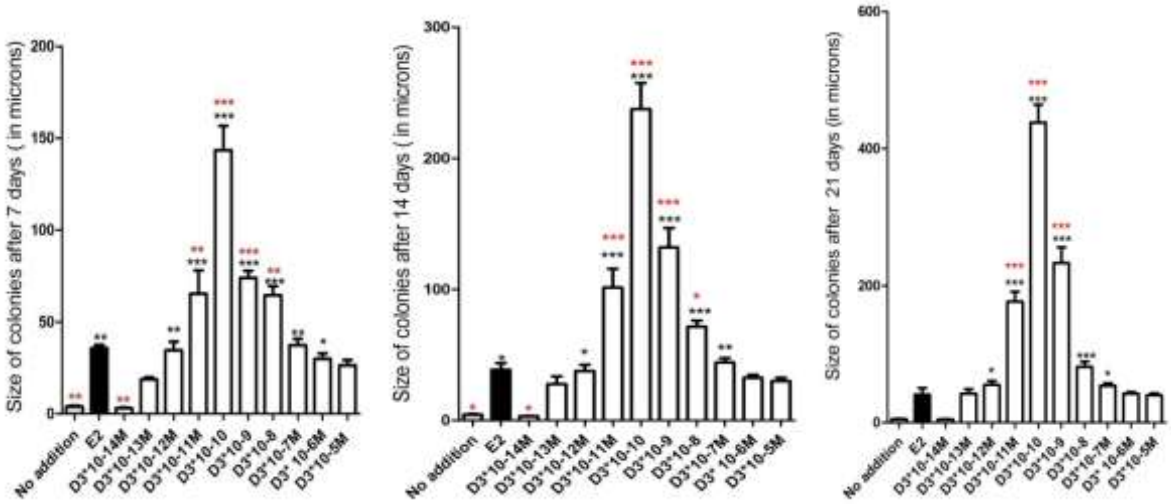
MCF10A and MCF10F cells were grown with17β-oestradiol or hexamethylcyclotrisiloxane (D3) in suspension culture for 7, 14 and 21 days. Colony size (***Figure 3.2*****),** colony number (***Figure 3.3*****)** and total cell number **(*Figure 3.4)*** were measured after 7, 14 and 21 days. Increased colony size demonstrated the enhanced ability of the cells to grow to a greater extent under non- adherent conditions. Increased colony number is an indication of a greater number of cells able to form colonies. Total cell count is an independent measure of number of cells growing in suspension. Increasing concentrations of D3 (10^{-14} M to 10^{-5} M) gave bell-shaped plots for all three parameters studied, with maximal colony size and colony number as well as total cell count, at 10^{-10}M in each case.

Colony size: **(*Figure 3.2*)**. Addition of 70nM 17β-oestradiol increased colony size for MCF10A cells after 7 days ($p<0.01$), 14 days ($p<0.05$) and but not 21 days ($p>0.05$) (***Figure 3.2 A***) and for MCF10F cells after 7 days, 14 days and 21 days ($p<0.001$ in all cases) **(*Figure 3.2 B*)** For MCF10A cells, colony size was increased from control at D3 concentrations of 10^{-13}M to 10^{-5}M inclusive. Size was greater compared to colonies in the presence of 70nM β-oestradiol for D3 concentrations of 10^{-11} M to 10^{-8} M (***Figure 3.2A*****)** For MCF10F cells, colony size was increased from control at D3 concentrations of 10^{-13}M to 10^{-5}M inclusive. Size was greater compared to colonies in the presence of 70nM β-oestradiol for D3 concentrations of 10^{-11} M to 10^{-8} M (***Figure 3.2B*****)**

Colony numbers **(*Figure 3.3*)**. There is a smaller increase in colony numbers and the histograms peak at 14 days, falling back slightly by 21days. High concentrations of D3, certainly 10^{-6}M and 10^{-5}M still show significant enhancement of colony numbers over the blank control, but less than the standard (70nM) E2. At all three time periods, 70 nM 17β-oestradiol stimulates most closely to 10^{-12} M D3.

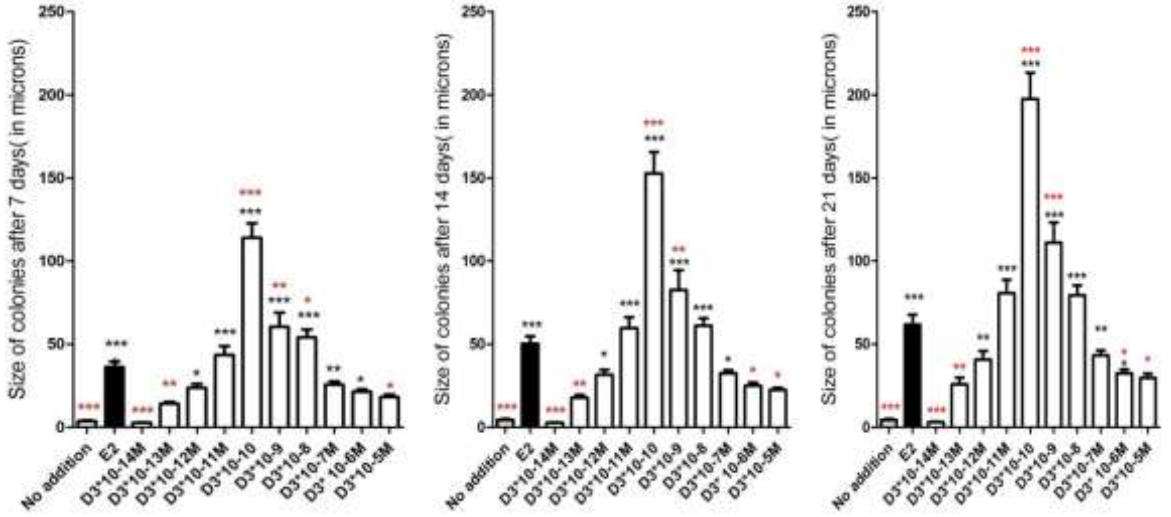
Cell counts **(*Figure 3.4*)**. The overall shape of the plots is similar between MCF10A and MCF10F cells and, indeed the previous plots in ***Figures 3.2*** *and* ***3.3*****.** Peak cell number is the same, at 10^{-10}M. Rather more cells were retrieved from MCF10A than MCF10F wells.

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells

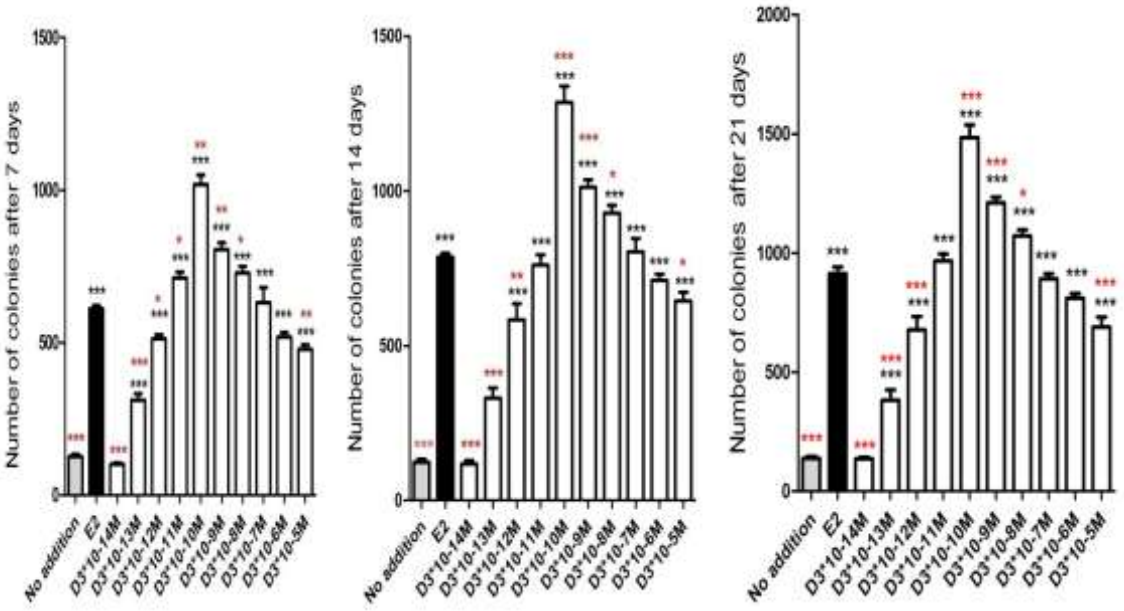


Day 7 Day 14 Day 21

FIGURE 3.2 Effect of different concentrations of hexamethylcyclotrisiloxane (D3) on the size of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 days to 21 days.

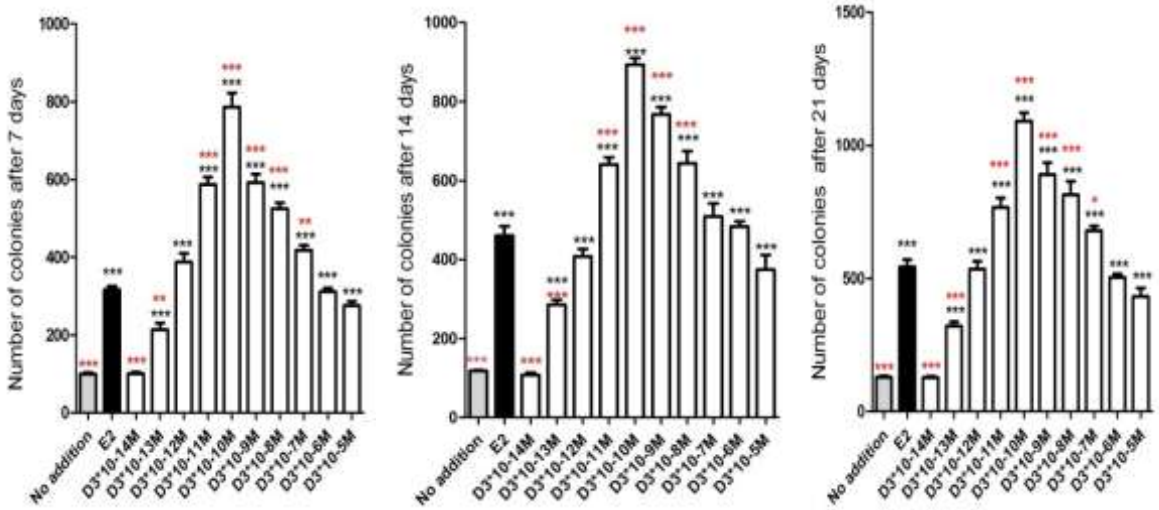
Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol or with D3 at concentrations from 10^{-14}M to 10^{-5}M. Average colony size was calculated from 15 fields per of view in each well measured and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. **** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells



Day 7 Day 14 Day 21

Figure 3.3 Effect of different concentrations of hexamethylcyclotrisiloxane (D3) on number of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 days to 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol,with D3 at concentrations from 10^{-14}M to 10^{-5}M. Colony growth is shown as the number of colonies per well from 7 days to 21 days, as determined under light microscope. Standard error of triplicates wells of replicate dishes. *** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

A. MCF10A cells

B. MCF10F cells

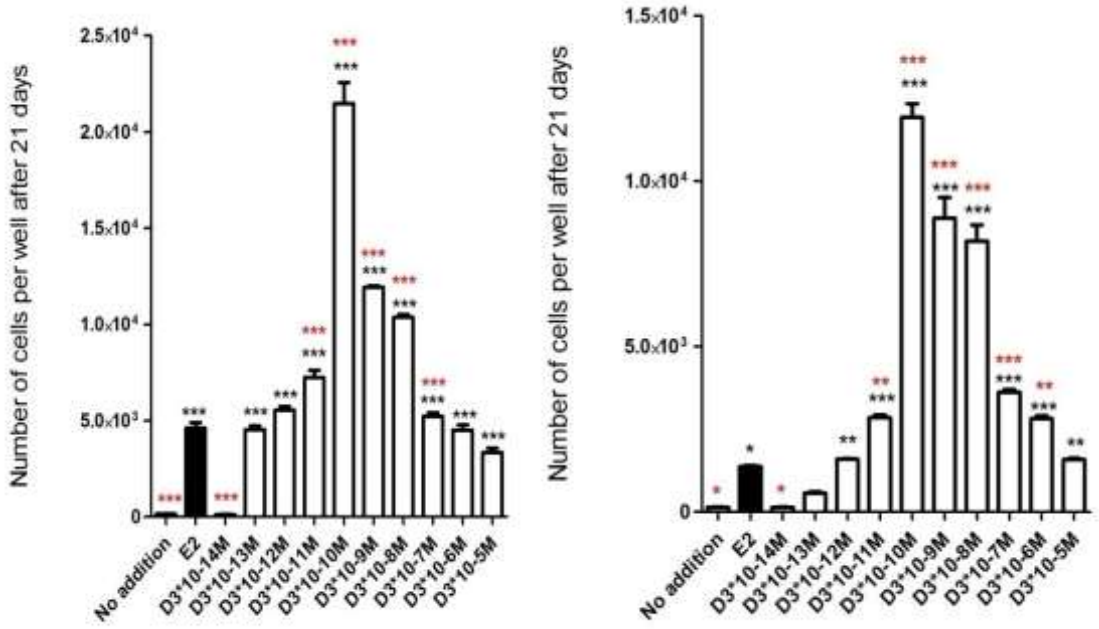


FIGURE 3.4 Effect of different concentrations of hexamethylcycloxane (D3) on the growth of MCF10A (A) and MCF10F (B) cells in semi-sold methocell suspension culture after 21 days

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol,with D3 at concentrations from 10^{-14}M to 10^{-5}M. Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

**** indicates p< 0.05,** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

3.1.3 Dose response effects of D4 on the size and number of MCF10A and MCF10F colonies in suspension culture

MCF10A and MCF10F cells were grown with 70nM 17β-oestradiol or octamethylcyclotetrasiloxane (D4) in suspension culture for 7, 14 and 21 days, colony size (***Figure 3.5***) colony number (***Figure 3.6***) and total cell number (***Figure 3.7***) were measured after 7, 14 or 21 days.

Colony size (***Figures 3.5*****)** Increasing concentrations of D4 (10^{-10} M to 10^{-5}M) gave increased colony size and colony number. This direct correlation had not reached saturation at the highest concentration tested (10^{-5}M).

Colony number **(*Figure 3.6*)** Colony counts mirrored the results for colony size. There was a direct association between D4 and colony number which persisted through to the 10^{-5}M highest concentration.

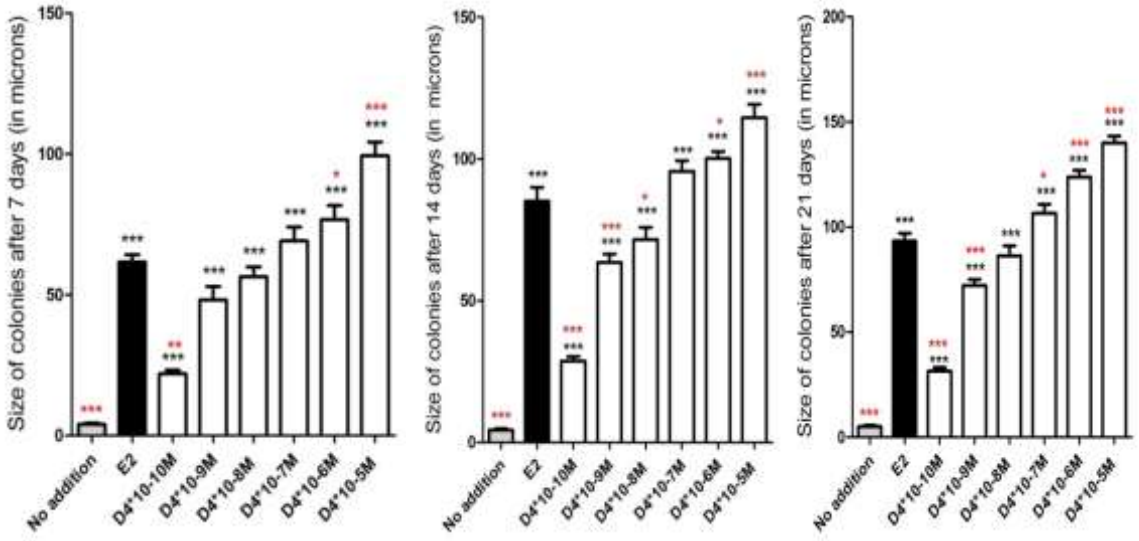
Cell counts ***(Figure 3.7*****)** Absolute cell numbers followed the same pattern, with a positive association between D4 concentration and cell count, continuing through the highest concentration.

Overall, therefore, the growth results for D4, however measured, are distinct from those using D3 in that stimulation of colony formation does not peak at 10^{-10}M. Conversely, absolute values for the E2 control are consistent with those obtained in the D3 experiments in terms of both size and numbers of colonies.

In all of the plots there are concentrations at which stimulation is lower or exceeds that achieved with 70nM 17β-oestradiol. At 7 days there is a wide range of non-significant pair wise comparisons .Blank values are minimal in all in plots of colony size and cell count. Only in colony numbers and for day 7 are p values against the weakest D4 solution in the 0.001-0.01 range (e.g. p=0.0075 for 10^{-6}M D4 against blank, day 7). Even that is in a range commonly ranked “highly” significant.

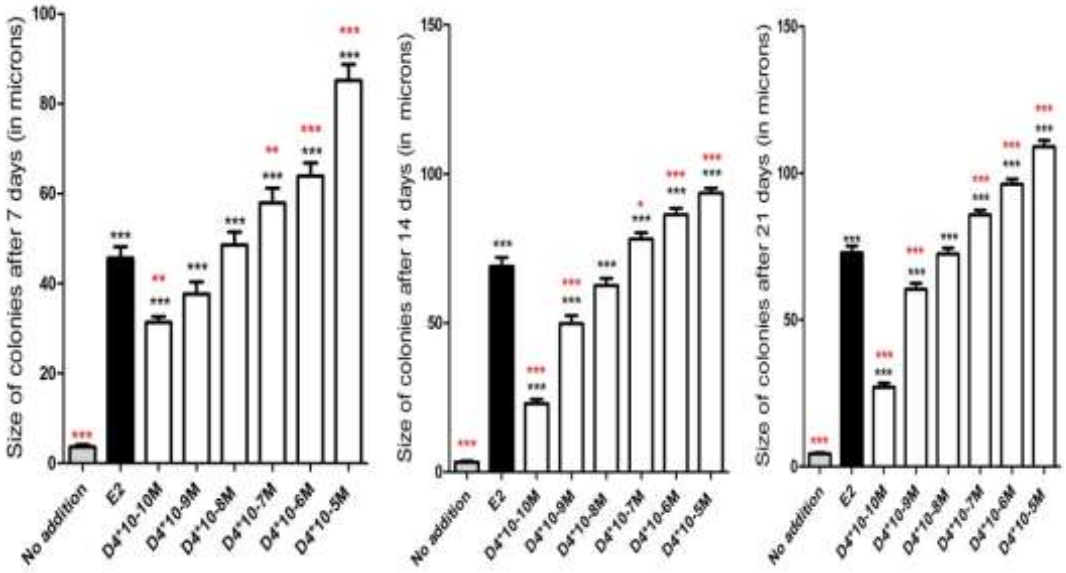
There is very little difference between the results for MCF10A and MCF10F cells which is consistent with robust experimentation and the strong common ancestry of the cell lines.

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells

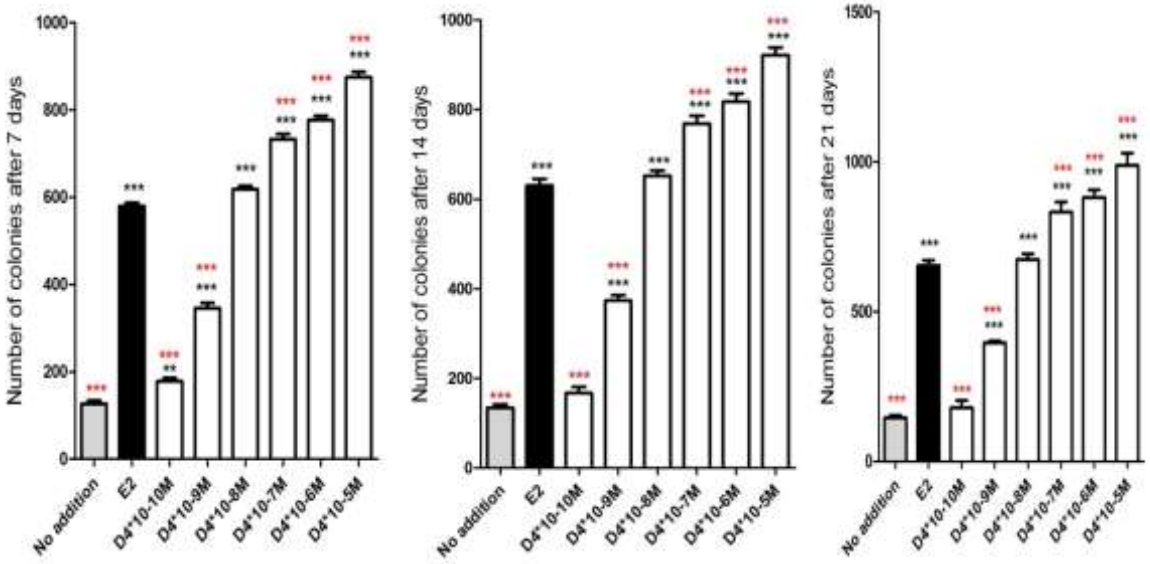


Day 7 Day 14 Day 21

FIGURE 3.5 Effect of concentrations of octamethylcyclotetrasiloxane (D4) on the size of colonies of MCF10A (A) and MCF10F (B) cells growing in methocel suspension culture from 7 days to 21 days.

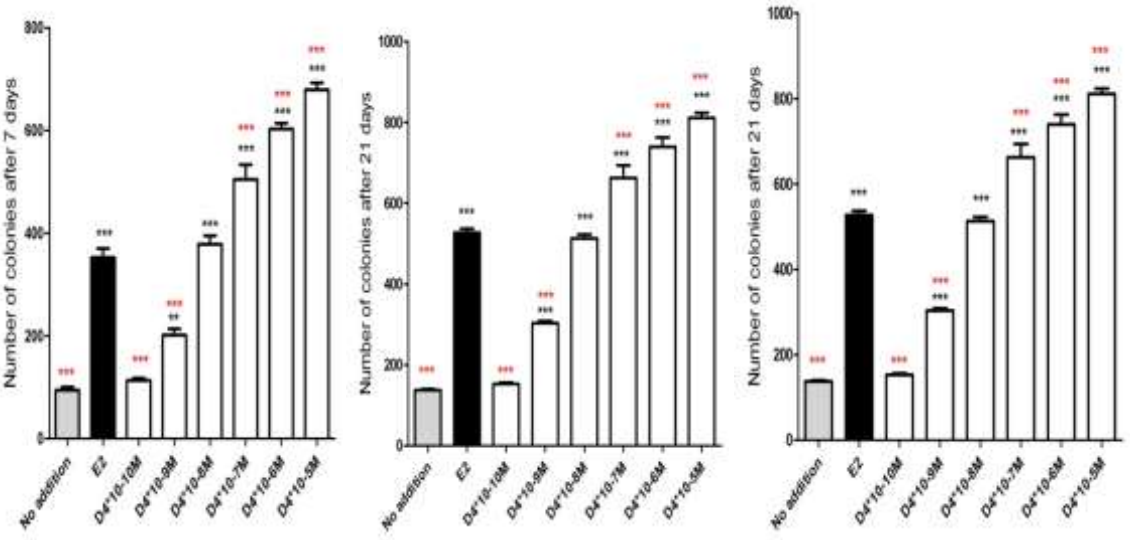
Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with D4 at concentrations from 10^{-10}M to 10^{-5}M. Average colony size was calculated from 15 fields per well of view and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. **** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

MCF10A cells



Day 7 Day 14 Day 21

A. MCF10F cells



Day 7 Day 14 Day 21

FIGURE 3.6 Effect of concentrations of octamethylcyclotetrasiloxane (D4) on number of colonies of MCF10A (A) and MCF10F (B) cells growing in methocel suspension culture from 7 days to 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with D4 at concentrations from 10^{-10}M to 10^{-5}M. Colony growth is shown as the number of colonies per well from 7 days to 21 days, under light microscope. Error bars are standard error of triplicate wells for each experiment. ***** indicates p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

A. MCF10A cells B. MCF10F cells

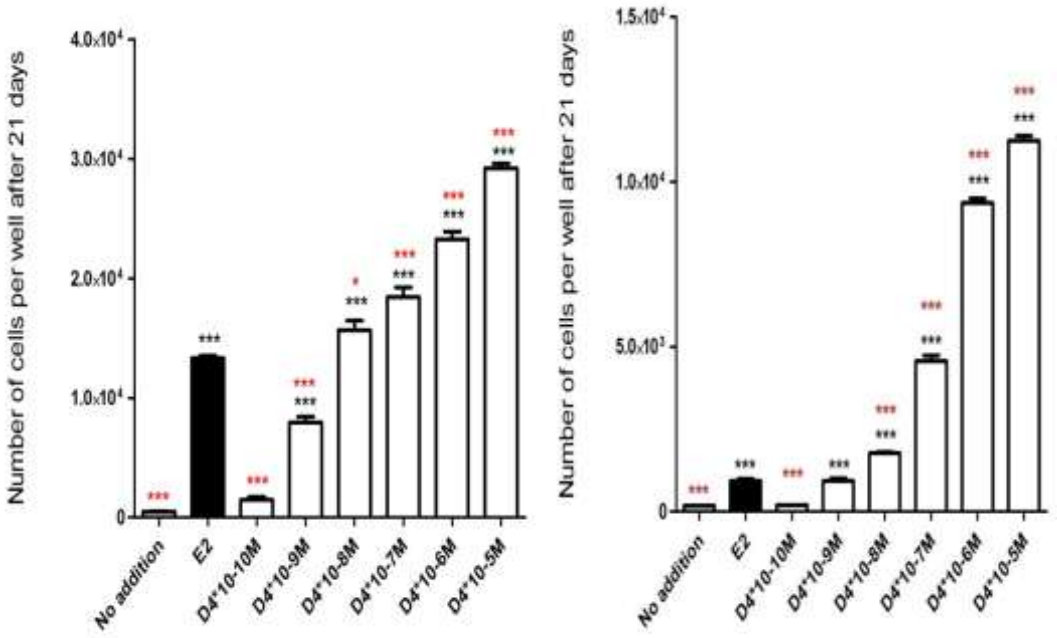


FIGURE 3.7 Effect of concentrations of octamethylcyclotetrasiloxane (D4) on growth of MCF10A (A) and MCF10F (B) cells in semi- solid methocel suspension culture after 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with D4 at concentrations from 10^{-10}M to 10^{-5}M.Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

**** indicates p< 0.05, and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

3.1.4 Dose response effects of D5 on the size and number of MCF10A and MCF10F colonies in suspension culture

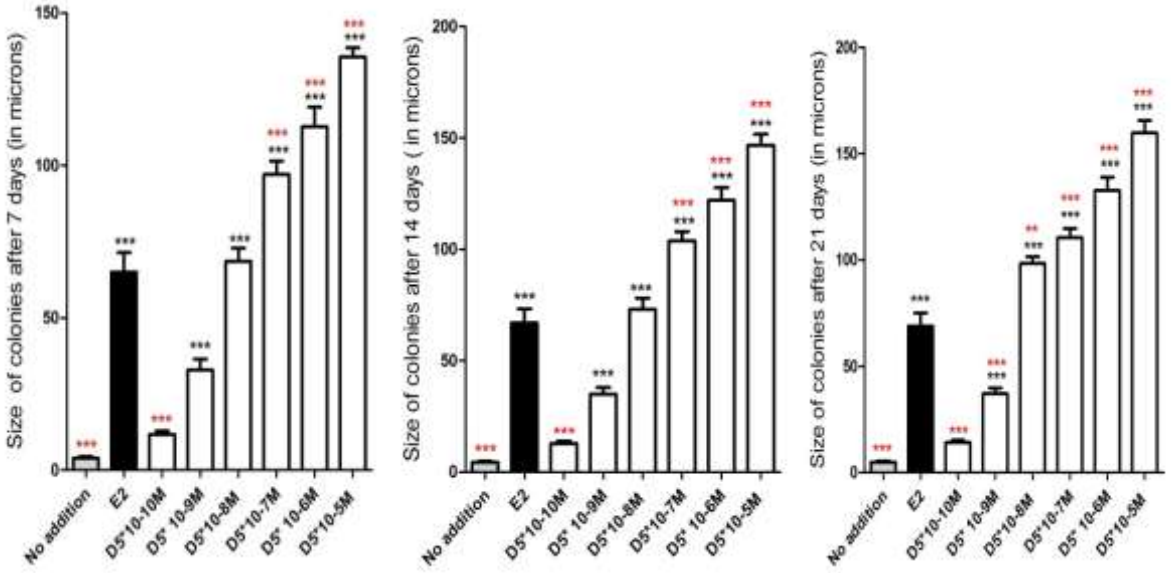
MCF10A and MCF10F cells were grown with *17β*- oestradiol or decamethylcyclopentasiloxane (D5) in suspension culture for 7, 14 and 21 days, Colony size (***Figure 3.8),*** colony number(***Figure 3.9***) and total cell number (***Figure 3.10***) were measured after 7, 14 or 21 days.

*Colony size (**Figure 3.8)*** Increasing concentrations of D5 (from 10^{-10} M to 10^{-5}) gave increased colony size and colony number. No saturation of this effect was observed continued to rise between 10^{-6}M and 10^{-5}M. As with D4, while significant stimulation of colony growth (however measured) is seen relative to blank controls for all concentrations, the lower (10^{-10} & 10^{-9}M) concentrations of D4 fail to stimulate to the level of 70nM 17β-oestradiol with statistical significance. The crossover into significantly enhanced colony stimulation over 70nM 17β-oestradiol happens around 10^{-8}M except for the 7 and 14 day cultures of MCF10F cells, where it occurs at 10^{-7}M. In absolute values, the colony size for MCF-10A and MCF10F are comparable. Unlike D3 and D4, 10^{-10}M D5 is ineffective in stimulating colony formation even at day 21. Otherwise D5 results are consistent with those obtained with D4. MCF10A and MCF10F also give mutually consistent results,

*Colony numbers **(Figure 3.9)*** Results for colony counts follow closely those for colony size. Increasing concentrations of D5 (from 10^{-10} M to 10^{-5}) gave increased colony size and colony number. No saturation of this effect was observed; numbers were continuing to rise through 10^{-5}M

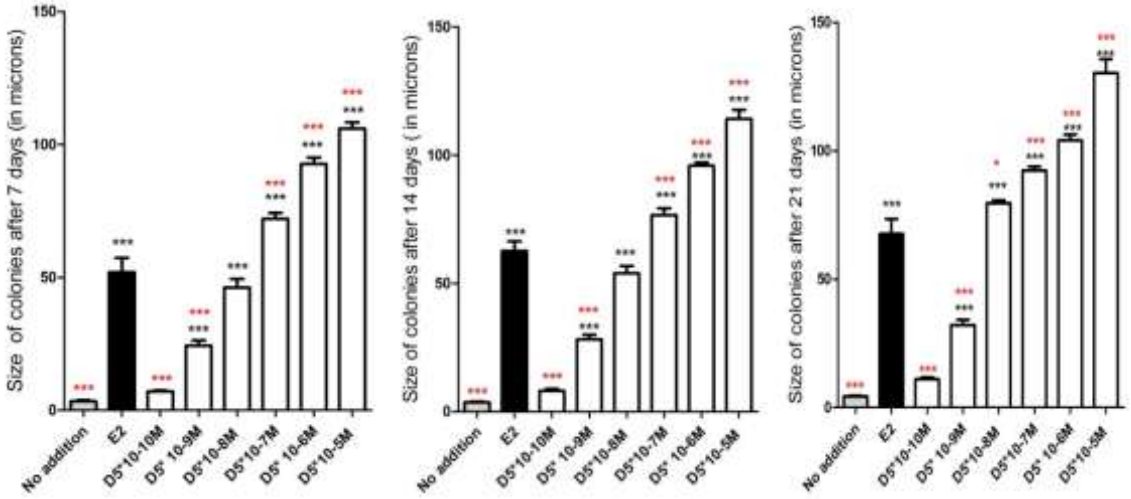
*Cell numbers **(Figure 3.10)*** the total cell counts of MCF10A cells tend to plateau between 10^{-7}M and 10^{-8}M, while MCF10F cell counts rise up to 10^{-5}M. Plotted as a line graph and subjected to curve-fitting, the coefficient of determination (r^2) value against an exponential fit is 0.948.

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells

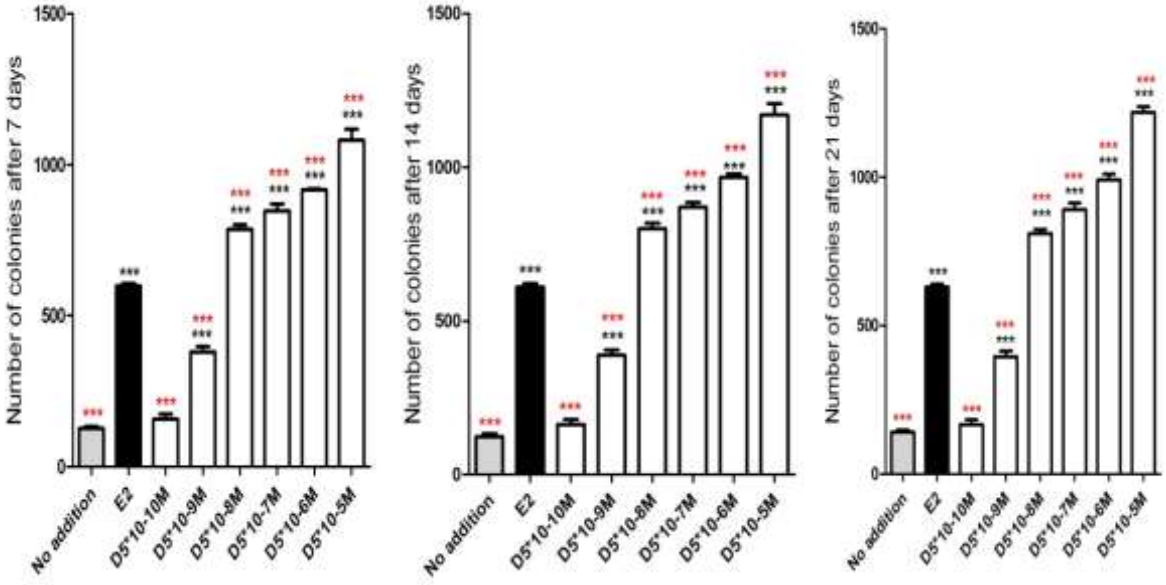


Day 7 Day 14 Day 21

FIGURE 3.8 Effect of concentrations of decamethylcyclopentasiloxane (D5) on size of colonies of MCF10A (A) and MCF10F (B) cells growing in methocel suspension culture from 7 days to 21 days.

