The Effects of Endosulfan on Human MCF-7 Breast Cancer Cells

by

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Abstract

Organochlorine pesticides (OCs) are environmental toxicants with important links to human health. They have been found to activate signalling pathways within cells and thereby affect cell survival and proliferation. Receptor Activator of Nuclear Factor kB (RANK) ligand and its receptor RANK are crucial for mammary epithelial proliferation in pregnancy and have recently been linked to hormone induced breast cancers. The objectives of this study were to confirm the proliferative effects of an OC (endosulfan) on human MCF-7 breast cancer cells, identify activated intracellular signaling pathways and investigate changes in RANK and RANKL gene expression. This study showed that endosulfan has a stimulatory effect on human MCF-7 cell proliferation, which may be invoked through activated intracellular signaling pathways (JNK, ERK1/2 and p38). In addition, there was a down regulation of RANK and upregulation of RANKL gene expression suggesting endosulfan is capable of modulating both cellular behavior and gene expression.

Keywords: Endosulfan, cell signaling, RANKL, breast cancer, proliferation

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List of Abbreviations

AP - Activator Protein

ATCC – American Type Culture Collection

DMSO – Dimethyl Sulfoxide

ECL – Enhanced Chemiluminescent

EDTA – Ethylenediaminetetraacetic Acid

MEM – Minimal Essential Media

ER – Estrogen Receptor

ERK - Extracellular Signal-Related Kinases

FBS – Fetal Bovine Serum

GAPDH – Glyceraldehyde 3-Phosphatase Dehydrogenase

IKK - Inhibitor of Kappa B Kinase

IL - Inerleukin

JNK - Jun Amino-Terminal Kinase

MAPK – Mitogen-Activated Protein Kinase

MPA - Medroxyprogesterone Acetate

NF-κB – Nuclear Factor Kappa B

OC - Organochlorine

OPG – Osteoprotegerin

p/s – Penicillin/Streptomycin

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PR – Progesterone Receptor

PVDF – Polyvinylidene Fluoride

RANK – Receptor Activator of Nuclear Factor Kappa B

RANKL – Receptor Activator of Nuclear Factor Kappa B ligand

SDS – Sodium Dodecyl Sulphate

STAT – Signal Transducer and Activator of Transcription

TGF – Transforming Growth Factor

TNF - tumor necrosis factor

1. – Introduction

1.1. - Organochlorine pesticides

1.1.1. - Organochlorines

Organochlorine (OC) pesticides are organic compounds known to cause adverse effects to human health and the environment. Organochlorines are cyclic hydrocarbon compounds that contain between one and ten carbon atoms covalently bonded to a chlorine atom (Turusov *et al*., 2002). They exhibit low water solubility, high lipid solubility, remain persistent in the environment and have a high bioaccumulative capacity (Coats 1990). OC pesticides demonstrate weak estrogenic or antiestrogenic effects. They have been found to alter normal cellular behaviour by mimicking a hormone, blocking the binding of endogenous hormones or modifying the synthesis, metabolism, or transport of hormones (Soto *et al*., 1995). OC compounds may act as a tumor promoter through hormone-mediated effects (Hansen and Matsumura, 2001) and have been linked with several hormone-related cancers including breast cancer (Calle *et al*., 2002). Due to the nature of persistence in the environment, bioaccumulation in the food chain, and possible human health effects, the use of most of OC pesticides were banned during the 1970s and 1980s. Despite the ban of these chemicals a few decades ago in several countries, measurable amounts of OC pesticides or their metabolites can still be found in human tissues (Cerrillo *et al*., 2005). Also, OC pesticides continue to be heavily used in some developing countries (Turusov *et al*., 2002). Therefore, the health effects of OC exposure remain an important global public health concern.

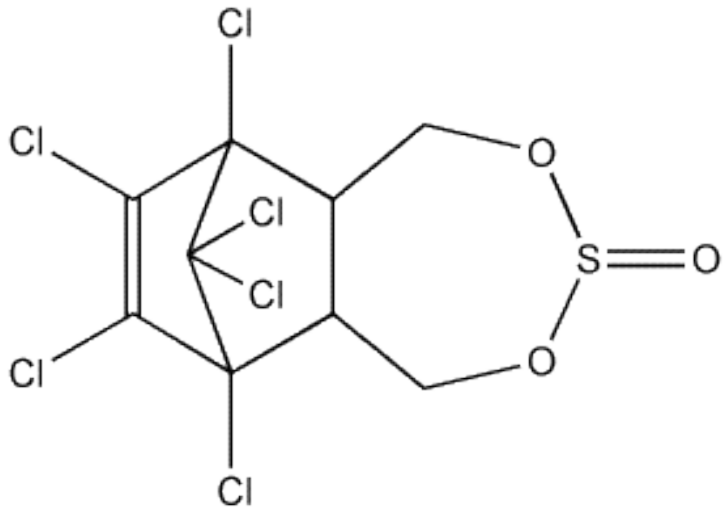


Figure 1: The structure of endosulfan (Ledirac *et al*., 2005)

1.1.2. - Endosulfan description and use

These pesticides can be divided into four groups: dichlorodiphenylethanes, cyclodienes, chlorinated benzenes and cyclohexanes. Endosulfan is a cyclodiene insecticide widely used throughout the world on crops like cotton, corn, coffee, tea and vegetables (Kidd and James, 1991) (Figure 1). It exists in two forms: alpha endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,3,4-benzodioxathiepin-3-oxide) and beta endosulfan (6,9-methano-2,4,3-benzodioxathiepin-6,7,8,9,10,10-hexachloro-1,5,5,6,9,9-hexahydro-3-oxide (Kidd and James, 1991). Endosulfan is applied as a mixture of alpha (α) and beta (β) isomers in the ratios 2:1 to

7:3 in agriculture (Wan *et al*., 2005a). Endosulfan (α) and (β) are primarily converted into the diol form in water and the sulfate form in soil and sediment. The endosulfan sulfate and diol further break down to the ether, hydroxyl ether, lactone, and alcohol forms (Hose *et al*., 2003). Endosulfan sulfate is the breakdown product considered to be as toxic as the parent isomers (Leonard *et al*., 2001). The half-lives of endosulfan (α) and (β) in water are on the order of days to months, whereas endosulfan sulfate is on the order of weeks to years (Leonard *et al*., 2001; Wan *et al*., 2005a).

1.1.3. - Endosulfan is persistent and bioaccumulates in the environment

Endosulfan is a ubiquitous environmental contaminant and persistent to degradation processes in the environment. Environmental endosulfan contamination is widespread and has been found in soil, ground and surface waters, marine sediments, air, rainfall, snow, and plants (Kumari *et al*., 2007; Tuduri *et al*., 2006; Herbert *et al*., 2004; Schulz *et al*., 2004; Tariq *et al*., 2004). Heaviest use of endosulfan was found in southern and western portions of Ontario including Lambton, Kent, Elgin, Haldimand-Norfolk, Niagara, Durham, York, and Simcoe counties (Harris *et al*., 2000). It reaches surface waters as a result of direct deposition and runoff from agricultural use. Aquatic animals periodically suffer from the lethal concentrations of endosulfan ranging from <0.01 mg/L to 0.54 mg/L in surface waters in southern Ontario (Harris *et al*., 2000). Endosulfan was found in both terrestrial and aquatic species in Greenland with significant bioaccumulation of endosulfan in tissue and biomagnification in wolfish and caplin (Vorkamp *et al*., 2007). The chemical is considered semi-volatile since 70% of endosulfan is able to volatilise within 2 days (Sutherland *et al*., 2004). Endosulfan is also

a ubiquitous air contaminant and regional atmospheric transport is prevalent within and into Ontario (Johnson *et al*., 1988). Both endosulfan (α) and (β) were detected in precipitation in northern portions of Lakes Ontario, Erie and Superior (Chan *et al*., 1994; Johnson *et al*., 1988). As well, high concentrations levels (5.71 ng/m^3) of endosulfan have been identified in Canada including Great Lakes Basin, the City of Toronto, the Fraser Valley of British Columbia, Prince Edward Island, Quebec, Ontario and Saskatchewan in 2003 (Yao *et al*., 2006).

Several European countries have banned the use of endosulfan and discontinuation of the use of endosulfan took effect in January 2011 in Canada (Health Canada). Despite the discontinuation of the use of endosulfan in Canada, it still remains a serious environmental concern due to its persistence and slow degradation processes in the environment. Endosulfan is one of the main causes of poisoning in humans in many countries (Kishi *et al*., 2002; Oktay *et al*., 2003; Roberts *et al*., 2004; Wesseling *et al*., 2005). The highest concentration of endosulfan (α) and (β) was found in female adipose tissue with a concentration of 17.72 ng/g lipid (Cerrillo *et al*., 2005, 2006; Ibarluzea *et al*., 2004; Hernandez *et al*., 2002), followed by breast milk with the concentration of 11.38 mg/ml (Cerrillo *et al*., 2005; Campoy *et al*., 2001); placenta at 7.74ng/mL (Cerrillo *et al*., 2005); umbilical cord blood at 6.11ng/mL (Cerrillo *et al*., 2005); and blood serum at 7.74 ng/mL (Vidal *et al*., 2002; Arrebola *et al*., 2001). Endosulfan diol was found in placenta with a mean concentration of 12.56 ng/mL and in blood at 13.23 ng/mL (Cerrillo *et al*., 2005). Endosulfan sulfate was found in placenta with a mean concentration of 3.57ng/mL and in umbilical cord at 2.82 ng/mL (Cerrillo *et al*., 2005). This indicates that endosulfan can be easily transferred to the fetus and newly-born infant. The mean

concentration of endosulfan (α) was found to be 2.44 ng/mL in cord serum and the mean concentration of endosulfan (β) was found to be 2.83 ng/mL in humans (Mariscal-Arcas *et al*., 2010). Although aquatic organisms near agricultural areas are known to be exposed to the highest levels of endosulfan, the primary source of exposure to OC pesticides in the human population is by ingestion (from eating contaminated food), inhalation, absorption through skin, and through pest control operations both at home and in resort areas (Wan *et al*., 2005b; Hose *et al*., 2003; Toppari *et al*. 1996; Naqvi and Vaishnavi, 1993). Other sources of exposure include dust, air, and soil.

1.1.4. - Endosulfan is highly toxic

Endosulfan (α) and (β) are both considered to be highly toxic. Doses as low as 35 mg/kg has been documented to cause death in humans (Abalis *et al*., 1986). The oral LD_{50} of endosulfan in rats was found to be 80 mg/kg, whereas the fish LC_{50} found in laboratory studies was 1 to 100 μg/L (Hose *et al*., 2003; Jonsson and Toledo, 1993). Endosulfan acts on the central nervous system as a non-competitive GABA antagonist causing inhibition of the gamma-aminobutyric acid (GABA)-gated chloride channels and uncontrolled excitation (Jia and Misra, 2007a). After exposure, acute symptoms such as hyperactivity, erratic swimming, and convulsions have been observed in adult fish (Jonsson and Toledo, 1993). It has also been hypothesized that endosulfan(α) causes inhibition of Ca+Mg-ATPase and Ca-ATPase activity (Coats, 1990; Srikanth *et al*., 1989). Endosulfan targets the prefrontal cortex of the brain altering levels of the neurotransmitters dopamine, noradrenalin and serotonin and causing disruptions in cognitive functions and memory (Cabaleiro *et al*., 2008). Endosulfan is extremely toxic to

fish, especially to juveniles (Dutta and Arends, 2003). It is particularly neurotoxic during the developmental stages in fish and rodents causing alterations in neurotransmitter and amino acid measurements and making organisms more vulnerable to the adverse effects of chemicals (Cabaleiro *et al*., 2008; Rosenthal, 1976). Of direct relevance to human health, evidence suggests that endosulfan may even increase the risk of Parkinson's disease (Wang *et al* 2006a; Jia and Misra 2007b).

Endosulfan induces immunotoxic and immunosuppressive effects in fish, rodents and humans. It causes suppression of phagocytic function of the immune cells of Australian freshwater fish (Harford *et al*., 2005). Female rats exposed to endosulfan *in utero* and through postnatal lactation displayed immunosuppression (Lafuente *et al*., 2006). It also induces the apoptosis of human T-cells (Kannan *et al*., 2000).

1.1.5. - Endosulfan causes reproductive and developmental defects

Chronic neural damage may result in abnormal hormonal release, defects in synthesis and feedback mechanisms and even impaired gonadal development and other reproductive dysfunctions (Pandey 1988; Haider and Inbaraj 1988; Matthiessen *et al*., 1984). Exposure to endosulfan during developmental phases has been shown to decrease fertility, reduce sperm count, cause degeneration of seminiferous tubule epithelium, increase abnormal sperm, and aspermatogenesis in rats (Saiyed *et al*., 2003; Sinha *et al*., 2001; Dalsenter *et al*., 1999). Adverse reproductive outcomes such as reduced male fertility, neonatal deaths and congenital birth defects were also found in humans exposed to endosulfan (Rupa *et al*., 1991). Increased incidences of cryptorchidism have been observed in populations exposed to endosulfan (Ferrando *et al*., 1992). Reproductive

dysfunctions were also evident in fish due to bioaccumulation of endosulfan. The transgenerational effect was shown when fish eggs exposed to sublethal concentrations of endosulfan were delayed in hatching, had reduced hatchability and and upon reaching maturity, produced fewer eggs which took longer to hatch (Gormley and Teather, 2003). The transgenerational transfer of endosulfan was also shown when fertility and hatch rates were found to be significantly reduced in two subsequent generations of rainbow trout after parental exposure to endosulfan (Holdway *et al*., 2008).

1.1.6. - Endosulfan is an endocrine disruptor

Endosulfan has adverse endocrine-disrupting effects on humans (Scippo *et al*., 2004). It has been found to evoke estrogenic responses in human breast cells by mimicking estrogen (Li *et al*., 2006) and interfering with the normal levels of estrogen receptors (Grunfeld & Bonefeld-Jorgensen 2004; Soto *et al*., 1995, 1994). Endosulfan is comprised of lipophillic phenolic rings and other hydrophobic components also found in steroid hormones necessary to activate the estrogen receptor (Watson *et al*., 2007). It has been shown to cause proliferation, transformation, differentiation and migration of human estrogen-sensitive breast cancer cells (Li *et al*., 2006; Bonefeld-Jorgensen *et al*., 2005; Soto *et al*., 1994; 1995), and estrogen-sensitive ovarian cells (Wong and Matsumara, 2006). The simultaneous exposure of breast cancer cells to endosulfan and growth factors has resulted in cell growth (Cossette *et al*., 2002). Lemaire *et al.* (2006) proposed that endosulfan activates estrogen receptor (ER) α but weakly antagonises ERβ. This further enhances the proliferative effects of endosulfan because activation of ERβ is involved in attenuating cell proliferation. In addition to mimicking estrogenic responses, endosulfan

has also been found to be anti-androgenic (Chatterjee *et al*., 2008; Kojima *et al*., 2004; Andersen *et al*., 2002). It induces the activation and proliferation of progesterone receptors in human breast cancer cells (Hunter *et al*., 1999; Soto *et al*., 1995).