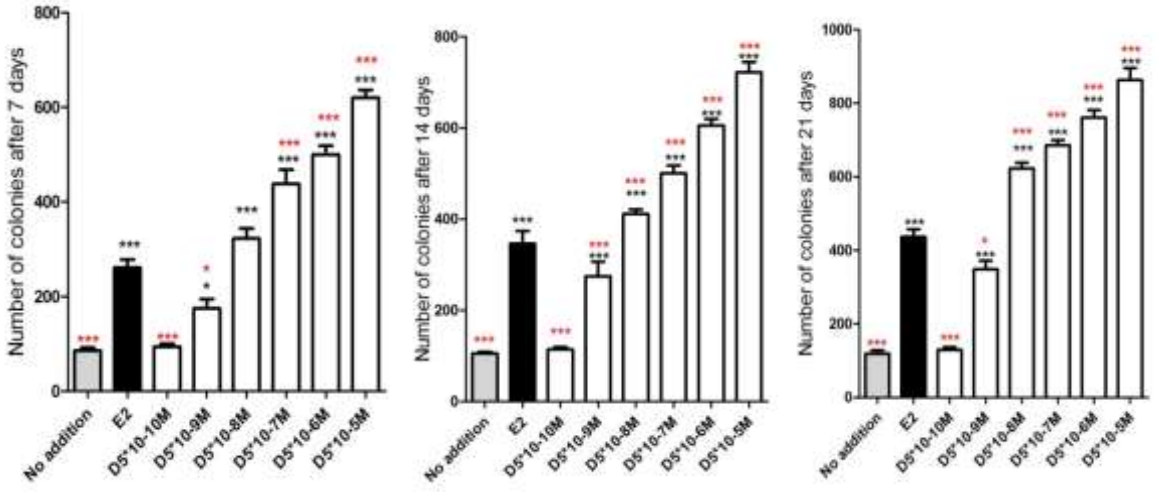
Cells were grown in stock medium with no addition, with70nM 17β-oestradiol, with D5 at concentrations from 10^{-10}M to 10^{-5}M. Average colony size was calculated from 15 fields per well of view and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. *** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk.)**

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells



Day 7 Day 14 Day 21

FIGURE 3.9 Effect of concentrations of decamethylcyclopentasiloxane (D5) on number of colonies of MCF10A (A) and MCF10F (B) cells growing in methocel suspension culture from 7 days to 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with D5 at concentrations from 10^{-10}M to 10^{-5}M. Colony growth is shown as the number of colonies per well from 7 days to 21 days, under light microscope. Error bars are standard error of triplicate wells for each experiment. **** indicates p< 0.05, ** p< 0.01 and *** p <0.001compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

A. MCF10A cells B. MCF10F cells

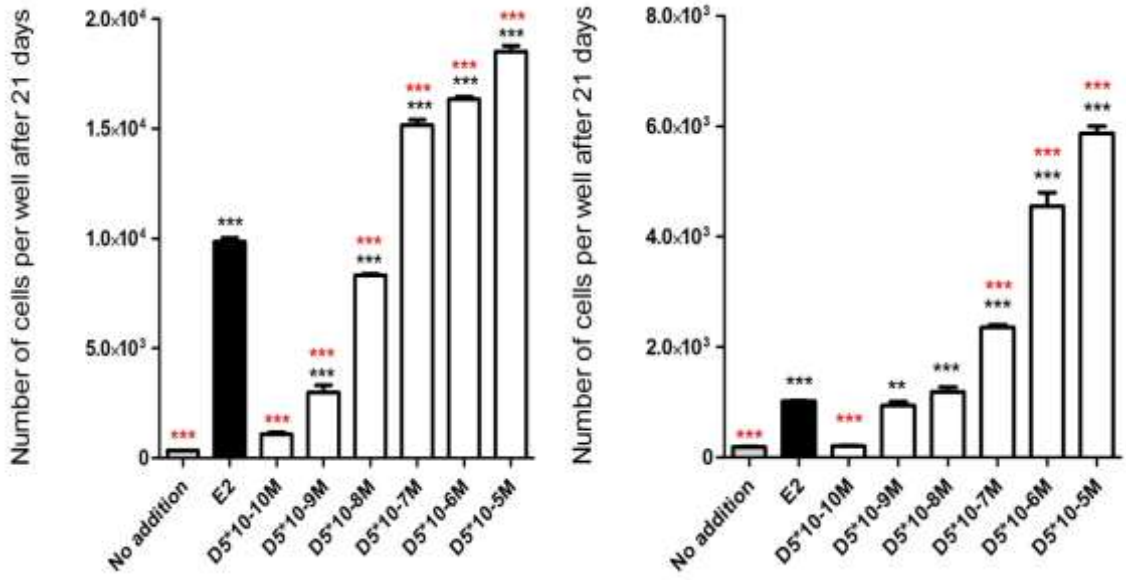


FIGURE 3.10 Effect of different concentrations of decamethylcyclopentasiloxane (D5) on growth of MCF10A (A) and MCF10F (B) cells in semi- solid methocel suspension culture after 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with D5 at concentrations from 10^{-10}M to 10^{-5}M. Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

***** p< 0.01 and *** p <0.001compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

3.1.5 Comet assay of DNA damage after exposure to Cyclosiloxanes (D3, D4 and D5)

An alkaline single-cell gel electrophoresis (SCGE) technique was employed to detect single strand and labile DNA adducts in the immortalised human breast epithelial cell lines MCF10A and MCF10F following exposure to D3, D4, D5 for periods of between 1 hour and 24 hours. Cells were lysed and embedded into agarose on microscope slides. Following electrophoresis and staining the DNA was visualised either as high molecular weight DNA contained within the area of the cell or with low molecular DNA bands which appear as a comet tail from the cell. Comets were analysed as average length of comet tail or as % of cells showing any comet tail.

MCF10A and MCF10F cells were exposed to 10^{-5}M concentrations of diethylstilboestrol or cyclosiloxanes for 1 hour or 24 hours and representative photographs are shown for MCF10A cells in Figure 3.11.Cells in the photomicrograph in ***Figure 3.11*** Panel C (D3) show strong classical comet formation after 1 hour of exposure to 10^{-5}M D3. Some indications of comet formation are seen in ***Figure 3.11*** panels B and D.

The bar charts show the effect on DNA tail length and % of cells with comets following exposure of MCF10A cells and MCF10F cells to D3 **(*Figure 3.12*)**, D4 **(*Figure 3.13*)** and D5 **(*Figure 3.14*)**.

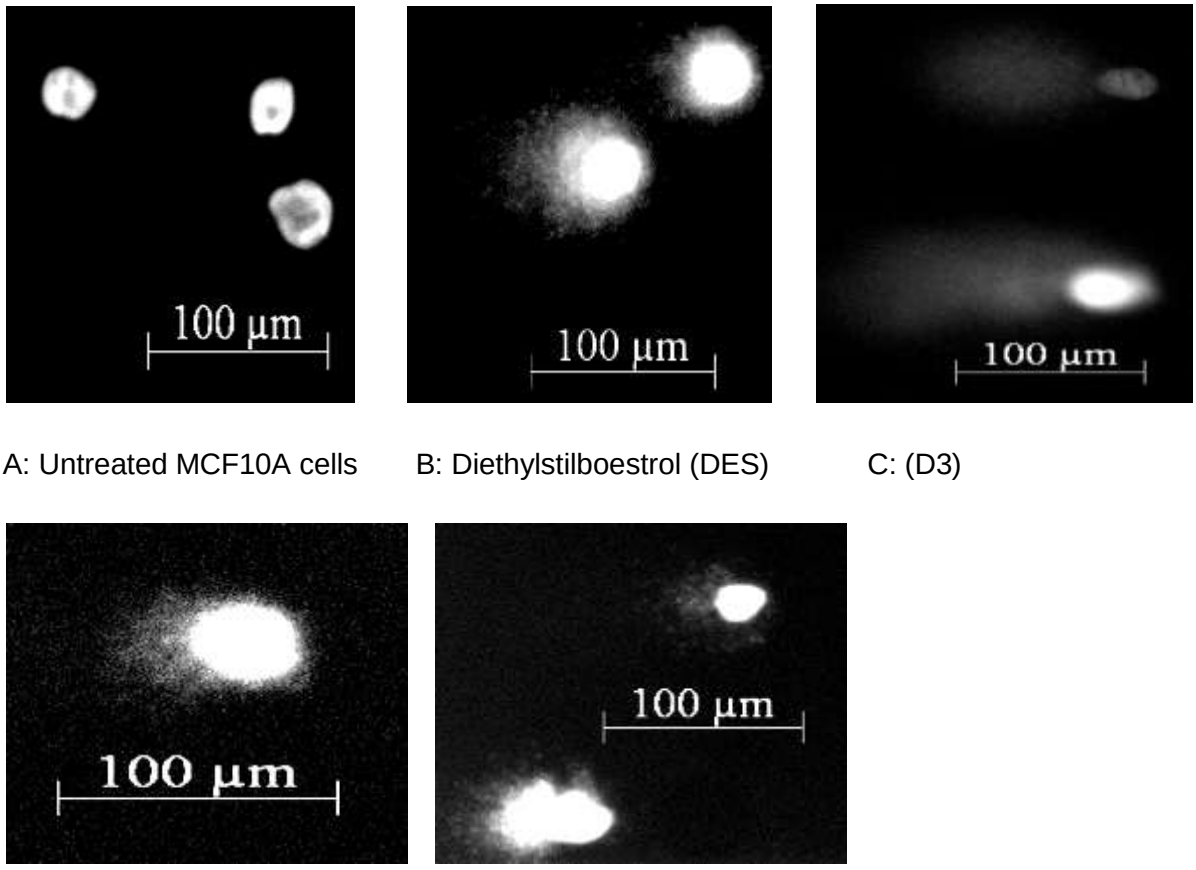
For MCF10A cells, there was no detected response at dilutions to 10^{11}M but the tail length rose dramatically at 10^{-10}M and fell thereafter at higher concentrations of D3 **(*Figure 3.12*)**. The exception being DES, which yielded large numbers of poorly developed comets in both cell lines. The single concentration comets were measured in MCF10F cells at 10^{-5}M concentrations **(*Figure 3.12 C ,D*)** series of assays using MCF10F cells show increased tail length and numbers (%) over the blank control, but as noted above, DES stimulated large numbers of weak comets. The numbers in the treated MCF10F cultures are comparable to those for MCF10A cells at the same concentration. However, this was at 10^{-5}M which was at the end of a sharp decline from its peak at 10^{-10}M in MCF10A.Tail length was also poor with MCF10F, peaking at 80 microns compared to 300 microns for MCF10A cells.

Numerical comet parameters for octamethylcyclotetrasiloxane (D4) are shown in **(*Figure 3.13)*** and decamethylcyclopentasiloxane (D5) (***Figure 3.14***) after 1 and 24 hours exposure for MCF10A 10^{-5} and 10^{-6}M concentration) and 10-5M only for MCF10F***.***

The bar charts show comet tail length to correlate with percent of cells with damaged DNA, the exception being DES, which yields large numbers of poorly developed comet cells. The

range of actual numbers of comets, as assessed by the maximum y-axis values were similar to those obtained with D3.

A difference in response to D4 **(*Figure 3.13*)** and D5 **(*Figure 3.14*)** was that D4 gave large numbers of well-formed comets at 1hr but not at 24 hours. The opposite was true for D5, where length was not overall different to control levels, although the maximum number of comets formed was less than half of that in response to D4.



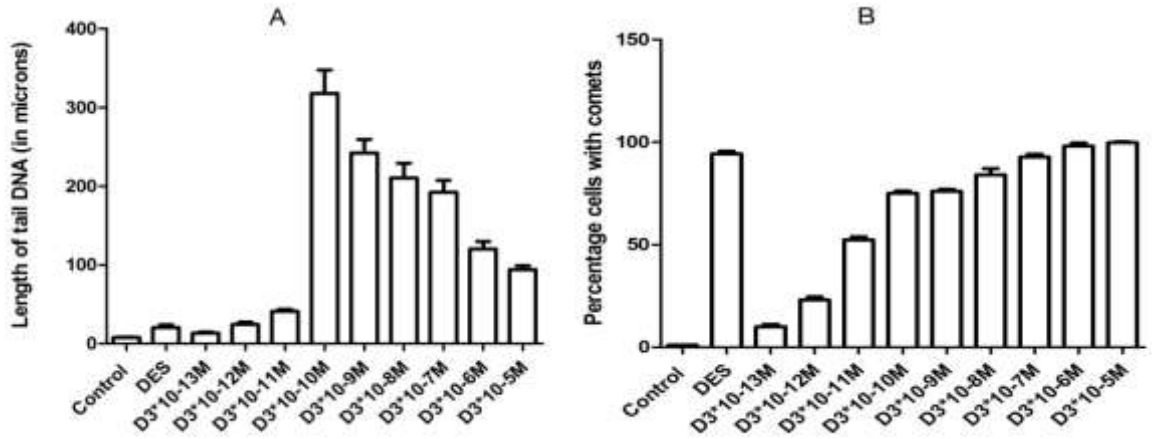
A: Untreated MCF10A cells B: Diethylstilboestrol (DES) C: (D3)

D: (D4) E: (D5 after 24 hrs)

FIGURE 3.11 Comet assay for detection of DNA damage in MCF10A cells after exposure to cyclosiloxanes (D3, D4 and D5).

Panel A is of untreated cells (control). B) Cells treated with 10-5 M Diethylstilboestrol (DES) for one hour, (positive control). C) Cells treated with 10^{-5}D3 for one hour. D3) Cells treated with 10^{-5}M D4for one hour. E) Cells treated with 10^{-5}M D5 for 24 hours. Cells viewed by fluorescence microscopy.

MCF10A cells



MCF10F cells

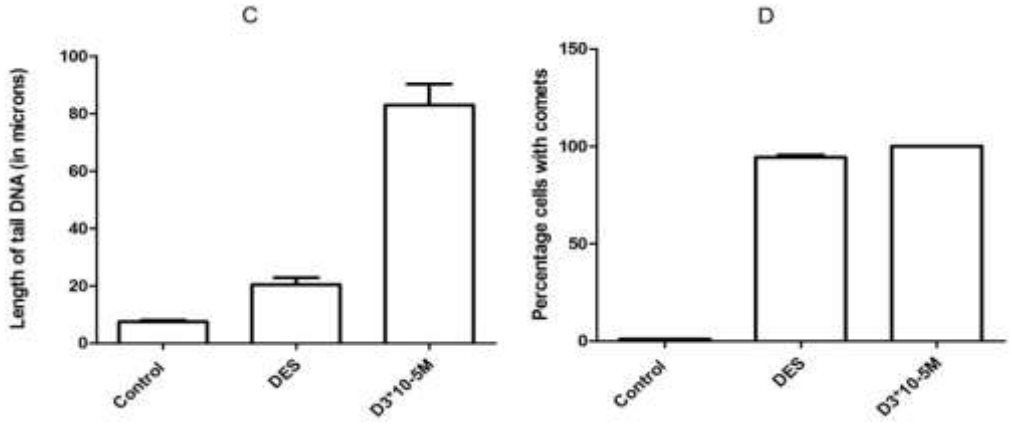
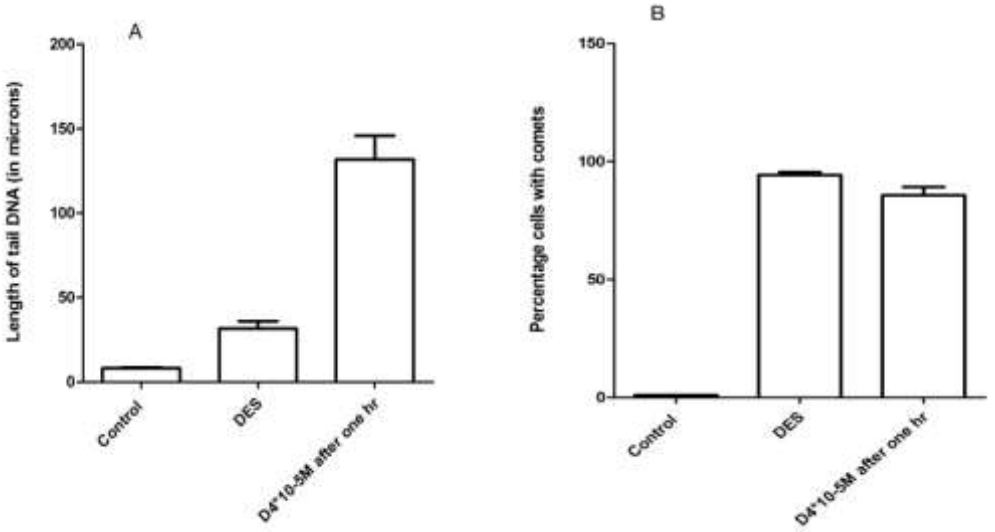


FIGURE 3.12 Effect of hexamethylcyclotrisiloxane (D3)) on DNA damage in MCF10A (A, B) and MCF10F (C, D) human breast epithelial cells as assessed by a comet assay measuring DNA tail length (A,C) or % of cells with comets (B,D).

Cells were grown in stock medium and treated for 1 hour as untreated cells (control) and DES treated cells. Treatment was with 10^{-5} M DES (positive control) and 10^{-5}M D3. Error bars represent standard deviation of 50 comets scored.

A. MCF10A cells



B. MCF10F cells

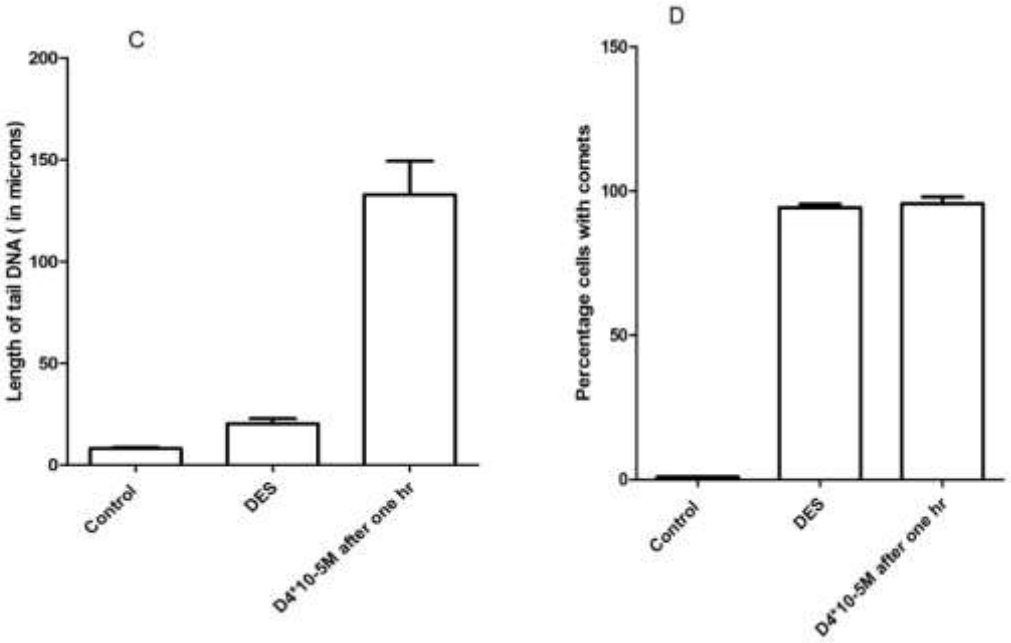
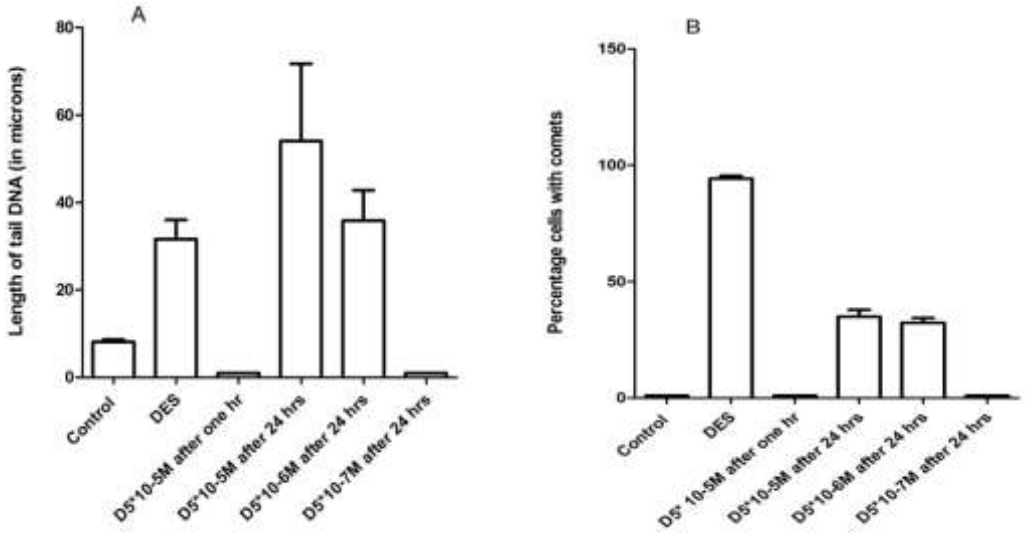


FIGURE 3.13 Effect of octamethylcyclotetrasiloxane (D4) on DNA damage in MCF10A (A) and MCF10F (B) human breast epithelial cells as assessed by a comet assay measuring DNA tail length (A, C) or % of cells with comets (B, D)

Cells were grown in stock medium and treated 1 hour with untreated cells (control). Treated cells with 10^{-5} M diethylstilboestrol (DES), (positive control) for one hr .Treated cells with 10^{-5}M D4 for one hr. Error bars represent standard deviation of 50 comets scored.

A. MCF10A cells



B. MCF10F cells

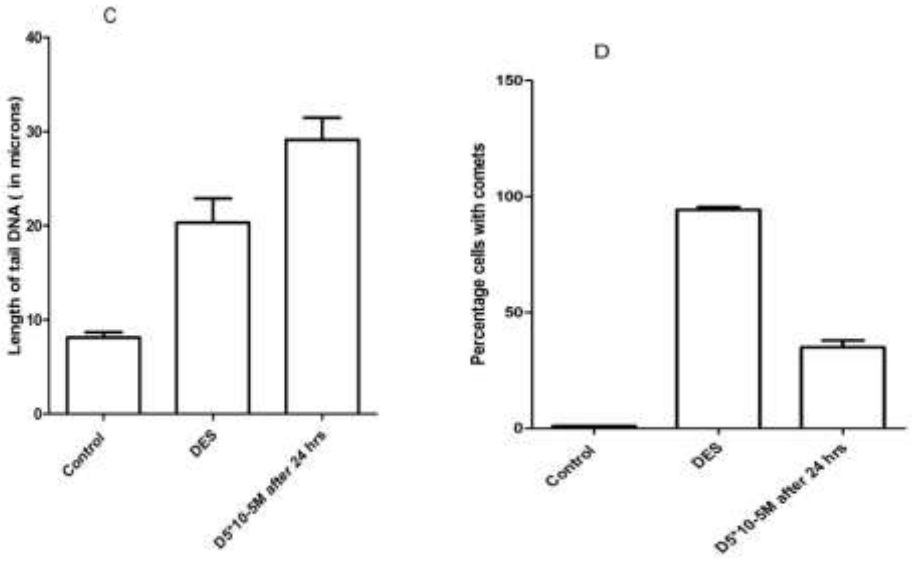


FIGURE 3.14 Effect of decamethylcyclopentasiloxane (D5) on DNA damage in MCF10A and MCF10F human breast epithelial cells as assessed by a comet assay measuring DNA tail length (A, C) or % of cells with comets (B, D)

Cells were grown in stock medium and treated 1 hour with untreated cells (control). Treated cells decamethylcyclopentasiloxane (D5) for 24 hrs. Treated cells 10^{-6}M decamethylcyclopentasiloxane (D5) for 24 hrs. Treated cells 10^{-7}M decamethylcyclopentasiloxane (D5) for 24 hrs. Error bars represent standard deviation of 50 comets scored.

3.1.6 Cyclosiloxanes: Effect on mRNA expression of DNA repair genes:

The ability of cyclosiloxanes hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) to enable growth of non-transformed MCF10A and MCF10F cells in suspension culture and to bring about damage to DNA in a Comet assay suggests that they may have genotoxic properties. However, a further important question is whether they might also impair DNA damage detection and DNA repair pathways. In an initial investigation, levels of mRNA for BRCA1, BRCA2, ATM, ATR, BRIP1, CHK1, CHK2, p53,PALB2, PARP1, PTEN ,Rad50,Rad51 and STK111 have been investigated in the MCF10A and MCF10F cells after short term (1 week) and longer term (30weeks) exposure to these cosmetic chemicals.

Levels of BRCA1 mRNA after exposure to D3, D4 and D5

Short (1 week) and long term (30 weeks) effects of exposing MCF10A cells (***Figure 3.15***) and MCF10F cells (***Figure 3.16***) to hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4), and decamethylcyclopentasiloxane(D5), in terms of levels of mRNA for BRCA1, was investigated using RT-PCR. Significant ($p<0.001$) elevation of BRCA1 mRNA was found after short term exposure to 10^{-5} M concentrations of D3 and D4 exposure in MCF10A cells (***Figure 3.15A***). However, after long term exposure to 10^{-5}M concentrations of chemicals D3, D4 and D5 there was significant reduction of BRCA1 mRNA in MCF10A cells for these chemicals ($p<0.001$ for D3, <0.01 for D4 and D5).

Short term (1 week) exposure to 10^{-5}M D3, D4 or D5 did not increase BRCA1 mRNA levels in MCF10F cells ***(Figure 3.16).*** However***,*** it must be acknowledged that error bars were large in these experiments. Long term exposure of 30 weeks showed a significant reduction in expression of BRCA1 mRNA in MCF10F cells with D4 and D5 ($p <0.01$) but not with D3 ($p <0.01$).

MCF10A cells

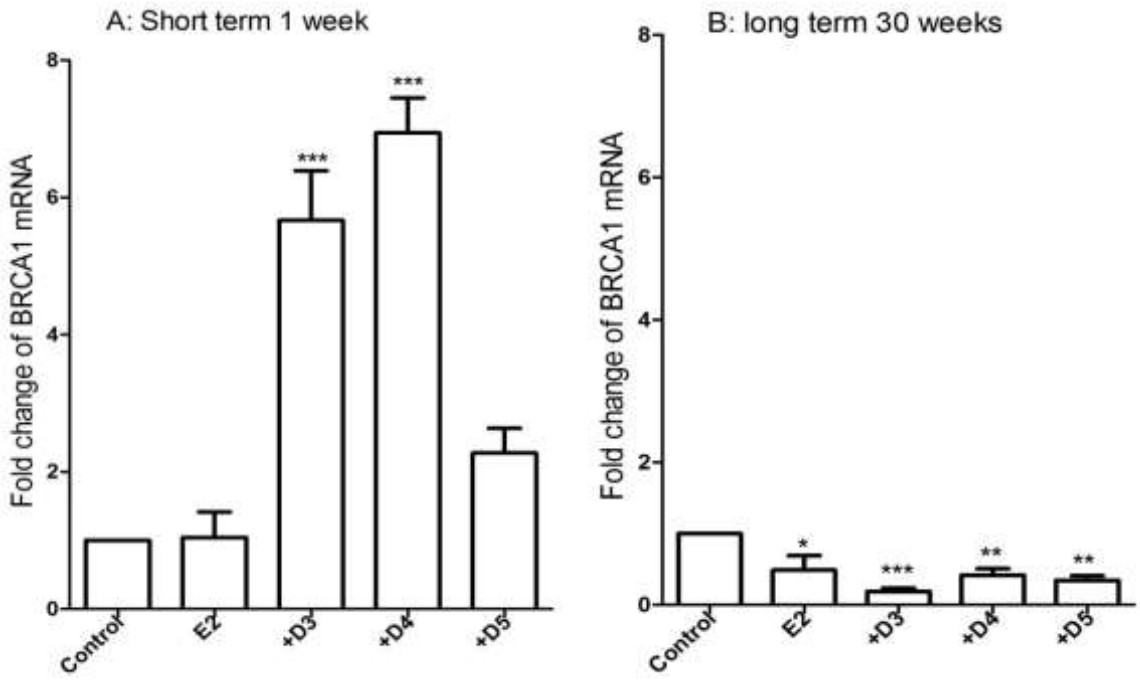


FIGURE 3.15 Real -time RT- PCR analysis of BRCA1 mRNA in MCF10A cells following short term (A) or long term (B) exposure to cyclosiloxanes D3, D4 and D5.

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2), *or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4) or 10^{-5}M decamethylcyclopentasiloxane (D5). The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* **** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

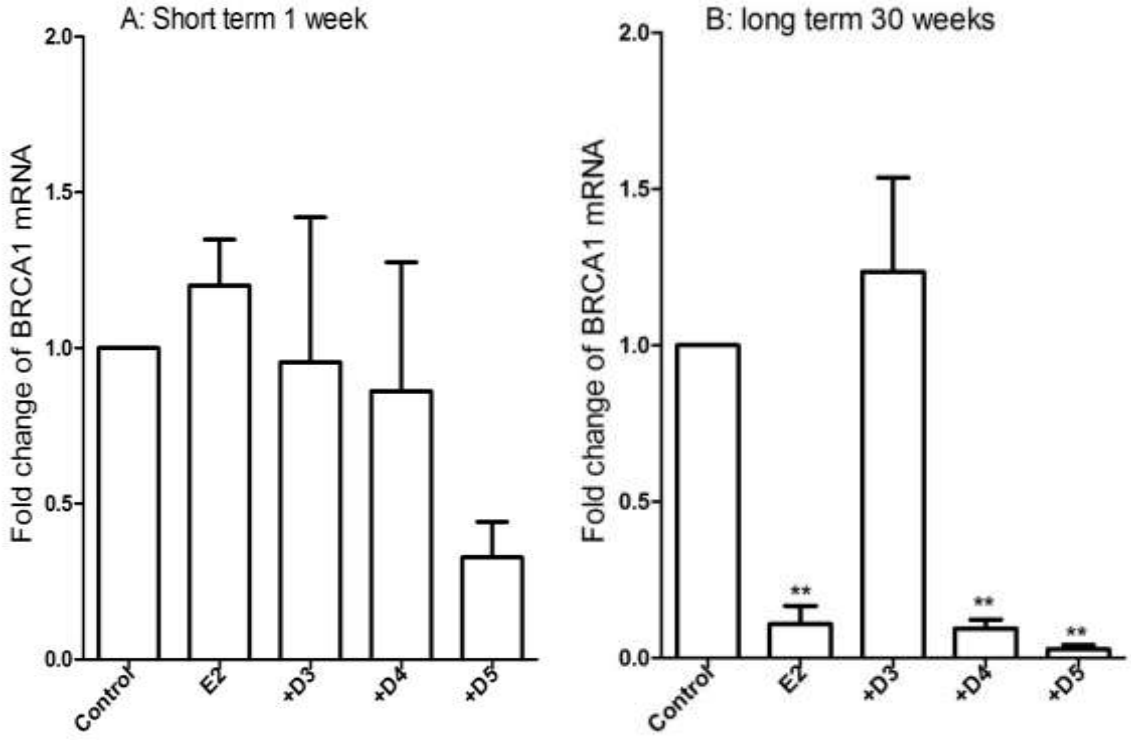


FIGURE 3.16 Real -time RT- PCR analysis of BRCA1 mRNA in MCF10F cells following short term (A) or long term (B) exposure to cyclosiloxanes (D3, D4 and D5).

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4) or 10^{-5}M decamethylcyclopentasiloxane (D5). The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* ***** indicates p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

Levels of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111 mRNA after exposure to cyclosiloxanes D3, D4 and D5

The effect of D3 in MCF10A cells on short (1 week) exposure of MCF10A cells (**Figure 3.17A**) to hexamethylcyclotrisiloxane (D3) was no significant change in the measured mRNA levels. Long term exposure of MCF10A cells yielded mixed responses. BRCA2, BRIP1, PALB2 and PARP1 mRNAs were reduced after 30 weeks exposure to 10^{-5}M D3 (all $p<0.05$), mRNA levels for RAD51 were significantly increased (***Figure 3.17B***). Results for effects of D3 in MCF10F cells with short-term exposure gave lowered mRNA levels for CHK2 and raised mRNA levels for RAD50 (***Figure 3.18A***). On long-term exposure mRNA for PALB2 was reduced ($p<0.05$), CHK1, CHK2, PTEN and STK111 were all lowered with a two-star ($p<0.01$) level of significance (***Figure 3.18B).***

High mean values in these plots are associated with large variances and do not reach statistical significance. The effects on mRNA levels of Short (1 week) and long term (30 weeks) exposure of MCF10A and MCF10F cells to octamethylcycloterasilxane (D4) are shown in ***Figures 3.19 and 3.20*** respectively. In MCF10A cells, For ATM, ATR, BRCA2, BRIP1, PALB2 and PARP1 there were significant reductions in mRNA after on long term exposure to 10^{-5}M D4. No significant effects were seen after one week of incubation. MCF10F cells did yield significant results after one week incubation with D4. There was a significant reduction in expression of BRCA2 and CHK2 mRNA. Long term exposure to D4 produced more extensive change in gene expression. Thirty weeks of incubation with D4 showed a significant reduction in expression of BRIP1, CHK1, CHK2, PARP1, PTEN and Rad51 mRNA in MCF10F cells. All changes except BRCA2 ($p<0.05$) were highly significant ($p<0.01$). Physical DNA damage (comet assay) wascnot measured after these long exposure periods.

Effects on mRNA levels after short (1 week) and long term (30 weeks) exposure of MCF10A and MCF10F cells to decamethylcyclopentasiloane (D5) are shown in ***Figures 3.21 and 3.22*** respectively. In the short-term experiments a significant elevation in expression of CHK1 mRNA was seen in MCF10A cells. The long-term effect of exposure to D5 showed a significant elevation of Rad51 mRNA and a significant reduction in expression of ATM, ATR, BRCA2, CHK1, CHK2, PALB2, and PARP1 mRNA. In MCF10F cells the effect of 1-week exposure to decamethylcyclopentasiloane (D5) was a significant reduction in expression of CHK2 mRNA. The long-term effect of exposure showed a significant reduction in expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells, with BRCA2, PALB2 and PARP1 achieving a significance level $p<0.001$, the highest of any treatment in this group of experiments. ***Table 3.1*** shows the differences in response between MCF10A and MCF10F cells

MCF10A cells**D3**

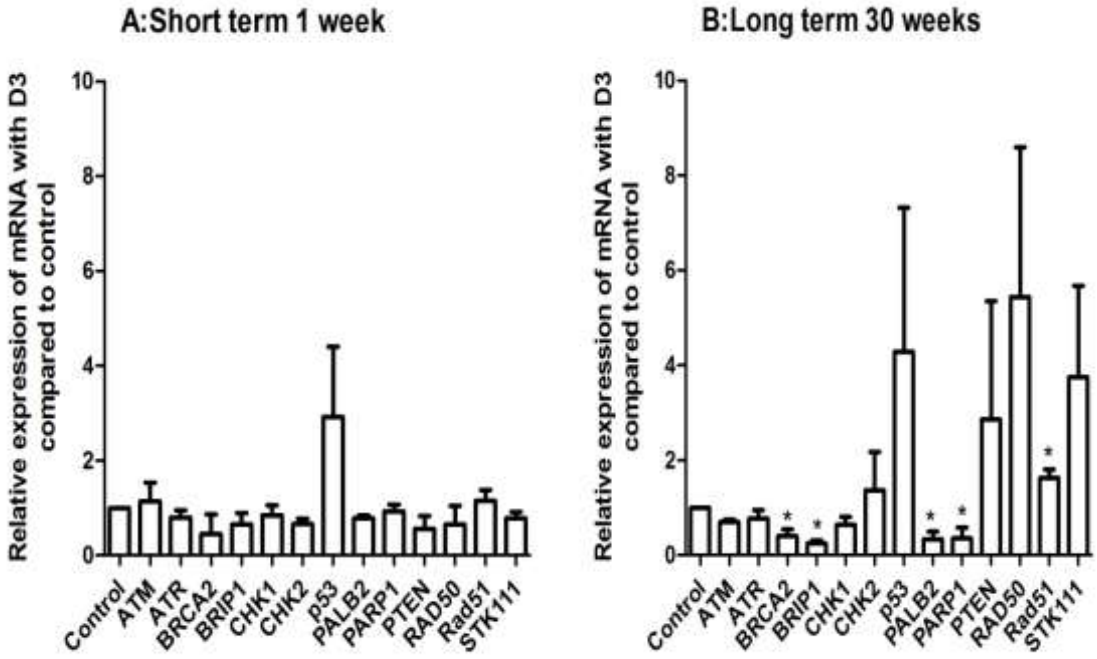


FIGURE 3.17 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells following short term (A) or long term (B) exposure to hexamethylcyclotrisiloxane (D3).

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3).The relative expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values****. * indicates p< 0.05 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells**D3**

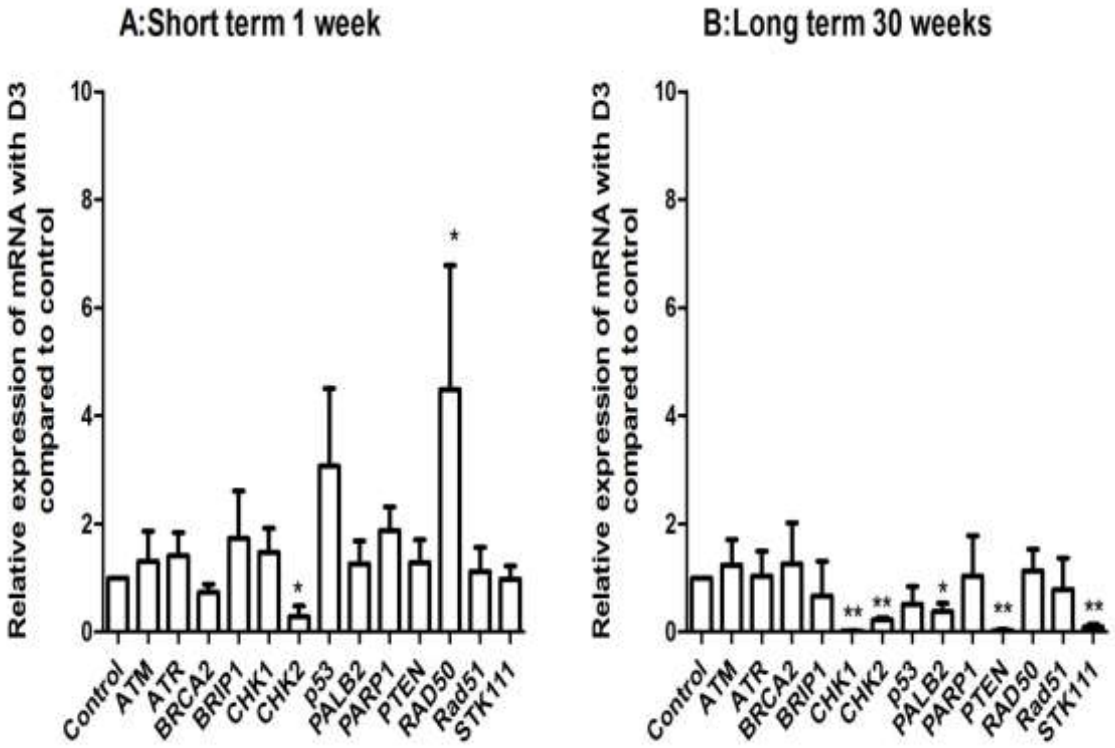


FIGURE 3.18 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells following short term (A) or long term (B) exposure to hexamethylcyclotrisiloxane (D3)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3), the relative expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* **** indicates p< 0.05, **p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10A cells**D4**

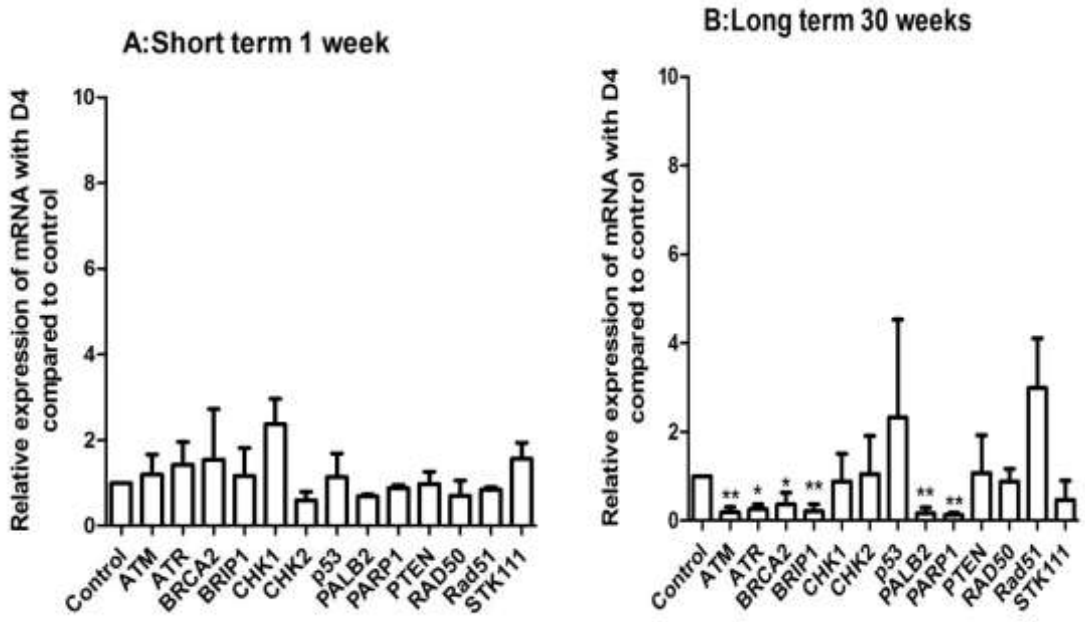


FIGURE 3.19 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells following short term (A) or long term (B) exposure to octamethylcycloterasilxane (D4)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-5}M octamethylcycloterasilxane (D4), *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* **** indicates p< 0.05, ** p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells**D4**

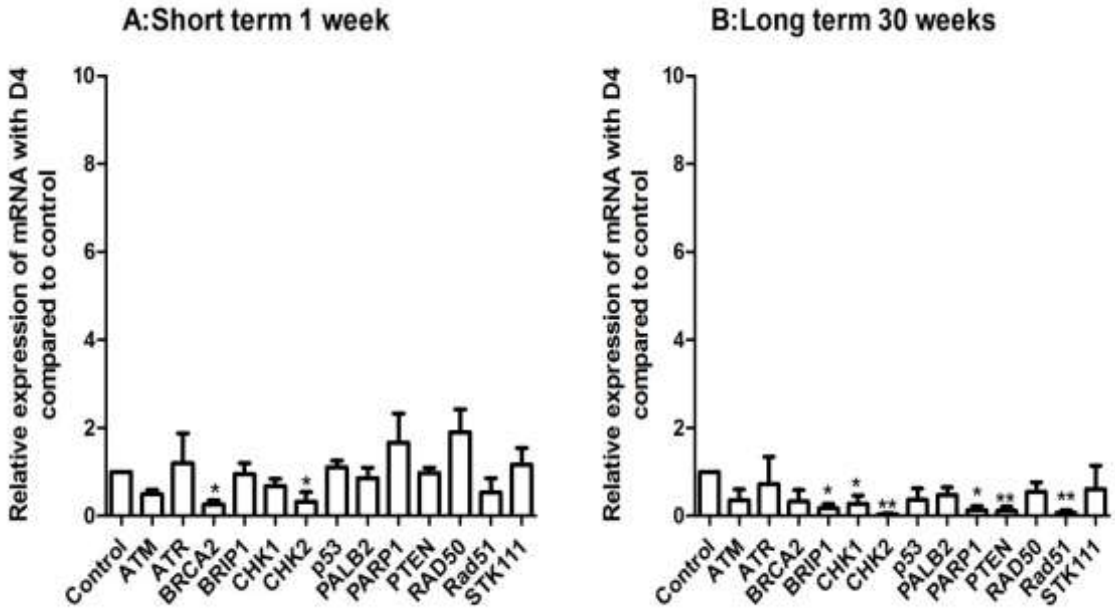


FIGURE 3.20 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells following short term (A) or long term (B) exposure to octamethylcycloterasilxane (D4)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10-5M octamethylcycloterasilxane (D4), *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* **** indicates p< 0.05, ** p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10A cells**D5**

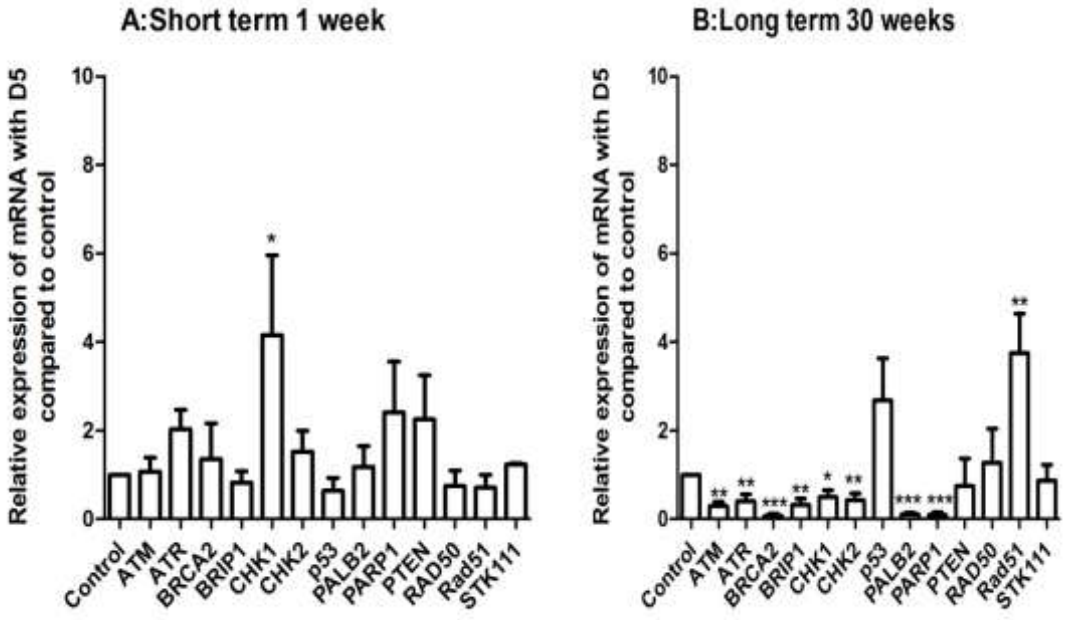


FIGURE 3.21 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells following short term (A) or long term (B) exposure to decamethylcyclopentasiloane (D5)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of $10^{-5}M$ decamethylcyclopentasiloane (D5). *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values****. * indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells**D5**

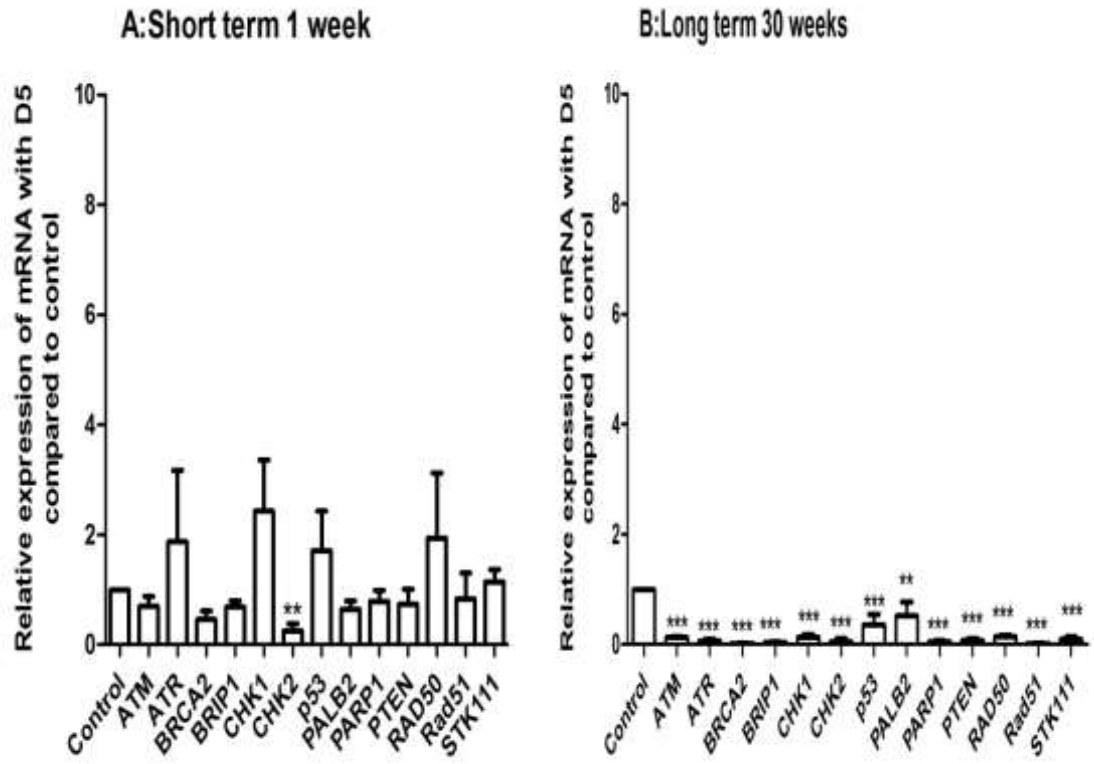


FIGURE 3.22 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells following short term (A) or long term (B) exposure to decamethylcyclopentasiloane (D5)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of $10^{-5}M$ decamethylcyclopentasiloane (D5). *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values****. * indicates p< 0.05, ** p< 0.01, ***p< 0.001compared to control by one way ANOVA with post-hoc Dunnett test.***

3.1.7 Level of BRCA1 protein after exposure to D3, D4 and D5

The effect of long term exposure to cyclosiloxanes on expression of BRCA1 protein was investigated in MCF10A and MCF10F cells using Western Immunoblotting. By comparison with molecular weight markers, a band at 220KDa was identified as BRCA1 protein. For MCF10A cells, a representative immunoblot is shown in ***Figure 3.23A.*** ***Figure 3.23B*** shows calculated values from three biological replicates taken from independent cell cultures. BRCA1 protein levels were reduced after long-term exposure to 10^{-8}M 17β-oestradiol, or to 10^{-5}M concentrations of D4 or D5 (***Figure 3.23B).***

For MCF10F cells a representative immunoblot is shown in ***Figure 3.24 A.*** ***Figure 3.24B*** shows calculated values from three biological replicates taken from independent cell cultures***.*** BRCA1 protein levels were significantly reduced after long-term exposure to 10^{-5}M concentrations of D3, D4 or D5 ($p<0.5$, <0.5 and <0.001) respectively ***(Figure 3.24B)***

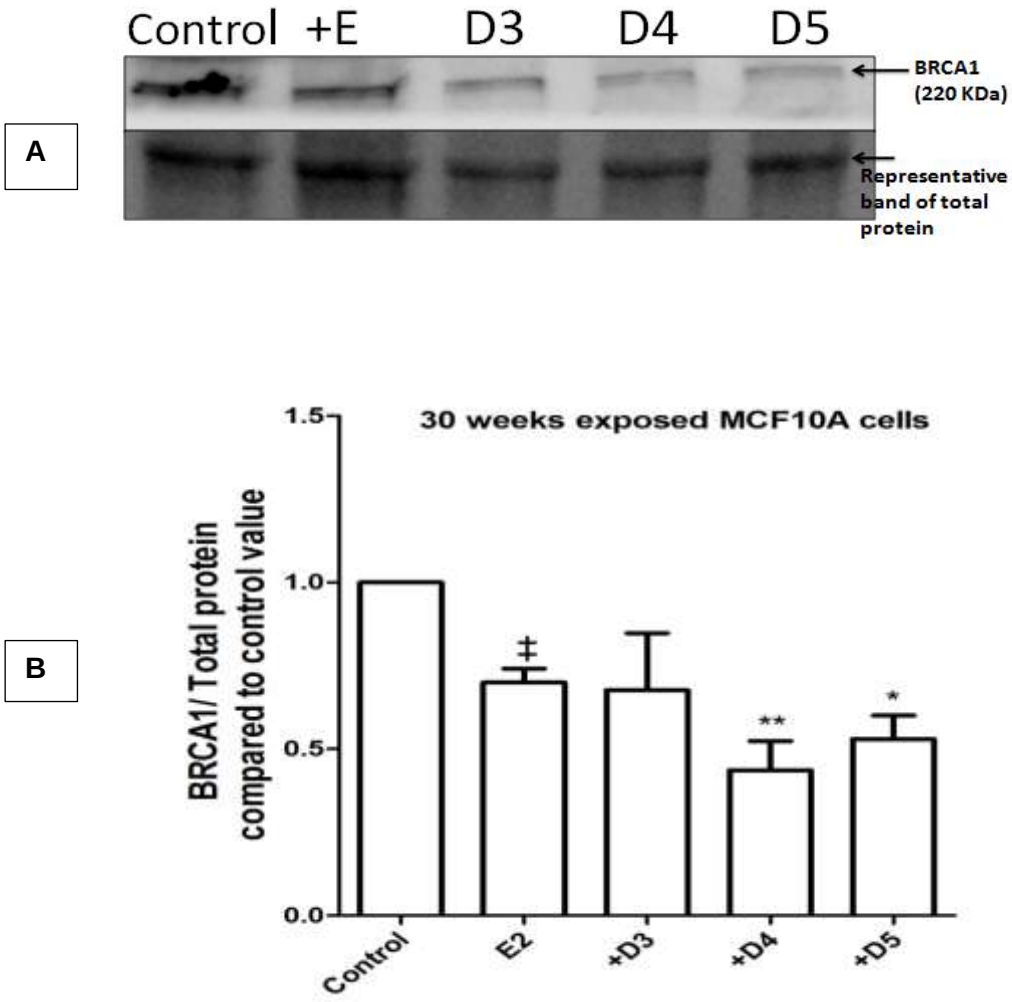


FIGURE 3.23 Level of BRCA1 protein after long term exposure to cyclosiloxane (D3, D4 and D5) in MCF10A cells using western Immunoblotting. A - Photograph of excised bands, B - quantitative plot.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10A human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4), 10^{-5}M decamethylcyclopentasiloxane (D5) compared to 10^{-8}M oestradiol (E2). **** indicates p< 0.05, ** p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test. ‡ indicated p< 0.05 compared to control by t-test.***

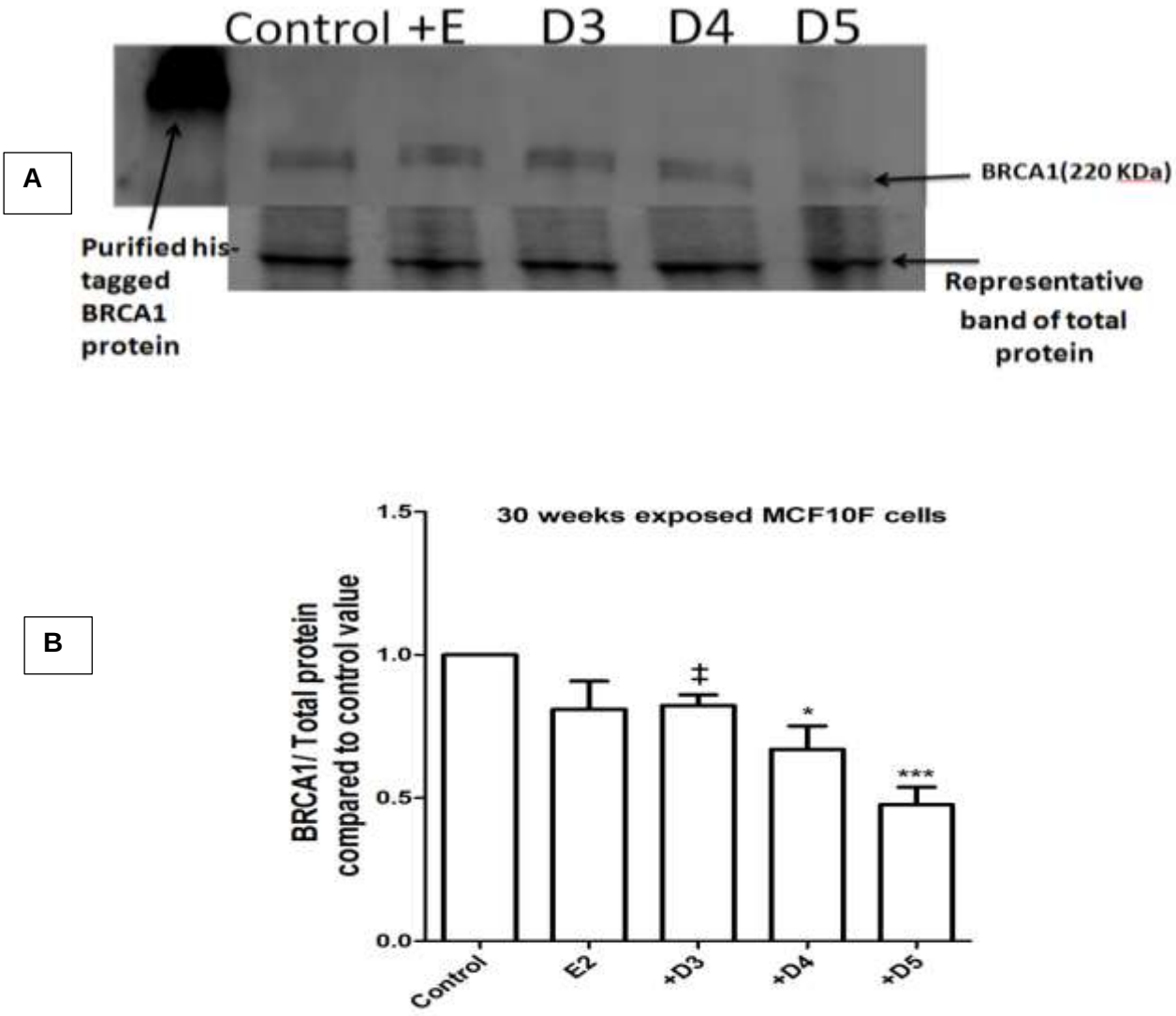


FIGURE 3.24 Level of BRCA1 protein after long term exposure to cyclosiloxanes (D3, D4 and D5) in MCF10F cells using western Immunoblotting. Upper - photograph of excised bands, lower, quantitative plot.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10F human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M hexamethylcyclotrisiloxane (D3), 10^{-5}M octamethylcyclotetrasiloxane (D4), 10^{-5}M decamethylcyclopentasiloxane (D5) compared to 10^{-8}M oestradiol (E2). **** indicates p< 0.05, *** p< 0.001 and compared to control by one way ANOVA with post-hoc Dunnett test.‡ indicated p< 0.05 compared to control by t-test. N.B. Western blot data showed that the his-tagged BRCA1 band had a higher molecular weight in comparison to BRCA1 bands of breast cell lysates, which is likely due to extra molecular weight of his-tag molecules that altered the protein mobility on the gel. His-tag modification increases effective size by 30 KDa. (Zhao et al., 2010)***

3.1.8. Summary of Results

The results are encapsulated in **Table 3-1** below. Both MCF10A cells and MCF10F cells perform comparably in the anchorage independent growth assay. Moreover the various ways of quantifying the results show the same effects, be it by colony number, colony size or total cell number in the colonies. D3 yields a dose response peaking at 10-10M, D4 and D5 show a continued rise in colony formation parameters with concentration of agent up the maximal amount tested. Comet assays give a positive indication of DNA damage. Reductions in DNA repair capability as measured by either mRNA or protein expression were seen after prolonged exposure to agents.

TABLE 3-1 Summary of cyclosiloxane results

ASSAY	MCF10A	MCF10F
Colony growth in methocel: number or size of colonies	D3 Bell shaped response curve D4, D5 exponential positive correlation with concentration	D3 Bell shaped response curve D4, D5 exponential positive correlation with concentration
Colony growth in methocel: total cell count	Mirrors colony growth	Mirrors colony growth
Comet assay	D3, D4 strongest response at 1 hour incubation. D5 strongest response after 24 hour incubation	Similar to MCF10A, except responses not as strong and the single dose chosen was not the strongest modifier with MCF10A
BRCA1 mRNA & protein	*70 nM E2* caused significant reduction over 30 weeks. D3 did not reduce m-RNA or protein (large *70 nM E2*) D5 reduced values more than D4	Reduction in m-RNA and protein after 30 weeks showed reductions D5>D4>D3. DES caused no reduction.
Other DNA repair mRNAs	Most m-RNAs suppressed by 30 weeks exposure to D5, exceptions being P53, RAD50, RAD51, STK111 Patchy reductions achieved by D3 & D4	All m-RNAs reduced after 30 weeks exposure to D5. Patchy reductions achieved bt D3 & D4

3.1.9 Discussion of results of exposure of MCF10 cells to Cyclosiloxanes D3, D4 &D5 *in vitro.*

The results demonstrate that exposure of non-transformed human breast epithelial cells to cyclosiloxanes can enable anchorage-independent growth in methocel culture, can cause DNA damage as measured by comet assays and can reduce levels of mRNAs encoding DNA repair proteins. Most notably, levels of both BRCA1 mRNA and BRCA1 protein were reduced after long-term exposure to the cyclosiloxanes. Differences were noted in the dose-responses of the three cyclosiloxanes and effects in the short-term (1 week) were not equivalent to those after longer exposure times (30 weeks). An overall summary of the results is given in **Table 3-1**

3.1.9.1 Anchorage-independent colony formation

Exposure to cyclosiloxanes D3, D4 and D5 all caused anchorage-independent colony formation and did so in both the immortalised non-transformed human breast epithelial cell lines MCF10A and MCF10F. Colonies of MCF10A and MCF10F cells were observed after 7 days which increased in size and number up to 14 days and then 21 days. Cyclosiloxane D3 gave colonies at and above concentrations of 10^{-13}M with maximal size and number observed at 10^{-10}M for both cell lines. Cyclosiloxane D4 gave colonies at and above 10^{-9}M with maximal size and number at 10^{-5}M for both cell lines. Cyclosiloxane D5 gave colonies at and above concentrations of10^{-7}M after 7 days and some increases at 10^{-8}M visible after 14-21 days, with maximal size and number at 10^{-5}M for both cell lines. In a publication from the same laboratory where this work was performed, comparable results have been obtained using parabens (esters of para-hydroxybenzoic acid with antimicrobial/antifungal properties and used as preservatives in products for domestic use) on the MCF10A cell line (Khanna and Darbre, 2013). The ability of anchorage-dependent cells to grow under anchorage-independent conditions has been shown to be a property closely related to transformation (Soule et al,1990) thus by inducing colony growth in methocel, cyclosiloxanes as well as parabens have the ability to induce a transformed phenotype

In the present study the results for anchorage-independent colony growth show D3 to be distinct from D4 and D5 in that D3 gives peak activity at 10^{-10}M in a bell-shaped curve, whereas D4 and D5 both yield a strong positive association between effect and concentration, not peaking or plateauing out at 10^{-5}M, the highest concentration used. Interestingly, in the 2010 European Scientific Committee on Consumer Safety (SCCS/1450/2016) report D3 is also considered as having separate effects from D4 and D5, although no reason is given. Whatever the differences in the detail of dose responsiveness it is clear that all of the cyclosiloxanes tested can increase the size and

number of colonies, an effect that is widely regarded as indicative of transformation towards neoplasticity, as discussed in the introduction to this thesis.

3.1.9.2 DNA damage as measured by a comet assay

Exposure to cyclosiloxane D3 caused DNA damage in MCF10A cells measurable on a comet assay after 1 hour of exposure to concentrations at and above 10^{-10}M. A single concentration experiment using MCF10F cells showed DNA damage was visible after 1 hour of exposure to 10^{-5}M D3. DNA damage was visible in both cell lines after exposure to 10^{-5}M D4 for 1 hour. For cyclosiloxane D5, DNA damage was only visible after 24 hours exposure to 10^{-5}M concentrations.

The comet assay results in the present study were not visually strong and the lengths of tail quantified were inversely related to the percentage of cells affected. The agents showed increasing efficacy with time between 1 and 24 hours exposure, which is consistent with susceptibility being maximal when DNA strands are exposed and given typical cycling times of cells in culture (NCBI bookshelf: https://www.ncbi.nlm.nih.gov/books/NBK9876)

The validity of the comet assay for the compounds tested was tested in an overview of over 95 compounds in which 88% of known carcinogens were comet positive. Specificity (non-carcinogens yielding comets) was 64% (Anderson et al 1998). The assay was also successfully employed in an investigation of dietary protection from reactive oxygen species damage (Erkekoglu 2014). The protocol was also validated by Darbre in studies on cyclosiloxanes (Farasani and Darbre 2016 & section 2.10 above). The continuity with previous studies in the Reading laboratories is the reason for using the non-steroidal, synthetic product DES (Elks, 2014) as control in these experiments, as opposed to the physiological E2 used elsewhere in these studies. DES has a chequered history, having been proposed and used in the mid-20th century as a preventive agent against pregnancy problems, but subsequently having its licences withdrawn due to associations with cancer in treated patients (Apfel, 1984). However, it is a potent synthetic oestrogen which has been shown previously to cause comets in the comet assay (Anderson et al. 1998).

The overall implication of this study is that effects on DNA damage were observed on human breast cells not seen previously in the Ames test (Ames et al,1973). The Ames test uses genetically manipulated bacteria that are sensitive to nutrient substrate changes so that genotypic changes cause failure to thrive (Ames et al 1973, Mortemans and Zeiger 2000). Phenotypic, DNA clastogenic and molecular expression changes consistent with a tumour promoting effect are seen with these compounds when applied to near-normal

mammary epithelial cell lines, suggesting a role for them in mammary cancer initiation. The use of eukaryotic and tissue specific cells from the organ of interest in this study may account for the positive results obtained here rather than the negative outcome of the tests based on bacteria.

3.1.9.3 BRCA1 mRNA and protein

Loss of heterozygosity in the recessive ('tumour suppressor') BRCA genes lead to defective double strand DNA break repair, which in turn constitutes a well-established risk factor for inherited susceptibility to breast carcinogenesis (Roy et al., 2012) In this project, using MCF10A cells, exposure for 1 week to 10^{-5}M of cyclosiloxanes D3, D4 or D5 caused increased levels of BRCA1 mRNA but decreased levels of BRCA1 mRNA after longer-term exposure - for 30 weeks. In MCF10F cells, there were no significant increases in BRCA1mRNA after 1 week or 30 weeks exposure to any of the cyclosiloxanes, but D5 caused an immediate decrease, already apparent after 1 week and decreases were observed after exposure to D4 or D5 after 30 weeks. This was one of the few instances of marked differential responsiveness between the two cell lines, and there are known differences in properties of their lines such as proliferation rate and adhesion (Russo et al, 2006). Western Immunoblotting confirmed that there was a loss of BRCA1 protein after long-term (30 weeks) exposure to 10^{-5}M concentrations of D4 or D5 in both cell lines. Inherited loss of BRCA1 is associated with increased breast cancer risk (Roy et al 2012) but these results suggest that BRCA1 function can also be lost by exposure to cyclosiloxanes. The different results observed after 1week and 30 weeks are very important in interpreting these results into the environmental situation where the human breast would be exposed also in the long term and not just for a few days.

3.1.9.4 DNA repair related mRNAs

DNA double strand break repair processes are associated with increased risk of breast cancer when lost (Kitagishi et al., 2013). Of the panel of 14 DNA repair related mRNAs, MCF10A cells showed no changes in expression after one week exposure to **D5** except for a rise in CHK1, but universally depressed expression at 30 weeks. MCF10A cells also were unaffected at one week; reduced expression at 30 weeks was seen with all genes except P53, PTEN, RAD51, RAD52 and STK111. D4 exposure for 1 week caused reductions in BRCA2 and CHK2; long term exposure caused reductions in all mRNAs except ATM, ATR and STK111. D4 exposure yielded no changes at one week in MCF10A cells and reductions only in ATM, ATR, BRCA2, BRIP2, PALB2 and PARP after 30 weeks. D3 reduced mRNA levels for CHK2 after one week, CHK1, CHK2,

PALB2, PTEN and STK111 at 30 weeks in MCF10F cells; no changes at one week and reductions in BRCA2, BRIP1 PALB2, PTEN and RAD51 in MCF10A cells. Overall, D5 caused disruption of expression in more repair genes than D4 or D3. In these experiments the differences in short and long term changes in repair gene expression between the two cell lines largely reproduce those described above for the BRCA1 gene. The results are therefore indicative of a more generalised loss of DNA repair in the cells beyond BRCA genes following exposure to the cyclosiloxanes.

3.1.9.5 Cyclosiloxanes give effects at concentrations measurable in human tissues.

In discussing the above results, it is of note that there are relatively few publications giving plasma concentrations of cyclosiloxanes. Hanssen (Hanssen et al., 2013) quotes maximum values of 12.7ng/ml (median is 40nM ($4x10^{-8}$M) for D4 in a 2013 publication measuring levels in human plasma in Norwegian women. Values for other cyclosiloxanes listed fall into the same range. In a study from Germany ((Fromme et al., 2015) concentrations are mostly given as ng/g l.w. (lipid weight), however a value of 8µg/l is given for plasma D6; representing 21.6nM ($2.16x10^{-8}$M). These values are towards the middle of the range of molar concentrations tested in this study supporting the physiological relevance of the work. The relevance of plasma concentrations is not necessarily representative of levels experienced by cells in intact epithelia, still less breast cancer foci, which are often encased in a very hard and dense matrix. The density of matrix in normal, pre-cancerous breasts is a risk factor for subsequent tumour development (Pettersson et al., 2014). Therefore, these results necessitate that future work includes measurements of the cyclosiloxanes in human breast tissue itself.

3.1.9.6 Socio-political & regulatory considerations relating to commercial cyclosiloxane use

Cyclosiloxanes are used as solvents in conditioning/spreading agents in a wide range of personal care products (Johnson et al., 2011; Dudzina et al., 2014). Octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) are combined and termed cyclomethicone which may also include, as an impurity, hexamethylcyclotrisiloxane (D3) (Johnson et al.,2011). Due to their extensive use in consumer products, these substances are ubiquitous in the environment (Genualdi et al., 2011; Wang et al., 2013), to the extent that D4 and D5 are defined as very persistent very bioaccumulative substances of environmental concern (Brooke et al., 2009) triggering a proposal for their restriction in Europe (ECHA, 2016). The results presented in this thesis

suggest that future regulation should consider not only the environmental impact of these compounds but also their effects on human breast cells and the potential for them to enable breast cancer development if sufficient concentrations are demonstrated to be entering breast tissues.