1.1.7. - The role of endosulfan in cancer

Exposure to endosulfan at very low concentrations may play a significant role in breast biology and breast cancer by causing alterations in hormonal mechanisms. Laville *et al.* (2006) found that endosulfan at low concentrations activates aromatase (an enzyme for catalyzing androgens into estrogen) in cancer cells further enhancing the estrogenic effect. MCF-7 breast cancer cells treated with endosulfan displayed significant increase in the ratio of 16-hydroxyestrone (a tumour promoting oestrogen) to 2-hydroxyestrone (non-genotoxic), amplifying cell proliferation (Bradlow *et al*., 1995). Endosulfan mimicks estrogen by building up cellular calcium levels, causing rapid secretion of prolactin and increasing cell proliferation (Watson *et al*., 2007; Wozniak *et al*., 2005; Rousseau *et al*., 2002)

Hence, endosulfan is postulated to act as a promoter of tumour growth through hormone-mediated effects (Hansen and Matsumura, 2001). Endosulfan (α) has been found to be a tumour promoter in hepatocytes (Dubois *et al*., 1996; Warngard *et al*., 1996; Fransson-Steen *et al*., 1992). Even though the evidence to classify endosulfan as carcinogenic is inconclusive, human epidemiologic and animal studies have shown that the exposure to OCs is positively correlated with several hormone-related cancers, including breast cancer (Calle *et al*., 2002), prostate cancer (Mills and Yang, 2003), endometrial cancer (Weiderpass *et al*., 2000), and testicular cancer (Biggs *et al*., 2008).

Elevated adipose tissue levels of endosulfan in women have been associated with an increased risk of breast cancer (Ibarluzea *et al*., 2004). Some studies have shown that exposure to endosulfan increases the total number of malignant tumours, pulmonary adenomas, carcinomas, hepatic carcinomas and sarcomas in female rats and lymphosarcomas in male rats (Reuber, 1981).

1.2. - Organochlorines and Activation of the Mitogen-Activated Protein Kinase Kinase Signaling (MAPKK) Cascade

1.2.1. - Mitogen-activated protein kinase kinase signaling (MAPKK) cascade

Organochlorines have been found to activate intracellular signalling pathways independent and in conjunction with the estrogen receptor activation (Hatakeyama *et al*., 2002) (Figure 2). Therefore, OCs can also cause the activation of mitogen-activated protein kinase kinase (MAPKK) signalling cascades ultimately altering cell behaviour (Ledirac *et al*., 2005). The MAPKK cascades are found in all eukaryotes. This pathway is comprised of three core enzymes that act in sequential phosphorylating steps: a MAP kinase kinase kinase (MAPKKK or Raf), a MAP kinase kinase (MAPKK or MEK), and a MAP kinase (MAPK or ERK) (English *et al*., 1999). MAPKs are important serine/threonine signal transducing enzymes connecting cell-surface receptors to critical regulatory targets responsible for altering gene expression within the cytoplasm and the nucleus of cells. Therefore, MAPK signalling cascades play a critical role in the control of gene expression, cell proliferation and programmed cell death (Chang and Karin, 2001).

The MAP kinase signaling cascade responds to a wide variety of extracellular signals such as binding of growth factors/hormones or estrogen mimicking compounds (xenoestrogens) to a membrane receptor (reviewed by Karp 2010). The MAP kinase kinase cascade starts off by

phosphorylating cytoplasmic components such as Raf. Raf is a serine-threonine protein kinase. One of its substrates is a MAPKK /MEK and phosphorylation of Raf activates

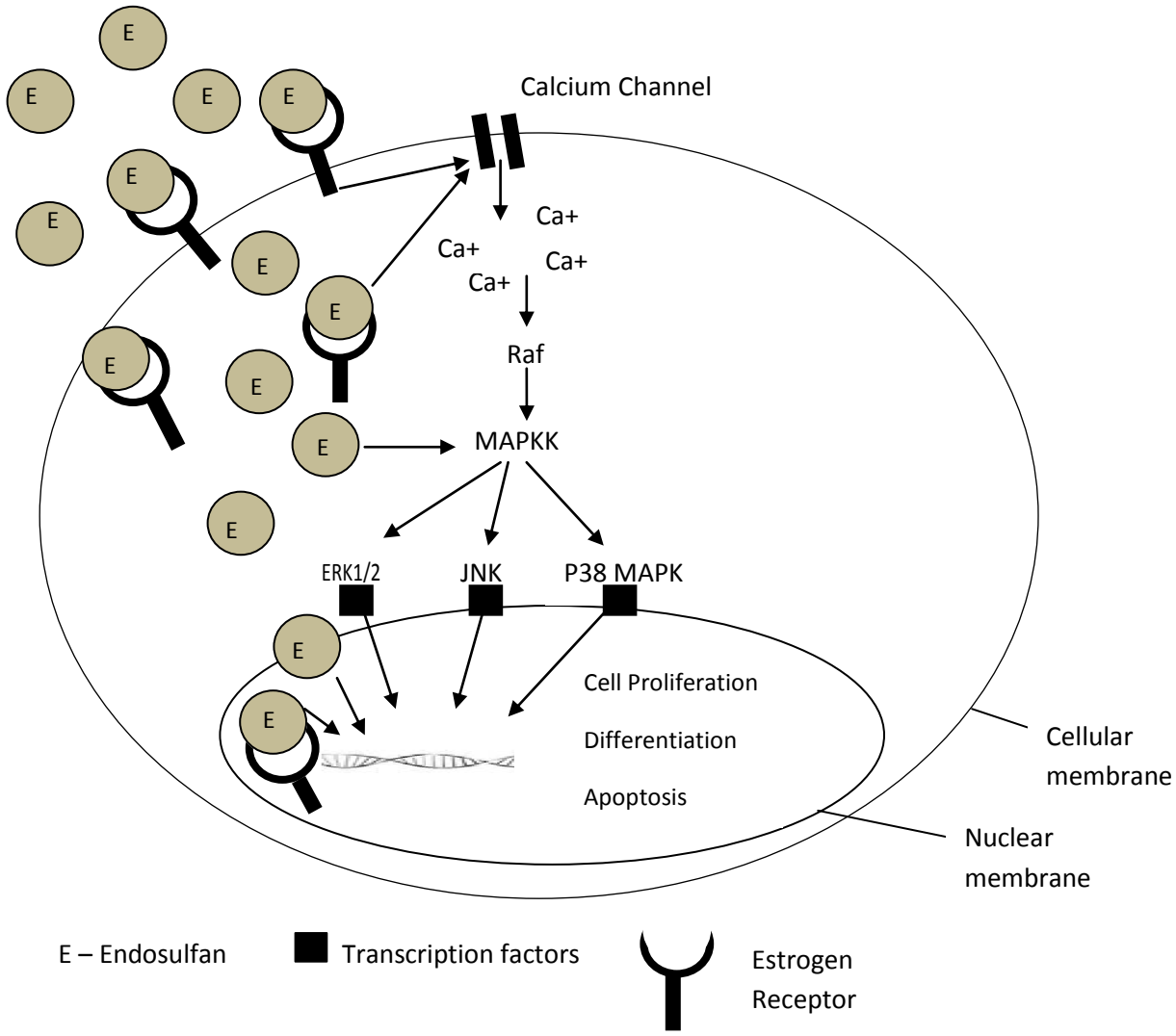


Figure 2: Activation of MAPKK pathway by organochlorines alters cell behaviour

MAPKK. MAPKKs are dual specificity kinases since they can phosphorylate tyrosine as well as serine and threonine residues (Shen *et al*., 2001). All MAPKs have a tripeptide near their catalytic site with the sequence Thr-X-Tyr. MAPK activates two MAP kinases named ERK1 and ERK2 via phosphorylation of both the threonine and tyrosine residues (Schramek, 2002). Phosphorylated ERK1/ERK2 have been found to activate a number of nuclear transcription factors and other nuclear proteins. This pathway activates cyclin D1, which plays a key role in driving a cell from G1 into S phase, and eventually causes activation of genes involved in cell proliferation (reviewed by Karp 2010).

Mammals express four distinctly regulated MAPKK subfamilies: extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38 MAPK (p38α/β/γ/δ), and ERK5 (Gutkind, 2000).

1.2.2. – Extracellular signal-related kinases (ERK1/2)

The ERK1/2 group is activated mainly by mitogenic stimuli, and it has been linked to cell survival. ERK1/2 stimulates DNA synthesis through phosphorylation of a rate-limiting enzyme in pyrimidine nucleotide biosynthesis (Graves *et al*., 2000). ERKs also promote cell-cycle progression by inactivating a cell-cycle inhibitory kinase (Palmet *et al*., 1998) and ERKs can stimulate cell proliferation by indirectly inducing cyclin D1 (Treinies *et al*., 1999). ERK may also induce growth factors that promote cell survival and ERK1 deficient mice ($Erk1^{-/-}$) have defective T-cell development (Pages *et al*., 1999)). Therefore, ERKs play a significant role in regulating cell proliferation.

1.2.3. - C-Jun amino-terminal kinases (JNK1/2/3) and p38 mitogen- activated protein kinase

The activation of JNK and p38 MAPK pathways is primarily caused by stress stimuli such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They are linked to the induction of cell differentiation and apoptosis (Xia *et al*., 1995). Both JNK1 and JNK2 are required to induce the apoptosis of ultraviolet-irradiated fibroblasts (Tournier *et al*., 2000). Importantly, induction of apoptosis did not occur in JNK deficient ($JNK3^{-/-}$) mice (Yang *et al*., 1997); while the JNK1 deficient (($JNK1^{-/-}$) mice exhibited defective T-cell differentiation to T-helper cell type-2 (Th2 cells) (Dong *et al*., 1998), JNK2 deficient (($JNK2^{-/-}$) mice exhibited defective T-cell differentiation to T-helper cell type-1 (Th1 cells) (Yang *et al*., 1998). Therefore, the JNK signalling pathway is important in T cell differentiation, regulating inflammatory responses and cellular apoptosis in mammals.

According to one study, p38α MAPK deficiency resulted in lethality in mice before day 11 of gestation due to defective placental developments (Adams *et al*., 2000). In additon, p38α deficient mice show low kidney production of the hormone erythropoietin and subsequently insufficient red blood cell production (Tamura *et al*., 2000). Hence, these studies underscore the importance of p38 MAPK in normal cell biology.

1.2.4. - Organochlorines activate MAPKK signaling cascades

Organochlorines have been found to activate MAPKK signaling pathways causing abnormal cell proliferation and tumorigenesis (Okoumassoun *et al*., 2003). An

organochlorine, heptachlor, which is structurally similar to endosulfan has been found to trigger significant proliferation in rat hepatocytes by the induction of ERK phosphorylation and inhibition of apoptosis (Okoumassoun *et al*., 2003). Heptachlor has also been shown to increase phosphorylation of ERK1/2 in human lymphocytic cells (Chuang and Chuang, 1998). Hence it is reasonable to postulate that endosulfan will also activate MAPKK signalling pathways in other human cells and alter gene expression.

RANKL and its receptor RANK play a crucial role in controlling the development of lactating mammary glands during pregnancy (Fata *et al*., 2000). In addition, induction of RANKL gene after stimulation with hormones increased proliferation in mammary epithelium (Beleut *et al*., 2010). Since OCs alter normal cellular behaviour by mimicking a hormone (Soto *et al*. 1995) and induce proliferation and differentiation of human breast cancer cells (Soto *et al* 1994, 1995; Bonefeld-Jorgensen *et al.*, 2005; Li *et al*., 2006), stimulation with OCs may lead to changes in RANK and RANKL gene expression in human breast cells (Figure 3).

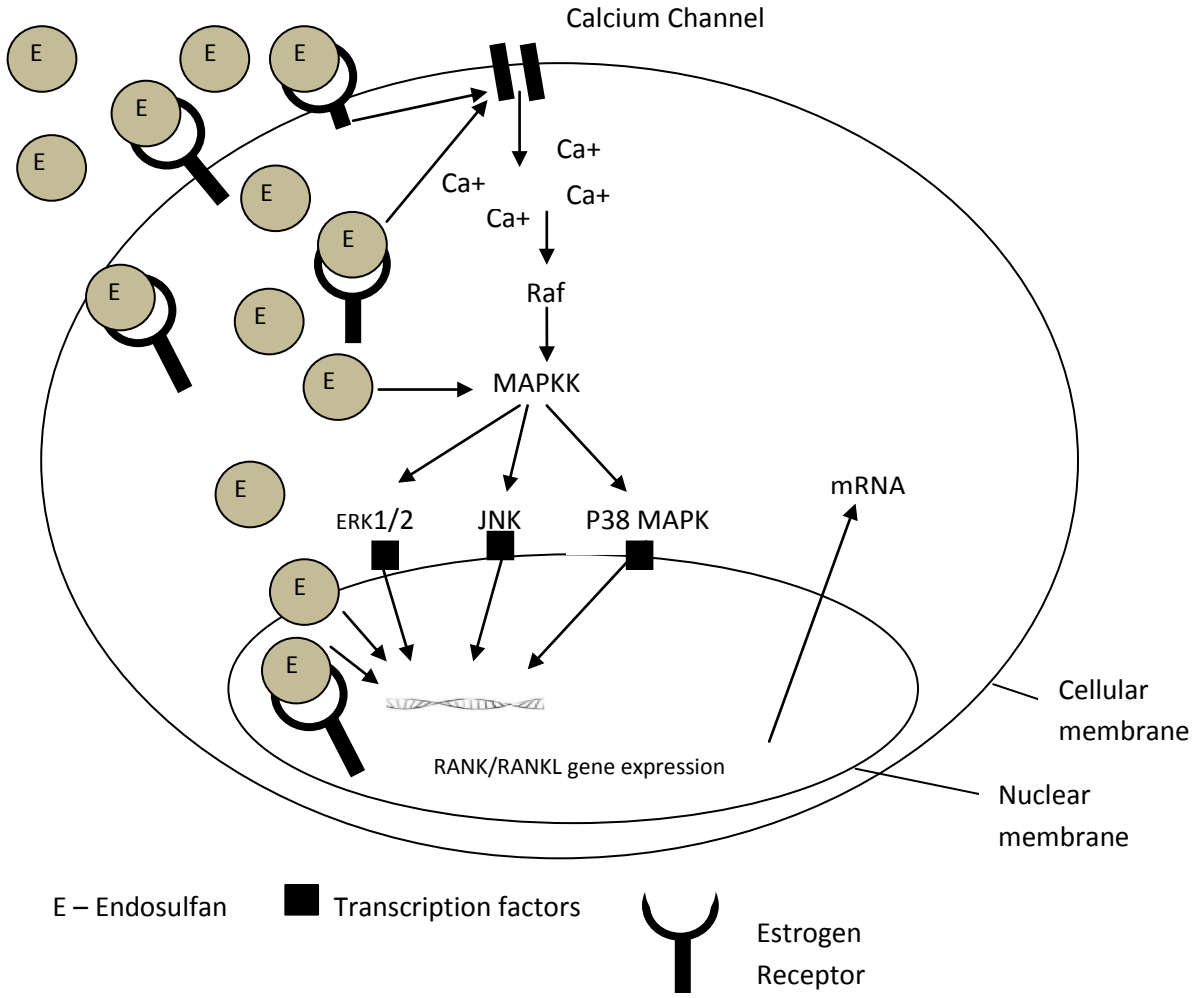


Figure 3: Exposure to organochlorines may alter RANK & RANKL mRNA expression in human breast cells.