Cyclosiloxanes are the objects of periodic regulatory scrutiny as they are incorporated into a number of cosmetic formulations in quite high concentrations. Sixty-two percent appears to be taken by the authorities as a standard degree of incorporation. Of that, absorption through skin is reported by the SCCS (*Scss/1450/2016)* as 0.94%, with the possibility of release back to the exterior through 2-way solvent traffic. The most recent SCCS report suggests Low Observed Adverse Effects Levels of around 100mg/kg, measuring changes in vital organ weight. (*Scss/1450/2016*). There is therefore the potential for high exposure in the human population and these results showing adverse effects on breast cells should be taken into consideration in future risk assessment.

Previous work has not reported any ability of the cyclosiloxanes to damage DNA but most previous work has been based on the Ames test using bacteria and not human breast cells as used in this study. The paragraph on mutagenicity of D5 in the 2010 European Union Opinion (SCCS_1241/10) - (ec.europa.eu/health/scientific_committees/consumer.../sccs_o_029.pdf) reads as follows under the heading Mutagenicity:-*"The negative results obtained in bacteria (reverse mutation assay) or in mammalian cells, i.e. in vitro chromosomal aberration and SCE [Sister Chromatid Exchange] test, along with an in vivo micronucleus assay and dominant lethal test, indicate that D5 does not possess mutagenic or genotoxic potential."*

The results presented in this thesis are not in complete agreement with this conclusion, a possible reason for the discrepancy being the very different time-frames involved using bacteria which divide very much more quickly than eukaryotic cells (doubling time in minutes compared with between 1 and 2 divisions per day respectively). This difference is also relevant to results using the Ames test, discussed below. In real life situations the exposure to these compounds is characteristically chronic and at low concentration levels. In this study, the D3 and D4 comet assay results proved positive.

PubMed™ searches using the terms cancer and cyclosiloxane together or any of the full or abbreviated names of D3 – D5 yield no results. Cyclosiloxane on its own yields 14. Eight are to do with physical chemistry and/or nanostructures, six have clinical implications (one of these is written in Japanese, concerning cycloxilanes in food & baby products, such as teats and will not be further discussed). Three are technically driven publications concerning breast implant materials ((Ali et al., 1998)) characterising cyclosiloxanes using infra-red and Raman spectroscopy purely to acquire data on implant material as a "starting

point for …surveillance studies". Shimono et al (Inoue et al., 1997, Shimono et al., 1997) described the use of these compounds for coating membranes in arterial bypass equipment, without adverse comments on safety. This demonstrates that there has been very little research on the cyclosiloxanes, arguably an inadequate amount, given their extensive use and potential for widespread exposure of the human population.

More physiologically, Kala (Kala et al., 1998) studied distribution in mice after sub-cutaneous injection and found it to be "wide". In another rodent study, Lieberman et al (Lieberman et al., 1999) described fatal liver and lung damage in treated mice, with 20x normal free radical presence in the liver and 7xin the lungs. The study was controversial however; that this is the only animal study highlights the need for more such in vivo work. It is a limitation of the work described here that monocultures of cell lines can never fully reproduce effects seen interacting multi-organ system such as exist in whole animals.

Much of the work in this area relies on the Ames test. This test uses bacteria that are genetically modified to carry mutations in genes coding for histidine biosynthesis. They therefore require histidine for growth. The method tests for mutations that can result in a reversion back to the wild type, permitting growth on a histidine-free medium. However, the test has limitations. For example, mutagens may act differently in a bacterial strain-dependent manner, compared to their effects in more complex eukaryotic cells. The DNA is organised differently, in chromosomes, as compared to the ring structures found in bacteria. Also the test is geared towards frame-shift mutations; mutagens having other effects on the genome point mutations may not be detected. The conversion of pro-mutagenic compounds by metabolism in the host will necessarily remain undetected. Mutagens identified in the Ames test need not necessarily be carcinogenic, an example being nitroglycerin (Benigni and Bossa, 2011). Combining the results from the present work and that published in the literature, it can be concluded that cyclosiloxanes show potentially mutagenic properties in mammary epithelial cells in addition to the effects apparent in bacterial based studies.

In summary, all of the three cyclosiloxanes tested showed consistent genotoxic and growth pattern modulating effects on the near-normal breast epithelial cell lines studied. While the experimental model itself has limitations, the results should act as a stimulus for more physiological studies probably necessarily using animals until practical tissue engineering evolves to a more mature discipline than presently exists.

3.2 Butylphenylmethylpropional (Lilial)

Chemical constituents of personal care products may be contributory to carcinogenesis in the breast (Darbre and Harvey, 2008). Butylphenylmethylpropional also known as 2-(4-tertbutylbenzyl) proplonaldehydeLilial (CAS 80-54-6) is added for purposes of fragrance at up to 2.5% concentration to a range of cosmetic products. (International Fragrance Association, Code of Practice 1999, Geneva). It has been demonstrated to exhibit oestrogenic activity and exposure to oestrogen itself is a risk factor for breast cancer (Charles and Darbre, 2009a). However, it has yet to be investigated in breast cells for effects on genomic stability which is an enabling characteristic underlying cancer development. Its absorption and accumulation in breast tissue is as yet unknown.

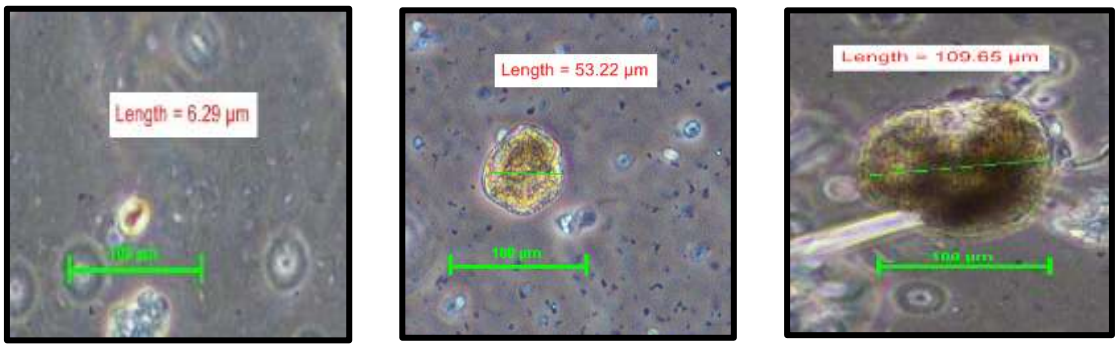
3.2.1Suspension growth of MCF10A & MCF10F cells after exposure to butylphenylmethylpropional (Lilial): morphology

MCF10A and MCF10F cells were grown in semi-solid suspension culture with 10^{-8}M 17β-oestradiol and varying concentration of butylphenylmethylpropional (Lilial). Photomicrographs of colonies after 21 days are shown in ***Figure 3.25***. In the absence of treatment, only small colonies of a few cells were present, having diameters less than 20 microns. A high proportion of much larger colony sizes were found with all the treatments shown in ***Figures 3.25*** B-C. Colony shapes were irregular, but maximal diameters exceeded 100 microns in each case

3.2.2 Suspension growth parameters in the presence of Lilial: quantitation

MCF10 A and MCF10F cells were grown with 17β-oestradiol or Lilial in suspension culture for up to 21 days, colony size (***Figure 3.26***), colony number (***Figure 3.27)*** and total cell number (***Figure 3.28***) were measured after 7, 14 or 21 days. Increasing concentrations of Lilial. (10^{-10} M to 10^{-5}M) gave increased colony size and colony number. Responses were similar in both cell lines and also similar to those for cyclosiloxanes D4 and D5. The concentrations at which the effect of Lilial exceeded that of E2 were 10^{-8}M for colony size and number. Probability values for concentrations higher than E2 were in all but 2 cases <0.001, the weaker responses at higher dilutions tended to be less robustly demonstrated, but within the standard <0.05 convention.

Cell numbers assessed by coulter counter also were higher than control values in all cases and exceeded E2 values ($p<0.001$) by 10^{-8}M. The trend was of increasing cell numbers with concentration through the lowest dilution used (10^{-5}M).

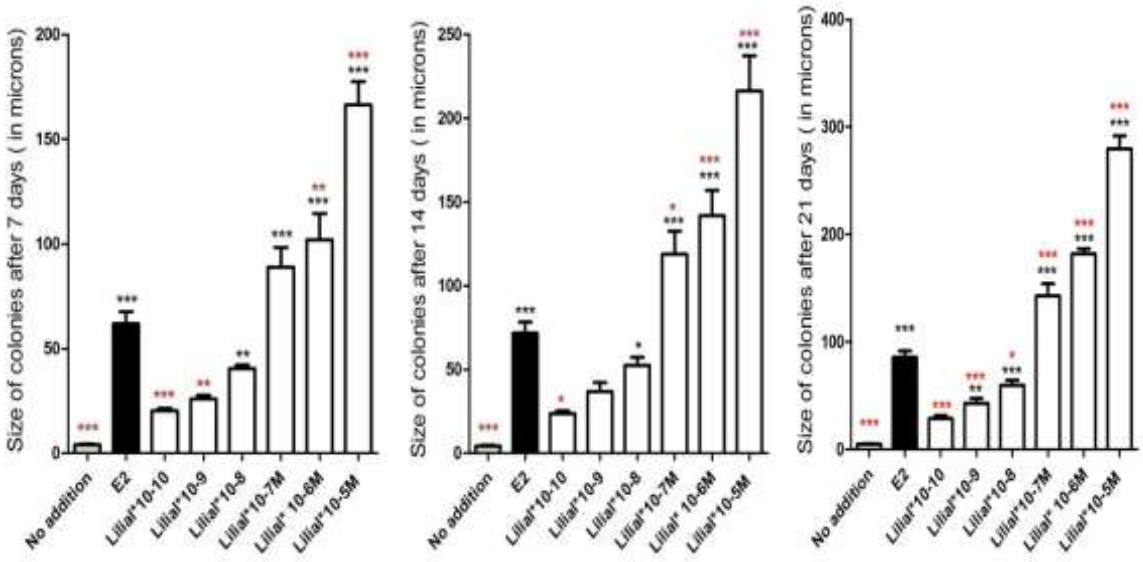


(A) No treatment (B) 17β- oestradiol (E2) (C) Lilial

FIGURE 3.25 Colonies of MCF-10A cells growing in semi-solid methocel suspension culture after 21 days exposure to Lilial

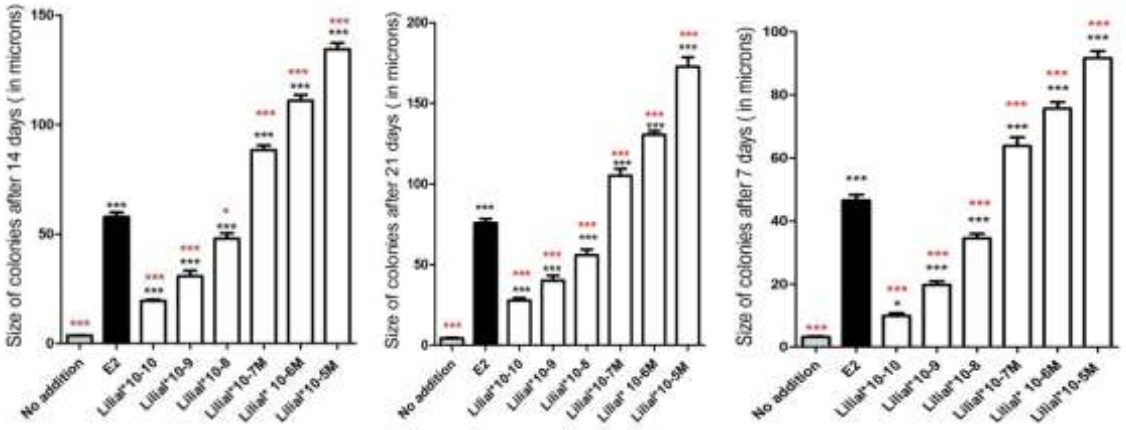
Cells were grown with: - (A) no treatment (B), 70 nM 17β- oestradiol (C), 10-5M butylphenylmethylpropional (Lilial). Phase contrast images.

A. MCF10A cells:



Day 7 Day 14 Day 21

B. MCF10F cells

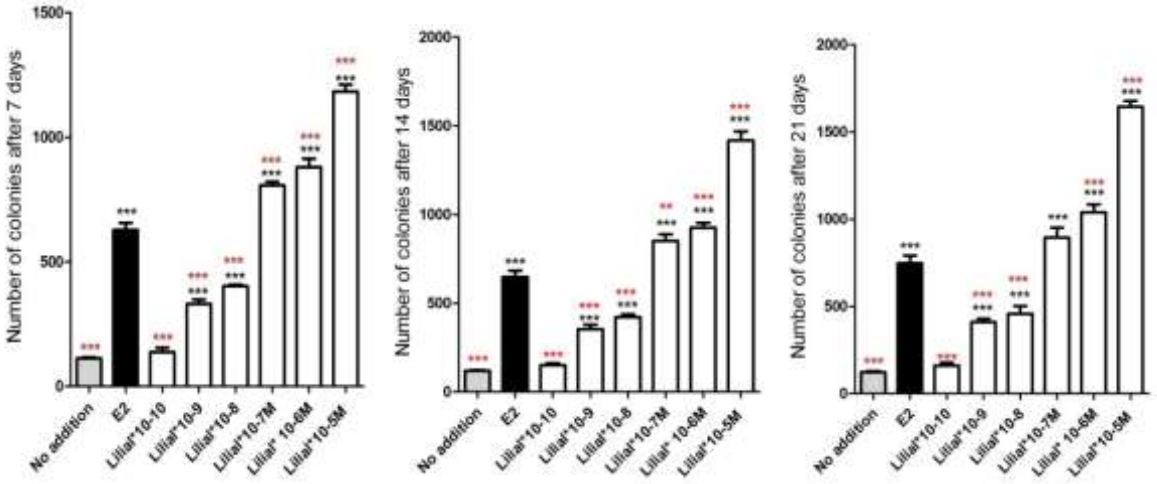


Day 7 Day 14 Day 21

FIGURE 3.26 Effect of concentrations from 10-10M to 10-5 M of Lilial on the size of colonies of MCF10A (A) and MCF10F (B) cells growing in methocel suspension culture from 7 days to 21 days.

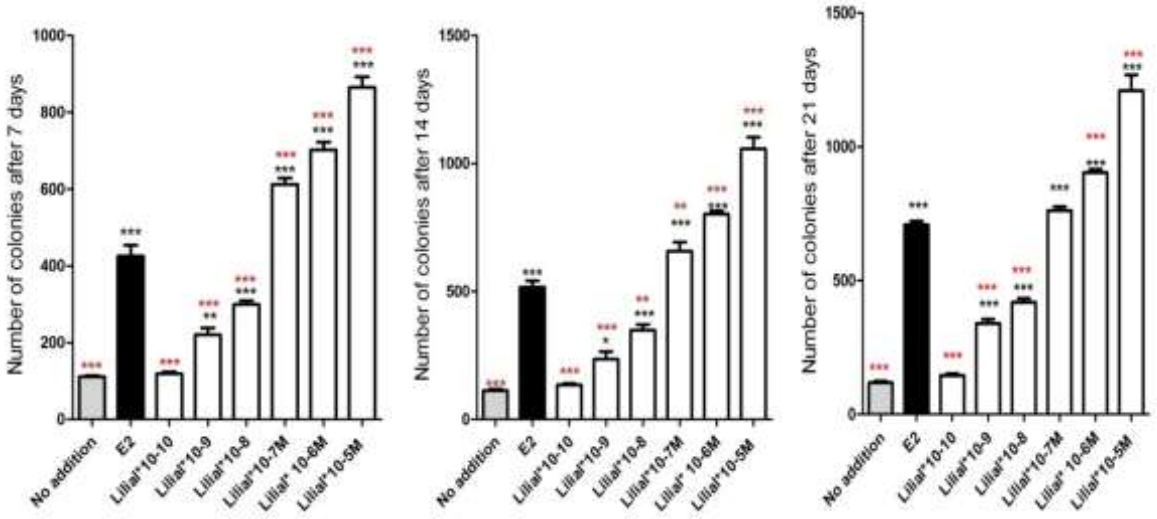
Cells were grown in stock medium with no addition, with70nM 17β-oestradiol, with10^{-5}M Lilial, with10^{-6}M Lilial, with10^{-7}M Lilial, with10^{-8}M Lilial, with10^{-9}M Lilial or with10^{-10}M Lilial. Average colony size was calculated from 15 fields per well of view and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. *** indicates p< 0.05, ** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)**

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells



Day 7 Day 14 Day 21

FIGURE 3.27 Effect of different concentrations from 10-10M to 10-5 M Lilial on number of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 to 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with10^{-5}M Lilial, with10^{-6}M Lilial, with10^{-7}M Lilial, with10^{-8}M Lilial, with10^{-9}M Lilial or with10^{-10}M Lilial. Colony growth is shown as the number of colonies per well from 7 days to 21 days, as determined under light microscope. Error bars are standard error of triplicate wells for each experiment. **** indicates p< 0.05, ** p< 0.01 and *** p <0.001compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

A. MCF10A cells B. MCF10F cells

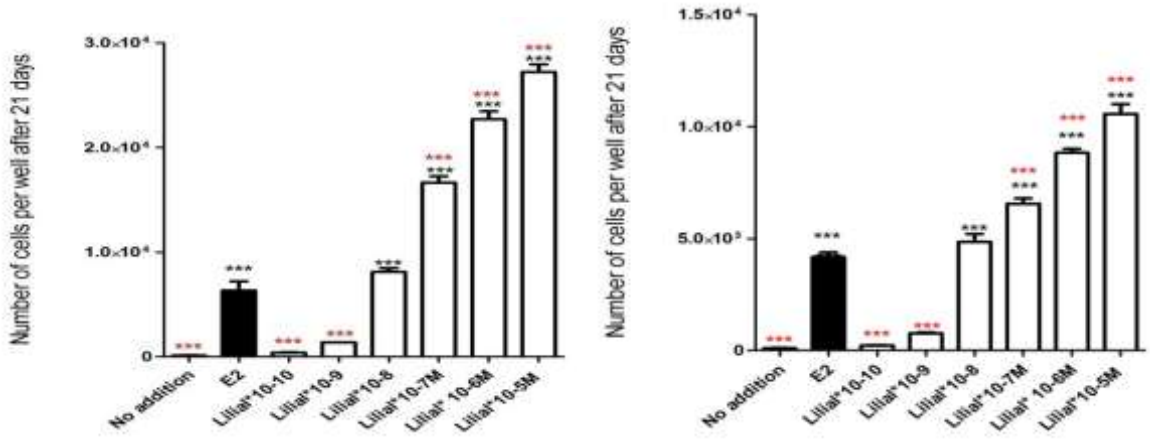


FIGURE 3.28 Effect of concentrations of Lilial on growth of MCF10A cells (A) and MCF 10F cells (B) in semi- solid methocel suspension culture after 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with10^{-5}M Lilial, with10^{-6}M Lilial, with10^{-7}M Lilial, with10^{-8}M Lilial, with10^{-9}M Lilial or with10^{-10}M Lilial. Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

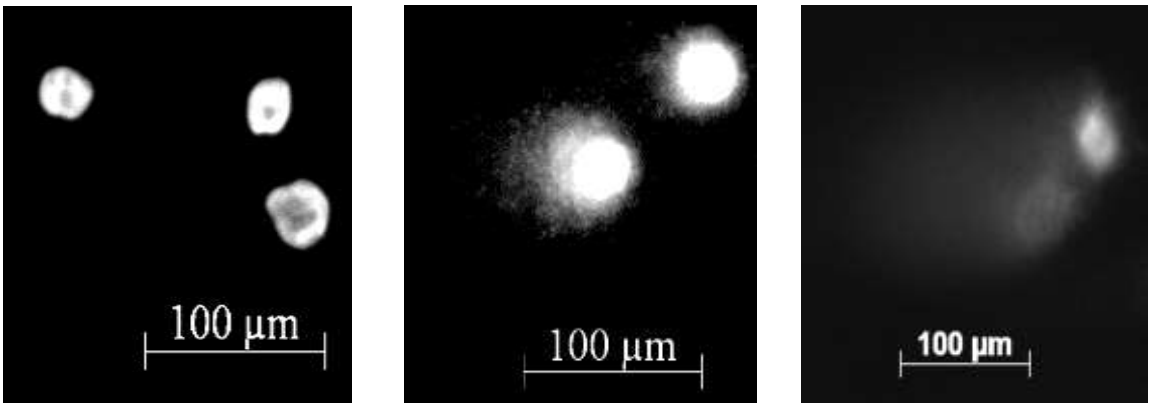
****** indicates p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

3.2.3 Comet assay of DNA damage after exposure to Lilial

An alkaline single-cell gel electrophoresis (SCGE) technique was employed to detect single strand and labile DNA adducts in the immortalised human breast epithelial cell lines MCF10A and MCF10F after exposure to Lilial at 10^{-5}M concentration.

Cells in the photomicrograph in ***Figure 3.29 panel C*** (Lilial) show strong classical comet formation in MCF10A cells. Some indications of comet formation are seen in panel B (DES).

The bar charts for the comet assays, shown in ***Figure 3.30*** show both comet numbers as percent of total count and tail length in MCF10A cells to correlate positively with concentration of agent. Lilial at 10^{-8} M did not produce comets. DES at 10^{-5}M yielded large numbers of poorly developed comet cells. MCF10F cells developed a high proportion of comets with long tails at 10^{-5} M Lilial. In both cell lines 10^{-5} M DES produced more comets and with longer tails than 10^{-5} M Lilial.

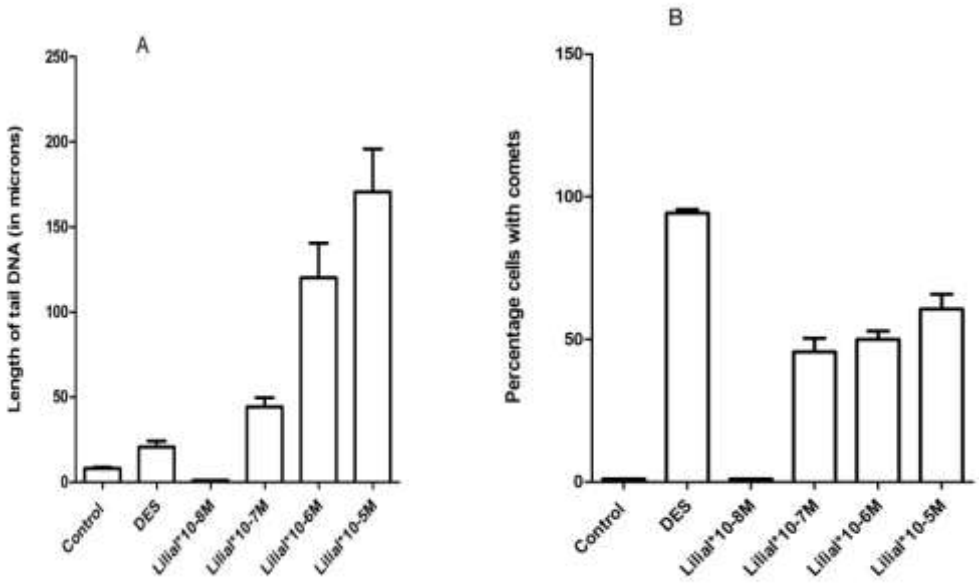


A: Untreated MCF10A cells B: Diethylstilboestrol (DES) C: Lilial

FIGURE 3.29 Comet assay for detection of DNA damage in MCF10A cells exposed to Lilial

Panel A is of untreated cells (control). B) Cells treated with 10-5 M Diethylstilboestrol (DES) (positive control). C) Cells treated with 10^{-5}M butylphenylmethylpropional (Lilial)*. All cells were treated for one hour. Cells viewed by fluorescence microscopy.*

MCF10A cells



MCF10F cells

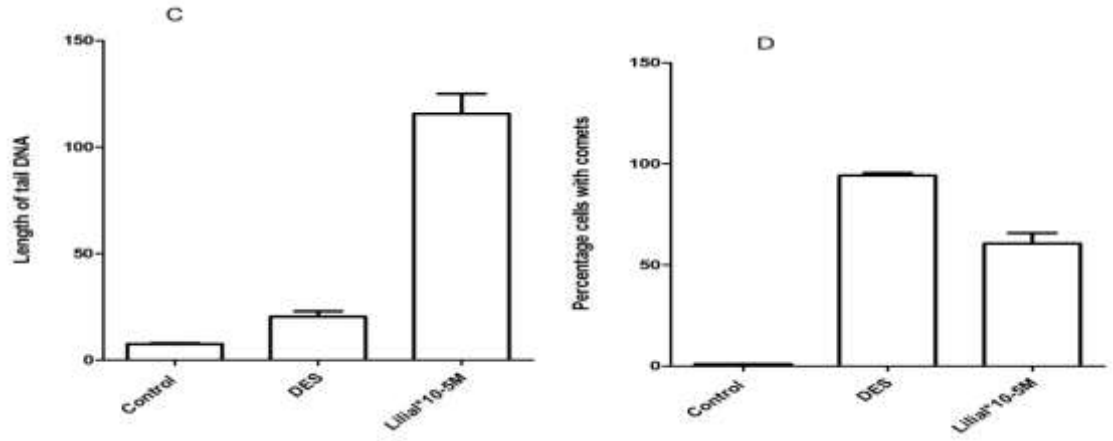


FIGURE 3.30 Effect of butylphenylmethylpropional (Lilial) on DNA damage in MCF10Aand MCF10Fcells as assessed by comet assay. Measuring DNA tail length (A, C) or % of cells with comets (B, D)

Cells were grown in stock medium and treated one hour with untreated cells (control). Cells were treated with DES (positive control) or Lilial at concentrations between 10^{-5} and 10^{-8}. Error bars represent standard deviation of 50 comets scored.

3.2.4 Lilial: Effect on mRNA expression of DNA repair genes:

Effect of Lilial were studied in the short term (1 week) and long term (30 weeks) using 10^{-5}M concentration because these were the highest concentration studied on suspect growth which did not cause toxicity over 21 days. This concentration has also been published as not detrimental to proliferation of MCF7 cells (Charles and Darbre, 2009a).

Levels of BRCA1 mRNA after exposure to Lilial

Short (1 week) and long term (30 weeks) effects on BRCA1 mRNA expression of exposing MCF10A (***Figure 3.31)*** and MCF10F (***Figure 3.32***) cells to Lilial in terms of levels of mRNA for BRCA1, was investigated using RT-PCR. Expression was halved after long term exposure to E2 and Lilial in MCF10A cells ($p<0.001$ in both cases). A drop at 1 week exposure was not statistically significant.

The long-term effect of exposure of MCF10F cultures to both E2 and Lilial showed a greater reduction in relative expression of BRCA1 mRNA ($p<0.001$ in both cases). Standard errors were much tighter than in the short exposure experiment.

MCF10A cells

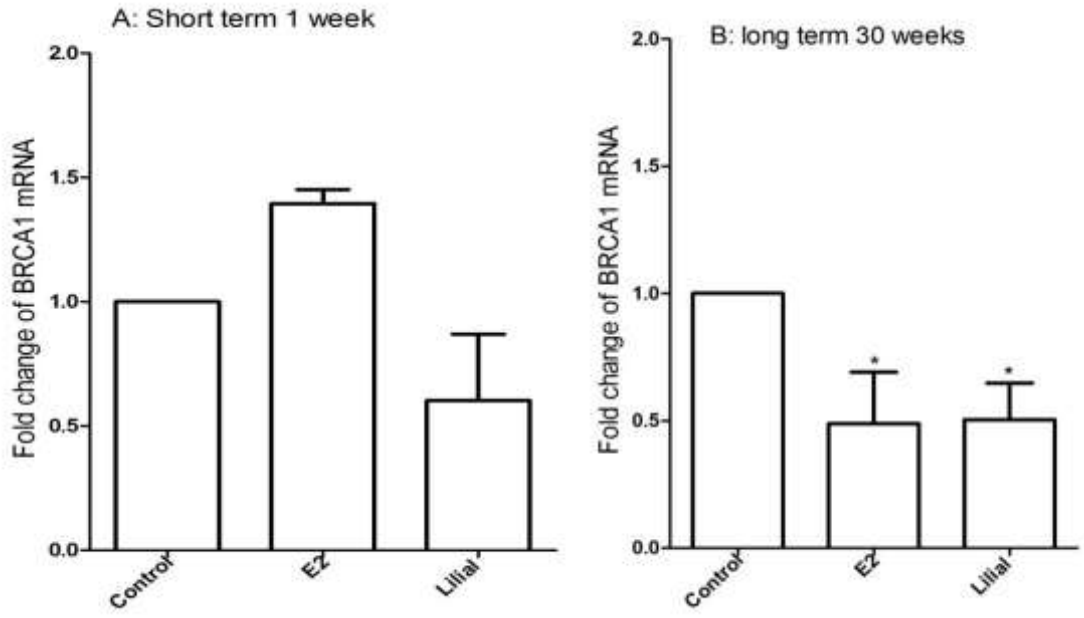


FIGURE 3.31 Real-time RT- PCR analysis of BRCA1 mRNA in MCF10A cells following short term (A) or long term (B) exposure to Lilial.

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of* 10^{-5}*M* Lilial*. The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

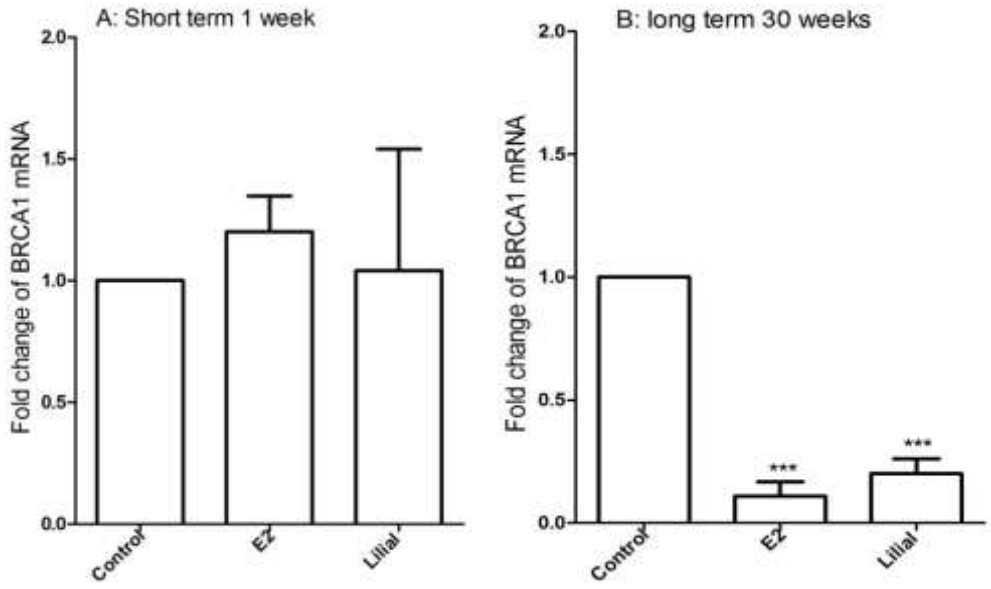


FIGURE 3.32 Real-time RT- PCR analysis of BRCA1 mRNA in MCF10F cells following short term (A) or long term (B) exposure to butylphenylmethylpropional (Lilial).

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2)*or in the presence of* 10^{-5}*M* Lilial*. The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05,*** p <0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

Levels of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111 mRNA on after exposure to Lilial

In MCF10A cells, short (1 week) and long term (30 weeks) effects of exposure to Lilial are shown in ***Figure 3.33.*** The short-term effect of exposure to Lilial showed a significant elevation in expression of CHK1, PALB2 and PARP1 mRNA in MCF10Acells.The long-term effect of exposure showed a significant elevation of p53 and Rad51 mRNA and a significant reduction in expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, PALB2, PARP1 and RAD50 mRNA.

The effects of exposure of MCF10F cells to Lilial are shown in ***Figure 3.34*****.** MCF10F cells were more affected by short-term exposure to Lilial. One week incubation resulted in a significant reduction in expression of ATM, BRIP1, PALB2 and STK111 mRNA in MCF10F cells (all p values <0.01). However, the long-term effect of exposure showed a significant reduction in expression of BRIP1, CHK2, p53, PALB2, PARP1, PTEN and Rad51 mRNA. The net result was that over time ATM expression lost its significant reduction, while CHK2, PARP1 and RAD51 expression became reduced contrasting with the results at one week.

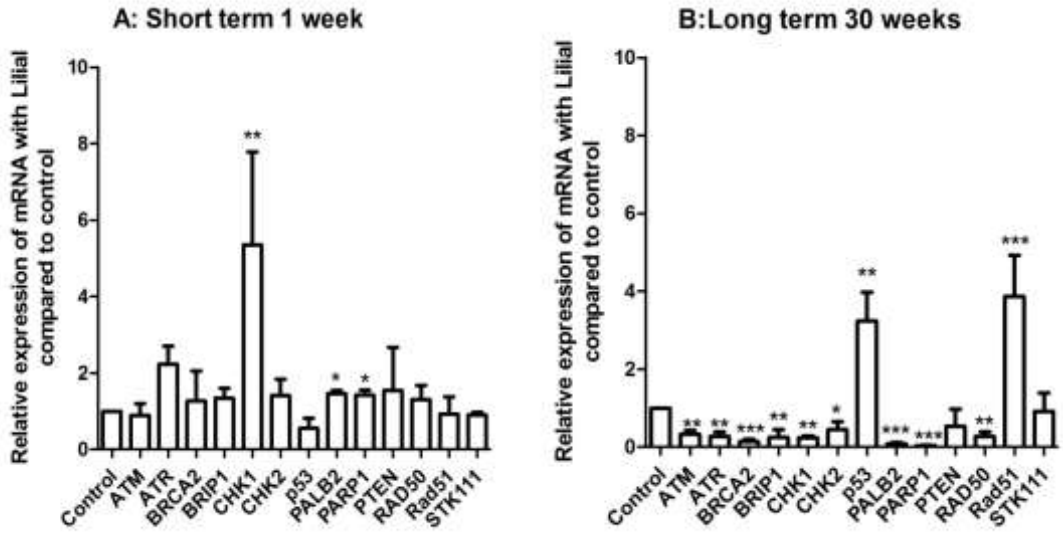


FIGURE 3.33 Real-time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells after short (A) or long term (B) exposure to Lilial

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of $10^{-5}M$ Lilial. *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

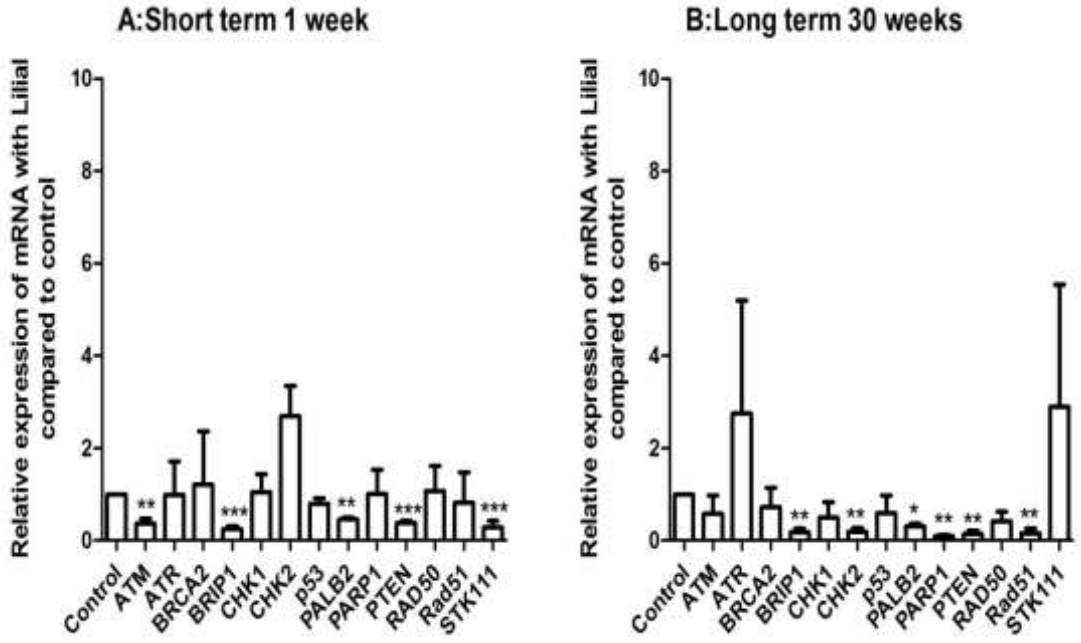


FIGURE 3.34 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells following short term (A) or long term (B) exposure to Lilial

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-5}*M* Lilial. *The relative expression of* ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

3.2.5 Level of BRCA1 protein after exposure to Lilial

Long term exposure of **MCF10A** cells to Lilial and E2 showed expression of BRCA1 protein using western Immunoblotting to be significantly reduced (***Figure 3.35)*** ($p<0.001$). The reduction with Lilial was greater than with E2

MCF10F cells showed, on long term exposure to Lilial but not E2, a significant reduction in expression of BRCA1 protein by Western blotting, both qualitatively (A) and quantitatively, (B) in ***Figure 3.36***

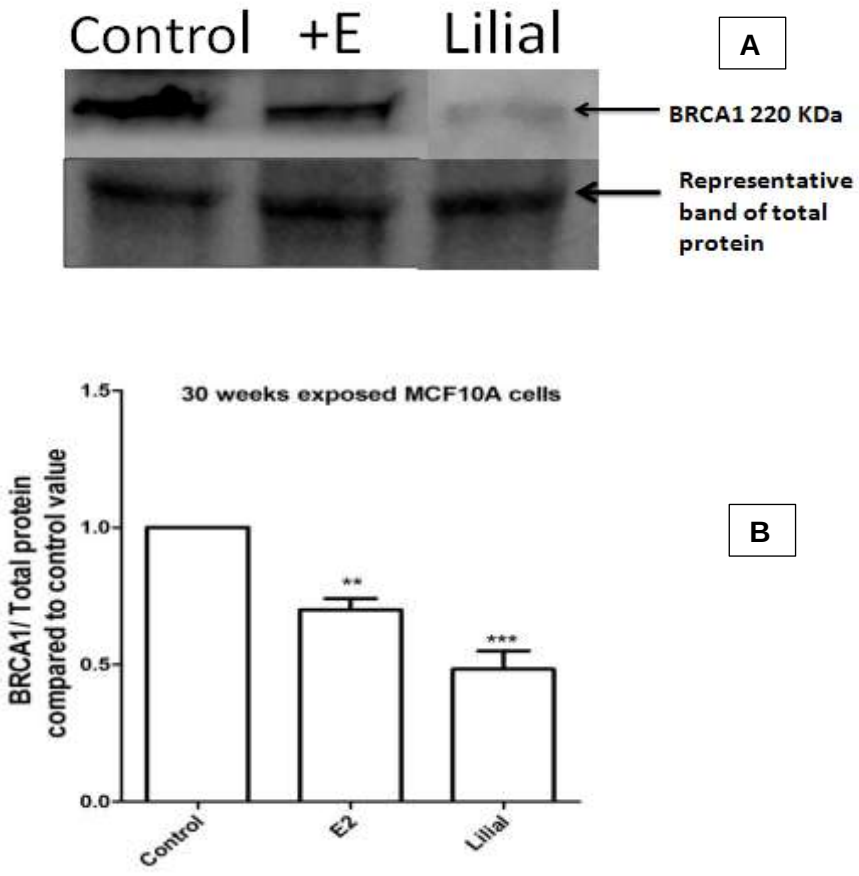


FIGURE 3.35 Level of BRCA1 protein in butylphenylmethylpropional (Lilial) treated MCF10A cells using western Immunoblotting. A- Photograph of excised bands, B, quantitative plot.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10A human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M butylphenylmethylpropional (Lilial) compared to 10^{-8}M oestradiol (E2). **** indicates p< 0.05, ** p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test.***

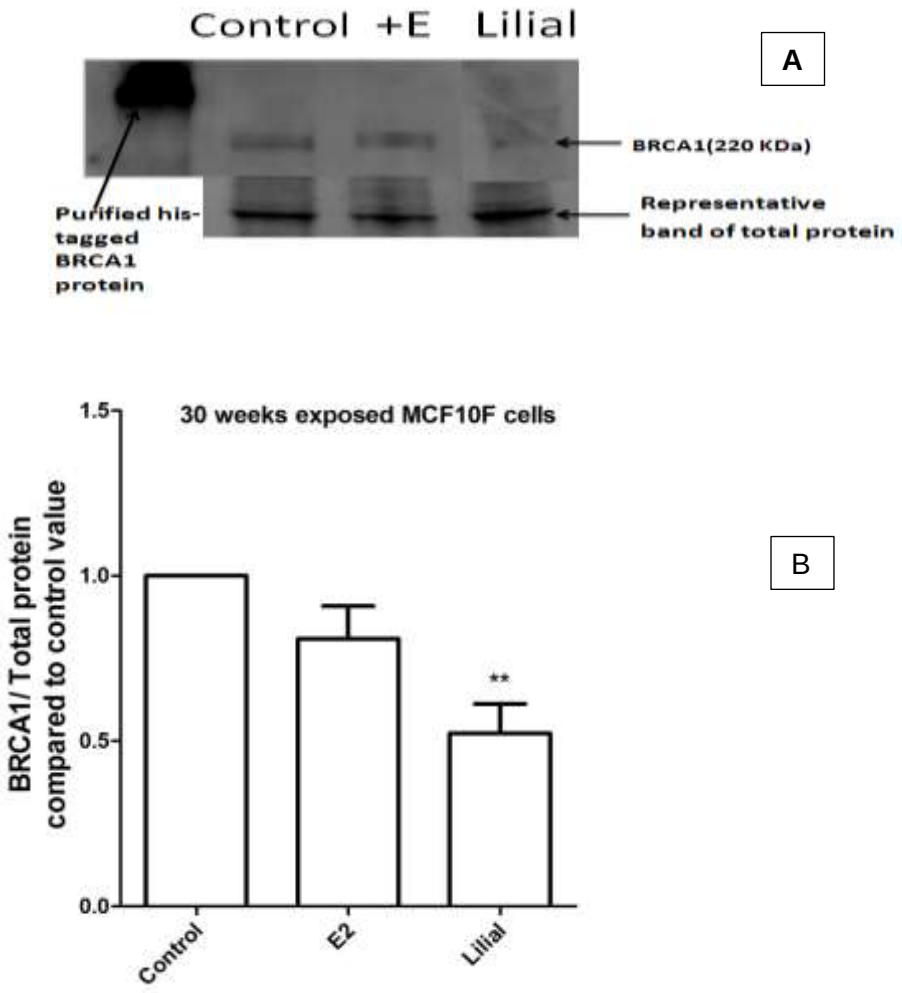


FIGURE 3.36 Level of BRCA1 protein in butylphenylmethylpropional (Lilial) treated MCF10F cells using western Immunoblotting. Upper - photograph of excised bands, lower, quantitative plot.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10F human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M Lilial compared to 10^{-8}M oestradiol (E2). ***** indicates p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test. N.B. Western blot data showed that the his-tagged BRCA1 band had a higher molecular weight in comparison to BRCA1 bands of breast cell lysates, which is likely due to extra molecular weight of his-tag molecules that altered the protein mobility on the gel. His-tag modification increases effective size by 30 KDa. (Zhao et al., 2010)***

3.2.6 Summary & discussion of Lilial results

The results with Lilial are tabulated below (***Table 3-2***). In this tabulation there is a continuous correlation of agent concentration with `anchorage independent growth across all concentrations. The comet assays show actual DNA damage and expression of DNA repair related mRNA and protein reflect a reduction in homoeostatic capacity.

TABLE 3-2 Summary of Lilial results

ASSAY	MCF10A	MCF10F
Colony growth in methocel: number or size of colonies	Positive correlation with max $^{10-5}$ M concentration of Lilial	Positive correlation with max $^{10-5}$ M concentration of Lilial
Colony growth in methocel: total cell count	Mirrors colony growth	Mirrors colony growth
Comet assay	Lilial induces particularly classical comet formation. Tail length increased vs DES, numbers of comets less than DES but showing positive dose response	Similar to MCF10A, except responses not as strong and only a single concentration tested
BRCA1 mRNA & protein	Both Lilial and E2 caused a reduction in protein expression. mRNA expression was reduced after 30 weeks exposure.	Lilial, but not E2 caused a reduction of protein mRNA expression was reduced after 30 weeks exposure
Other DNA repair mRNAs	One week exposure - increased expression in CHK1, PALB2 & PARP. 30 weeks – all expression reduced except P53, RAD51 increased and STK111 unchanged	At one week – reduced ATM, BRIP1, PALB2 PTEN & STK111 expression. At 30 weeks BRIP1, PALB2 PTEN & CHK2 were reduced

3.2.7 Discussion of results of exposure of MCF10 cells to Lilial

The results presented here demonstrate that exposure of non-transformed human breast epithelial cells to Lilial can enable anchorage-independent growth in methocel culture, can damage DNA in comet assays and can alter, generally reducing levels over time, mRNAs encoding DNA repair proteins. Most notably among these changes levels of both BRCA1 mRNA and BRCA1 protein were reduced after long-term exposure to Lilial. Differences were noted in the dose-responses to Lilial and effects in the short-term (1 week) were not equivalent to those after longer exposure times (30 weeks). An overall summary of the results is given in **Table 3-2**.

3.2.7.1 Anchorage-independent colony formation

The results presented above demonstrate that exposure of non-transformed human breast epithelial cells to Lilial can enable anchorage-independent growth in methocel culture. The colony forming response of both MCF10A and MCF10F cells showed a clear positive association, with dose response across the range used, measuring average colony size. The association neither disappeared at lower concentrations nor tended to plateau at high concentrations. The plots for colony numbers mirror those for colony size. There was no peak or plateau effect. The effect noted for the size and numbers of colonies was comparable – i.e. an approximately tenfold increase in measurement over the five orders of magnitude in dose applied the when assessed as total individual cell numbers at 7, 14 and 21 of days culture.

As with the all the agents studied here, the colony formation assay may rely on actions other than genotoxicity. Usta (2013) detected the fragrance chemicals lyral and Lilial decreasing viability of HaCat cells by increasing free radical production and lowering antioxidant protection. These HaCat cells are transformed but the effects were not seen in MCF-7 cells which are also tumour-derived. They postulated lyral and Lilial as being toxic to mitochondria, disrupting the electron transport chain, increasing ROS production, thus affecting mitochondrial membrane potentials, decreasing ATP concentrations, culminating in cell death.

3.2.7.2 DNA damage as measured by comet assay

The comet assays were conducted in alkaline conditions which detects single strand as well as double strand DNA breaks. The comet tails were markedly bigger and of better conformation in the Lilial treated cells than in those exposed to diethylstilboestrol, but a smaller percentage of cells were affected. There is no clearly demonstrable cause for this,

but it could be could be interpreted as evidence for more extensive toxicity of Lilial, leading to disintegration of some cells making them unavailable for comet formation.

Previous work by Di Sotto et al (2014) showed no genotoxicity at chromosomal level measured by DNA fragmentation (comet assay) or abnormal chromosome count or conformation (aneuploidy) using cancer-derived cells. Such cells are abnormal to start with, compared to the near-normal cells used with this study. These results therefore underline the importance of using non-cancerous breast cells to assess the potential for compounds to damage DNA.

3.2.7.3 BRCA1 mRNA and protein

Assessing the molecular results, BRCA-1 mRNA detected was quantitatively reduced after long-term exposure of MCF-10A cells to Lilial. The MCF10F cell line gave unchanged m-RNA levels at one week irrespective of treatment but again exposure to Lilial or E2 reduced expression in the long term. Viewing the gene as a promoter of DNA repair, it would be intuitive to see short-term up-regulation but once repair mechanisms were overcome, it would be reasonable for production to slow. Reduced protein expression for BRCA1 after exposure to Lilial is consistent with reduced m-RNA levels at the same time period.

BRCA1 mRNA and protein rose in the short term on treatment of MCF10A or MCF10F. Long-term exposure to Lilial or E2, however, resulted in mRNA level reduced to below blank control levels. This is suggestive of an initial burst of repair activity which receded on being overwhelmed by the continued burden of damage. This study is the first to examine such changes in non- transformed cells and to do so over the longer term.

3.2.7.4 DNA repair related mRNAs

Such a trend was also seen with the panel of repair-related genes in MCF10A cells, with CHK1 m-RNA being particularly elevated at one week exposure. The long-term trend was for expression to be below that of controls, except for P53 and Rad-51. MCF10F cells gave few statistically significant differences in m-RNA, but short-term increases with relatively high variability rendering the results below the p=0.05 significance threshold.

As with the BRCA genes, previous studies are largely confined to the work of Di Sotto (2014). Single strand breaks were observed and a lack of mutagenicity was observed for Lilial in all of the Ames tests using different bacterial strains, albeit in neoplastic MCF-7 cells. His group further showed no effects in MCF7 cells and that, in the presence of exogenous metabolic activators, no genotoxic derivatives were produced by CYP450-mediated biotransformations such as might occur in the liver.

3.2.7.5 Socio-political and environmental studies relating to Lilial

Lilial can occur in personal care products at concentrations up to 2.5%, approximately equivalent to 0.1M, given a relative density for the product close to unity. Sgorbini et al (2010) have estimated by thermal desorption gas chromatography-mass spectrometry following contact sorptive tape extraction that skin absorption is high, at 52.3%, from a standard cream formulation. The highest concentration used in this study of 10^{-5}M, diluting to 10^{-10}M cannot be considered supraphysiological.

A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the human biomonitoring of non-occupational exposure to the fragrance is reported by Pluym 2016). This was undertaken within the framework of the Cooperation Project of the German Federal Ministry for the Environment, Nature Conservation, Building and Nuclear Safety (BMUB) and the German Chemical Industry Association (VCI). It is indicative of the early-stage nature of research in this area that authorities in developed countries are still working on development of acceptable methodologies and that there still exist no measurements of the extent to which Lilial is entering human breast tissue.

The overall message to be taken from the present study is that Lilial, at these concentrations produces effects on two closely related breast epithelial cell lines that are consistent with its being an initiator or promotor of oncogenesis. Over the range of Lilial concentrations studied here, the colony forming response of both MCF10A and MCF10F showed a clear positive dose response when measured either by colony numbers or size irrespective of the length of incubation; this effect was reproduced when measured as total individual cell numbers at 21 days culture. None of these plots indicated that the effect had plateaued at 10^{-5}M.

There is little in the literature covering comparable ground; the only recent work on Lilial & cancer is that of Di Sotto (2014) in the breast cancer cell line MCF-7. Differences in using genotoxicity (Ames) assays for mutagenicity and a micronucleus assay for clastogenicity are likely to account for the differences in their results from those presented here. Bacteria present convenient high-throughput models for assessing genotoxicity and breast carcinoma cells, especially those derived from metastatic deposits, will differ from normal epithelial cells. The present results using near-normal cell lines demonstrate that effects can sometimes be observed in human epithelial cells where they are not evident in bacteria.

There is insufficient research to link definitively effects seen in laboratory studies such as this with clinical evidence of harm. There is, however, enough concern over the potentially carcinogenic, mutagenic or reprotoxic (CMR2) (https://echa.europa.eu/documents/10162/13562/cmr_report_en.pd) status of Lilial that

chemists such as Schroeder (2014) record efforts to find replacement substances (such as 5,7,7-Trimethyl-4-methyleneoctanal) and attempt to define the structural requirements in compounds for a 'Lilial' odour. The results reported in this thesis should add to this concern and future risk assessment.

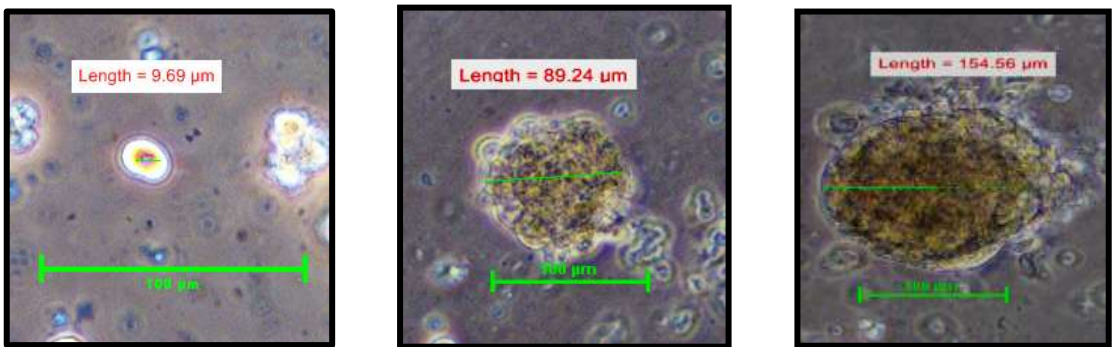
3.3 Triclosan

Triclosan is used as a synthetic broad-spectrum antimicrobial agent. It is extensively used in pharmaceutical and personal care products and household cleaners, also in commercial products including textiles and plastics (Liu et al., 2002). Studies from the Reading laboratories have established that triclosan can exert oestrogenic and androgenic effects on breast cancer cells in vitro (Gee et al., 2008a, Charles and Darbre, 2009a) Triclosan is among a group of Endocrine Disrupting Compounds that are taken seriously as a risk, with both biochemical (Taboada-Puig et al., 2015) and ultraviolet radiation based techniques (Carlson et al., 2015) piloted for its industrial scale removal from waste waters.

For female physiology, a rat model used by Manservisi (Manservisi et al., 2015) yielded profound mammary changes, assessed by gene expression, on triclosan treated rats, which subsequently impacted on survival of the young exposed by drinking the milk. Clinically, negative associations have been found between urinary triclosan and thyroid hormonal parameters in obese patients (Geens et al., 2015). In men, high internal body concentrations of endocrine disrupting chemicals, including triclosan, suffer an increased risk of infertility along with disturbed hormone balance (Den Hond et al., 2015)

3.3.1 Suspension growth of MCF10A and MCF10F cells and colonies exposed to triclosan: morphology.

MCF10A and MCF10F cells were grown in semi-solid suspension culture with varying concentrations of triclosan and 10-8M 17β- oestradiol for up to 21 days. Photomicrographs of colonies after21 days are shown in Figure 3.37 in the absence of treatment, only small colonies were seen (Figure 3.37A). Large colonies were evident are growth in the presence of 10-8M E2 or 10-7M triclosan, shown in Figure 3.37 panels B and C respectively

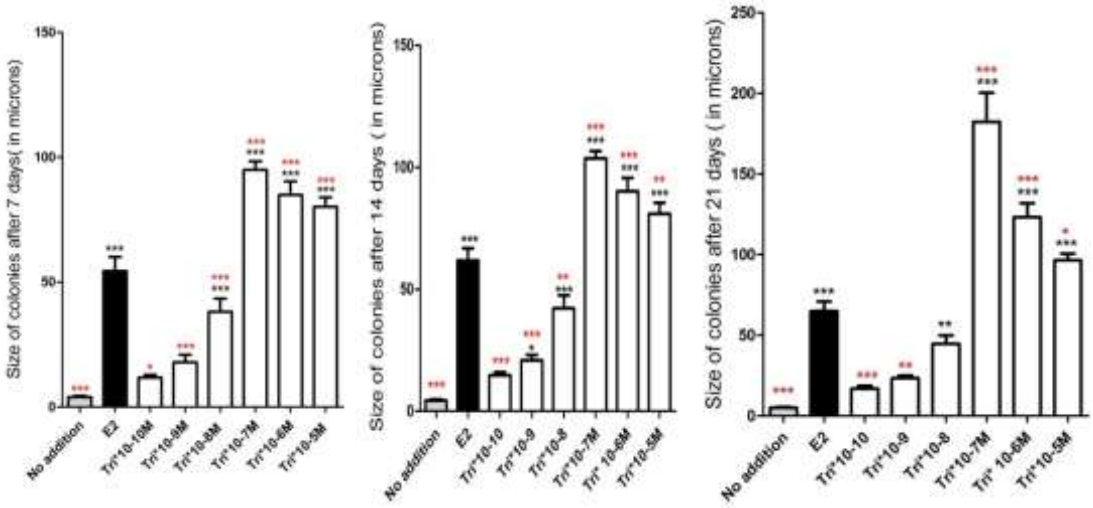


(A) No treatment (B) 17β- oestradiol (E2) (C) Triclosan

FIGURE 3.37 Colonies of MCF10A human breast epithelial cells growing in semi-solid methocel suspension culture after 21 days exposure to triclosan.

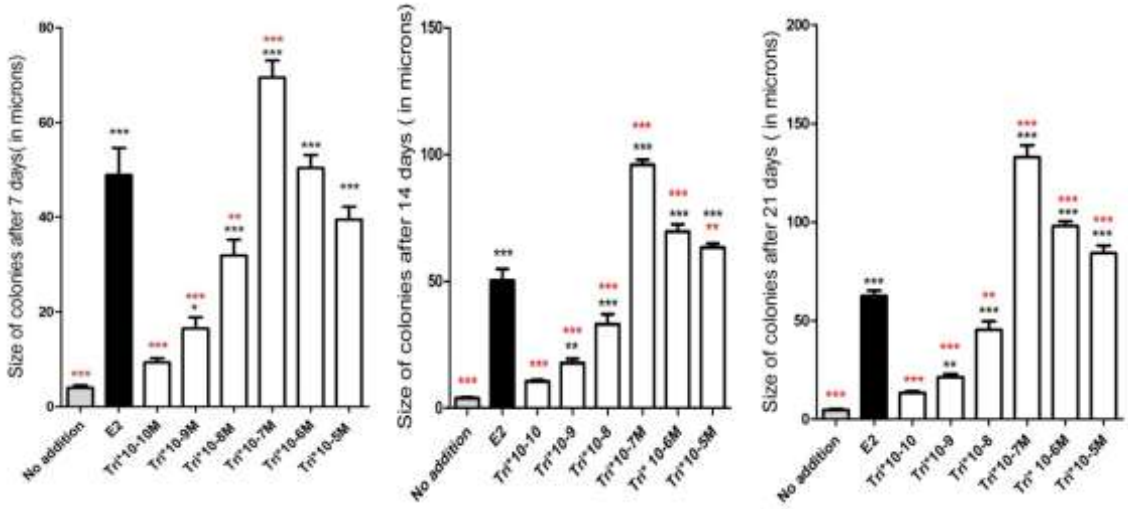
Cells were grown with: - (A) no treatment (B), 70 nM 17β- oestradiol (C), 10^{-7}M triclosan*. Phase contrast images.*

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells

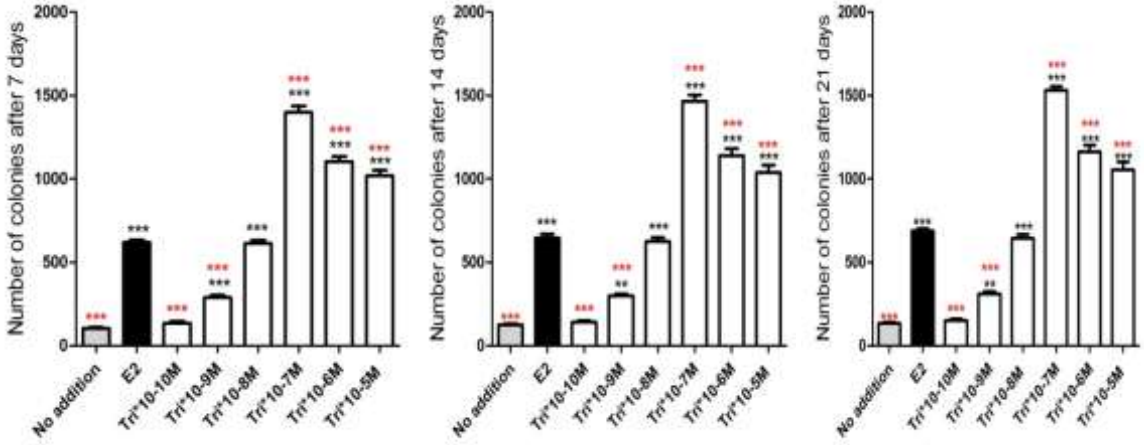


Day 7 Day 14 Day 21

FIGURE 3.38 Effect of different concentrations of triclosan on the size of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 days to 21 days.

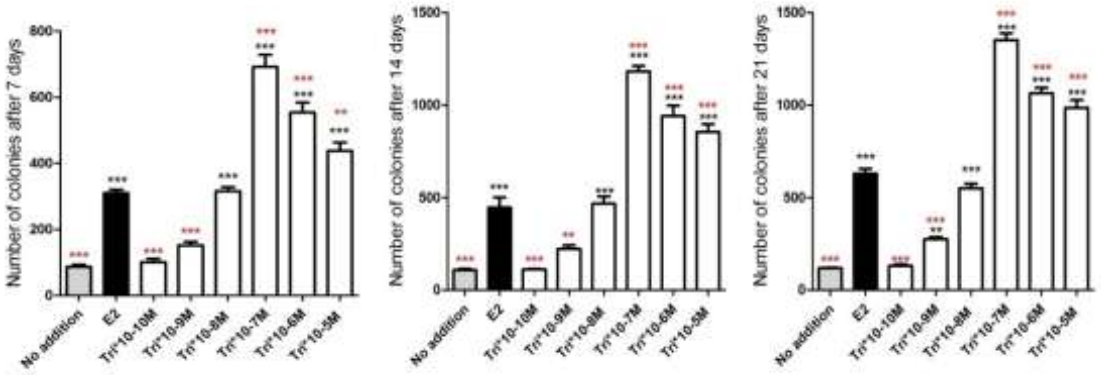
Cells were grown in stock medium with no addition, with70nM 17β-oestradiol, with10^{-5}M triclosan, with10^{-6}M triclosan, with10^{-7}M triclosan , with10^{-8}M triclosan, with 10^{-9}M triclosan or with10^{-10}M triclosan. Average colony size was calculated from 15 fields per well of view and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. *** indicates p< 0.05** p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).**

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells



Day 7 Day 14 Day 21

FIGURE 3.39 Effect of concentrations of triclosan on number of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 days to 21 days.

Cells were grown in stock medium with no addition, with10^{-5}M triclosan, with10^{-6}M triclosan, with10^{-7}M triclosan, with10^{-8}M triclosan, with 10^{-9}M triclosan or with10^{-10}M triclosan. Colony growth is shown as the number of colonies per well from 7 days to 21 days, as determined under light microscope. Error bars are standard error of triplicate wells for each experiment.

***** indicates p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

A. MCF10A cells

B. MCF10F cells

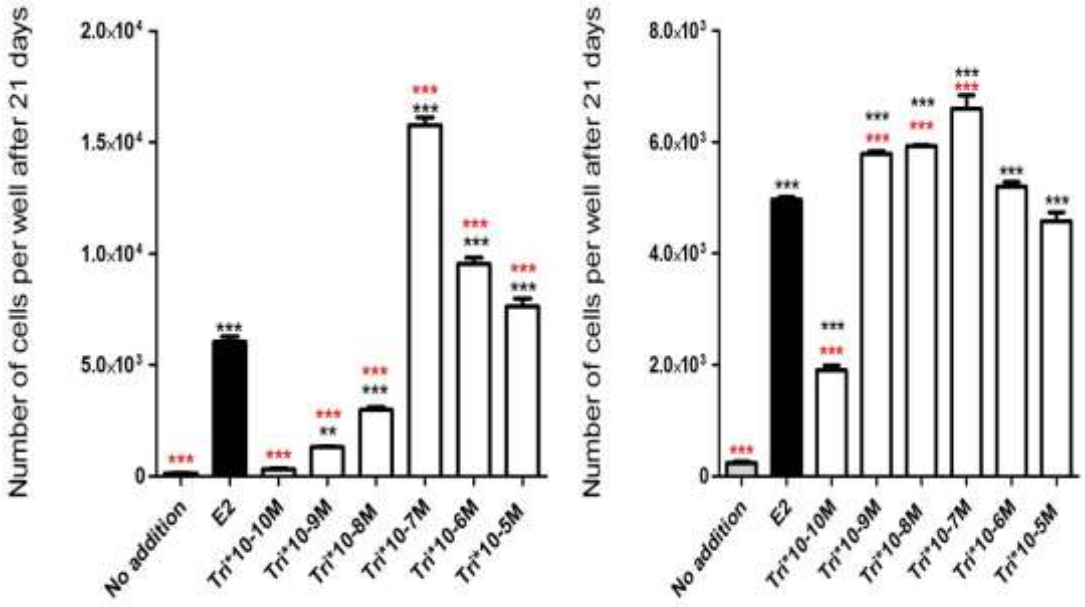


FIGURE 3.40 Effect of concentrations of triclosan on total numbers of MCF10A (A) and MCF10F (B) cells in semi- solid methocel suspension culture after 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with10^{-5}M triclosan, with10^{-6}M triclosan, with10^{-7}M triclosan , with10^{-8}M triclosan, with 10^{-9}M triclosan or with10^{-10}M triclosan. Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

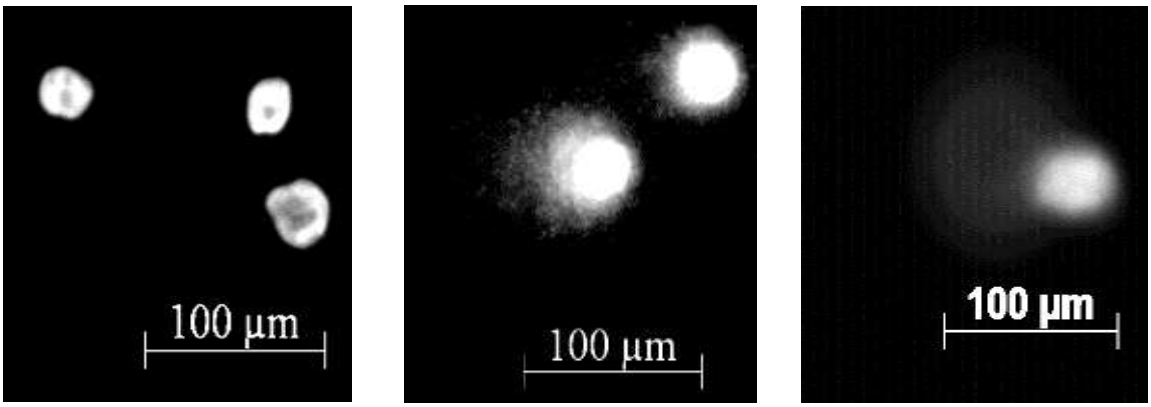
**** indicates p< 0.01 and ***p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)**

3.3.2 Comet assay of DNA damage after exposure to triclosan

An alkaline single-cell gel electrophoresis (SCGE) technique was employed to detect single strand and double strand breaks in DNA and alkaline labile DNA adducts in the immortalised human breast epithelial cell lines MCF10A and MCF10F after exposure to triclosan.

Cells in the photomicrograph in ***Figure 3.41*** panel D (triclosan) show comet formation with long tail. Some indications of comet formation are seen in panel B.

The bar charts for MCF10A (***Figure 3.42***) show comet tail length peaking at 10^{-7}M ($p<0.001$ vs control) while the numbers of comets as % of total cells decreases with increasing concentrations of triclosan after 10^{-8}M. The lowest concentration (10^{-9}M) was completely ineffective, giving comet parameters at control levels. MCF10F cells show increased tail length using triclosan compared to DES, but a smaller proportion of cells are classified as comet-forming.

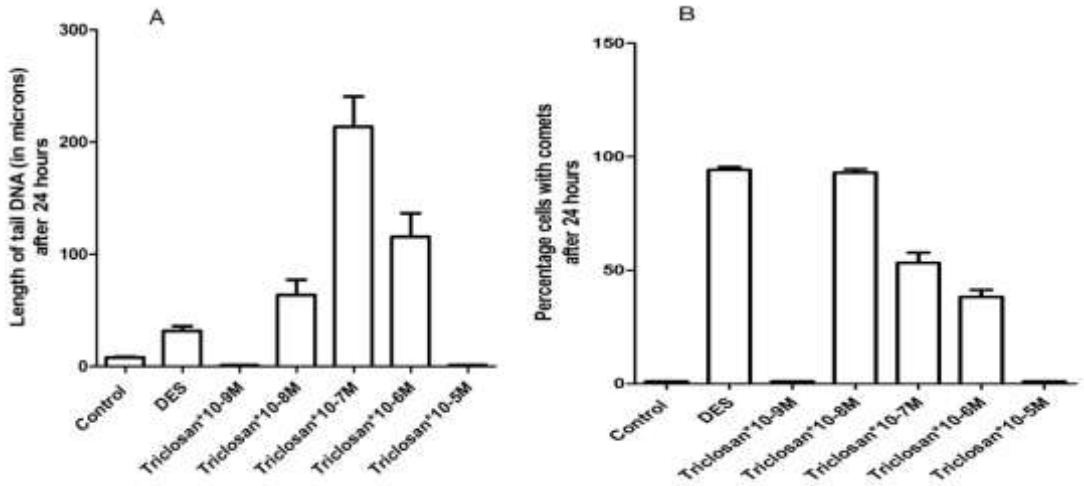


A: Untreated MCF10A cells B:Diethylstilboestrol (DES) C: Triclosan treated 24 hours

FIGURE 3.41 Comet assay for detection of DNA damage in MCF10A cells exposed to triclosan.