1.3 - RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B - RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND SIGNALING AXIS

1.3.1 - RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B

The Receptor Activator of Nuclear Factor kappa B (RANK), also known as TRANCE receptor is a member of the tumor necrosis factor (TNF) cell surface receptor superfamily (Wei *et al.*, 2001). RANK is found to be an effective activator of the

transcription factor Nuclear Factor Kappa-B (NF – kB) and activator protein-1 (Anderson *et al*., 1997; Ross, 2000). Therefore, it controls transcription of its target genes via an intracellular cascade of signals (Wei *et al*., 2001; Cao *et al*., 2001). The RANK transmembrane receptor protein is comprised of 616 amino acid residues, of which 383 amino acids reside in the intracellular domain. RANK mRNA is ubiquitously expressed in human tissues including dendritic cells, T cells, fibroblasts (Anderson *et al*., 1997) and mammary epithelial cells (Srivastava *et al*., 2003).

1.3.2 - RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND

RANKL is a member of the TNF cytokine superfamily that exists in both soluble and membrane-bound forms (Lasey *et al*., 1998; Yasuda *et al*., 1998). RANKL is a type II transmembrane protein consisting of 317 amino acid residues, and is found on the surface of stromal cells, osteoblasts and activated T cells (Simonet *et al*., 1997; Anderson *et al*., 1997). RANKL is expressed as an osteoclast differentiation factor (Yasuda *et al*., 1998), as an apoptosis regulatory protein (Wong *et al*., 1997), and as a ligand for the soluble TNFR family member osteoprotegerin (Lacey at al., 1998). RANKL binding to RANK causes RANK to interact with TNF-receptor associated factors (TRAFs) such as TRAF2, TRAF3, TRAF5, and TRAF6 initiating a cascade of kinases. RANKL binding to RANK activates osteoclast maturation (Wei *et al*., 2001). The RANKL gene is found on the human chromosome 13q14 (Anderson *et al*., 1997). The RANKL gene exists in three RANKL isoforms: hRANKL1, hRANKL2, and hRANKL3. The hRANKL1 isoform is a 45 kDa membrane- associated protein comprised of intracellular, transmembrane and extracellular domains and spans approximately 36 kb of genomic DNA with six exons. Whereas, the hRANKL2 form is a 31 kDa soluble protein lacking the N-terminus

intracellular domain and the hRANKL3 isoform is a 39.5 kDa soluble protein lacking both the intracellular and transmembrane domains (Suzuki *et al*., 2004).

1.4. - Functions of the RANK/RANKL Pathway

1.4.1. - Role in bone remodeling and bone disease

The RANK/RANKL pathway is a key regulator of bone metabolism through its effects on development and activation of osteoclasts. Bone growth and remodeling is a tightly regulated and a dynamic process. Bone matrix is synthesized by osteoblasts while resorption of bone occurs by multinucleated osteoclasts (Kong *et al*. 1999a; Takahashi *et al*., 1999).

The process of osteoclast maturation and bone resorption is regulated by three main factors: RANKL, downstream signaling receptor for RANKL (RANK) and a decoy receptor osteoprotegerin (OPG) (Kong *et al*., 1999b; Lacey *et al*., 1998; Yasuda *et al*., 1998; Wong *et al*., 1997). RANK is expressed by multinucleated osteoclasts (Burgess *et al*., 1999). RANKL mRNA is expressed by bone osteoblasts (Simonet *et al*., 1997). RANKL binds specifically to the RANK receptor on osteoclast or osteoclast precursor cells and causes activation and maturation of osteoclasts (Yasuda *et al*., 1998; Lacey *et al*., 1998) and inhibition of osteoclast apoptosis (Fuller *et al*., 1998). RANKL expression is upregulated by many soluble factors affecting bone resorption, including the proinflammatory cytokines, interleukin-1 and TNF-α (Hofbauer *et al* ., 1999; Martin *et al*., 1998). RANKL can also be cleaved by metalloproteinases into a soluble form (Nakashima *et al*., 2000). The RANK/RANKL causes activation of NF-κB, which is a

transcriptional regulator present in mammalian cells. It plays an important role in the regulation of genes involved in apoptosis, cell cycle control, immune responses and many other cell events (Dolcet *et al*., 2005; Cao *et al*., 2001). RANKL binds to the RANK receptor ultimately causing activation of the catalytic subunit IKKα and subsequent activation of NF-κB is involved in modulating normal cell cycle progression (Cao *et al*., 2001). Although the exact mechanism of this transduction pathway is not fully elucidated, it is nevertheless believed to be important in regulating osteoclasts and osteoclast precursor cells.

The natural decoy receptor of RANKL, OPG, is a RANK homolog that works by binding to RANKL on osteoblast/stromal cells, thus inhibiting RANK/RANKL interaction and osteoclast activity (Figure 4). Knockout studies in mice have been the major contributor to knowledge about the functions of the RANK/RANKL pathway in bone. RANKL -/- mice have been shown to suffer osteopetrosis, stunted growth and a defect in tooth eruption due to failed osteoclast development. The study concluded that RANKL deficient osteoblasts were not able to support normal osteoclastogenesis, and therefore mice showed decreased osteoclast production and severely impaired bone remodeling. However, administration of OPG dramatically reduced the bone loss mediated by RANKL (Kong *et al*., 1999a) and effectively inhibited osteoclastogenesis both *in vitro* and *in vivo* (Simonet *et al*., 1997). Thus RANKL levels must be regulated simultaneously with OPG levels, as the balance of the two will determine whether osteoclastic or osteoblastic activity dominates.

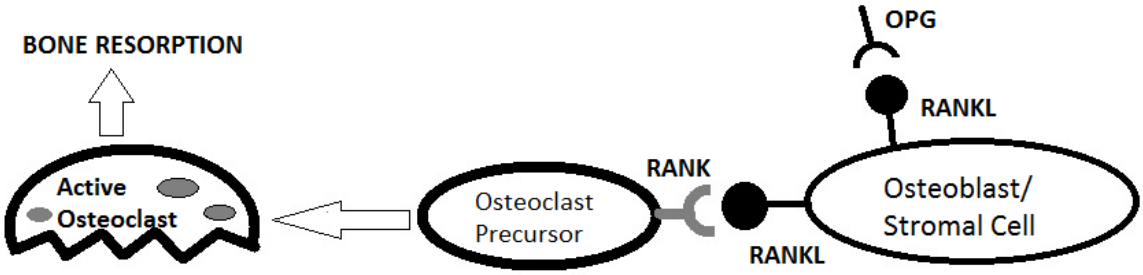


Figure 4: Activation of osteoclasts through RANKL binding to the RANK receptor causing bone resorption. OPG binds to RANKL and inhibits osteoclast activity.

1.4.2. - Role in immunology

The cell surface membrane bound RANKL is made and expressed by activated T cells and functions as an osteoclast differentiation factor (Hofbauer *et al*., 1999; Martin *et al*., 1998). RANKL knockout mice are immuno-compromised with inadequate development of lymph nodes and lack Peyer's patches (Kong *et al*., 1999b). RANK and RANKL are also expressed on B cells and dendritic cells. RANKL has been identified as a crucial factor involved in T cell–dendritic cell interactions and in dendritic cell survival (Bachmann *et al*., 1999; Anderson *et al*., 1997). RANKL is not only expressed on activated T cells but T cell-derived RANKL appears to be a link between inflammation and bone loss (Figure 5). T cell-derived RANKL regulates the development and activation of osteoclasts resulting in bone loss (Kong *et al*., 1999a) and T cells from inflamed joints also express RANKL. Increased expression of RANKL due to chronic systemic activation of T cells appears to play a prominent role in viral infections, autoimmune diseases or chronic local inflammation involving bone and joints such as in arthritis (Nakashima *et al*., 2003). Proinflammatory cytokines that stimulate

osteoclastogenesis such as IL-1ß, IL-6, IL-11, IL-17, and TNF-α, upregulate the expression of RANKL and downregulate RANK expression. On the other hand, cytokines that inhibit osteoclastogenesis, such as IL-13, interferon-γ, and transforming growth factor (TGF)-ß1 increase the expression of RANKL but decrease the expression of its inhibitor OPG (Nakashima *et al*., 2000). The wide use of corticosteroids to treat multisystem autoimmune disease and arthritis have resulted in bone loss since they have been found to strongly induce RANKL expression and decrease expression of its decoy receptor, OPG (Yasuda *et al*., 1998; Vidal *et al*., 1998).

Synovial tissue from patients with rheumatoid arthritis and osteoarthritis has been shown to express high levels of RANKL (Kong *et al*., 1999a). High levels of RANKL were also found in affected synovial tissue of patients with spondyloarthropathies (Haynes *et al*., 2003). Co-culturing activated CD4+T cells and bone marrow cells resulted in the induction of osteoclastogenesis. This effect was blocked by the addition of OPG. Soluble RANKL produced by T lymphocytes also activated functional osteoclasts and once again administration of OPG was shown to protect both cartilage and bone in rats with adjuvant-induced arthritis. Therefore, administration of OPG in animal models of arthritis was found to play a prominent role in bone protection.

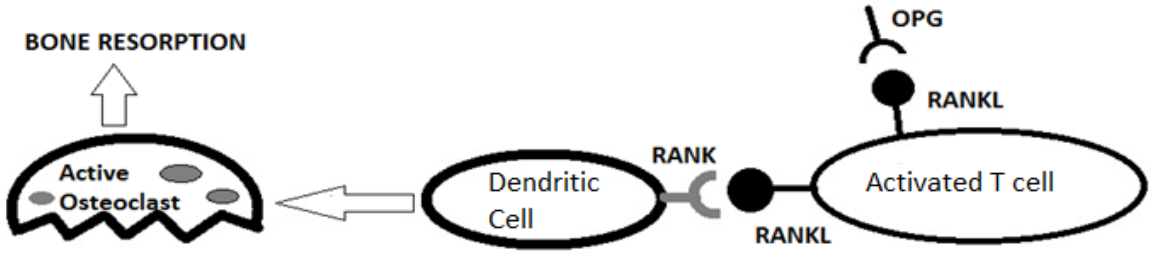


Figure 5: Activation of osteoclasts by binding of RANKL on activated T cells to RANK on dendritic cells indicating a link between activated T lymphocytes and osteoclastogenesis.

1.4.3. - Role in lactating mammary gland development

Along with the significance of RANK/RANKL signaling pathway in the regulation of osteoclastogenesis and the immune system, it is essential in the morphogenesis of the lactating mammary gland. RANK and RANKL signaling axis is crucial for the formation of the lobulo-alveolar structures during pregnancy (Fata *et al*., 2000). Expression of RANKL is not only restricted to lymphocytes, monocytes, macrophages, dendritic cells, and synovial fibroblasts but it is also found on mammary epithelial cells of pregnant and lactating females (Theill *et al*., 2002; Kotake *et al*., 2001; Kong *et al*., 1999b). RANKL deficient mice not only exhibited defects in early differentiation of T and B lymphocytes and showed lack of active osteoclasts, but also failed to form the lobulo-alveolar mammary structures during pregnancy and displayed increased apoptosis of mammary epithelium (Fata *et al*., 2000).

1.4.3.1. - Development and structure of the mammary gland

The human mammary gland undergoes many stages of development, growth and differentiation. These stages include embryonic, prepubertal, pubertal, pregnancy,

lactation and involution. The ectoderm forms the epithelial layer of the gland, while mesoderm forms the surrounding stroma during the embryonic stages (Shackleton *et al*., 2006). Mammary stem cells are activated during the developmental periods of puberty and pregnancy (Harmes and DiRenzo, 2009; Shackleton *et al*., 2006; Smith, 2005). The epithelial cells of the mammary gland develop into a branched ductal system during puberty. The collecting duct branches into smaller ductules, eventually terminating into ductal-lobular units or lobules that develop mainly during pregnancy. These lobules consist of alveoli, which are the actual secretory units that produce milk during the lactation stage. The lobulo-alveolar compartment develops and undergoes functional differentiation during pregnancy in response to reproductive hormones such as progesterone and prolactin (reviewed by Henninghausen and Robinson, 2001).

1.4.3.2. - Steroid hormones control ductal and alveolar development

Women are repeatedly exposed to ovarian hormones during the reproductive lifespan (Bernstein, 2002; Apter *et al*., 1989; Trichopoulos *et al*., 1972). Mammary gland development becomes hormone dependent at the onset of puberty. Estrogen and progesterone are primarily known to play a significant role in mediating signaling processes in the development of the mammary gland and the reproductive system. Steroid hormones function via binding to the specific nuclear receptors and activating genes in a ligand dependent manner. Estrogen receptors and progesterone receptors are found at high levels in mammary glands. Progesterone receptor deficient mice have an inability to ovulate and have severely limited mammary gland development (Henninghausen and Robinson, 2001). A recent study has shown that mammary stem cells increase 14-fold

during the luteal diestrus phase of the menstrual cycle in mice when the progesterone levels are the highest (Joshi *et al*., 2010).

1.4.4. - Proliferation of mammary gland cells through activation of the osteoclast differentiation factor RANKL

Along with the systemic hormones, the osteoclast differentiation factor, RANKL, plays a crucial role in mammary development. Recent studies demonstrating the new role of RANK/RANKL pathway in lobular-alveolar development emerged from the study of mice lacking these proteins through genetic ablation. Fata *et al*. (2000) uncovered the role of RANKL and its receptor RANK in controlling the development of lactating mammary glands during pregnancy. RANK was found to be constitutively expressed in normal mammary gland epithelial cells in mice, however RANKL expression was induced by sex hormones after day 12.5 of pregnancy. RANK and RANKL deficient mice displayed reduced pregnancy-induced proliferation and differentiation of the alveolar buds into functional mammary glands. These mice lacked lactating mammary glands and were unable to feed their offspring (Hofbauer *et al*., 2008; Fata *et al*., 2000). Administration of recombinant RANKL induced lobulo-alveolar development in mice by stimulating proliferation of alveolar bud epithelia and restored milk production.

Earlier studies showed that signal transducer and activator of transcription 5a (Stat5a) protein is involved in the development of mammary gland. Phosphorylation of Stat5a and Stat5b proteins was found to be very low in mammary tissue of virgin mice, however a drastic increase was observed in pregnant mice (Lui *et al*., 1996). Stat5a was

also known for its major role in the expression of the milk-secreting proteins by regulating gene expression (Liu *et al*., 1997). RANKL upregulation during pregnancy and lactation was shown to be under the control of Stat5a activation by prolactin stimulation (Srivastava *et al*., 2003). In addition, it is also postulated that Stat5a activation regulates genes involved in proliferation and cell cycle progression.

1.4.5. - Steroid hormones in the development of breast cancer through induction of RANKL

Breast cancer is one of the most common cancers in humans, affecting approximately 207, 090 women in the United States every year (Jemal *et al*., 2010). As mentioned earlier, women are repeatedly exposed to ovarian hormones during the reproductive lifespan; the higher number of menstrual cycles also correlates with increased risk of developing breast cancer (Bernstein, 2002; Apter *et al*., 1989; Trichopoulos *et al*., 1972). Hormone replacement therapy has been positively correlated with an increased risk and incidence of breast cancer (Rossouw *et al*., 2002; Beral *et al*., 2003).

Hormones and hormone derivatives have been shown to increase RANKL expression in the breast (Gonzalez-Suarez *et al*., 2010; Schramek *et al*., 2010). Synthetic progesterone derivatives (progestins) such as medroxyprogesterone acetate (MPA) used for hormone replacement therapy and contraceptives significantly increased the risk of developing breast cancer in mice (Schramek *et al*., 2010). Stimulation of luminal cells with progesterone increased RANKL expression by several thousand-fold (Joshi *et al*., 2010). In addition, in vivo administration of medroxyprogesterone (MPA) triggered massive induction of RANKL in mouse mammary gland epithelial cells (Schramek *et al*.,

2010). Induction of RANKL after stimulation with progesterone increased proliferation in mammary epithelium during mammary gland lactational morphogenesis (Beleut *et al*., 2010). However, when RANK is genetically inactivated in these mammary gland epithelial cells, epithelial proliferation is attenuated and cells are driven towards preprogrammed apoptosis (Schramek *et al*., 2010).

The notion that the downstream mediator of progesterone is RANKL was further validated when progesterone stimulated luminal cells of breast ducts to increase RANKL expression by several thousand-fold (Joshi *et al*., 2010). Progesterone seems to play a prominent role in the expansion of mammary stem cells during the reproductive cycles, where mammary stem cells may be the seeds that accumulate mutagenic hits, possibly leading to breast cancer (Joshi *et al*., 2010).

Additionally, the activation of RANK/RANKL signaling pathway in the absence of hormones can also induce proliferation of mammary epithelial cells (Fernandez-Valdivia *et al*., 2010; Gonzalez-Suarez *et al*., 2010). Mouse mammary tumour virus-RANK transgenic mice exhibited accelerated epithelial cell growth (pre-neoplasias) and increased mammary tumour formation after treatment with a carcinogen, 7, 12-dimethylbenzanthracene, or progesterone derivative medroxyprogesterone acetate (MPA). Cyclin D1 expression has been proven to be involved in mammary gland development and breast carcinogenesis (Yu *et al*., 2001) and has been shown to be induced by NF-κB signaling pathway activated by RANK/RANKL binding (Guttridge *et al*., 1999). RANKL inhibition reduced mammary epithelial proliferation and cyclin D1 levels caused by stimulation with hormone or carcinogen and subsequently attenuated the

mammary tumour development in wild-type mice and hormone treated mouse mammary tumour virus-RANK (mmtv- RANK) transgenic mice (Gonzalez-Suarez *et al*., 2010). This links the RANK/RANKL signaling pathway to mammary tumorigenesis and illustrates the potential role of RANK and RANKL inhibition in mediating breast cancer.

Although regulation of RANKL expression in the breast by the hormones prolactin and progesterone has been reported to occur through Stat5a activation, no current data exists on how other signalling pathways may contribute to the regulation of its expression. RANKL upregulation has been reported through MAPK pathways in osteoblasts (Zhou *et al*., 2010), but no research on how MAPK pathways in breast cancer cells may regulate RANK/RANKL gene expression has been reported.

1.4.6. - RANKL inhibition prevents bone metastasis

If mammary stem cells may be the 'seeds' that accumulate mutagenic hits that could lead to breast cancer (Joshi *et al*., 2010), then Stephen Paget (1889) was the first to propose that bone microenvironment provides an especially fertile 'soil' for seeding circulating cancer cells. Bone is the most common site of metastatic invasion in breast cancer (Lau *et al*., 2009; Mundy, 2002; Sloan and Anderson, 2002; Chambers *et al*., 2000). Approximately 80% of patients with advanced breast cancer suffer from bone metastasis (Lau *et al*., 2009). Jones *et al.* (2006) observed that RANKL induces the migration of various RANK expressing human breast cancer cell lines including MDA-MB-231, Hs578T and MCF-7. However, addition of RANKL inhibitors such as OPG

caused reduction in RANKL-induced migration of cells (Jones *et al*., 2006). Blockade of RANK/RANKL signalling was found to slow down metastasis of breast cancer to bone (Canon *et al*., 2008). Inhibition of this pathway by OPG administration reduced the skeletal tumour burden by 50% (Jones *et al*., 2006) and 75% (Morony *et al*., 2001) and prevented tumour-induced paralysis (Jones *et al*., 2006) in mice.

The inhibition of RANK/RANKL pathway seems to be a potential target for therapeutic intervention in bone-related diseases. An inhibitor of the RANK/RANKL signalling pathway, Denosumab, was developed with high affinity for RANKL (Pageau, 2009). It is a fully human monoclonal antibody to RANKL which prevents the binding of RANKL to its receptor RANK and thereby prevents bone resorption both in vitro and in vivo (Gerstenfeld *et al*., 2009). It is effectively used in the treatment of osteoporosis induced bone loss, bone metastases, rheumatoid arthritis and multiple myeloma and giant cell tumor of bone (Ellis *et al*., 2008; McClung, 2006) and has completed clinical trials for the management of skeletal related events in women with breast cancer (Stopeck *et al*., 2010).

1.5. - Human MCF-7 Breast Cancer Cells Used in this Study

An *In vitro* model was used to study cancer cells behaviour as it allows for a detailed analysis at the cellular and molecular levels. Cell culture allows for an in depth and controlled study of behavioural responses, stimulation of molecular signaling and pathways and changes in gene expression in human cells. This study used human MCF-7 breast cancer cells; human breast epithelial adenocarcinoma cells derived from a pleural effusion from a breast cancer patient. MCF-7 cells retain several characteristics of

differentiated breast epithelium and are known to contain high amount of estrogen receptors (Brooks *et al*., 1973). They also contain high levels of prolactin receptors and progesterone receptors.

1.6. - Research Objectives

Organochlorine pesticides (OCs), such as endosulfan, are organic compounds that persist in the environment, bioaccumulate through the food chain, and pose a risk of causing adverse effects to human health and the environment by evoking estrogenic responses (Soto *et al*., 2005; Coats, 1990). Xenoestrogens have been found to activate intracellular signalling pathways independent and in conjunction with estrogen receptor activation (Hatakeyama *et al*., 2002; Hatakeyama *et al*., 2003) causing changes in gene expression levels and ultimately altering cell behaviour. The objectives of the study were to confirm the proliferative effects of organochlorine pesticide (endosulfan) on human MCF-7 breast cancer cells, identify activated intracellular signaling pathways and investigate changes in specific gene expression profiles. Since organochlorines have been found to stimulate intracellular signalling, the mitogen-activated protein kinase kinase (MAPKK) pathway, and in particular three major arms of this pathway, ERK1/2, JNK and p38 MAPK, were studied. Activation of these signalling pathways is crucial for changes in gene expression that alter cellular behaviour (Chang and Karin, 2001). Therefore, the effects of endosulfan on the Receptor Activator of Nuclear Factor kB (RANK) and RANKL gene expression were examined. RANK ligand and its receptor RANK are crucial for mammary epithelial proliferation in pregnancy (Fata *et al*., 2000)

and also found to be associated with hormone mediated breast cancers (Schramek *et al*., 2010) making them important mediators in breast biology.

2. – Materials and Methods

2.1. - Growth of Cell Lines

Human MCF-7 breast cancer (MCF-7) cells were purchased from American Type Culture Collection (ATCC) and maintained in Minimal Essential Media (MEM) containing Earle's salts and 2mM L-glutamine (ATCC) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 0.1% penicillin/streptomycin (p/s) (Hyclone). These cells were incubated at 37º C in a water jacketed incubator (Fisher) in 5% CO_2. Human intestinal epithelial (HT-29) cells (kind gift of Dr. J. Green-Johnson) were maintained in RPMI-1640 medium (Hyclone) supplemented with 10% FBS and 0.1% p/s at 37º C in 5% CO_2.

Both of these cells lines were adherent cultures grown in 75 cm^2 Sarstedt cell culture flasks with 20 mL of culture media. Media was replaced every two days and cells were passaged after 70-75% confluency was reached. Cells were usually passaged every 5-7 days. In order to passage the cells, media was aspirated and the cells were washed three times with 1X PBS. About 2mL 0.5% Trypsin/EDTA (Hyclone) was added to the 75cm^2 flask and incubated for 2 minutes at 37ºC until the cells were detached from the bottom of the plate. Cells were rinsed with 10 mL of cell culture media and placed in a 50 mL polystyrene tube (Benton Dickinson). Cells were centrifuged at 1200 x g for 5 minutes and the pellet was resuspended in 5 mL of media. One hundred µL of media suspension containing cells was stained with ½ dilution of trypan-blue (MP Biomedicals, Inc) and counted using a haemocytometer. After counting, about 1 million cells were added to each new flask. In order to freeze the cells, about 9 million cells were added to 100 µL DMSO (Sigma) and 300 µL FBS in cryovials (Fisher Scientific). First they were

placed in a Nalgene "Mr.Frosty" Cryo container at -80º C overnight and then transferred to liquid nitrogen.

2.2. - Stimulation of MCF-7 cells with Endosulfan

A stock solution of 10 mM endosulfan (Sigma, Product Number: PST-501) was prepared by dissolving in DMSO. The compound was further diluted in MEM media with 10% charcoal dextran-stripped fetal bovine serum (FBS, Fisher) to nominal concentrations of 1µm and 0.1 µm. Cells were stimulated for up to 3 days before cell counting or RNA isolation and for 30 minutes or 45 minutes prior to protein isolation for MAPK phosphorylation detection.

2.3 - Proliferation of MCF-7 cells in the Presence of Endosulfan was Tested Using Flow Cytometry (Millipore's Guava Instrument)

Proliferation in the presence of endosulfan was measured using Millipore's Guava (flow cytometer) instrument that counts cell viability. It is a technique used for counting and examining cells by suspending them in a stream of fluid and passing them through the electronic detectors. A beam of light of a single wavelength is directed onto a hydrodynamically-focused stream of fluid containing cells. Each viable suspended cell passing through the beam scatters the ray and emits fluorescent light (cells are incubated in a reagent containing fluorescent dye prior to counting). This fluorescent light is picked up by the detectors and generates viable cell count. For experimentation, cells were counted with a haemocytometer and 300, 000 cells were plated in each of the four 25 cm^2

flasks (Sarstedt) with 5 mL of charcoal dextran-stripped media and incubated at 37° C in 5% CO_2. After 24 hours, the media was removed and cells were exposed with 1 µM endosulfan or 0.1 µM endosulfan diluted in the appropriate media. This experiment comprised of two control conditions. The cells in the negative control flask were stimulated with 0.1% DMSO diluted in the media since this was the calculated percentage of DMSO found in the diluted concentration of 1 µM endosulfan. The cells in the control condition were incubated in just MEM media with charcoal dextran-stripped (FBS). Initially, 300 000 cells were plated in 12 flasks (4 flasks for each day). Media was not removed or changed from the flasks until the cells were extracted to measure cell growth. The cell growth was observed over 3 days.

2.3.1. - Preparing the cell suspension with Guava ViaCount reagent and generating absolute cell count and viability

The Guava ViaCount assay was used to distinguish between the viable and non-viable cells based on the differential permeability of DNA-binding dyes in the ViaCount Reagent. The Guava ViaCount Reagent was purchased from the Guava Technologies. Cells from each flask were detached using trypsin and re-suspended in 5 mL of media (cells + trypsin + 5 mL of media = original cell suspension). The original cell suspension was diluted by a factor of 10. Fifty µL of that original cell suspension volume was transferred to 1 mL tube along with 450 µL of the Guava ViaCount reagent. The solution was vortexed for 5 minutes and the absolute cell count and viability measurements was generated using the Guava instrument. The entire endosulfan stimulation experiment was

repeated three times and a graph was generated. SigmaPlot was used to generate linear regressions and used to generate p values using a student's T-test.

2.4 - Protein Analysis

2.4.1 - Protein isolation

For experimentation, 1 million cells were plated in three 25 cm^2 flasks with 5 mL of charcoal dextran- stripped media and incubated at 37° C in 5% CO_2. One flask comprised of the control condition, while the remaining two flasks were stimulated with 0.1 µM endosulfan in MEM media. The lysis reagent in Pro-Q Diamond Phosphoprotein Enrichment Kit (Invitrogen) was used for the isolation of protein from the MCF-7 cells. About 1 million cells were harvested and lysed with 500 µL of lysis reagent along with endonuclease and protease inhibitor (Applied Biosystems). After lysis, a cell pellet was obtained through centrifugation at 15 000 g in a table top microfuge (Thermo Scientific) for protein and was extracted after 30 minutes and 45 minutes.

The pellet was resuspended in the lysis reagent until the pellet was completely dissolved and incubated for 30 minutes on ice. The cell lysate was centrifuged at +4 degrees Celsius for 30 minutes at 15,000 g. The supernatant cytoplasmic lysate was retained and unphosphorylated proteins were quantified using the Bradford Assay.

In order to isolate phosphorylated proteins, the cytoplasmic lysate was loaded onto a column containing a phosphoprotein-binding resin and the phopho proteins were subsequently eluted using the elution buffer. The sample was concentrated using Tris and CHAPS in the Vivaspin filtration concentrator provided in the kit. To ensure maximum

recovery of protein, the sample was precipitated using the methanol-chloroform-water method. About 600 µL of methanol, 150 µL of chloroform, and 450 µL of deionized water were added to the sample in a microcentrifuge tube in sequence and vortexed after the addition of each solution. The sample was centrifuged for 5 minutes at 13 000 g and the upper aqueous phase was removed. About 450 µL of methanol was added to the interface layer containing protein and the mixture was vortexed. The sample was centrifuged again for 5 minutes at 13 000 g and a protein pellet was obtained. The pellet was left to air-dry for about one hour and resolubilized in 100 µL of HEPES buffer. The phosphorylated protein sample was quantified using the Bradford Assay.

Samples were diluted in 2X SDS-PAGE buffer containing 20 mM dithiothreitol, 6% SDS, 0.25 M Tris (pH 6.8), 10% glycerol, 10 mM NaF and bromophenyl blue (all Sigma). The samples were heated for 10 minutes at 95º C for western blot analysis or frozen at -20º C for storage.