Panel A is of untreated cells (control) treated for one hour. B) Cells treated with 10^{-5} M Diethylstilboestrol (DES) (positive control) treated for one hour. C) Cells treated with 10^{-7}M triclosan *treated for 24 hours. Cells viewed by fluorescence microscopy.*

MCF10A cells



MCF10F cells

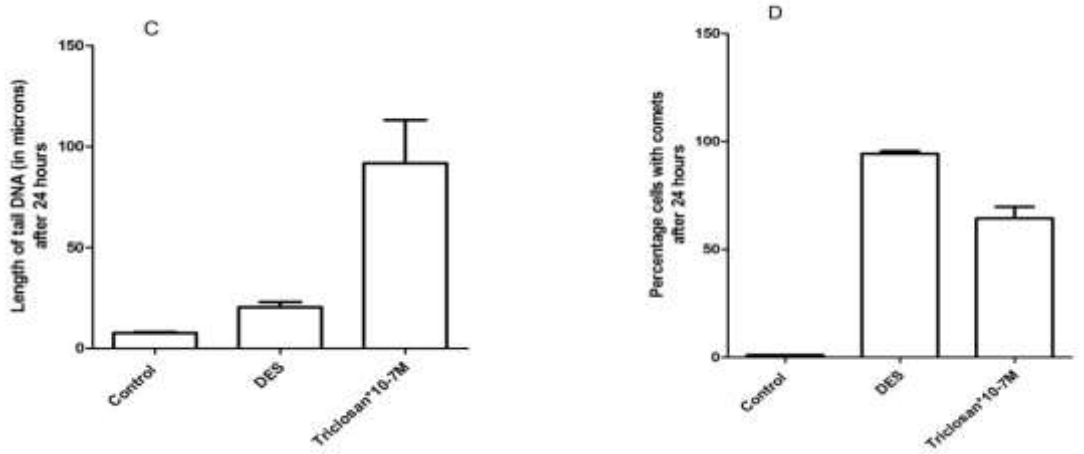


FIGURE 3.42 Effect of triclosan on DNA damage in MCF10A and MCF10F human breast epithelial cells as assessed by a comet assay. Measuring DNA tail length (A, C) or % of cells with comets (B, D)

Cells were grown in stock medium and treated one hour with untreated cells (control).Plots A and C illustrate tail length, plots B and D, comet numbers as percent of cells counted. Cells were treated with 10^{-5} M diethylstilboestrol (DES), (positive control).Treated cells with 10^{-5}M triclosan*. Treated cells with 10^{-6}M triclosan. Treated cells 10^{-7}M* triclosan*. Treated cells 10^{-8}M triclosan*. *Treated cells 10^{-9}M* triclosan*. Error bars represent standard deviation of 50 comets scored.*

3.3.3 Triclosan: mRNA expression of DNA repair genes

Following proliferation studies (data not shown), concentration of 10^{-6}M and above were found to be toxic to MCF10A and MCF10F cells. Therefore, the long term studies used 10^{-7}M triclosan and not 10^{-5}M as for the previous chemicals

Levels of BRCA1 mRNA after exposure to triclosan

Short (1 week) and long term (30 weeks) effects on BRCA1 mRNA expression of exposing MCF-10A ***(Figure 3.43***) and MCF10F ***(Figure3.44***) cells to 10^{-7}M triclosan in terms of levels of mRNA for BRCA1, was investigated using RT-PCR. Significant reduction was found after long term triclosan exposure in MCF10A cells. A similar trend with E2 was not statistically significant. In MCF10F cells the long-term effect of exposure to E2, and triclosan was a significant reduction in expression of BRCA1 mRNA in both cases.

MCF10A cells

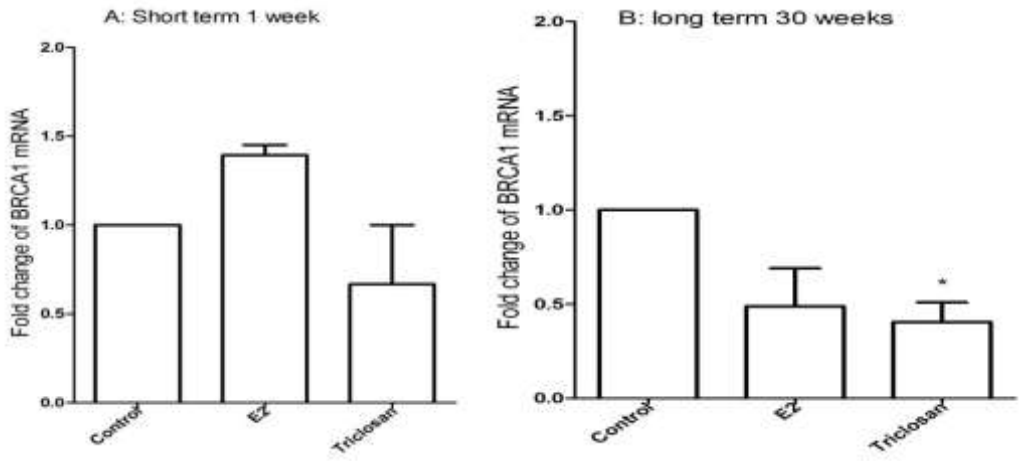


FIGURE 3.43 Real -time RT- PCR analysis of BRCA1 mRNA in MCF10A cells following short term (A) or long term (B) exposure to triclosan.

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of 10^{-7}M* triclosan*. The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

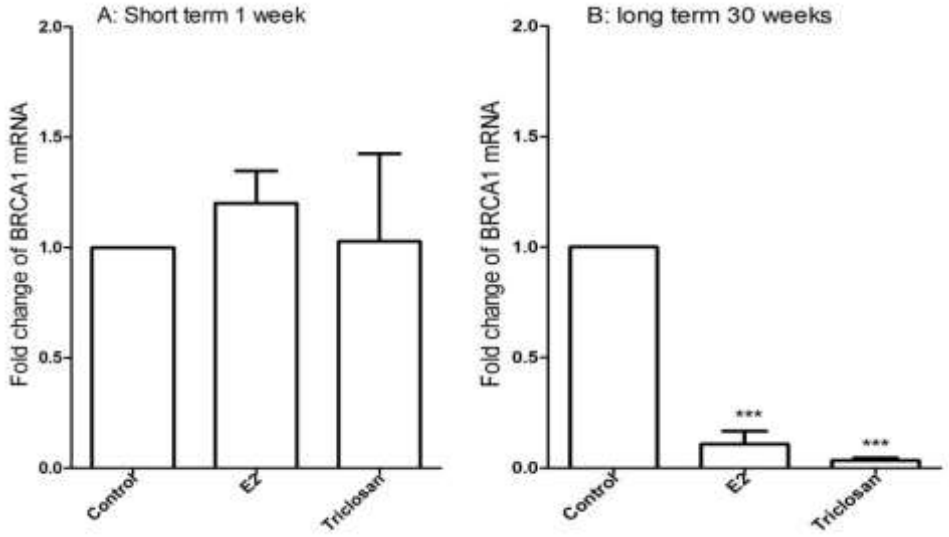


FIGURE 3.44 Real -time RT- PCR analysis of BRCA1 mRNA in MCF10F cells following short term (A) or long term (B) exposure to triclosan.

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of* 10^{-7}*M* triclosan*. The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

****** indicates p< 0.001, compared to control by one way ANOVA with post-hoc Dunnett test.***

Levels of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, RAD50, Rad51 and STK111 mRNA after exposure to triclosan

In MCF10A cells short (1 week) and long term (30 weeks) effects of exposure to triclosan are shown in ***Figure 3.45.*** The short-term effect of exposure to $^{10\text{-}7}$M triclosan showed a significant reduction in expression of p53 mRNA in MCF10A cells. The long-term effect of exposure to $^{10\text{-}7}$M triclosan showed a significant reduction in expression of ATM, ATR, BRCA2, BRIP1, CHK1, PALB2, PARP1, PTEN, RAD50 and STK111 mRNA in MCF10A cells

Effect of exposure of MCF10F cells are shown in ***Figure 3.46***. The short-term effect of exposure to 10^{-7}M triclosan showed a significant reduction in expression of ATM and BRIP1 mRNA in MCF10F cells. However, long-term exposure to $^{10\text{-}7}$M triclosan there was a significant reduction in expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, and p53, PALB2, PARP1, PTEN, Rad50 and Rad51 mRNA in MCF10F cells.

MCF10A cells

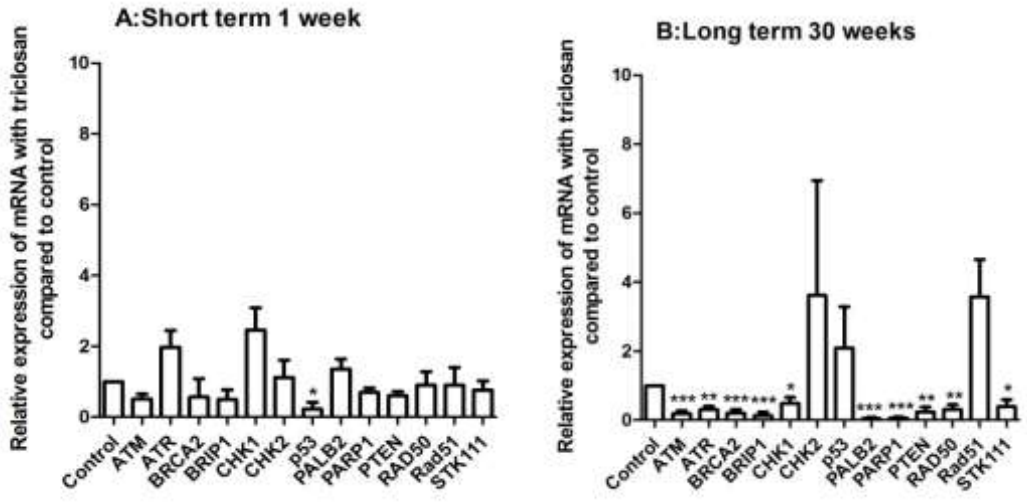


FIGURE 3.45 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells following short term (A) or long term (B) exposure to triclosan

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-7}M triclosan*. The relative expression of mRNAs were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

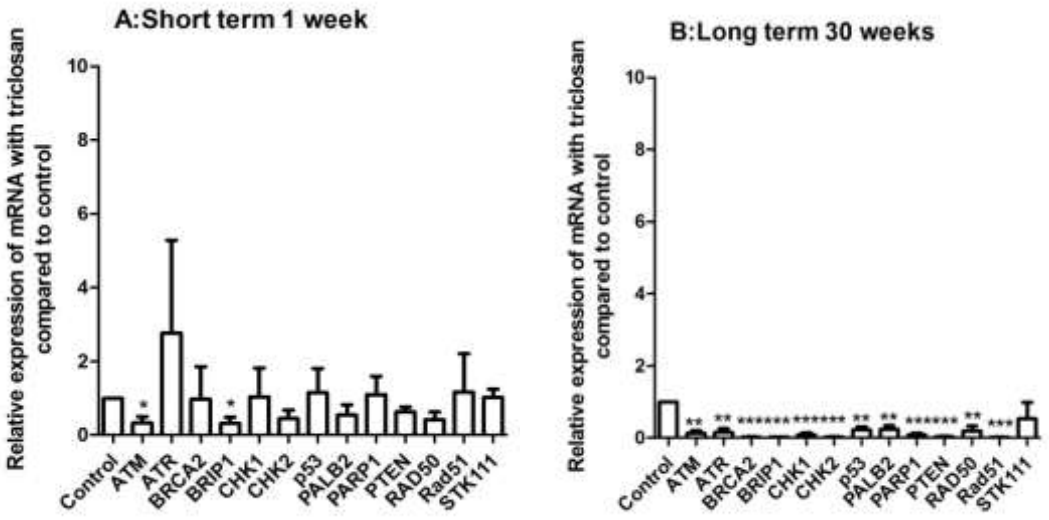


FIGURE 3.46 Real -time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10F cells short term (A)or long term (B) exposure to triclosan

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10^{-7}M triclosan. *The relative expression of mRNAs were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

3.3.4 Level of BRCA1 protein after exposure to triclosan

The effect of long term exposure to triclosan on expression of BRCA1 protein using western Immunoblotting in MCF10A cells is shown in ***Figure 3.47***. A representative of the western immunoblot is shown in ***Figure 3.47A***. ***Figure 3.47B*** shows a quantitative plot. The BRCA1 band is visibly and quantitatively weaker than E2 or control ($p<0.01$ against control value). ***Figure 3.48*** shows the effect of long term exposure to triclosan on expression on BRCA1 protein using western Immunoblotting in MCF10Fcells. Both qualitative and quantitative analyses are shown. The results mirror the mRNA levels, with control values > E2 > triclosan ($p<0.01$ vs control)

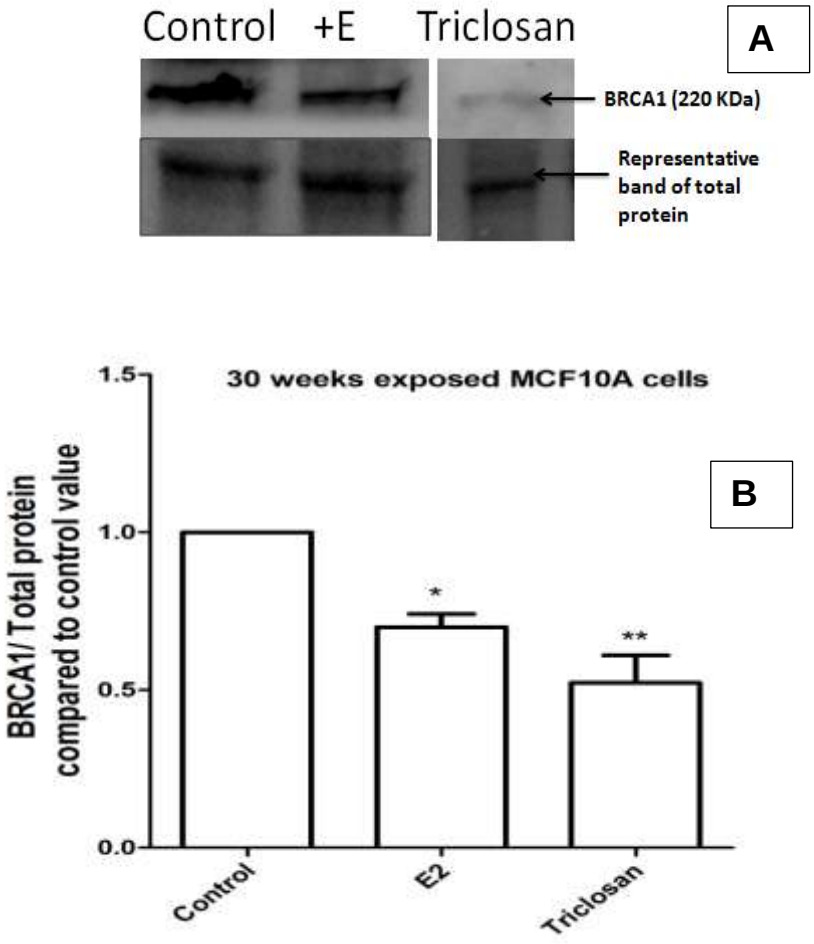


FIGURE 3.47 Level of BRCA1 protein in triclosan treated MCF10A cells using western Immunoblotting.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10A human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-7}M triclosan compared to 10^{-8}M oestradiol (E2). **** indicates p< 0.05, ** p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test.***

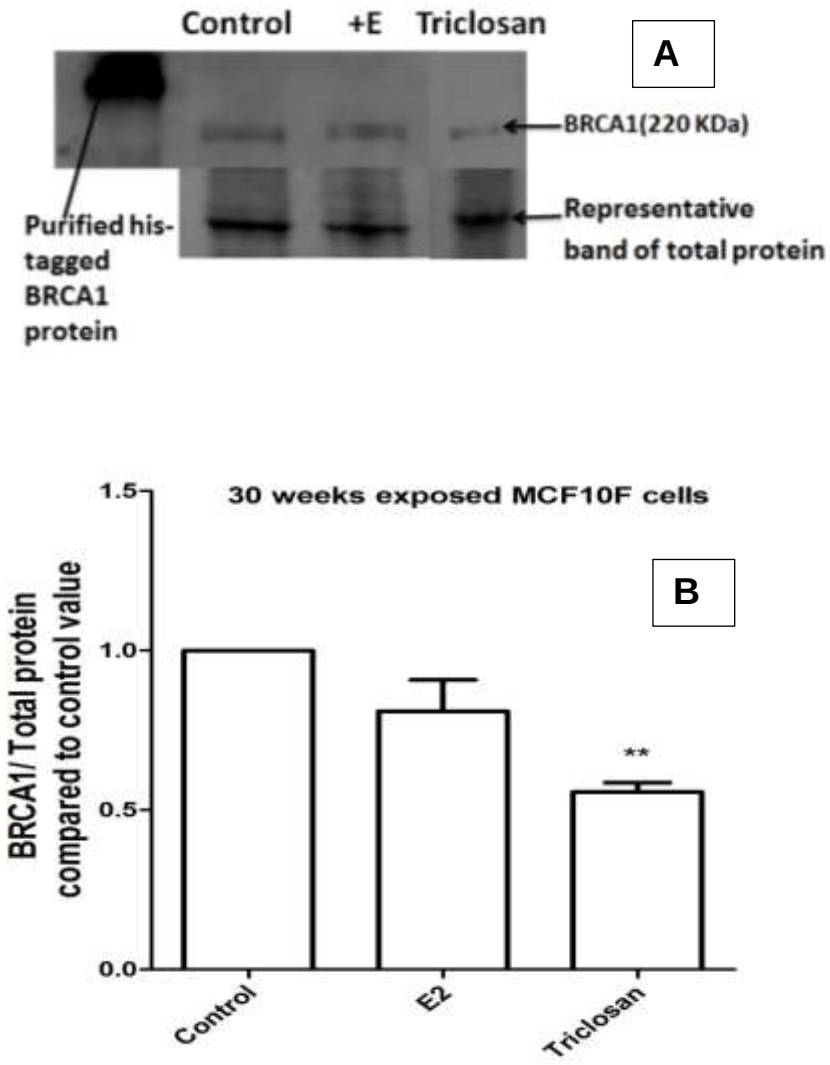


FIGURE 3.48 Level of BRCA1 protein in triclosan treated MCF10F cells using western Immunoblotting.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10F human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-7}M triclosan compared to 10^{-8}M oestradiol (E2).

**** indicates p< 0.05, ** p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test.***

3.3.5 Summary of results using triclosan

The exposure of both cell lines to triclosan give comparable results as set out in ***Table 3.3***. They constitute clear evidence that triclosan has transforming potential to breast epithelial cells and is potentially genotoxic

Table 3-3 SUMMARY OF TRICLOSAN RESULTS

ASSAY	MCF10A	MCF10F
Colony growth in methocel: number or size of colonies	Dose response for all parameters showing peak growth promotion at 10^{-10}M	Dose response for all parameters showing peak growth promotion at 10^{-10}M
Colony growth in methocel: total cell count	Mirrors colony growth	Mirrors colony growth
Comet assay	Not clear comet formation morphologically. Peak tail length at 10^{-7}M Numbers fall off with increasing concentration.	Tail length increased over DES and control. Numbers reduced at concentration tested
BRCA1 mRNA & protein	mRNA only significantly reduced after 30 weeks of triclosan. E2 ineffective E2 and triclosan show reduced protein product	E2 and Triclosan effective in reducing mRNA. Only triclosan reduced expression of protein product.
Other DNA repair mRNAs	mRNAs suppressed by 30 weeks exposure, except for CHK2, P53 & RAD51. High values associate with large standard deviations.	Extremely consistent and strong suppression of mRNA by 30 weeks

3.3.6 Discussion of results of exposure to Triclosan

The outcome demonstrates that exposure of non-transformed human breast epithelial cells to triclosan can enable anchorage-independent growth in methocel culture, can cause DNA damage as measured by comet assays and can reduce levels of mRNAs encoding DNA repair proteins. Particularly, levels of both BRCA1 mRNA and BRCA1 protein were reduced after long-term exposure to the triclosan. Alterations were found in the dose-responses of the triclosan and effects in the short-term (1 week) were not comparable to those after longer exposure times (30 weeks). An overall summary of the results is given in **Table 3-3**

3.3.6.1 Anchorage independent growth

Triclosan is an antimicrobial with ubiquity of exposure in a number of populations (Pycke et al 2014). The results presented here demonstrate that exposure of MCF-10A cells and MCF10F cells to triclosan can enable anchorage-independent growth in methocel culture. This has been shown to be a property closely related to transformation (Soule et al,1990), thus by inducing colony growth in methocel, triclosan has demonstrated an ability to induce a transformed phenotype.

In the present study, the actions of triclosan on colony size are to promote growth of individual colonies, peaking at 10^{-7}M and reducing at higher concentrations. This maximal effect contrasts with the cyclosiloxanes D4 and D5 and with Lilial which show continuously rising growth stimulation to 10^{-5}M (the highest dose tested). Triclosan may be toxic at the higher concentrations because there is sharp fall-off in comet numbers and tail length between 10^{-6} and 10^{-5}M these assays. A similar effect was observed by Henry (2013) who showed triclosan to be toxic (to MCF-7 cells) above 100µg/ml ($3x10^{-8}$M), making those neoplastic cells more sensitive to the agent. In the Reading laboratories, proliferation studies indicated that concentrations of 10^{-6}M and above were toxic to the immortal but not transformed MCF10A and MCF10F cells using relatively short exposure times, measured in days. Therefore, the long term studies here used 10^{-7}M triclosan and not 10^{-5}M as for the previous chemicals

The dose response is similar irrespective of the measure used. As with the cyclosiloxanes and with Lilial, the results measuring numbers closely resemble those measuring size. The peak stimulation is also at 10^{-7}M. In the present study, the actions of triclosan on colony size are to promote growth of individual colonies, reducing at higher concentrations, evident irrespective of the measure used.

3.3.6.2 DNA damage assessed by comet assay

DNA clastogenicity as measured by the alkaline comet assay resulted in peak tail length for MCF10A cells at 10^{-7}M. Numbers decreased with increasing concentration, suggesting total breakdown of cells. Numbers of comets recorded were not greater with triclosan than with DES, a synthetic oestrogen which was adopted here (as opposed to the natural oestrodiol used in other assay systems) to make results comparable with previous studies from the Reading laboratories. However, the tail length – i.e. the extent of damage represented by shorter more mobile fragments – was higher in the triclosan treated cultures. These results might reflect less efficient repair in the presence of triclosan, despite a similar cell death rate. There are a number of issues surrounding the interpretation of comet assays. It is possible that repair mechanisms activated during the incubation may alter the percentage and physical characteristics of the comets (Lorenzo et al., 2013)). Standardization of assay methodology is important both on intra- and inter-laboratory basises (Valverde and Rojas, 2009)). Statistical analysis should, according to Lovell (Lovell and Omori, 2008), incorporate power and sample size calculations and interpretation based upon size of effects and their confidence intervals and meaningfulness is as important as reporting based purely on statistical significance tests.

3.3.6.3 DNA repair and proliferation control gene expression

BRCA mRNA and protein expression long term treatment with 10-7M triclosan and E2 resulted in reduced BRCA1 mRNA expression. Protein results indicated that both E2 and triclosan reduced measurable levels of BRCA1 protein in MCF10A cells, but only triclosan did so in MCF10F cells. In both cases the reduction by triclosan was significant at the $p=0.01$ level. The importance of the BRCA1 in the context of the comet assay results, is that BRCA1 is one of the first lines of defence against DNA strand breaks, so that if the cell attempts to repair DNA BRCA1 will be expressed, if it fails fragmentation will occur and comet tails will form.

mRNA from the panel of other DNA repair related genes resulted in uniform reduction of expression in all cases with MCF-10F cells at 30 weeks; no effects were observed for short term exposure to triclosan. MCF10A cells reacted similarly, except that CHK2, P53 and RAD51 were unchanged at 30 weeks. Such a circumstance is suggested by the gene panel results, where, certainly with MCF-10F there were reduced BRCA1 levels, significant to $p<0.01$. mRNA from the panel of DNA repair related genes resulted in uniform reduction of expression in all cases with MCF10F cells at 30 weeks; no effects were observed for short term exposure to triclosan. MCF10A cells reacted similarly, except that CHK2, P53 and RAD51 were unchanged at 30 weeks.

It is not clear whether this apparent cytotoxic combination effect is additive, supra-additive or depends on some other type of interaction between two compounds simultaneously applied. Differences in reported action may well result from variations in exposure times. This study has highlighted differences between short and long term (30 week) exposure *in vitro*. The study by Gee, for example was limited to 21 days. Although cells as monolayers might be expected to be more sensitive to agents directly applied to them, the human social use of cosmetics is likely to involve decades of continual use which would be expected to result in continued build-up of any toxic agents.

Like the other agents tested in the present study, the effects of triclosan are exacerbated by long exposure times. As with many bioactive compounds, triclosan is beneficial for its antimicrobial properties but its application in uncontrolled, non-clinical settings such as personal hygiene or is arguably unwise. Johnson (Johnson 2011), applying the *Navigation Guide Systematic Review Methodology*, concluded that there was "sufficient" non-human evidence and "inadequate" human evidence of an association between triclosan exposure and human reproductive and developmental health. Early development, like cancer, is heavily dependent on highly regulated proliferation and differentiation.

3.3.6.4 Physiological relevance of these studies

To address the absorption of triclosan and the relevance of the concentrations used in this study:- In experiments on skin absorption (Moss et al., 2000) Radiolabelled triclosan was applied to the skin of rats; twenty-four hours after application 12% of label was recovered in the faeces, 8% in the carcass 1% in the urine, 30% in the stratum corneum and 26% could be rinsed from the skin surface. In a human study on 2,517 urine samples (Calafat et al., 2008), using automated solid-phase extraction coupled to isotope dilution–high-performance liquid chromatography–tandem mass spectrometry, concentrations of total (free plus conjugated) triclosan occurred in in 74.6% of samples at concentrations of 2.4–3,790 µg/L. There was more in the urine of individuals from higher income households appearing maximally in the third decade of life. This very wide range of concentrations is consistent with those applied to the breast cancer cells in this study. The high-end figure quoted by Calafat is in the 10^{-6}M region. In this study activity on MCF10 cells *in vitro* is observed at lower concentrations. Triclosan 10^{-7} M concentation (289.54 x 10^{-7} g/l), represents 28.954 x 10-7 µg/l, Triclosan 10^{-6} M equates to 2.8954 x 10-7 µg/l. This demonstrates the physiological relevance of this work. With breast cancer tissue being often composed largely of hard matrix it is difficult to equate values in homogenised material to cellular exposure and clearly the only relevance of excreted concentrations is that they point to circulating levels over the period of collection and are subject to variations

in urine strength, which can be compensated for by comparing to the relatively continuous and stable levels of creatinine excretion. (Stevens et al., 2013)

Triclosan accumulates in the liver and in fatty tissue (Geens, 2012), which is characteristic of breasts. Much work on this compound centres on reproduction, so estimates of prevalence in the community tend to focus on pregnant women. Measurable levels in urine from pregnant women occurred in 87% of samples tested by Weiss et al (2015) and half of serum samples (Ye 2011).

Urine levels may reflect concentration by the kidneys, however and fluid intake levels (Stevens et al., 2013). Measuring tissue samples, Geens (Geens et al., 2012) found highest concentrations in rat livers (3.14ng/g and fat 0.61ng/m). This group consider that, despite concentrations in adipose tissue, these compounds seem to have 'a low bioaccumulation potential' (Geens et al 2012). Measurements have however been made in human breast milk. Although the recent paper by Azzouz (Azzouz et al., 2016) is largely a technical report, highlighting pretreatments of fluids such as milk to deplete them of protein (which might remove some bound agent), they have measured concentrations between 3.3 and 50,000 ng/l in human milk.

In conclusion, Triclosan has been shown to have qualitatively similar genotoxic properties and influences on three dimensional growth characteristics as the cyclosiloxanes BPA and Lilial. This might be expected of a compound that shares with those compounds a profile in the literature of physiological effects often characterized by endocrine mimicking/disrupting properties (Gatidou et al., 2007).

3.4 Bisphenol A

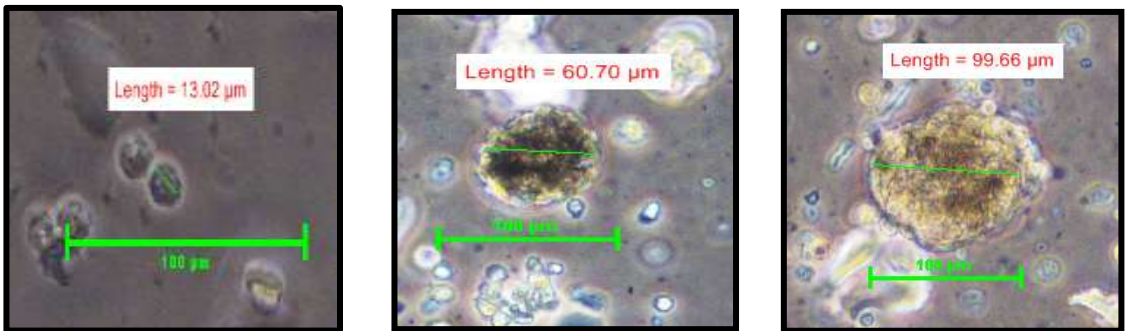
Bisphenol-A (BPA) is an environmental contaminant because it is polymerized in the production of bottles including those for feeding neonates. Epoxy resins coat the insides of almost all food and drink cans. BPA is therefore ingested by leaching from these products, the rate of leaching increasing with damage or on storage. BPA, known to enter the human breast (Bonefeld-Jorgensen et al., 2007), has been included in this study because it is currently under regulatory consideration. Although not directly a cosmetic ingredient many personal care products are stored long term in plastic containers. The experiments followed the same pattern as for the previous cosmetic ingredients tested.

3.4.1 Suspension growth of MCF10A and MCF10F cells and colonies exposed to BPA: morphology.

MCF10A and MCF10F cells were grown in semi-solid suspension culture with of 17β-oestradiol and varying concentration Bisphenol-A (BPA). Photomicrographs of colonies after 21 days are shown in ***Figure 3.49***. In the absence of treatment, only small colonies were found, compared with the additional large colonies induced by E2 and by the even larger and denser colonies induced by BPA, as shown in ***Figure 3.49, panels B-C***

3.4.2 Suspension colony parameters in the presence of BPA: quantitative.

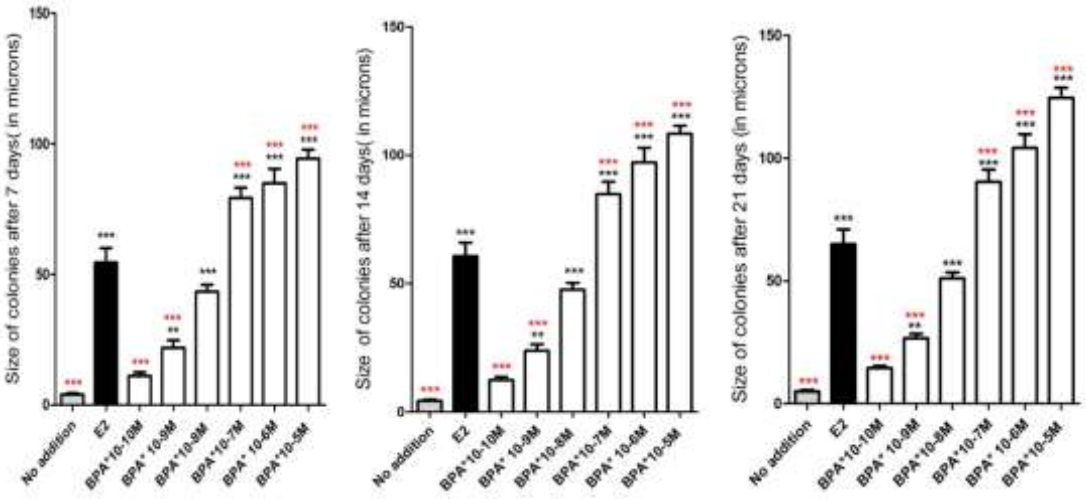
MCF10A and MCF10F cells were grown with 17β-oestradiol or BPA in suspension culture for 7, 14 and 21 days. Colony size ***(Figure 3.50)***, colony number (***Figure 3.51*****)** and total cell number (***Figure 3.52)*** were measured after 7, 14 or 21 days. Increasing concentrations of Bisphenol-A (BPA) (10^{-10} M to 10^{-5}M) gave increased colony size and colony number. The increase had not peaked by 10^{-5}M. All twelve plots, covering both cell lines show a similar dose- response pattern.



(A) No treatment (B) 17β- oestradiol (E2) (C) Bisphenol A (BPA)

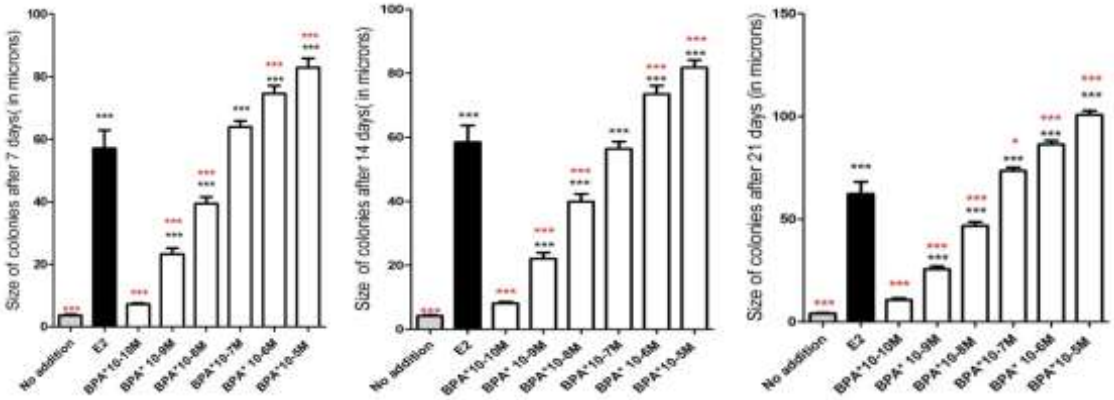
FIGURE 3.49 Colonies of MCF10A cells growing in semi-solid methocel suspension culture after 21 days. *CELLS WERE GROWN WITH:- (A) NO TREATMENT (B), 70 NM 17B-OESTRADIOL (C), 10^{-5}M BISPHENOL A (BPA). PHASE CONTRAST IMAGES.*

A. MCF10A cells



Day 7 Day 14 Day 21

B. MCF10F cells

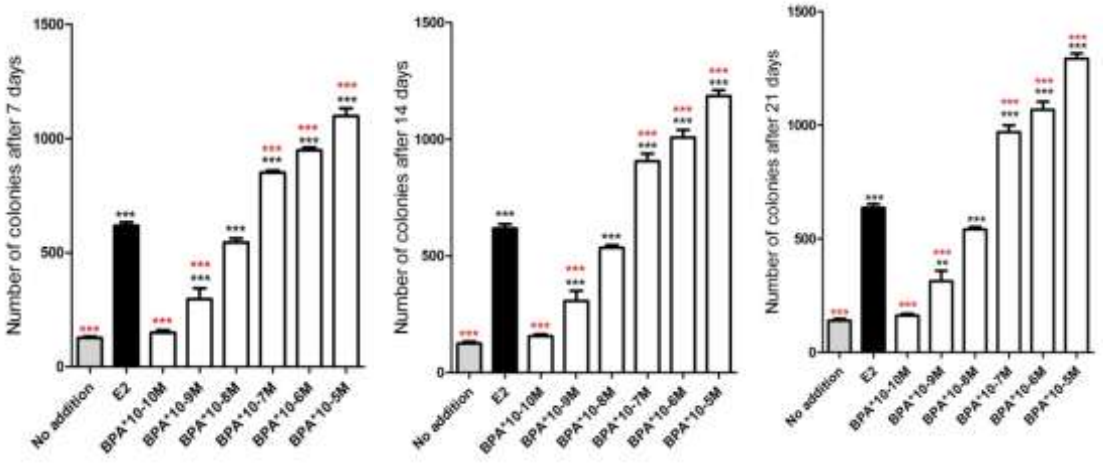


Day 7 Day 14 Day 21

FIGURE 3.50 Effect of different concentrations of BPA on the size of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 to 21 days.