2.4.2. - Western blot analysis

Thirty micrograms of protein sample was loaded and separated using a Mini-PROTEAN Precast Gels (BioRad). The gels were run in the BioRad Mini-PROTEAN Tetra Cell at 200 V for about 45 minutes in the 1X electrode buffer containing 0.30% Tris-base (Sigma), 0.14% glycine (MP Biomedicals, Inc) and 0.1% SDS. Correct loading and running of the protein gels was determined by staining with Brilliant Blue G Concentrate (Sigma) for 20 minutes. The gel was de-stained using 50% methanol (Sigma) and 10% acetic acid (Sigma) for 5 hours. The de-staining solution was changed

several times until the background was washed away and the protein bands were clearly visible.

Protein was transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (BioRad) using the BioRad Mini Trans-Blot Electrophoretic Transfer Cell. The transfer was carried out at 4° C for 30 minutes at 100 V in the transfer buffer containing 1X electrode buffer, 10% methanol and 0.1% SDS. Transfer was confirmed by staining the membrane with 0.1% Ponceau S in 5% acetic acid (Sigma) for 15 minutes and washing the background with distilled water. After transfer was confirmed, the membrane was washed with the blotting buffer, containing 25 mM Tris (pH 7.4) 0.15 NaCl and 0.1% Tween, until the stain was removed.

2.4.3. - Detection of proteins

Antibodies specific for ERK1/ERK2, phospho-ERK1/ERK2, JNK, phospho-JNK, and phospho- p38 MAPK were purchased from R&D Systems. Antibodies specific for p38 MAPK and β-actin were purchased from BioLegend. A secondary goat anti-rabbit IgG, horseradish peroxidase (HRP) - conjugated antibody was used for detection of the primary antibody (R&D Systems).

ERK1(MAPK3)/ERK2(MAPK1) are rabbit polyclonal antibodies that bind to 44 kDa peptides. To detect unphosphorylated ERK1/ERK2, the PVDF membrane containing transferred protein was incubated with 0.1 µg/mL rabbit anti-human ERK1/ERK2 antibody in a blocking solution for one hour at room temperature with gentle agitation. To detect for phospho-ERK1 (T202/Y204)/ERK2(T185/Y187), the membrane was

incubated with 0.05 µg/ml rabbit anti-phospho-ERK1/ERK2 antibody in a blocking solution for one hour at room temperature with gentle agitation.

JNK(SAPK1) is a rabbit polyclonal antibody that binds to 46 kDa and 54 kDa peptides. To detect unphosphorylated JNK, the PVDF membrane containing transferred protein was incubated with 0.2 µg/mL rabbit anti-human JNK antibody in a blocking solution for one hour at room temperature with gentle agitation. To detect for phospho-JNK (T183/Y185), the membrane was incubated with 0.5 µg/mL rabbit anti-phospho-JNK antibody in a blocking solution for one hour at room temperature.

P38 MAPK is a rabbit polyclonal antibody that binds to 38 kDa peptides. To detect unphosphorylated p38 MAPK, the PVDF membrane containing transferred protein was incubated with 0.5 µl/mL rabbit anti-human JNK antibody in a blocking solution for one hour at room temperature with gentle agitation. To detect for phospho-p38 MAPK (T180/Y182), the membrane was incubated with 0.5 µg/mL rabbit anti-phospho-p38 MAPK antibody in a blocking solution for one hour at room temperature.

Beta-Actin is a rabbit polyclonal antibody that binds to 45 kDa peptides. To detect β-actin, the PVDF membrane containing transferred protein was incubated with 0.5 µl/mL rabbit anti-human β-actin antibody in a blocking solution for one hour at room temperature with gentle agitation.

After incubation with each of the primary antibodies, the membranes were washed at room temperature for one hour with 5 changes of blotting buffer containing 25 mM Tris (pH 7.4), 0.5 M NaCl and 0.1% Tween. The membranes were incubated in with 0.5 µg/ml anti-rabbit IgG-HRP secondary antibody for one hour at room temperature.

The membranes were washed again at room temperature for one hour with 5 changes of blotting buffer.

Chemiluminescent peroxidase substrate working solution (Sigma) was prepared by mixing 1 part of the chemiluminescent reagent with 2 parts of the chemiluminescent reaction buffer. The working solution was poured over the membrane and left for 5 minutes in the absence of light. The excess solution was drained, the membrane wrapped in a plastic wrap and placed in an autoradiography cassette. The membrane was exposed to autoradiography film (Kodak) for 30 seconds. After exposure, the film was developed using the Kodak X-OMAT 1000A Processor.

2.5. - Detection of mRNA

Since the expression of both RANK and RANKL have been observed in breast cancer cell lines and linked to mammary tumorigenesis (Gonzalez-Suarez *et al*., 2010). RT-PCR analysis was conducted using RNA isolated from MCF-7 breast cancer cells to examine the changes in RANK and RANKL gene expression with 0.1 µM endosulfan exposure.

2.5.1. - Preparing cytoplasmic lysates and RNA purification

For experimentation, one million cells were plated in four 25 cm^2 flasks with 5 mL of media and incubated at 37° C in 5% CO_2. One flask comprised of the control condition, while the remaining three flasks were stimulated with 0.1 µM endosulfan. Media was not removed or changed from the flasks until RNA was extracted. RNA was extracted after 24 hours, 48 hours and 72 hours.

About 1 million cells were harvested and lysed with 600 µL of CytosALL reagent (Thermo Scientific) and mixed with 6 µL of RNase inhibitor (Applied Biosystems). The pellet was resuspended in water until completely dissolved and incubated for 5 minutes on ice. The cell lysate was centrifuged at 4º C for one minute at 15 000 g in a table top microcentrifuge. The supernatant cytoplasmic lysate was retained.

In order to purify the RNA, RNeasy Mini Kit was purchased from Qiagen. About 100 µL retained after RNA extraction was added to 350 µL Buffer RLT with 1% β-mercaptoethanol and homogenized. About 250 µL was added to the lysate. The sample was then applied to RNeasy spin column placed in a 2 mL collection tube. The lysate was centrifuged in a microcentrifuge for 15 seconds at 10000 g. Ethanol was added to allow total RNA binding to the membrane. After centrifugation, 500 µL of Buffer RPE was added to wash the spin column membrane and centrifuged for 15 seconds at 10000 g. The membrane was washed again with 500 µL of Buffer RPE for 2 minutes at 10000 g. RNA was eluted using 50 µL of RNase free water (Fisher) by centrifuging for 1 minute at 10000 g in 1.5 mL collection tube. RNA was quantified using the A_{260}/A_{280} reading on a UV spectrophotometer.

2.5.2 - RNA reverse-transcribed to cDNA

RNA was reverse-transcribed to cDNA using Superscript II RT enzyme and Oligo $(dT)_{12\text{-}18}$ primers (Invitrogen). One µg of isolated RNA was combined with 1 µl of Oligo $(dT)_{12\text{-}18}$ primers and 10 mM dNTPs. The reaction was heated to 65º C for 5 minutes in order for the primers to anneal to the template mRNA strand. The reaction was chilled on ice immediately and 5X First Strand Buffer and 0.1 M DTT was added. The cDNA

synthesis was carried out at 42° C for one hour using 200U Superscript II reverse transcriptase (Invitrogen). The reverse transcriptase was inactivated by heating at 70° C for ten minutes. The cDNA samples were stored at -20° C.

2.5.3 - Polymerase chain reaction

GAPDH, RANK, RANKL transcripts were amplified from constructed cDNA using RT-PCR. PCR reactions were carried out using a GoTaq Hotstart Polymerase (Applied Biosystems). GoTaq was activated with an initial incubation of 95° C for 2 minutes. One microlitre of cDNA was used as template for each amplification reaction.

Glyceraldehyde 3-phosphatase dehydrogenase($GAPDH_3$) is a housekeeping gene used as a genomic control for possible contamination of cDNA samples and a positive PCR control. The following primers were used for amplifications: 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense), to produce 555 bp from genomic DNA and 451 bp from cDNA. The reaction mixture included 2 mM dNTPs, 10 µM primers, 25 mM $MgCl_2$ and 0.2 UTaq. Following the initial activation, the reaction was followed by thirty five cycles of denaturing at 94° C for 45 seconds, primer annealing at 57° C for 1 minute and extension at 72° C for 45 seconds. The reaction was completed with final annealing for one cycle at 72° C for 10 minutes.

The following RANK primers were used for amplifications: 5' – GGGAAAGCACTCACAGCTAATTTG-3'(sense) and

GCACTGGCTTAAACTGTCATTCTCC-3' (antisense), to produce 452 bp fragments. The reaction mixture comprised of 2 mM dNTPs, 10 µM primers, 25 mM $MgCl_2$ and 0.2 UTaq. The reaction was started with an initial incubation of 95° C for 2 minutes, followed by thirty five cycles of denaturing at 94° C for 45 seconds, primer annealing at 57° C for 1 minute and extension at 72° C for 45 seconds. The reaction was completed with final annealing for one cycle at 72° C for 10 minutes.

RANKL primers used for amplification of 389bp fragments were as follows: 5'-ACTGGATCCGGATCAGGATG-3' (sense) and 5' –AGCTGCGAAGGGGCACATGA-3' (anti-sense). HT-29 cells were used as a positive control for RANKL gene expression. The reaction mixture for amplification included 2 mM dNTPs, 10 µM primers, 25 mM $MgCl_2$ and 0.2 UTaq. The reaction was started with an initial incubation of 95°C for 2 minutes, followed by thirty five cycles of denaturing at 94° C for 45 seconds, primer annealing at 57° C for 1 minute and extension at 72° C for 45 seconds. The reaction was completed with final annealing for one cycle at 72° C for 10 minutes.

2.5.4. - Electrophoresis of PCR products

The size of each PCR product was determined by electrophoresis through a 2% electrophoresis grade agarose (Sigma) gel in 1X TAE buffer at 100V for approximately 45 minutes. Gels were stained with 0.5 ng/mL ethidium bromide for fifteen minutes and visualized under UV using a FluorChem™ SP (Alpha Innotech) geldock, and images were captured for further analysis.

2.6 - Inhibition Studies

SB203580, a specific inhibitor of p38-MAPK pathway (Barancik *et al*., 2001) was used to inhibit the phosphorylation of p38 MAPK protein. SB203580 powder (Sigma) was reconstituted in DMSO to a concentration of 50 mM. SB203580 was further diluted with 0.1 µM endosulfan in charcoal dextran stripped media to a concentration to 50 µM. Cells were stimulated with and without inhibitor for 30 minutes and 45 minutes. Protein was isolated and p38 MAPK and phosphorylated p38 MAPK were detected as described previously.

3 - Results

In order to rule out the possible effects contributed by undefined hormones present in serum, cells were cultured in low quantities of charcoal-stripped serum 24 hours prior to the treatment with endosulfan. Charcoal dextran stripping reduces the serum concentration of many hormones and certain growth factors, such as estradiol, cortisol, corticosterone, the B vitamins, T3, T4 and prostaglandins. The removal of these hormones was essential prior to the treatment with endosulfan because they may interfere with the signalling pathways activated by the endosulfan and lead to synergistic or subtract from estrogenic responses.

3.1 - MCF-7 Cells Proliferation in the Presence of Endosulfan

The mean number of viable cells from all four conditions (Control, DMSO Control, 0.1 µM endosulfan, and 1.0 µM endosulfan) for three consecutive days at 24 hours intervals are presented in Figure 6. The experiment was repeated three times and a graph was generated. The viable cell count ranged from 300,000 to 2,445,094 in a 25cm^2 flasks with 5mL of media. The mean number of cells treated with 0.1 µM endosulfan was significantly more than the mean number of cells in the DMSO control condition ($p = 0.00157$) after 24 hours, after 48 hours ($p = 0.00864$) and after 72 hours ($p = 0.00052$). The mean number of cells treated with 1.0 µM endosulfan was also significantly greater than the mean number of cells in the DMSO control condition after 24 hours ($p = 0.03307$); 48 hours ($p = 0.00679$) and 72 hours ($p = 0.00028$). The mean number of MCF-7 cells treated with 1.0 µM did not significantly differ from the mean number of cells treated with 0.1 µM endosulfan after 24 hours or 48 hours. However, the mean

number of cells treated with the lower concentration of endosulfan (0.1 μM) was significantly higher than the higher concentration (1.0 μM endosulfan) after 72 hours.

Overall, the results indicate that treatment of MCF-7 cells with either endosulfan concentration shows a statistically significant increase in proliferative activity over control as measured at 24 through 72 hours of incubation. Interestingly, although no significant difference in total cell counts was measured between the two endosulfan concentrations after 24 hours and 48 hours, there was a significant increase in cell number for the lower endosulfan (0.1 μM) concentration over the higher (1.0 μM) endosulfan concentration (Figure 6) after 72 hours. This paradoxical situation of the lower concentration having more stimulatory effect on the cells than the higher concentration has also been seen with other organochlorines and will be discussed further in the next chapter.

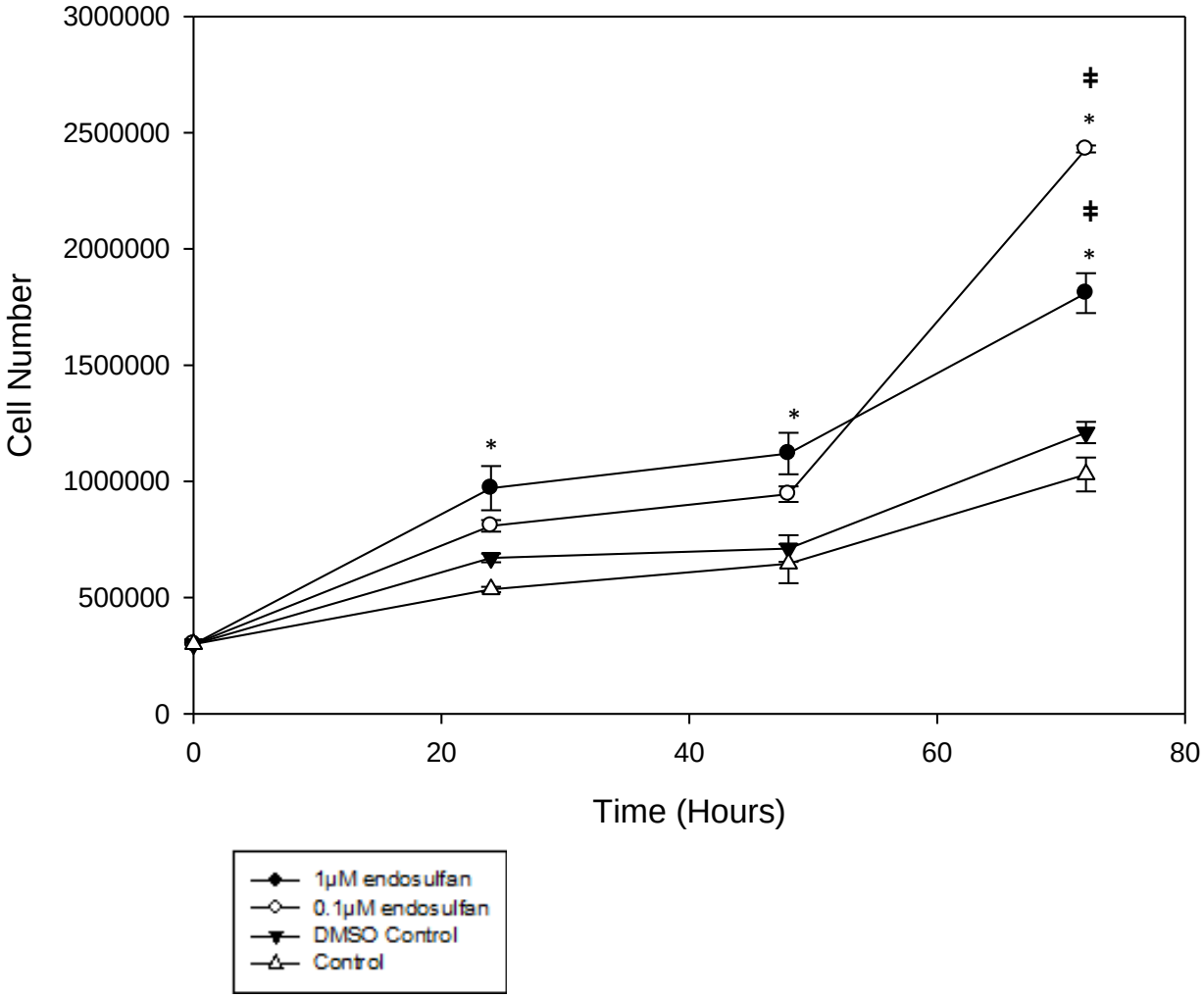


Figure 6: Endosulfan treatment significantly increases MCF-7 cell counts

Cells stimulated for 24, 48 and 72 hours with either 0.1 µM endosulfan or 1.0 µM endosulfan show a significant increase in cell number (*) ($p \leq 0.05$; n = 3) over cells cultured in DMSO as a vehicle control. Additionally, proliferation is greater for cells treated at lower concentration (0.1 µM) endosulfan for 72 hours (ǂ) from the higher 1.0 µM endosulfan stimulation.