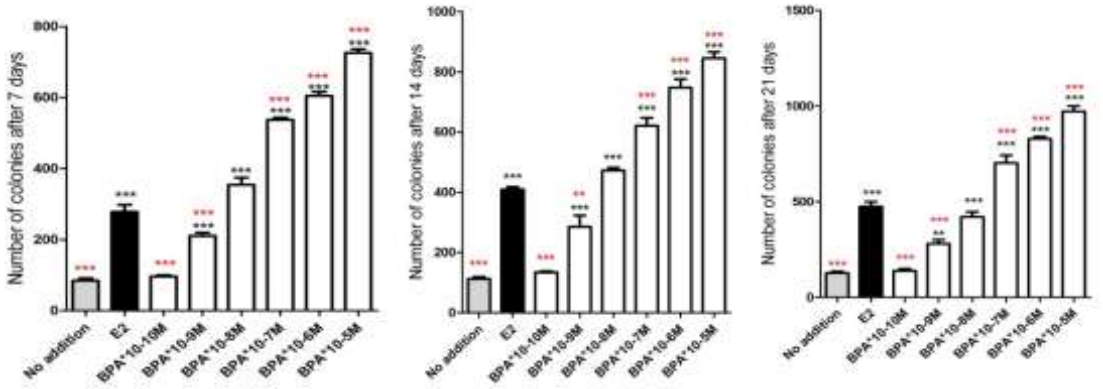
Cells were grown in stock medium with no addition, with70nM 17β-oestradiol or with10^{-5}M BPA, with10^{-6}M BPA, with10^{-7}M BPA, with10^{-8}M BPA, with10^{-9}M BPA or with10-10M BPA. Average colony size calculated from 15 fields per well of view and results are presented as the overall average of readings and standard error of triplicate wells from three replicate experiments. ***** indicates p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

MCF10A cells



Day 7 Day 14 Day 21

A. MCF10F cells



Day 7 Day 14 Day 21

FIGURE 3.51 Effect of different concentrations of BPA on number of colonies of MCF10A (A) and MCF10F (B) cells growing in semi- solid methocel suspension culture from 7 days to 21 days.

Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with10^{-5}M BPA, with10^{-6}M BPA, with10^{-7}M BPA, with10^{-8}M BPA, with10^{-9}M BPA or with10^{-10}M BPA. Colony growth is shown as the number of colonies per well from 7 days to 21 days. Error bars are standard error of triplicate wells for each experiment.

***** indicates p< 0.01 and *** p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk).***

A. MCF10A cells B. MCF10F cells

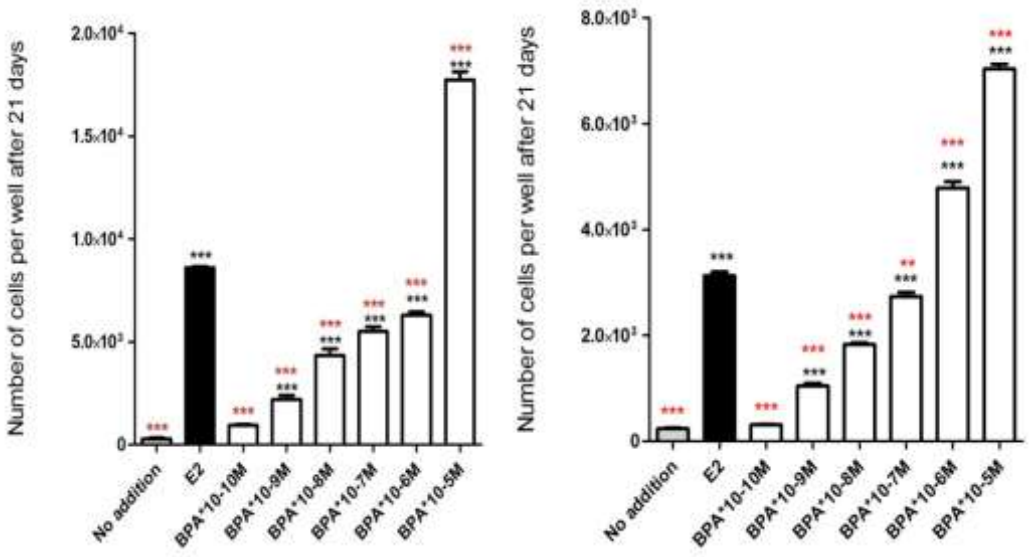


FIGURE 3.52 Effect of different concentrations of bisphenol A on growth of MCF10A (A) and MCF10F (B) cells in semi- solid methocel suspension culture after 21 days.

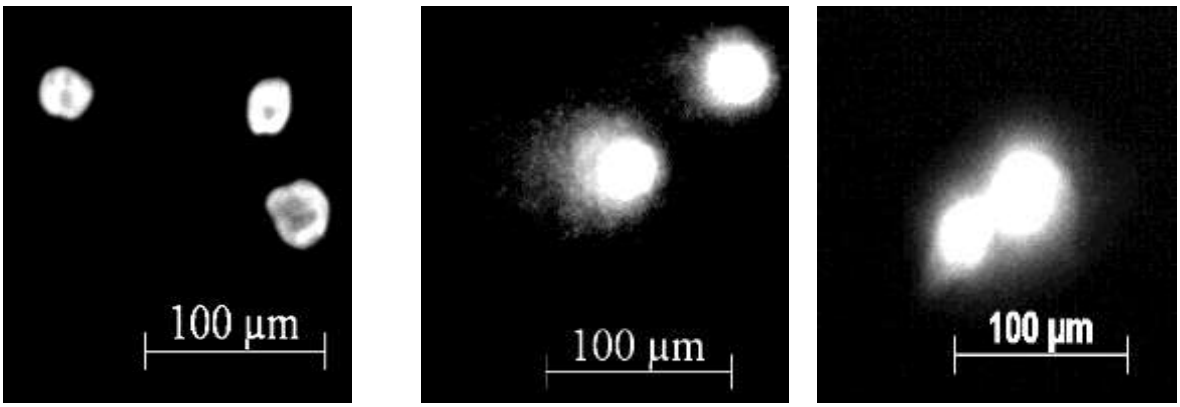
Cells were grown in stock medium with no addition, with70 nM 17β-oestradiol, with10^{-5}M BPA, with 10^{-6}M BPA, with10^{-7}M BPA, with10^{-8}M BPA, with10^{-9}M BPA or with10^{-10}M BPA. Cell growth is shown as the number of cells per well after 21 days, as counted using a Coulter counter. Error bars are the standard error of triplicate wells.

******indicates p <0.001 compared to no addition (grey bar, black asterisk) and cells with 17β-oestradiol (black bar, red asterisk)***

3.4.3 Comet assay of DNA damage after exposure to bisphenol A (BPA)

An alkaline single-cell gel electrophoresis (SCGE) technique was employed to detect single strand and labile DNA adducts in the immortalised human breast epithelial cell lines MCF10A and MCF10F cells after exposure to BPA.

Some cells in the photomicrograph in ***Figure 3.53*** panel D (bisphenol A (BPA)) show comet formation after 24 hours exposure to BPA. Some indications of comet formation are seen in panel B after exposure to DES. The bar charts in ***Figure 3.54*** show comet tail length and percentage of cells. Comets are less frequent with BPA treatment than with the other agents tested in this study: less than half in both cell types. However, comets are present at 1 hour of incubation. BPA at 10^{-5}M evokes the longest tails. DES also produced a large percentage of small comets. Comet formation is, however apparently induced by BPA.

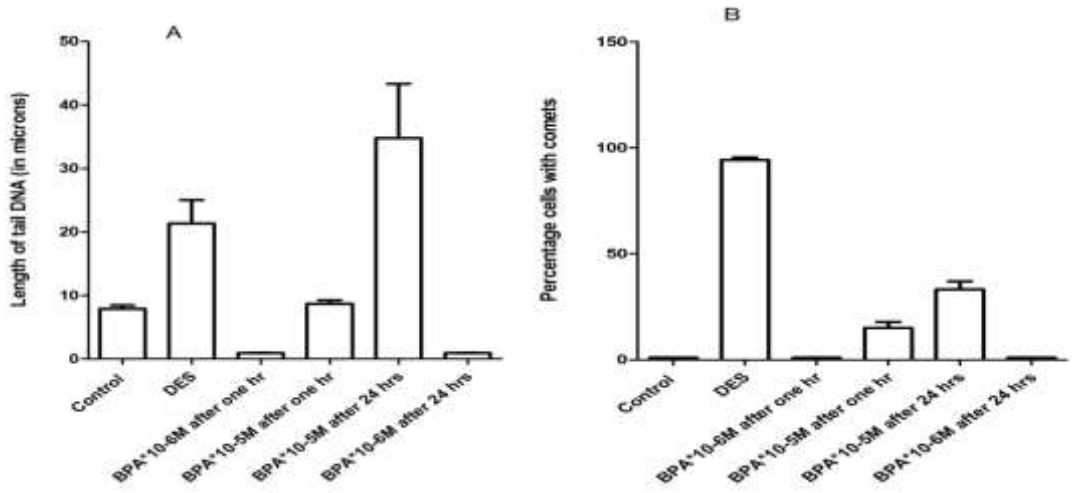


A: Untreated MCF10A cells B: Diethylstilboestrol (DES) C: Bisphenol-A (BPA) treated 24 hours

FIGURE 3.53 Comet assay for detecting DNA damage in MCF10A cells exposure to bisphenol A (BPA).

Panel A is of untreated cells (control) treated for one hour. B) Cells treated with 10^{-5} M Diethylstilboestrol (DES) (positive control) treated for one hour. C) Cells treated with 10^{-5}M bisphenol A (BPA) *treated for 24 hours. Cells viewed by fluorescence microscopy.*

MCF10A cells



MCF10F cells

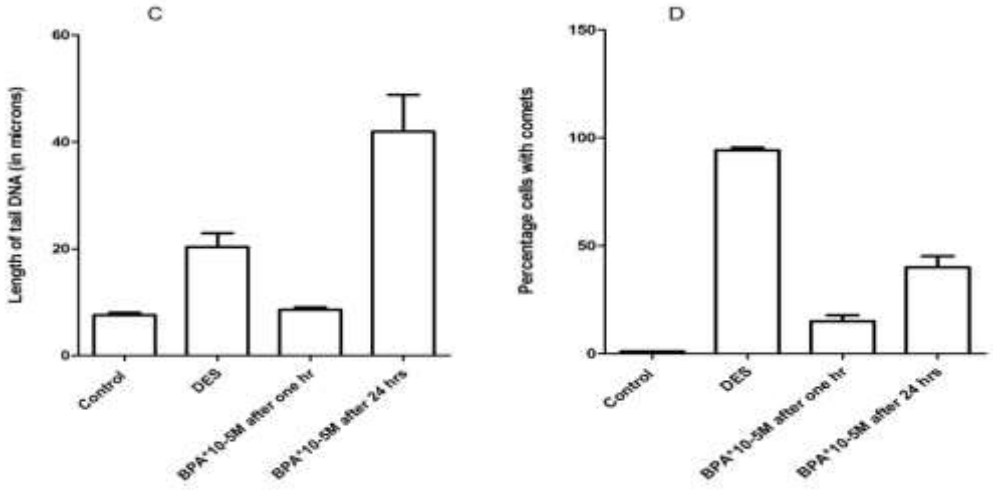


FIGURE 3.54 Effect of bisphenol A (BPA) on DNA damage in MCF10A and MCF10F human breast epithelial cells as assessed by comet assay. Measuring DNA tail length (A,C) or % of cells with comets (B,D)

Cells were grown in stock medium and treated one hour with untreated cells (control). Treated cells with 10^{-5} M diethylstilboestrol (DES), (positive control).Treated cells with 10^{-5}M Bisphenol A (BPA)*.Treated cells with 10-6M* Bisphenol A (BPA)*. Error bars represent standard deviation of 50 comets scored.*

3.4.4 Bisphenol A: Effect of mRNA expression of DNA repair genes:

Levels of BRCA1 mRNA after exposure to bisphenol A (BPA)

Short (1 week) and long term (30 weeks) effects of exposing MCF10A ***(Figure 3.55*)** and MCF10Fcells ***(Figure 3.56***) to10^{-5}M bisphenol A (BPA), in terms of levels of mRNA for BRCA1, was investigated using RT-PCR. Significant reduction was found in the long term for E2 and bisphenol A (BPA) exposure in MCF10A cells ***(Figure 3.55*)**. In MCF10F cells, the short-term effect of exposure to bisphenol A (BPA) showed a significant increase in expression of BRCA1 mRNA in MCF10F cells but in the long-term the effect of exposure to E2, and bisphenol A (BPA) showed a significant reduction in BRCA1 mRNA expression ***(Figure 3.56***).

MCF10A cells

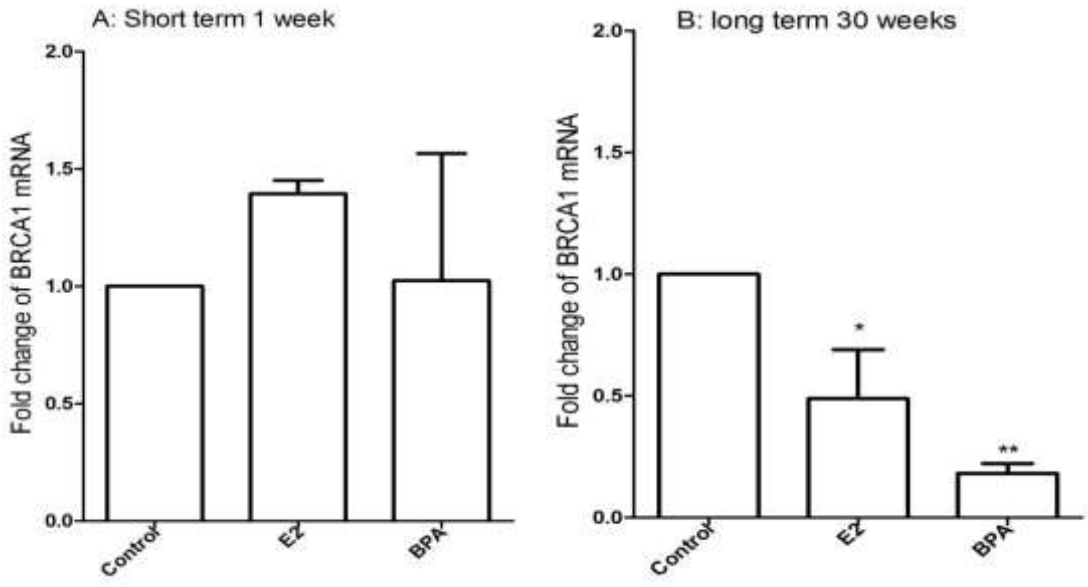


FIGURE 3.55 Real-Time RT- PCR analysis of BRCA1 mRNA in MCF10A cells following short term (A) or long term (B) exposure to bisphenol A (BPA).

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of 10^{-5}M bisphenol A (BPA). The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

**** indicates p< 0.05, **p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

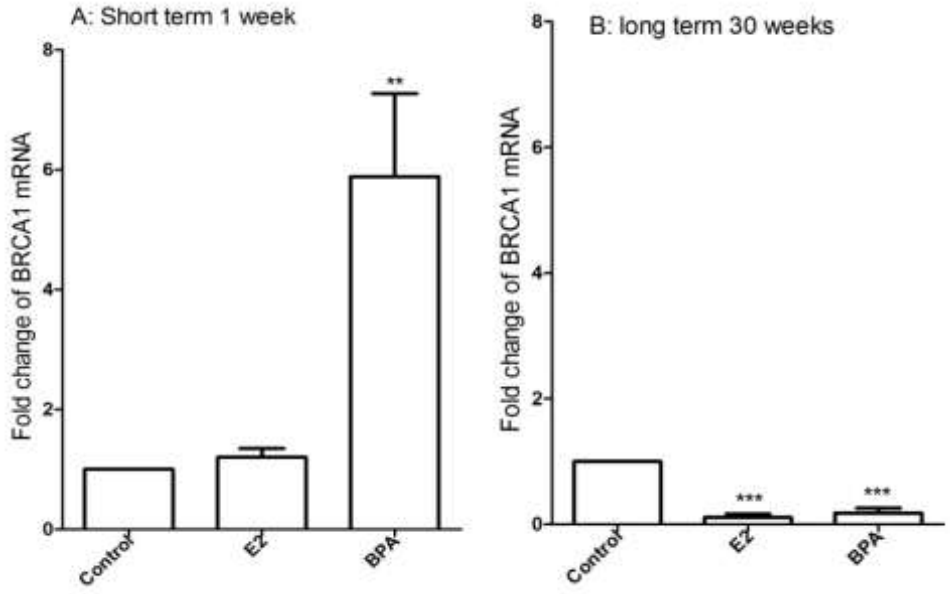


FIGURE 3.56 Real-time RT- PCR analysis of BRCA1 mRNA in MCF10F cells following short term (A) or long term (B) exposure to bisphenol A (BPA).

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the present of 10^{-8} 17β- oestradiol (E2) *or in the presence of 10^{-5}M bisphenol A (BPA). The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.*

****** indicates p< 0.001, compared to control by one way ANOVA with post-hoc Dunnett test.***

Levels of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, RAD50, Rad51 and STK111 mRNA after exposure to bisphenol A (BPA)

Short (1 week) and long term (30 weeks) effects of exposure to bisphenol A (BPA) are shown for MCF**10A** cells in ***Figure 3.57*** and MCF**10F** cells in ***Figure 3.58***. The short-term effect of exposure showed a significant reduction in expression of PTEN mRNA in MCF10A cells. The long-term effect of exposure to BPA was a significant reduction in expression of ATM, ATR, BRCA2, BRIP1, CHK1, PALB2 and PARP1 mRNA in MCF10A cells; in all but one of these (CHK1) $p<0.001$.

In MCF**10F** cells the short-term effect of exposure to bisphenol A (BPA) was a significant reduction in expression of CHK2 mRNA. However, the long-term effect of exposure was a significant reduction in expression of BRIP1, CHK2, PALB2, PARP1, PTEN, Rad50 and Rad51 mRNA in the same cells. P53, ATR and STK111 gave high mean values with poor variance.

MCF10A cells

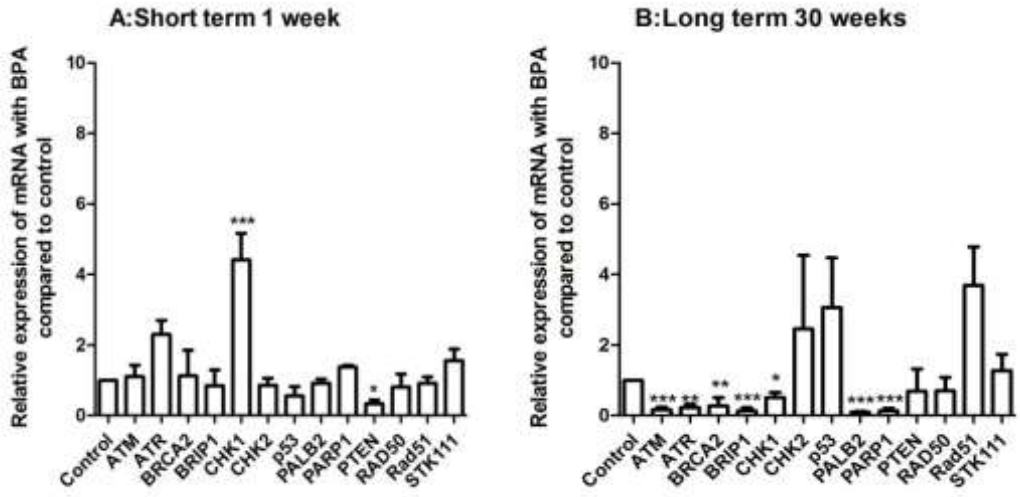


FIGURE 3.57 Real-time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10A cells following short term (A) or long term (B) exposure to bisphenol A (BPA)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10-5M bisphenol A (BPA). The relative expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111*mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.* **** indicates p< 0.05, ** p< 0.01 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.***

MCF10F cells

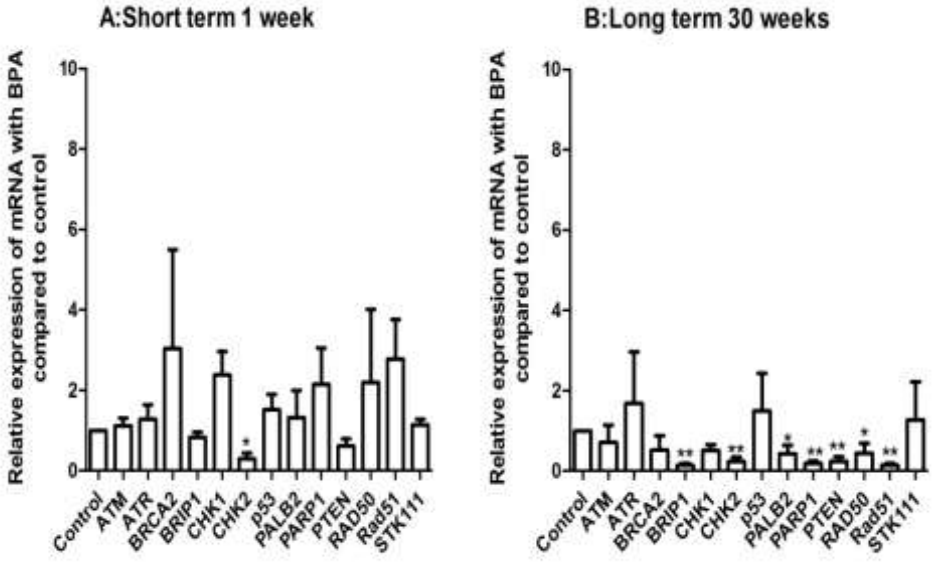


FIGURE 3.58 Real-time RT- PCR analysis of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN, Rad50, Rad51 and STK111 mRNA in MCF10Fcells following short term (A) or long term (B) exposure to bisphenol A (BPA)

Cells were grown in stock medium for 1 week (A) or 30 weeks (B) with no addition (control) or in the presence of 10-5M bisphenol A (BPA). The relative expression of ATM, ATR, BRCA2, BRIP1, CHK1, CHK2, p53, PALB2, PARP1, PTEN,Rad50, Rad51 and STK111mRNA were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values.

**** indicates p< 0.05, ** p< 0.01 compared to control by one way ANOVA with post-hoc Dunnett test.***

3.4.5 Level of BRCA1 protein after exposure to bisphenol A (BPA)

Figure 3.59 shows the effect of long term exposure to bisphenol A (BPA) on expression of BRCA1 protein using western Immunoblotting in MCF10A cells. The image at A is a qualitative photograph shown above a quantitative plot (B).***Figure 3.60*** shows the effect of long term exposure to bisphenol A (BPA) on expression of BRCA1 protein using western Immunoblotting in MCF10F cells. Both qualitatively (A) and quantitatively (B).In plot B for MCF10A cells both E2 and BPA significantly reduce protein accumulation; for MCF10F cells only BPA induces a reduced protein level.

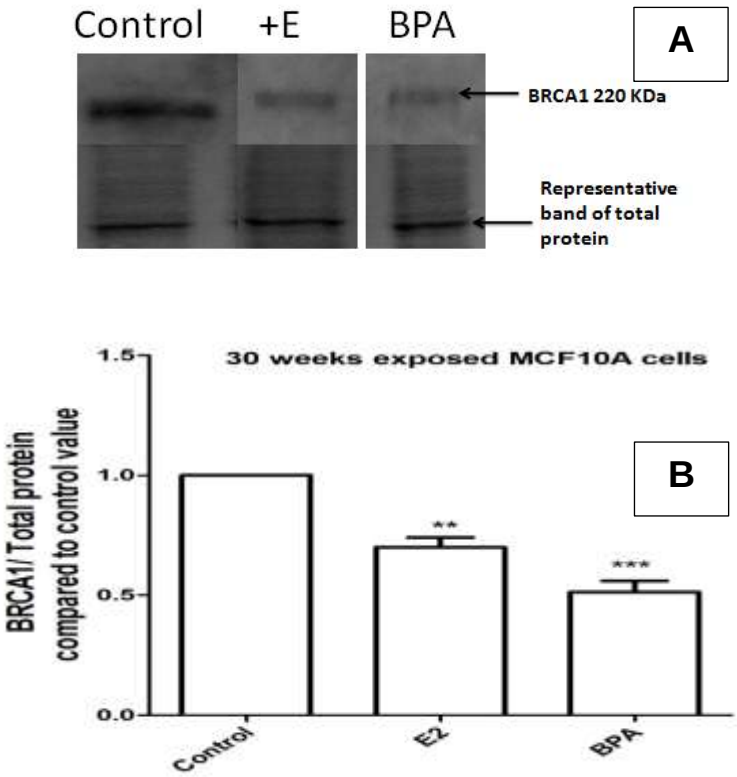


FIGURE 3.59 Level of BRCA1 protein in BPA treated MCF10A cells using western Immunoblotting.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10A human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M bisphenol A (BPA) compared to 10-8M oestradiol (E2).

***** indicates p< 0.01, *** p< 0.001 and compared to control by one way ANOVA with post-hoc Dunnett test.***

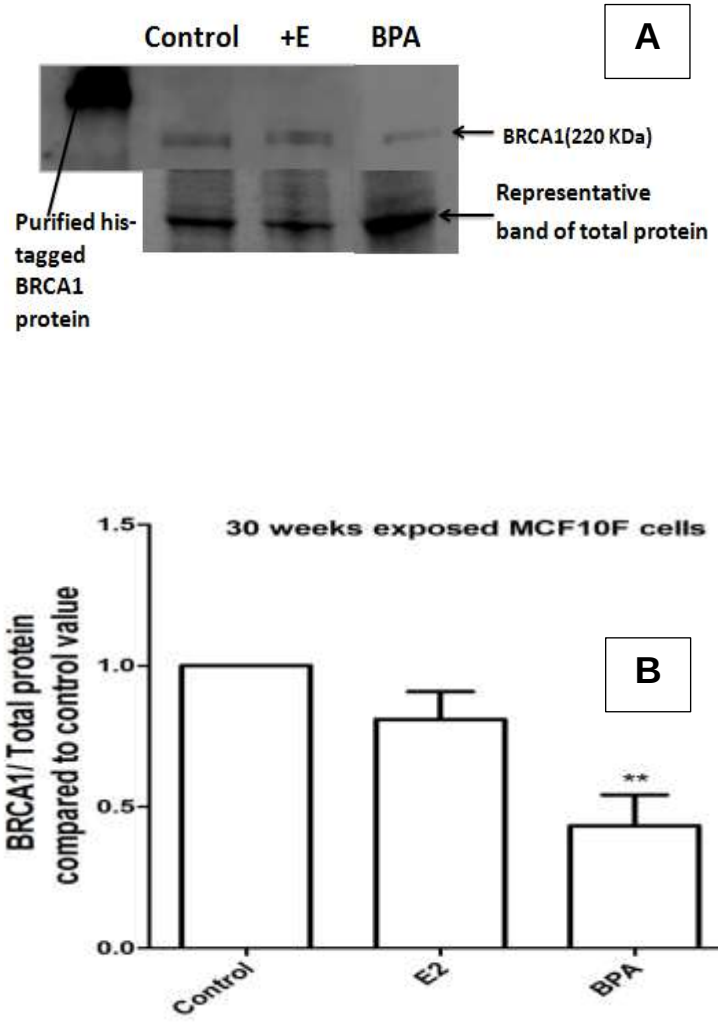


FIGURE 3.60 Level of BRCA1 protein in BPA treated MCF10F cells using western Immunoblotting.

BRCA1 protein levels were normalised to total protein using BioRad stain-free technology. BRCA1 protein levels in MCF10F human breast epithelial cells were observed after 30 weeks of no addition (control) or in the presence of 10^{-5}M bisphenol A (BPA) compared to 10^{-8}M oestradiol (E2).

***** indicates p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test.***

3.4.6 Summary of results using Bisphenol-A

A visual overview of the relevant results appears in **Table 3.4**. As with the other agents tested neoplastic transformation is exhibited on exposure and DNA repair mechanism-related mRNA and protein levels change after 30 weeks, consistent with a reduction in the cells' homoeostatic capacity.

TABLE 3-4 Summary of Bisphenol-A results

ASSAY	MCF10A	MCF10F
Colony growth in methocel: number or size of colonies	Dose response for all parameters showing exponential rise with concentration	Dose response for all parameters showing exponential rise with concentration
Colony growth in methocel: total cell count	Mirrors colony growth	Mirrors colony growth
Comet assay	Only significantly longer tails at 24h. Comet numbers with BPA < with DES but >control	Tail length increased by BPA over DES and control. Numbers reduced at concentration tested
BRCA1 mRNA & protein	mRNA drops at 30 weeks. Protein expression reduced at 30 weeks by E2 & BPA	Short term rise in BRCA mRNA followed by drop below controls at 30 weeks Protein falls at 30 weeks in response to BPA only
Other DNA repair mRNAs	ATM, ATR, BRCA2, BRIP1, CHK1, PALB2, PARP1, suppressed after 30 weeks exposure. CHK1 raised. High values associate with large variances	CHk2 significantly lower at 1 week. Consistent suppression of mRNAs by 30 weeks except or ATR, P53 and STK111

3.4.7 Discussion of results of exposure to Bisphenol A (BPA)

The results described here demonstrate that exposure of non-transformed human breast epithelial cells to BPA yield results strikingly similar to those obtained using the other chemicals the cyclosiloxanes, Lilial and triclosan. BPA is described here enabling anchorage-independent growth of three-dimensional colonies in methocel-based medium, increasing in efficacy between 10^{-10} and 10^{-5}M. It can also cause comet formation, indicating DNA damage and can reduce levels of mRNAs encoding DNA repair proteins. Most notably, levels of both BRCA1 mRNA and BRCA1 protein were reduced after long-term exposure to 10^{-5M} BPA. Dose-responses of BPA and after1 week exposure were not equivalent to those after longer exposure times (30 weeks). An overall summary of the results is given in **Table 3-4**

3.4.7.1 Anchorage independent colony formation

The results demonstrate that exposure of non-transformed human breast epithelial cells to Bisphenol A (BPA) can enable anchorage-independent growth in methocel culture. The reactions to BPA encountered in this study were similar to those for triclosan in that there was a clear positive association between concentration and colony growth, whether measured as colony size, or colony numbers or cell count between 10^{-10} and 10^{-5}M.

BPA has been shown to cause proliferation of breast cancer cells in monolayer culture (Soto et al., 2008), but these experiments show that BPA can enable growth of non-transformed breast cells in suspension culture. This is an indication that the cells have attained a transformed phenotype and can now grow unattached to a matrix in the manner of cancer cells. (Soule et al., 1979)

3.4.7.2 DNA damage assessed by comet assay

Comet tail length was enhanced by 10^{-5}M BPA treatment. Comet assays with BPA included different exposure times from 1 hour to 24 hours. A 24 hr exposure yielded more comets and longer tails than a 1-hour exposure. The type and irreversibility of DNA damage by BPA due to repair failure was defined using comet assays by Tayama et al 2008. They were, however, not able to explain their observation that different DNA damage was effected by E2 (chromosome abnormalities but not DNA migration), compared to DES and the oestrogen-like chemicals tested – which included BPA. Timing is important in all such studies, as while initial DNA damage may cause genetic signs of enhanced repair activity in the short-term, longer continued insults may cause cells to switch to a suicide

mode. In terms of cancer induction it is important to distinguish between damage that can be repaired, damage that induces cell death and the critical medium level and gene specific pro-carcinogenic damage that causes aberration of cell cycle control but is not fatal to the cell clone.

3.4.7.3 DNA repair gene mRNA and protein products

BPA shows more differential molecular genetic responses between the two cell lines than the other agents. There is a sharp short-term increase in BRCA1 mRNA as well as smaller long-term rises in CHK2 and P53 in MCF10F cells. In both animals and humans, loss of normal BRCA1 function increases sensitivity to genotoxic insults, through defective DNA repair (Jones et al., 2010). BPA is included in this study as, although it is not a cosmetic ingredient, it is used in the packaging of many consumer products and it is currently undergoing intense regulatory scrutiny as a potential environmental endocrine disrupter and mutagen. Over 6 billion pounds per year of BPA are used to manufacture polycarbonate plastic products, in resins lining metal cans, in dental sealants, and in blends with other plastics (Welshons et al., 2006). The ester bond linking BPA molecules can undergo hydrolysis, resulting in the release of free BPA into food, beverages, and the environment. *In vitro* dose responses to BPA resemble oestradiol, causing changes in a range of cell functions at concentrations between 1 pm and 1 nm, according to a systematic review by Vom Saal (Vom Saal 2007).

Russo et al (2012) published molecular genetic studies on MCF10F cells that bears some direct comparison to the work presented here. They used microarrays to quantify the expression of mRNA to from a panel of DNA repair related and apoptosis controlling genes after a 2 week exposure to BPA at 10-5 and 10-6M concentration. The cells showed an increased expression of BRCA1, BRCA2, BARD1, CtIP, RAD51, and BRCC3 (DNA repair related genes) and down-regulation of PDCD5 and BCL2L11, both apoptosis pathway genes. The DNA methylation analysis indicated hypermethylation of BCL2L11, PARD6G, FOXP1, and SFRS11, and hypomethylation of NUP98 and CtIP (RBBP8) after exposure to BPA. The overlap between genes studied by Russo and in this work is not extensive, but both indicate that normal human breast epithelial cells exposed to BPA tend to increase the expression of genes involved in DNA repair in order to overcome the DNA damage induced by this chemical, at least in the short term (1 or 2 weeks). In the present study although this was clearly a trend over the range of genes studied, high variability limited the number of statistically significant rises at 1 week. The subsequent drop below control levels

after 30 weeks exposure was statistically more robust but the stand-out genes (ATR, P53 and STK111) that countered this trend with elevated results also suffered high variability.

Despite its different source as an environmental hazard, the reactions to BPA encountered in this study were similar to those for Lilial or triclosan in terms of anchorage independent colony growth and comet formation. Comet tail length was particularly enhanced by BPA treatment. However, BPA shows more by way of differential molecular genetic responses to the two cell lines than the other agents. There is a short-term sharp increase in BRCA1 mRNA as well as more modest long-term rises in CHK2 and P53 in MCF-10F cells. This is of particular interest as Dairkee (2013) shows, in HRBEC cell lines, inactivation of the "p53 axis" by this material, leading to deregulation of proliferation kinetics.

3.4.7.4 Relevance of this work to human exposure

There are many studies which have been conducted to address the issue of the potential for BPA to impact human health as well as wildlife: more research is clearly needed. The formulation of hypotheses underpinning epidemiological and ecological studies can be facilitated by extrapolation from laboratory animal studies when potential mechanistic pathways are similar in laboratory animal models, wildlife and humans (Michalowicz, 2014, Vom Saal 2007). This review highlights the circumstance that while traditional toxicological studies – mostly on animals – involve high dosages, these do not reflect the lifelong exposure to low concentrations of BPA that characterise the human experience in developed countries.