3.2 - Activation of Intracellular MAPKK Pathways with Endosulfan Exposure

After examining the effects of endosulfan stimulation on MCF-7 breast cancer cells, in particular proliferative effects, intracellular changes at the molecular level were investigated using protein isolation and Western blot analysis to detect changes in phosphorylation (activation) status of key proteins in the MAP kinase signalling cascade. Since the lower concentration (0.1 µM) was found to have more stimulatory effect on the cells, this endosulfan concentration was used for protein analysis studies. The phosphorylation status of three MAPKK pathways (ERK1/2, JNK and p38MAPK) after stimulation with low endosulfan concentration (0.1 µM) was explored.

Protein isolated from unstimulated MCF-7 cells showed little phosphorylation of ERK2 protein (42 kDa) (Figure 7A). There was a slight increase in detectable phosphorylated ERK1/2 after 30 minutes of endosulfan stimulation but a darker band of phosphorylated ERK1/2 was observed after 45 minutes of endosulfan exposure. Protein isolated from unstimulated cells displayed mostly unphosphorylated forms of ERK2 protein (42 kDa) (Figure 7B). There was a slight decrease in detectability of the unphosphorylated form of ERK2 after 30 minutes of endosulfan exposure but a much lighter band of unphosphorylated ERK2 was observed after 45 minutes of endosulfan exposure which correlates with the change in its state to the phosphorylated form.

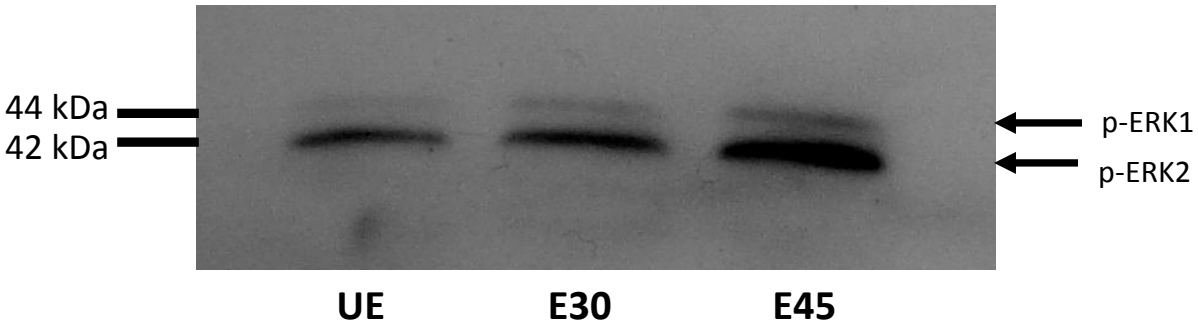
Unstimulated MCF-7 cells expressed little phosphorylated JNK protein (46 kDa) (Figure 8A) but JNK protein was clearly detected in its unphosphorylated form (Figure 8B). Although there were no large changes in phosphorylated JNK protein after 30

minutes, the band for phosphorylated JNK appeared much darker after 45 minutes of endosulfan exposure. There was a decrease in the intensity of the band for unphosphorylated JNK after 30 minutes and after 45 minutes of endosulfan exposure which relates to the change in form to the phosphorylated state.

Unstimulated MCF-7 cells showed negligible expression of phosphorylated p38MAPK protein (38 kDa) (Figure 9A). However, the band for phosphorylated p38MAPK appeared darker after 30 minutes and after 45 minutes of endosulfan exposure. Consequently, protein isolated from unstimulated cells displayed only the unphosphorylated form of p38 MAPK protein (38kDa) (Figure 9B). The band for unphosphorylated p38 MAPK appeared lighter after 30 minutes and after 45 minutes of endosulfan exposure.

Beta actin was used as a positive control as it is a ubiquitous protein found in eukaryotic cell cytoskeletons. Beta actin was present in unexposed and endosulfan exposed MCF-7 cells and the band for beta actin protein in unexposed cells appeared similar in intensity compared to the band for beta actin protein in endosulfan exposed cells. Hence, there appears to be no difference in the expression of this protein in these two conditions using Western blot analysis (Figure 10).

A)



B)

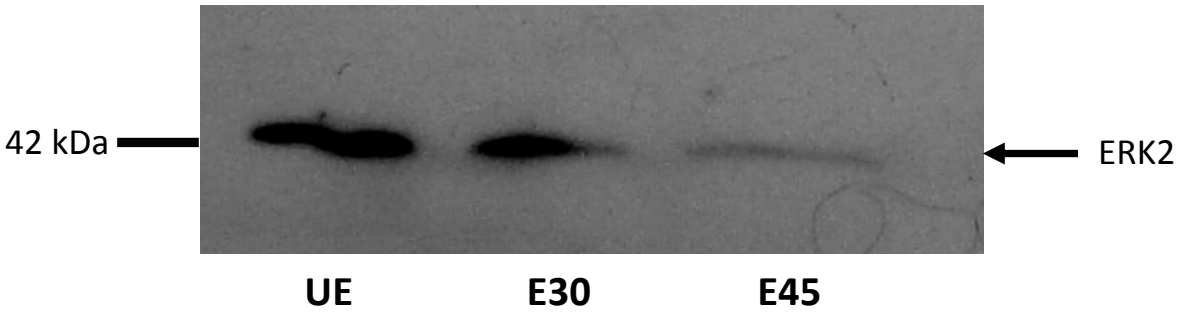
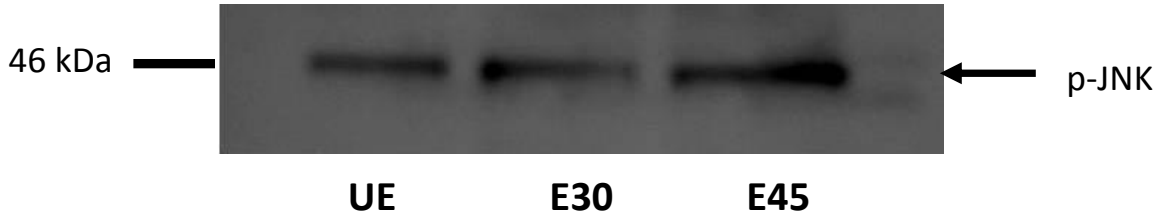


Figure 7: Western blot analysis of protein isolated from endosulfan exposed MCF-7 breast cancer cells shows ERK1/2 phosphorylation.

A - Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (UE) and endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for the phosphorylated form of ERK1/2 (44 kDa and 42 kDa respectively) shows a slight increase in detectable phosphorylated ERK1/2 after 30 minutes and a greater increase in phosphorylated ERK1/2 after 45 minutes. **B** - Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (UE) and exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for ERK2 (42 kDa) shows a slight decrease in unphosphorylated ERK2 after 30 minutes and a greater decrease in unphosphorylated ERK2 after 45 minutes.

A)



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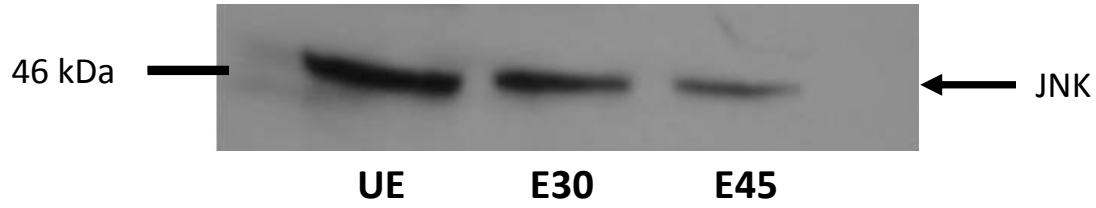
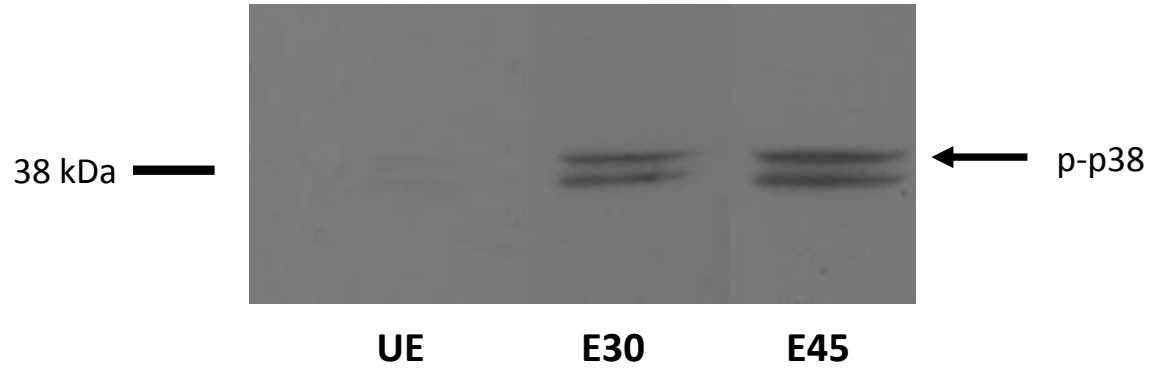


Figure 8: Western blot analysis of protein isolated from endosulfan exposed MCF-7 breast cancer cells shows JNK phosphorylation.

A - Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (UE) and 0.1 µM endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for phospho-JNK (46 kDa) shows no increase in phosphorylated JNK after 30 minutes but a slight increase in phosphorylated JNK after 45 minutes. **B** - Western blot analysis of whole protein samples isolated from unstimulated MCF-7 cells (UE) and endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for JNK (46 kDa) shows a slight decrease in unphosphorylated JNK after 30 minutes but a greater decrease in unphosphorylated JNK after 45 minutes.

A)



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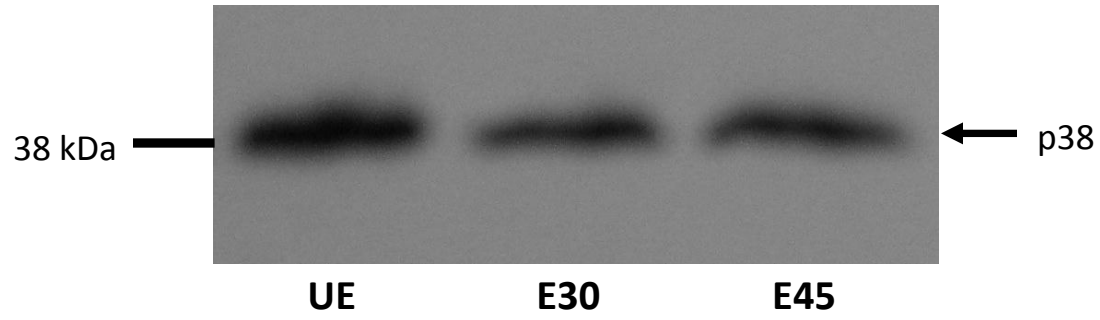


Figure 9: Western blot analysis of protein isolated from endosulfan exposed MCF-7 breast cancer cells shows p38 MAPK phosphorylation.

A - Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (UE) and endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for phospho-p38MAPK (38 kDa) shows an increase in the intensity of the band for phosphorylated p38 MAPK after 30 minutes and after 45 minutes. **B** - Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (UE) and exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and probed with antibodies specific for p38MAPK (38 kDa) shows a slight decrease in unphosphorylated JNK after 30 minutes and after 45 minutes.

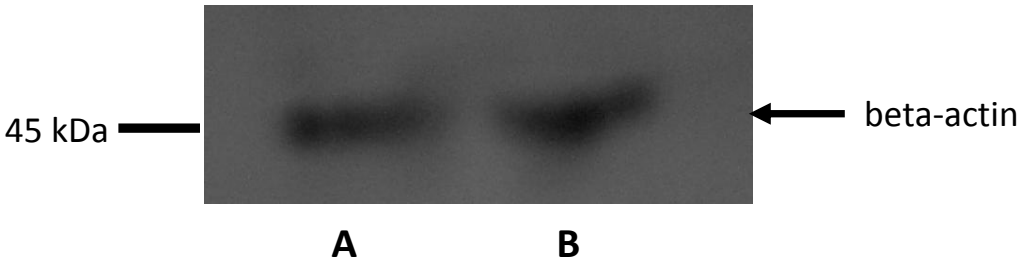


Figure 10: Western blot analysis of beta actin protein isolated from both unexposed and endosulfan exposed MCF-7 cells.

Western blot analysis of whole protein samples isolated from unexposed MCF-7 cells (A) and endosulfan exposed MCF-7 cells (B) and probed with antibodies specific for beta-actin (45 kDa) showed no difference between the two conditions in terms of the intensity of the band.

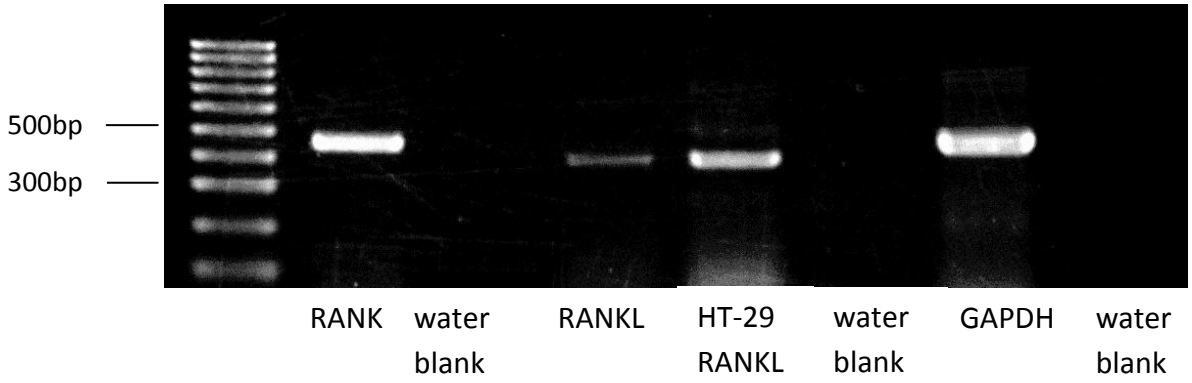
3.3 - Modulation of Gene Expression of RANK and RANKL with Endosulfan Exposure

The presence of RANK (452 bp) and RANKL (389 bp) cDNA was detected in unstimulated MCF-7 cells (Figure 11A). HT-29 human intestinal epithelial cells were used as a positive control for RANKL gene expression as very little RANKL cDNA was expected to be detected in MCF-7 cells. GAPDH (451 bp) was used as a positive RT-PCR control as it is a ubiquitously expressed housekeeping gene always present in MCF7 cells. Water was used as negative PCR controls to detect any contamination of PCR reagents with cDNA. After MCF-7 cells were exposed to 0.1 µM endosulfan for 72 hours, the expression of RANK was no longer detected (Figure 11B) but RANKL cDNA expression levels appeared much greater. GAPDH expression levels did not appear to change in endosulfan exposed cells.

Three replicates of RANK and RANKL gene expression analysis from 0.1 µM endosulfan exposed MCF-7 cells are shown in Figure 12. Replicates were performed on three separate treatments of MCF-7 cells stimulated for 24 hours (S24), 48 hours (S48) and 72 hours (S72). Once again, HT-29 human intestinal epithelial cells were used as a positive control for RANKL gene expression. Unexposed MCF-7 cells displayed RANK, RANKL and GAPDH expression. Endosulfan exposed MCF-7 cells showed an overall gradual decrease in RANK detectability with the dissipation in the intensity of band, increase in RANKL detection with an increase in the intensity of the band and no change in GAPDH with no change in the intensity of the band. The gradual increase in RANKL gene expression was more evident in one of the replicates of RT-PCR analysis of MCF-7

cells exposed to 0.1 µM endosulfan (Figure 13). There was a slight increase in RANKL expression after 48 hours of exposure but a greater increase in the intensity of the band was observed after 72 hours.

A)



B)

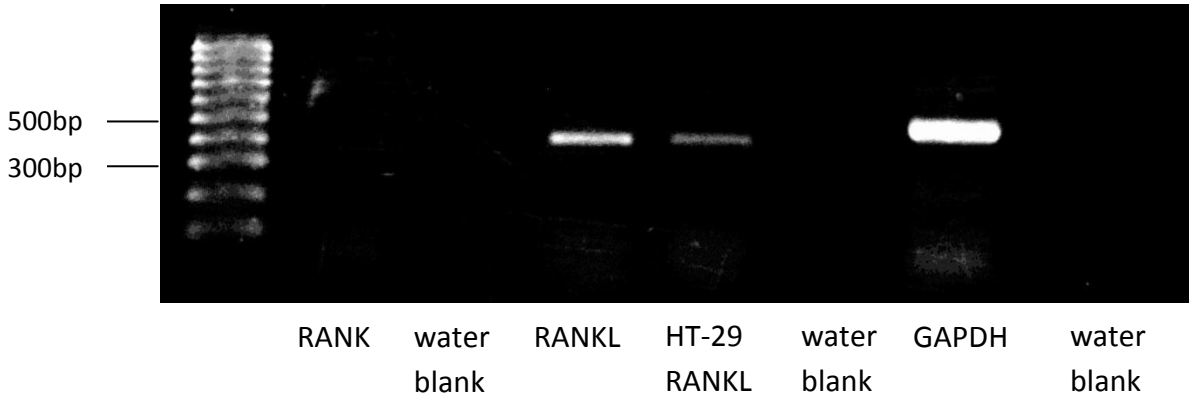


Figure 11: RT-PCR analysis of RANK and RANKL expression levels in unexposed and 0.1µM endosulfan exposed MCF-7 breast cancer cells.

A – RT-PCR showing the expression of RANK (452 bp) and RANKL (389 bp) cDNA in unexposed MCF-7 cells. HT-29 human intestinal epithelial cells used as a positive control for detection of RANKL gene expression. GAPDH (451 bp) used as a positive RT-PCR control in MCF7 cells. Water blanks used as negative PCR controls. **B** - RT-PCR analysis showing the absence of RANK expression in MCF-7 cells exposed to 0.1µM endosulfan for 72 hours. RANKL and GAPDH expression is detected in exposed cells. HT-29 cells used as a positive control for RANKL gene expression. Water blanks used as negative controls.

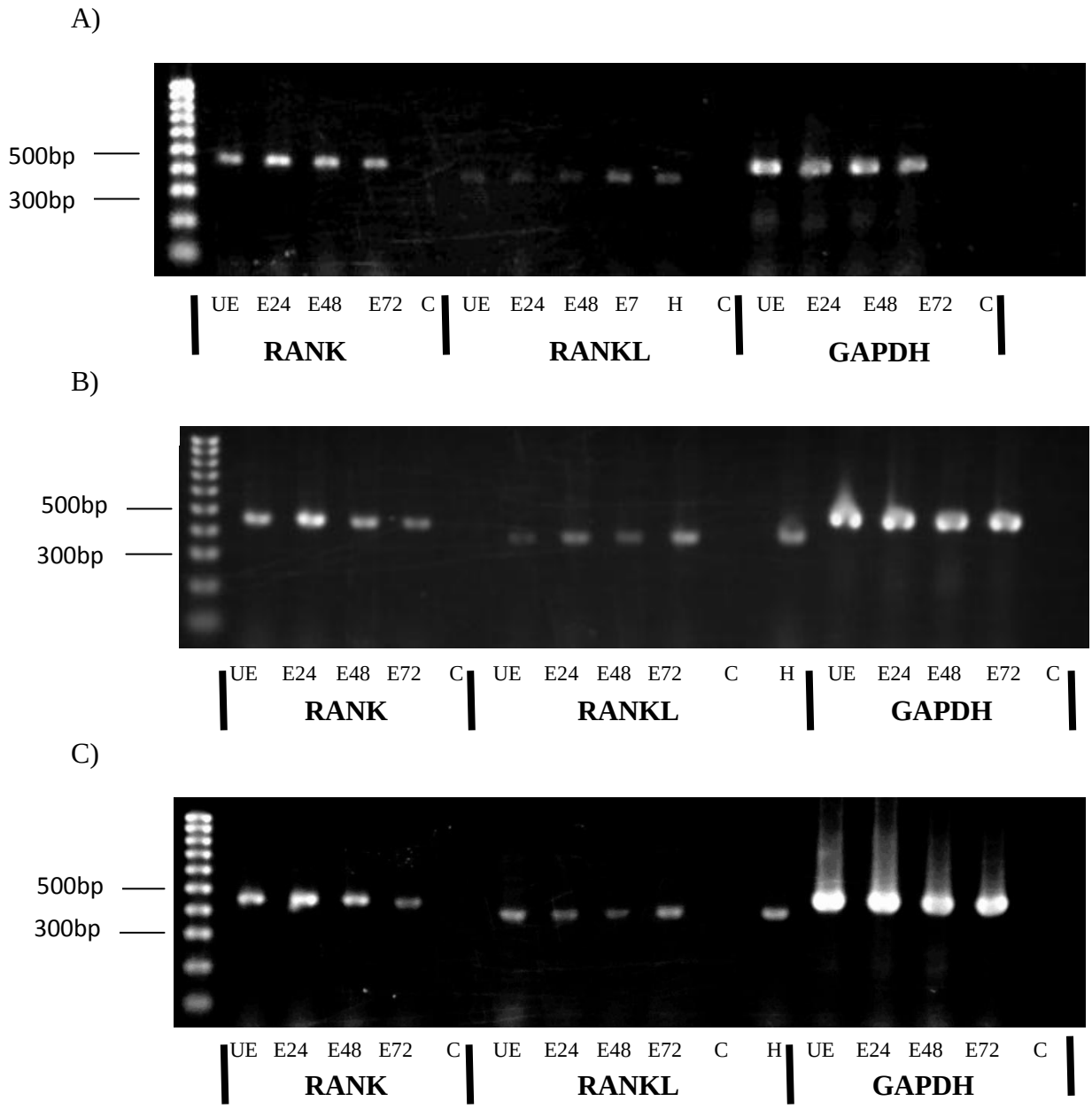


Figure 12: RT-PCR replicates of RANK and RANKL gene expression analysis from 0.1 µM endosulfan exposed MCF-7 cells. A)Unexposed cells (UE) MCF-7 cells show RANK (452 bp), RANKL (389 bp) and GAPDH (451 bp) expression. B) Endosulfan exposed MCF-7 cells for 24 hours (E24), 48 hours (E48) and 72 hours (E72) shows a decrease in detection of RANK cDNA, an increase in RANKL cDNA expression and no change in GAPDH cDNA detection. HT-29 human intestinal epithelial cells (H) used as a positive control for RANKL gene expression detection and water blanks used as negative controls (C) for RT-PCR reactions.

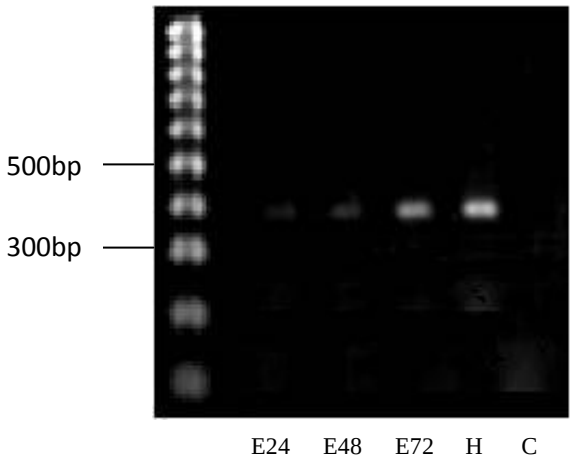


Figure 13: Increase in RANKL cDNA detection with 0.1 µM endosulfan exposure over the span of 72 hours.

RT-PCR analysis of MCF-7 cells exposed to 0.1µM endosulfan show a slight increase in detectable RANKL transcripts after 48 hours (E48) but the intensity of the band increased after 72 hours (E72). HT-29 cells (H) used as a positive control for RANKL gene expression detection. Water blank used as a negative control (C).

3.4- Endosulfan induced p38 MAPK Intracellular Signalling Inhibited by SB203580

SB203580, a specific inhibitor of p38-MAPK pathway (Barancik *et al*., 2001) was used to inhibit the phosphorylation of p38 MAPK protein. Protein isolated from 0.1µM endosulfan exposed MCF-7 cells that were also treated with SB203580 inhibitor showed a slight decrease in detectable phosphorylated p38 MAPK (38 kDa) after 30 minutes but there was a greater decrease in the intensity of the phosphorylated p38MAPK band after 45 minutes compared to cells exposed to endosulfan alone (Figure 9A). Accordingly, protein isolated from 0.1 µM endosulfan exposed MCF-7 cells treated with SB203580 inhibitor showed an increase in the intensity of the unphosphorylated p38 MAPK band after 30 minutes and after 45 minutes compared to only endosulfan exposed cells (Figure 9B). This indicates that inhibition of endosulfan induced phosphorylation of the p38 protein can be achieved with this select inhibitor.

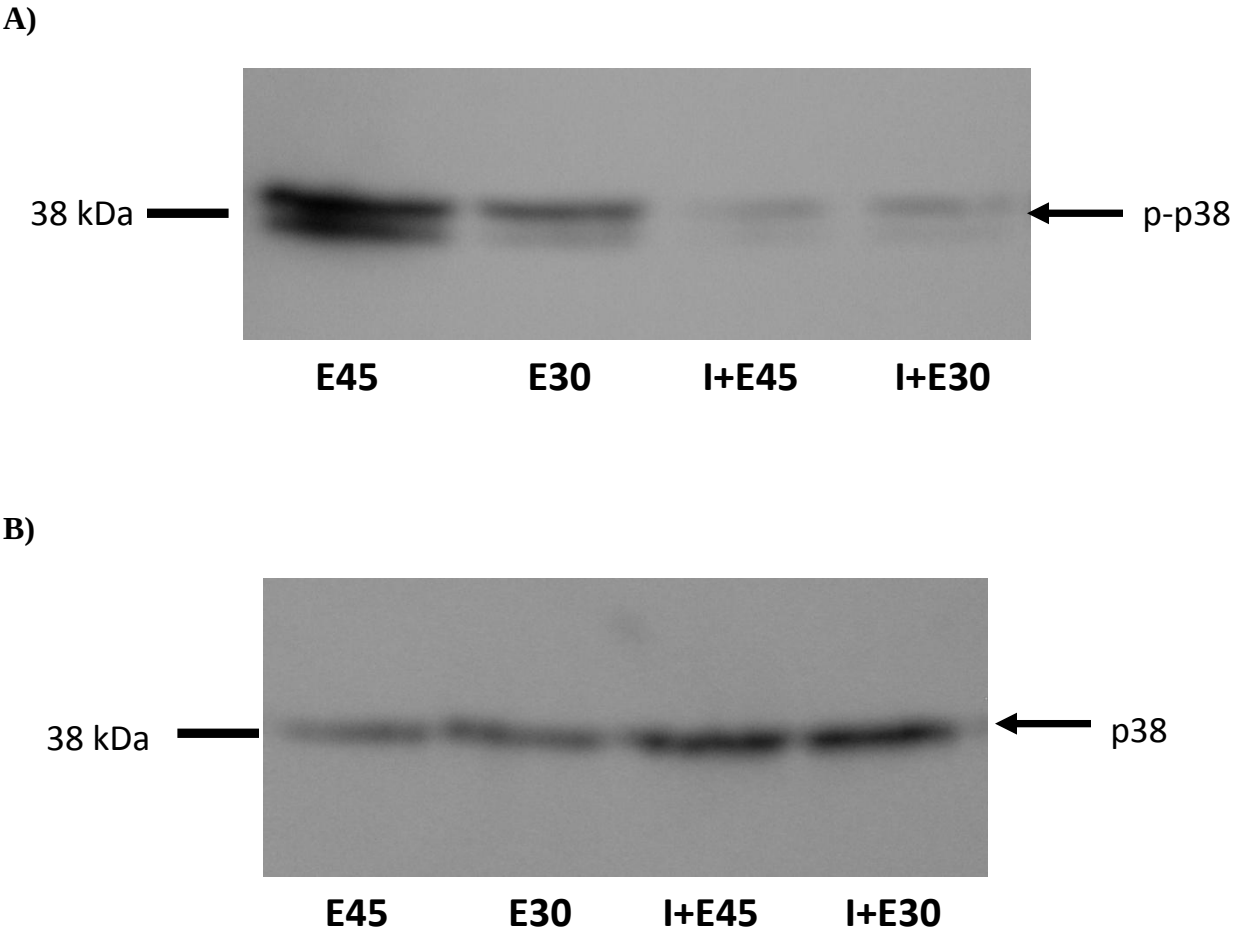


Figure 14: Inhibition of p38 MAPK phosphorylation induced by 0.1 µM endosulfan exposure with SB203580.