As a ubiquitous plastics constituent it is, in the modern era, difficult to assemble suitable control subjects for epidemiological studies. Similarly it is difficult to establish truly relevant exposure conditions in the design of cell culture experiments, especially where monolayers of a single cell type on a non-organic substrate are envisaged. It is necessary to construct mechanistic hypotheses by considering results from a range of approaches.

The American National Health and Nutrition examination Survey concludes that the average consumption of BPA was 34ng/kg/day (Lakind and Naiman, 2011). Uptake by individuals was not related to consumption of bottled water or canned tuna (which had been suspected), but was associated with soft drinks, school meals and meals "prepared outside the home" – presumably referring primarily to "fast food".

BPA has been detected and measured in urine and milk (Hines et al., 2015) in Californian women. BPA was found in >53% of urine samples, but less frequently in milk. Overall, Hine considers urine to be the most reliable (as well as more readily available) vector for exposure assessment. BPA exposure as so estimated did not correspond quantitatively to other measurements, including triclosan, suggesting “considerable variability” in exposures. Certainly the route of exposure to BPA and triclosan is different, the former being through leaching from plastic containers, the latter from direct skin application in cosmetic preparations or from oral intake in mouthwashes and toothpaste.

BPA is readily transported around the body and in pregnant mice it is found to accumulate in foetuses, leading to increased mammary cancer susceptibility in later life (Doherty et al, 2010). Exposure of prepubertal rats has similar consequences on proteomics and cancer incidence (Betancourt et al., 2012). However its role in human breast cancer is less well documented, with Yang (Yang et al., 2009) claiming that although there is a positive association between BPA and other risk factors, no direct effect was observed on exposure and cancer incidence between 167 cancer and control subjects. In this study, there does seem to be a direct relationship between exposure and cellular responses, particularly the colony assay, where a whole-cell and inter-cell phenotypic response is seen, rather than the individual narrow measures of genetic expression measurements. Taken together, all of the assay modes in this study point towards concerted pro-carcinogenic influences of BPA.

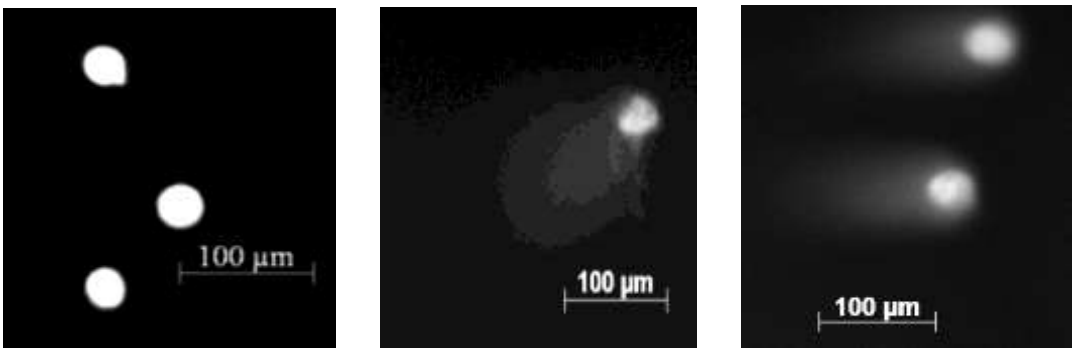
3.5 Aluminium chloride and aluminium chlorohydrate

This study has been published (Farasani and Darbre, 2015). The results demonstrated that exposure to aluminium salts causes DNA damage, as measured using a comet assay, and down regulation of five of the panel of mRNAs regarded as essential for detection and repair of DNA double strand breaks in MCF10A cells. The effect of the aluminium salts on suspension growth of MCF10A cells has already been previously documented (Sappino et al., 2012).

3.5.1 Comet assay of DNA damage after exposure to aluminium chloride and aluminium chlorohydrate

An alkaline single-cell gel electrophoresis (SCGE) technique was employed to detect single strand and labile DNA adducts in the immortalised human breast epithelial cell line MCF10A after exposure to Al chloride and Al chlorohydrate. Cells in the photomicrograph ***Figure 3.61*** panel B & C (Al chloride and Al chlorohydrate respectively) show comet formation after 24 hours exposure.

The bar charts in ***Figure 3.62*** show comet tail length to correlate with percent of cells forming comets (i.e. with damaged DNA) in MCF10A cells. However there is a shifted peak to the dose responses between the compounds, with 10^{-5}M Al chloride producing the longest tails and highest numbers of comets in MCF10A and 10^{-6}M affecting these parameters maximally for the chlorohydrate

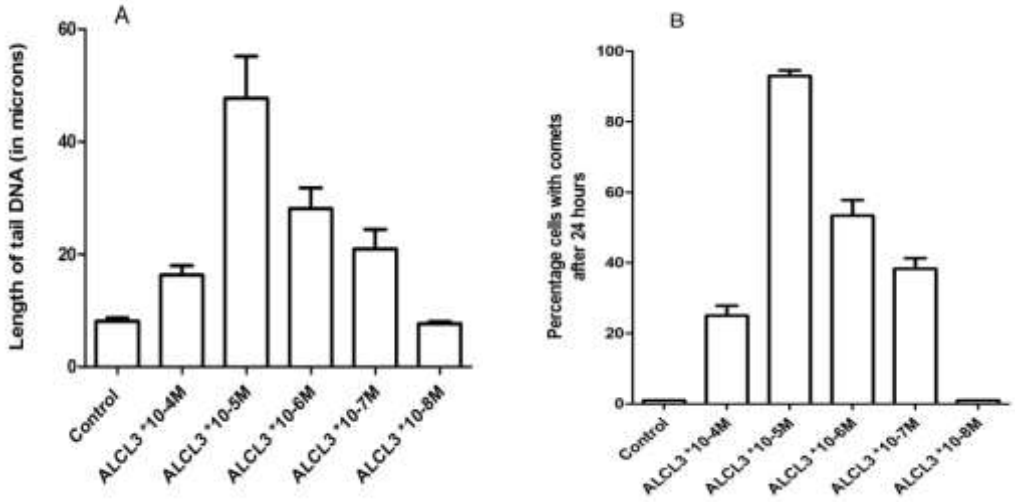


A)-Control B) Al chloride treated 24 hours C: Al chlorohydrate treated 24 hours

FIGURE 3.61 Comet assay for detection of DNA damage in MCF10A cells exposure to Al chloride and Al chlorohydrate.

Panel A is of untreated cells (control) treated for one hour. B) Cells treated with 10^{-5} M Al chloride treated 24 hours. *C) 10^{-5} M* Al chlorohydrate treated 24 hours*. Cells viewed by fluorescence microscopy.*

Al Chloride



AL chlorohydrate

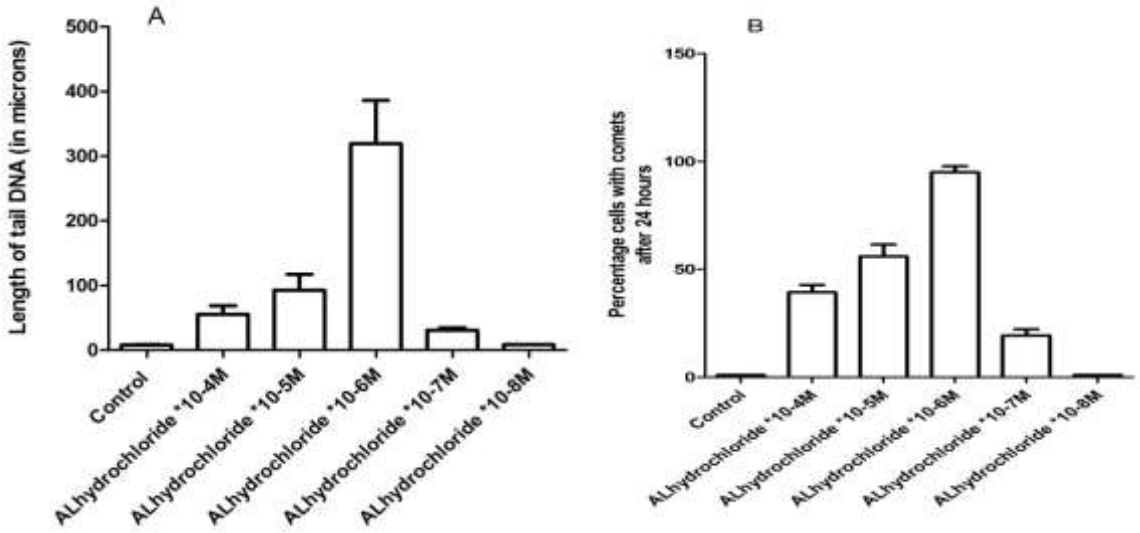


FIGURE 3.62 Effect of Al chloride and Al-chlorohydrate on DNA damage in MCF10A human breast epithelial cells as assessed by a comet assay.

Cells were grown in stock medium and treated one hour with untreated cells (control).Treated cells with 10^{-4}M Al chloride and Al chlorohydrate. Treated cells with 10^{-5}M Al chloride and Al chlorohydrate, 10^{-6}M Al chloride and Al chlorohydrate,10^{-7}M Al chloride and Al chlorohydrate, 10^{-8}M Al chloride and Al chlorohydrate. Error bars represent standard deviation of 50 comets scored.

3.5.2 Aluminium: Effect of mRNA expression of DNA repair genes:

A time period of 19–21 weeks was chosen in line with previously published gene expression studies on Al in human breast cells and mRNA/protein samples were harvested from independent cell cultures after 19, 20 and 21 weeks to ensure biological replicates. The concentration of 10−4 M Al was chosen for study as the highest concentration of Al which had previously been shown to have no detrimental effect on proliferation of human breast cells in the long term (Darbre, 2005a).

Levels of BRCA1 mRNA after exposure to Al chloride and Al chlorohydrate

Long term (19-21 weeks) effects of exposing MCF10A cells (Figure 3.61) to 10−4 M concentration of Al chloride and Al chlorohydrate in terms of levels of mRNA for BRCA1, was investigated using RT-PCR. Highly ($p<0.001$) significant reductions were found after long term (19-21 weeks) exposure to Al chloride and Al chlorohydrate in MCF10A cells.

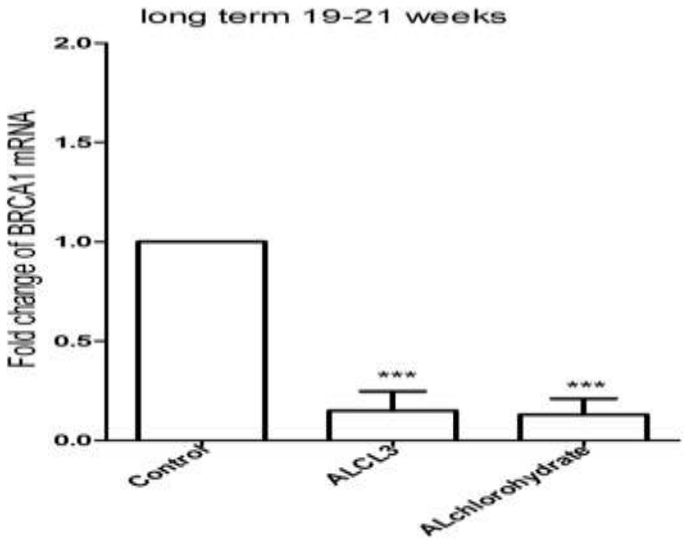


FIGURE 3.63 Real-time RT- PCR analysis of BRCA1 mRNA in MCF10A cells following long term (19-21 weeks) exposure to Al chloride and Al-chlorohydrate.

Cells were grown in stock medium for 19-21 weeks with no addition (control) or in the presence of 10-4M Al chloride and 10-4M Al chlorohydrate. The relative expression of BRCA1 mRNA was normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values. ***** indicates p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.**

Levels of ATR, BRCA2, CHK1, CHK2 and Rad51 mRNA after exposure to Al chloride and Al chlorohydrate

Long term (19-21 weeks) effects on MCF10A cells (Figure 3.64) of exposure to 10−4 M concentrations of Al chloride and Al chlorohydrate in MCF10A cells was then investigated on a panel of other mRNAs. Al chloride gave a significant reduction in expression of BRCA2, CHK2, Rad51 and ATR mRNA. The long-term effect of exposure to Al chlorohydrate showed a significant reduction in expression of BRCA2, CHK1, CHK2, Rad51 and ATR mRNA.

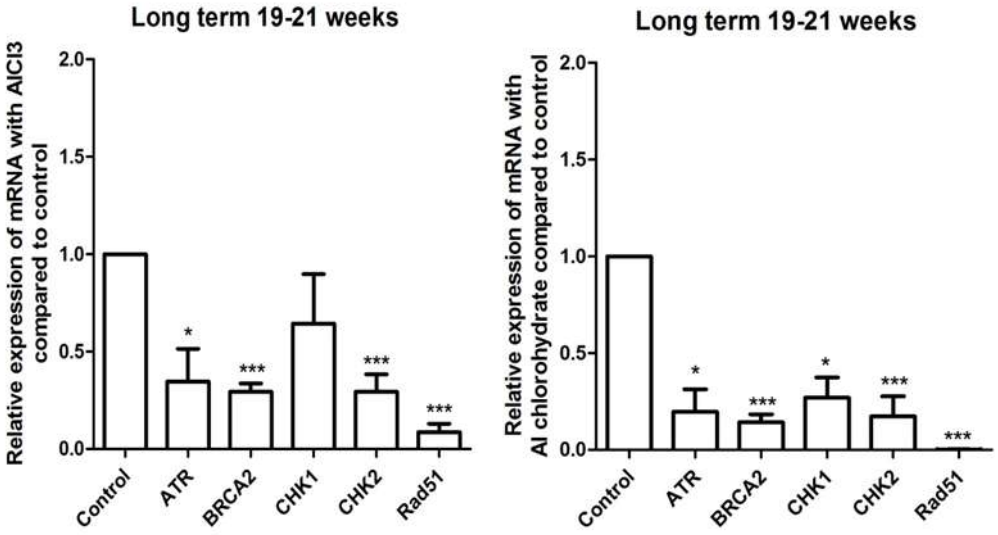


FIGURE 3.64 Real-time RT- PCR analysis of BRCA2, CHK1, CHK2, Rad51 and ATR mRNA in MCF10A following long term (19-21 weeks) exposure to Al chloride and Al chlorohydrate.

Cells were grown in stock medium for 19-21 weeks with no addition (control) or in the presence of 10-4M Al chloride and 10-4M Al chlorohydrate. The relative expression of mRNAs were normalised to that of the endogenous control β-actin mRNA, and average values calculated for triplicate technical replicates. Results are shown ± standard error for biological triplicate values. Results are shown ± standard error for biological triplicate values.

***indicates p< 0.05 *** p< 0.001 compared to control by one way ANOVA with post-hoc Dunnett test.**

3.5.3 Immunoblotting for BRCA1 protein after exposure of cells to Al

The effect of long term exposure to Al chloride and Al chlorohydrate on expression of BRCA1 protein using western Immunoblotting in MCF10A cells is shown in Figure 3.65. Both agents reduce expression, the chloride result ($p<0.05$) less strongly significant than the chlorohydrate ($p<0.01$). This is evident visually in the photograph (A) as well as quantitated the histogram (B). The higher level of significance in the chlorohydrate result is also consistent with a greater drop in expression**.**

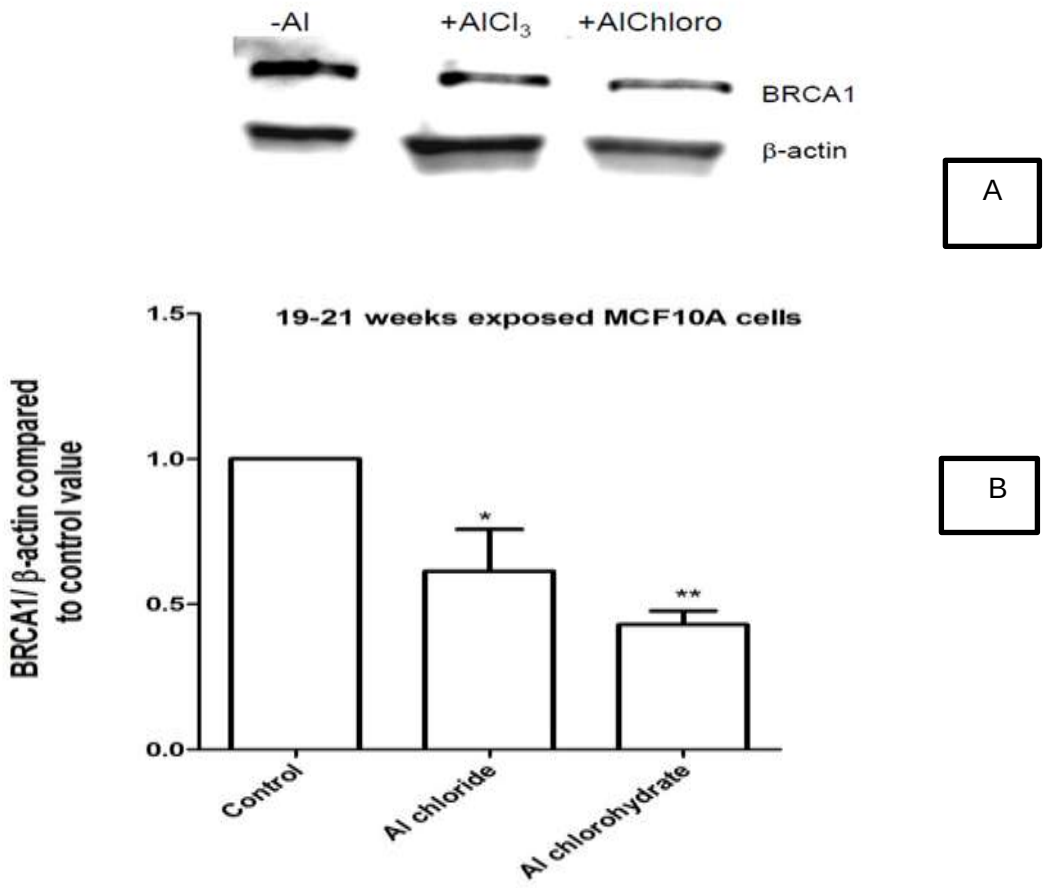


FIGURE 3.65 Level of BRCA1 protein in Al chloride and Al chlorohydrate treated MCF10A cells using western Immunoblotting.

BRCA1 protein levels were normalised to β-actin using BioRad stain-free technology. BRCA1 protein levels in MCF10A human breast epithelial cells were observed after 19-21 weeks of no addition (control) or in the presence of 10-4M Al chloride and 10-4 M Al chlorohydrate compared to control. *** indicates p< 0.05** p< 0.01 and compared to control by one way ANOVA with post-hoc Dunnett test.**

3.5.4 Summary of results using Aluminium salts

These results do not include the anchorage independent growth assay. The comet assay for DNA damage and the effects of exposure on mRNA and protein expression in a panel of DNA repair related genes indicate, as with the other cosmetic constituents tested, an ability to compromise cellular integrity and drive cells towards an immortal growth pattern.

TABLE 3-5 Summary of Aluminium results

ASSAY	MCF10A Al chloride	MCF10A Al Chlorohydrate
Comet assay	Both tail length and comet numbers peak at 10^{-5}M	Both tail length and comet numbers peak at 10^{-6}M
BRCA1 mRNA & protein	Expression reduced $p<0.05$	Expression reduced $p<0.01$
Other DNA repair mRNAs	mRNA drops markedly for all genes tested: ATR, BRCA2, CHK2, RAD51, Not CHK1	mRNA drops markedly for all genes tested: ATR, BRCA2, CHK1, CHK2, RAD51

3.5.5 Discussion of results of exposure to aluminium

3.5.5.1 The present study

The results demonstrate that non-transformed human breast epithelial cells exposed to Al chloride and Al chlorohydrate, can cause comet formation, i.e. DNA fragmentation and also reduces expression of mRNAs encoding DNA repair proteins. Both BRCA1 mRNA and BRCA1 protein were reduced, but only after long-term exposure to the Al chloride and Al chlorohydrate. The effect of the aluminium salts on suspension growth of MCF10A cells has already been previously documented (Sappino et al., 2012). An overall summary of the results is given in **Table 3-5**. The main differences between the two salts lies in the comet and BRCA1 gene assays, as indicated in detail below.

3.5.5.2 DNA damage assessed by comet assay

In the present study only the MCF10A cell line was assessed. Aluminium formulations produced visually classical comets. Both Al chloride and Al chlorohydrate induced comets. Cells were more sensitive to the chlorohydrate than the chloride in this assay, here being an order of magnitude between peak result concentrations (10^{-5}M for the chloride, 10^{-6}M for chlorohydrate. This adds weight to the previous report demonstrating that Al chloride could cause double strand breaks in the DNA of MCF10A cells (Sappino et al 2012).

3.5.5.3 DNA repair gene mRNA and protein product expression

Exposure of MCF10A cells to both forms of aluminium results in a reduction of intracellular levels of BRCA1 mRNA and of BRCA1 protein, changes that would work towards impaired DNA repair. Apart from BRCA1, all mRNAs measured in the 5-member repair-gene panel tested were significantly reduced by Al chlorohydrate after a 20 week exposure period. Incubating with Al chloride, only CHK1 was not significantly reduced. In the present study exposure of MCF-10A cells results in a reduction of intracellular levels of BRCA1 mRNA and of expressed protein, changes that would work towards impaired DNA repair. Results were similar with other members of the repair-gene panel over a long exposure period. Only CHK1 was not significantly reduced after exposure to aluminium chloride; the chlorohydrate results were all significantly reduced and to a greater extent. The results add weight to the concept of aluminium as present in antiperspirants may adversely affect cancer risks through impairing repair of DNA damage.

3.5.5.4 Physiological relevance of these studies

Despite the limited and apparently high concentrations of Al used, the Al concentration (10−4 M) used in these experiments is not inconsistent with measurements of Al in human

breast tissues, which can also be high, Levels of Al in ranging from 4–437 nmol/g dry weight of tissue (Exley et al, 2007). Assuming this is about 20% of wet weight and the relative density to be not far from unity these levels calculate out to 0.80×10^{-6} M–0.87×10^{-4} M. Nipple aspirate fluids have been recorded ranging from 150–520 µg/l (mean 268.4 ± 28.1 µg/l) (House et al, 2013) i.e. $0.56–1.93 \times 10^{-5}$ M. The concentrations of Al used in this study can therefore be seen in clinical situations.

The impact of aluminium on the breast microenvironment is likely to play an increased role in populations both living longer and using more cosmetics over a long period of time. However, so far only one out of three epidemiological studies has shown a link between aluminium and breast cancer (McGrath, 2003), with incidence of disease occurring at an earlier age with greater use. The negative studies are those of Mirick (Mirick et al., 2002) and Fakri (Fakri et al., 2006). The impact of aluminium in combination with other mutagens &/or endocrine disruptors has yet to be investigated in terms of the lowest observed effect concentrations (LOEC) or the equivalent parameter from no observed effect (NOEC).
There is however more aluminium found in breast tissue than in blood (Darbre, 2016), which suggests accumulation or a means of delivery other than through the circulation.
The effects of aluminium in combination with other mutagens is identified as an important topic for future research (Darbre 2015).

Chapter 4 General Discussion

This project reports experimental results demonstrating the ability of the selected potentially genotoxic compounds to influence genomic instability through DNA damaging activity and/or through compromising DNA repair processes. Many environmental compounds which can enter human breast tissue have been characterised as possessing activity which can drive the hallmark of sustained proliferative signalling in oestrogen responsive breast epithelial cells (Darbre, 2015). However, these results demonstrate that the compounds tested here (cyclosiloxanes, Lilial, triclosan and bisphenol A and aluminium salts) can also influence genomic instability, which is an enabling characteristic underpinning the development of the hallmarks of cancer (Hanahan and Weinberg, 2011).

Transforming properties of each of the endocrine disrupting chemicals were studied using the MCF10A and MCF10F cell lines by their ability to enable anchorage-independent growth of these non-transformed human breast epithelial cells. All three of the cyclosiloxanes, Lilial, triclosan and bisphenol A were found to induce dose-dependent increase in colony formation in semi-solid methocel suspension culture. Aluminium salts were not tested for this property because they had already been reported to induce growth of MCF10A cells in suspension culture (Sappino et al., 2012) In line with previously published data (Russo and Russo, 2006), exposure to 70nM 17*β*-oestradiol induced colony formation in each experiment. The ability of non-transformed epithelial cells to grow under anchorage-independent conditions has long been acknowledged as an in vitro property which correlates with tumour growth in vivo (Shin et al, 1975). Use of the MCF10A and MCF10F immortalised cell lines has been validated by Russo and Russo as an assay showing that exposure to high concentrations (70nM) of *17β*-oestradiol can induce transforming properties in these cells (Russo et al., 2006). Therefore, it can be concluded that exposure to these compounds can also induce a transformed phenotype in human breast epithelial cells.

Comet assays did demonstrate DNA damage following exposure to some of these compounds but the assay gave less clear-cut results, with comets not always being of a classical appearance and with disparity between tail length and percent of comet producing cells. Exposure to 10^{-5}M DES, which was used as a positive control, gave comets each time is line with previously published data (Anderson et al., 1998). However, the contrast between genotoxically stimulated and totally unstimulated cell populations is clear in that

exposure to such materials invariably resulted in enhanced comet formation. Tail length and proportions of comet-forming cells gave opposite dose responses to cyclosiloxanes, tails becoming more common but shorter with increasing concentration of agent. This pattern was also obtained with triclosan, but Lilial gave increasing tail length and comet numbers the positive association extending over the entire concentration range. These results presented here can be interpreted as variations in sensitivity between chemicals, illustrated well with the two aluminium formulations, where both gave a peaked dose response curve for comet assays, but the chlorohydrate one was shifted to the right – i.e. the sensitivity of the cells was lower to this compound.

RTPCR assays demonstrated that exposure to these compounds did result in alterations to mRNA levels of several DNA repair proteins. Importantly, effects after one week (short term) were often not the same as effects observed after 30 weeks (longer term) exposure. Exposure for one week did not give consistent and significant changes in mRNA from the panel of 14 DNA repair genes. However, in MCF10A BRCA1 mRNA expression was raised after one week, but lowered by 30 weeks of exposure to D3 and D4 relative to untreated cells. This could be interpreted as an initial attempt to correct damage, eventually the effort being withdrawn when damage limitation became impossible. Indeed, the reduction in BRCA1 mRNA was paralleled by loss of the BRCA1 protein. After long-term exposure, levels of several of the other members of the panel of mRNAs were reduced. If loss of the other mRNAs were found to also be paralleled by loss of the relevant protein, then this could imply reduced capacity within the cell for DNA repair which would lead to genomic instability. This loss of DNA repair capability, coupled with actual DNA damage visualised in the comet assays after prolonged exposure to these cosmetic chemicals is the feature of these results with greatest practical impact potential (Maqbool 2016).

Inheritance of loss of function of the BRCA1 gene is a well-established risk factor for susceptibility to breast cancer development (Betancourt et al., 2012). The BRCA1 gene was identified, and cloned in 1994 (Lalle et al., 1994) based on its linkage to early onset breast cancer and to familial multi-cancer syndromes that included breast cancer. It is rarely mutated in sporadic breast cancer (Rosen et al., 2003). It transpired to be recessive and therefore acting as a “tumour suppressor gene”, pathology being activated by loss of heterozygosity. The normal physiological significance of the gene transpired to be as a key factor in DNA repair, acting to prevent DNA polymerase stalling and facilitate chromatid recombination (Scully, 2000). This is a first report that BRCA1 gene expression can also be reduced following exposure to environmental chemicals which are used routinely in personal care products and to bisphenol A which is used widely in plastic products.

Work using the alkyl esters of *p*- hydroxybenzoic acid (parabens) has demonstrated that assessment of functional significance requires consideration of combined effects where multiple forms are present simultaneously. Barr et al (2012) detected five paraben esters in 60% of human breast tissues and whilst some parabens might be present individually at concentrations sufficient to stimulate proliferation of human breast cancer cells *in vitro*, parabens in other tissues reached functionally significant levels for proliferation only when all five esters were combined (Charles and Darbre, 2015). It is therefore possible that similar additive effects might occur for genotoxic end points if mutagenic compounds are co-located at lower concentrations than those needed for each to act individually. The main unknown element here remains as to how much of each of these compounds tested in this thesis are actually present in human breast tissue. Future studies are needed to measure concentrations of cyclosiloxanes, Lilial and triclosan in human breast tissue in order to inform future in *vitro* assay work.

In the present study, the effects of triclosan are consistent with those of the cyclosiloxanes, particularly D4 & D5 and with Lilial, showing similar dose responsiveness in tests for anchorage independent colony formation, DNA clastogenicity and suppression of DNA repair mechanism genes by both RT PCR and Western blotting techniques. Since the MCF10A/10F cells are non- transformed it would be interesting to repeat these studies in cancer cells, such as the MCF7 human breast cancer cell line. Although MCF7 cells grow in suspension culture and have a non –diploid genotype, it would be interesting to know whether DNA repair system can be compromised by these elements in cancer cells as well.

Overall, the study presented here demonstrates that the environmental genotoxic compounds tested modified gene and protein expression as well as behavioural characteristics of individual near-normal cells in monoculture models in a manner that is highly suggestive of a role for these compounds in breast cancer aetiology and progression. The experiments presented here do not prove carcinogenicity or tumour promoting properties, but are strongly suggestive of either or both of these actions if the effects translate into a whole-body situation. The chemicals studied here have been detected in the environment and have been shown possess endocrine disruption properties. However less is known about genotoxic properties.

Serious controversy arises when human activity and preferences are involved. At one level, there is also little doubt that breast cancer has some hormone dependence, it may regress on ovariectomy in premenopausal women (Ingle et al., 1986). However, the epidemiological impact of endocrine disrupting chemicals, including those of underarm cosmetic preparations, in the development of breast cancer is still controversial after a decade of study. A World Health Organisation report in 2013 authored by Bergman et al was cautiously supportive of there being a pathogenic effect, trending in parallel with exposure (WHO-UNEP, 2013). This drew harsh criticism from a large group of stakeholders (Lamb et al., 2014), sufficiently strident to attract an equally strongly worded rebuttal, describing Lamb's critique as "industry sponsored", from a group of authors headed by Bergman (Bergman et al., 2015).

A more academically oriented organisation, the Endocrine Society, published a 'scientific statement' in its journal ***Endocrine Reviews,*** where Diamante-Kandrakis (http://press.endocrine.org/edc) advocates a number of measures "invoking the precautionary principle" and the involvement of individual and scientific stakeholders to raise public awareness and implement changes in policy. This principle is discussed positively by Resnik and Kriebel (Resnik, 2004, Kriebel et al., 2001) but criticised by Peterson (Peterson, 2006). It is a principle that, if agreed upon, would apply to the projection of the results from this project, drive further lines of research and reach out to link with regulatory bodies and processes.

In Europe, the Danish Environmental project No. 1531 (2014) and the European Commission's Scientific committee on Consumer Safety (SCCS) have produced reports covering products including the cyclosiloxanes. The former concluded that "No human data regarding carcinogenic effects following exposure to D3, D4, D5 and D6 have been located". The SCCS (SCCS_1241_10) held a 3rd round-table meeting in May 2015 to evaluate progress in their investigations; this timing illustrates the complexity in achieving a consensus. There is a need to involve heads of academic faculties and research laboratories more widely and openly in discussing the way in which biochemistry and biology at levels through *in vitro* and animal models can inform the likely relevance of these absorbable chemicals. Co-operation with industry can touch on carcinogenic or cancer promoting effects and to promote rational research into the development of less harmful alternatives.

There are consistent risk/benefit balances to be struck and apparent paradoxes to solve in the exposure to compounds for legitimate purposes that might however have unintended consequences. For instance regarding antimicrobials and preservatives, while the present

study highlights risks to the general population from triclosan exposure, Sadowski (2016) suggests triclosan as a potential prostate cancer t**herapy,** due to its ability to inhibit fatty acid synthase. It is no coincidence that most cancer chemotherapeutics are toxic. They are used specifically because they kill or disable cells analyses are necessary in these circumstances.

All of the compounds tested here on the near-normal (immortalized) MCF10 breast cancer epithelial cell lines showed positive results in a phenotypic assay of anchorage independent 3-dimensional colony formation range of genotoxicity tests, including characteristics, DNA fragmentation and DNA repair gene function. It is therefore appropriate that these results should now be incorporated into risk assessment of long –term exposure to personal care products. Previous studies have tended to report only short effect over few days, but these provided a new relevance to long term exposure of the human breast to these chemicals. *In vitro* studies are always limited by the lack of physiological interaction between organ systems. Monoculture models, as used here, suffer the additional disadvantage of lacking contact based or paracrine interaction between cell types. However, monoculture models using established cell lines are useful due to the reproducibility of results and by lending themselves to high throughput assay systems. Future studies are needed for measurements in breast tissue.

The MCF-10A & 10F cells complement the earlier studies performed in Reading and elsewhere on the cancer-derived cell line MCF-7. Research programmes should in the future increasingly diversify from their origins in exogenous hormone mimicking or disruptive activity towards passage of chemicals through basal membrane constructs and to study how these chemicals gain access to cells, can be increasingly removed through efflux pumps or. Models including mesodermal cells such as fibroblasts, which can construct stromal elements around epithelial clusters, might also be informative.

They indicate potential for carcinogenesis rather than progression. It is appropriate to start at the simplistic end of the experimental hierarchy and work upwards. A further cell line exists in the MCF- series – MCF-12A. It is a spontaneously immortalised **normal** breast epithelial cell line (https://**www.atcc.org**/~/ps/CRL-10782.ashx), whereas, although non-tumorigenic, the MCF-10 cells originated from a 36 year old breast with fibrocystic disease. Spontaneous immortalization is where a primary culture goes through a “crisis” in it propagation; most cells die (Hayflick, 1984, Hayflick, 2000) but some adapt by expressing telomerase into immortality. Spontaneous immortalization entirely generated *in vitro* might give different response patterns than cells derived from breast tissue where proliferative

pathologies already exist and there is locally abnormal interaction with mesenchymal stroma producing cells.

Further experiments going up the experimental model hierarchy might involve co-culture with mesenchymal type cells such as fibroblasts. These could be subsequently cleared from the cultures using a selective medium containing D-valine (which fibroblasts are reported not to tolerate (Gilbert and Migeon, 1975) . Further up the scale, *in vivo* models using xenotransplantation of cells that will form tumours in immunodeprived ('nude' or 'scid' (Pantelouris, 1971, Bosma and Carroll, 1991)) mice or studies of mammary development in neonatal rodents after treatment of their parents, might be attempted. Human studies might make use of tissue arrays for high throughput gene or protein expression. Ultimately, epidemiological studies, although very hard to control, might with sufficient numbers of participants, provide answers that will be of use to regulatory authorities in determining safe usage of these compounds or banning them outright. In that event, replacement compounds will be sought and developed and the whole investigatory cycle repeated.

In conclusion, despite their limitation these studies provide evidence that these cosmetic chemicals can enable non- transformed cells to change towards a transformed phenotype with genomic instability. This raises the need for further research into their effects in combination at physiologically relevant doses and in animal models. These results should also be useful for including into future risk assessments.

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