A - Western blot analysis of whole protein samples isolated from endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and endosulfan exposed cells treated with SB203580 inhibitor for 30 minutes (I+E30) & 45 minutes (I+E45). The membrane was probed with antibodies specific for phospho-p38MAPK (38 kDa) and shows a decrease in phosphorylated p38 MAPK after 30 minutes and after 45 minutes of inhibitor treatment. **B -** Analysis of whole protein samples isolated from endosulfan exposed MCF-7 cells for 30 minutes (E30) & 45 minutes (E45) and endosulfan exposed cells treated with SB203580 inhibitor for 30 minutes (I+E30) & 45 minutes (I+E45). The membrane was probed with antibodies specific for p38MAPK (38 kDa) and shows a increase in unphosphorylated p38 MAPK after 30 minutes and after 45 minutes of inhibitor treatment.

4. - Discussion

4.1. Endosulfan Remains to be a Global Health Concern

Endosulfan is used as an insecticide all over the world. It is a ubiquitous contaminant in the environment and resistant to degradation processes. Many studies have shown that exposure to endosulfan is positively correlated with endocrine disruption (Lemaire *et al.*, 2006), reproductive and immune dysfunctions (Saiyed *et al*., 2003), and cancers such as those of the breast (Ibarluzea *et al*., 2004; Hansen and Matsumura, 2001). Despite the discontinuation of the use of endosulfan since January 2011 in Canada (Health Canada), it is expected to persist in the environment because the half-life of an endosulfan metabolite, endosulfan sulphate, is on the order of weeks to years (Leonard *et al*., 2001; Wan *et al*., 2005b). Hung *et al.* (2005) proposed that the atmospheric half-life for endosulfan (α) is 38 years in the Canadian Arctic. Endosulfan and its metabolites have regularly been detected in groundwater, surface water, sediment and rain and snow in many regions of the world (Kumari *et al*., 2007; Tuduri *et al*., 2006; Herbert *et al*., 2004; Schulz, 2004; Tariq *et al*., 2004). Due to its continued use in some developing countries (Jia *et al*., 2008) and widespread atmospheric transport and deposition, it still remains an important global public health concern.

Endosulfan is also a highly toxic substance. Low concentrations of endosulfan exert significant reproductive and endocrine-disruptive effects. In particular, endosulfan evokes estrogenic responses on human MCF-7 breast cancer cells (Li *et al*., 2006; Soto *et al*., 1995). Physiological concentrations of endosulfan (α) and (β) in humans range from 17.72 ng/g lipid found in female adipose tissue (Cerrillo *et al.*, 2005) to 2.44ng/mL found

in cord serum (Mariscal-Arcas *et al*., 2010). Therefore, comparable concentrations of 0.1 µM and 1 µM endosulfan were used in order to optimize the relevance of our study.

4.2. - Endosulfan Stimulates Proliferation in MCF-7 cells

Previous research has shown that exposure to endosulfan causes proliferation of MCF-7 human breast cancer cells at concentrations of 10-25 µM (Li *et al*., 2006; Soto *et al*., 1995). Our research confirms the proliferative effects of much lower concentrations of endosulfan on these same MCF-7 cell type (Figure 6). Endosulfan was found to be stimulatory at both 0.1 µM and 1.0 µM concentrations at 24 hours, 48 hours and 72 hours. However, there was no significant difference in terms of cell proliferation between these two concentrations at 24 hours and 48 hours. This can be attributed to the notion that both of these concentrations are relatively low and cause similar stimulatory effects at 24 hours and 48 hours. However, exposure to the lower concentration (0.1 µM) of endosulfan caused greater proliferation than the higher concentration (1.0 µM endosulfan) after 72 hours. Exposure to higher concentrations for a prolonged period of time may be toxic and cause cell death. The dose-response pattern is not yet fully understood; however, endosulfan sulphate, the breakdown product of endosulfan, is considered to be as toxic as the parent isomers (Leonard *et al*., 2001). Therefore, this greater accumulation of endosulfan metabolites at 72 hours in the cells exposed to higher concentrations may be toxic to the cells. Conversely, the lower concentrations may be more involved in activating estrogen receptor (ER) α and inducing smaller Ca^{2+} influx (Watson *et al*., 2007). Smaller accumulation of Ca^{2+} over a prolonged period may lead to

more proliferative responses in the long term. The notion that a lower concentration had a greater stimulatory effect than the higher concentration may also forecast the possibility that the lower concentration had a more subtle effect on activating gene expression. Exposure to low concentrations of endosulfan has been shown to increase the relative amount of the 16 alpha-hydroxyestrone metabolite which is involved in enhancing breast cell growth in comparison to 2-hydroxyestrone which inhibits breast cell proliferation (Bradlow *et al*., 1995).

The observation that very low concentrations of endosulfan can cause cancer cell stimulation is important because these concentrations of organochlorines and other estrogen mimicking compounds present in our environment may be an important etiological factor in the development and progression of breast cancer. This suggests that these low concentrations of endosulfan that were otherwise known to be subtoxic can have deleterious impact on human health.

4.3. - Endosulfan Activates Proteins of MAPKK Signaling Pathway

Activation of MAPKK signalling cascades can lead to opposing cellular responses such as proliferation, growth arrest, cell differentiation and apoptosis simultaneously depending on the duration and strength of the external stimulation and on the cell type (Shaul and Seger, 2007).

In MCF-7 breast cancer cell lines, low concentrations of endosulfan appear to be a potent activator of some non-genomic signalling pathways that can be connected to cell proliferation events. The non-genomic pathways activated by estrogen mimicking compounds independent of estrogen receptor activation are not yet fully elucidated

(Hatakeyama *et al*., 2002). Nevertheless, organochlorine pesticides cause the activation of MAPKK signalling cascades that play a critical role in cell survival, proliferation and differentiation, ultimately altering cell behaviour (Ledirac *et al*., 2005). The MAPKK cascades are found in all eukaryotic cells including human MCF-7 breast cancer cells. Our research shows that endosulfan causes activation of key proteins within the MAPKK signalling pathway including ERK1/2, JNK and p38 MAPK. Activation of MAPKK signalling cascades by organochlorine pesticides in breast cancer cells is a novel phenomenon which has not been explored in the past.

The presence of some detectable levels of phosphorylated ERK2 protein in non treated MCF-7 cells illustrates that these breast cancer cells already have slightly activated ERK2 signalling which is known to be involved in promoting cell-cycle progression by inactivating a cell-cycle inhibitory kinase (Palmer *et al*., 1998) and stimulating cell proliferation by indirectly inducing cyclin D1 (Treinies *et al*., 1999). After exposure to low concentrations (0.1 µM) of endosulfan, there was a marked increase in phosphorylated ERK1/2 proteins especially after longer exposure of 45 minutes (Figure 7). This signifies that endosulfan is a potent activator of ERK1/2 signaling pathway, further augmenting cell proliferation and possibly inactivating cell cycle inhibitory kinases. Our results are consistent with previous studies that reported activation of ERK1/2 by 50 µM heptachlor in human lymphocytes (Chuang and Chuang, 1998) and by both endosulfan and heptachlor in human keratinocyte (HaCaT) cells (Ledirac *et al*., 2005).

Similarly, although some phosphorylated JNK protein was present, most of the JNK protein was detected in unphosphorylated form in non treated MCF-7 cells. The

activation of this pathway is linked with the induction of cell differentiation and apoptosis (Xia *et al*., 1995). After exposure to endosulfan, there was a marked increase in phosphorylated JNK proteins especially after a longer exposure of 45 minutes (Figure 8). These findings correlate with a previous study illustrating the activation of JNK signaling pathway by endosulfan and heptachlor in human keratinocyte cells (Ledirac *et al*., 2005). Therefore, it appears that organochlorines, in particular endosulfan, is capable of activating JNK signaling pathway and consequently regulating gene expressions involved in cell differentiation and apoptosis.

Similar to the JNK pathway, the p38 MAPK protein is also involved in the cell differentiation and apoptosis. Contrary to the JNK protein discussed earlier, p38 MAPK signaling protein was found in an inactive state in untreated MCF7 cells. A striking phosphorylation of p38 MAPK protein occurred after 30 minutes exposure with low concentration of endosulfan illustrating that endosulfan is a potent activator of p38 MAPK signalling pathway (Figure 9). Supporting evidence from previous research also demonstrated the presence of non-activated form of p38 MAPK found in some tumours such as in breast cancer which could be stimulated with glypican-3, a proteoglycan involved in cell proliferation and survival (Buchanan *et al*., 2010). The transformation of non-activated form of p38 MAPK to the activated form by endosulfan in cancer cells depicts the importance of this pathway is mediating the mammary epithelium proliferation and differentiation in tumours.

Hence this shows the complex interplay of MAPKK signalling proteins activated by low concentrations of endosulfan which would in part explain the observed increase in cell proliferation (Figure 6). Activation of intracellular pathways by this ubiquitous toxin

is an important finding because it suggests the role of MAPKK pathway in regulating organochlorine- induced gene alternations and alterning cell behaviour. It raises attention on better understanding of the underlying mechanisms of this pathway suspected to be involved in mammary epithelium proliferation and tumorigenesis.

When the p38 MAPK pathway in endosulfan treated MCF-7 cells was inhibited with a specific inhibitor, SB203580, a marked decrease in phosphorylated p38 MAPK protein and a remarkable increase in unphosphorylated p38 MAPK protein was detected (Figure 14). This confirmed that the phosphorylation of p38 MAPK protein was caused by endosulfan exposure. Importantly, blocking endosulfan-induced phosphorylation of the p38 protein can be achieved with this select inhibitor in order to further investigate downstream mechanisms and effects of endosulfan exposure on gene expression. Since activation of p38 can be completely blocked in endosulfan exposed cells, future research could look into whether endosulfan induced RANK and RANKL gene expression is regulated through the p38 MAPK pathway. This will add to the understanding of the regulation of RANK and RANKL signalling axis and provide a relationship between the MAPKK pathway and RANK/RANKL expression in human breast cancer.

4.4. - Endosulfan Modulates RANK and RANKL Gene Expression

RANK is constitutively expressed throughout development in normal mammary epithelial cells (Srivastava *et al*., 2003). Although previous research stated that RANKL is only found in female mammary epithelial cells during pregnancy and lactation (Theill *et al*., 2002; Kotake *et al*., 2001; Kong *et al*., 1999), more recent evidence illustrates it is also weakly expressed in tissues isolated from human breast tumours (Reinholz *et al*.,

2002) and in metastatic cells (Bhatia *et al*., 2005). Our research supports this finding since RANKL is weakly expressed in MCF-7 cells (Figure 11).

Previous research showed that the induction of RANKL is essential for the formation of lobulo-alveolar mammary structures during pregnancy (Fata *et al*., 2000). This induction is mediated by reproductive hormones such as progesterone and prolactin (Schramek *et al*., 2010; Henninghausen and Robinson, 2001). A recent study showed that synthetic progesterones (progestins) such as MPA triggers a massive induction of RANKL in mammary gland (Schramek *et al*., 2010; Gonzalez-Suarez *et al*., 2010). Similarly, hormone mimicking endosulfan also caused upregulation of RANKL gene expression in MCF-7 cells (Figure 11). Additionally, exposure to a low concentration of endosulfan for 72 hours caused RANK expression to disappear in MCF-7 cells (Figure 11). Importantly, endosulfan stimulation of MCF-7 cells caused an overall gradual decrease in RANK and increase in RANKL over the course of a three day period. This indicates that endosulfan exposure causes specific changes in gene expression. Organochlorines have been shown to induce MAPK signaling pathway, in particular the p38 kinase cascade, stimulating activator protein (AP-1) gene expression in human endometrial Ishikawa cells (Frigo *et al*., 2004).

Similar to RANK and RANKL, AP-1 is involved in controlling a number of cellular processes including differentiation, proliferation, and apoptosis (Ameyer *et al*., 2003). This provides insight into the relevance and perhaps detrimental effects of exogenous compounds on altering gene expression and fate of the cell. It is known that hormones and hormone derivatives play a prominent role in the activation of RANK/RANKL signalling axis that drives the mammalian cells into the cell cycle

progression and differentiation (Schramek *et al*., 2010; Gonzalez-Suarez *et al*., 2010). Induction of RANKL after stimulation with progesterone or prolactin increases proliferation in mammary epithelium during mammary gland lactational morphogenesis (Beleut *et al*., 2010; Fata *et al.*, 2000).

However, induction of RANK/RANKL signalling axis in the absence of hormones can also induce hyper proliferation of mammary epithelial cells (Fernandez-Valdivia *et al*., 2010; Gonzalez-Suarez *et al*., 2010) linking this pathway to mammary tumorigenesis. Currently, Stat5a activation is the mechanism through which RANK/RANKL gene expression is thought to be regulated. No current data exists on how other signalling pathways may contribute to the regulation of RANK and RANKL gene expression in breast cells. Since our results show that endosulfan alters RANK and RANKL gene expression in human MCF-7 breast cancer cells and is also a potent activator of key proteins in the MAPKK signalling pathway, a study on how MAPKK pathways in breast cancer cells may regulate RANK/RANKL gene expression will contribute new knowledge in this field.

Therefore, the results of this study contribute to further understanding of the detrimental impact of endosulfan as an environmental toxin to humans, provide further important measurable connections between environmental toxins and breast cancer, elucidate the mechanisms of action of cell behavior changes with endosulfan exposure and provide new knowledge of RANK/RANKL regulation by intracellular pathways.

Low but significant concentrations of endosulfan and other estrogen mimicking compounds present in our environment can have adverse impact on humans. A direct

correlation between the level of exposure to organochlorines and the risk of breast cancer is not yet determined but it seems to be an important etiological factor in the development and progression of breast cancer. A better understanding of the underlying mechanisms causing cell proliferation after endosulfan exposure and also the mechanisms involved in mammary epithelium proliferation and tumorigenesis is required. Furthermore, the possibility of the regulation of RANK and RANKL genes through the activation of intracellular kinase pathways uncovers a new area of research of immense clinical relevance.

4.5 - Advantages and Disadvantages of MCF-7 Breast Cancer Cell Lines Used in this Study

Using an in vitro model to study the effects of endosulfan on MCF-7 breast cancer cells presented a number of advantages and disadvantages. MCF-7 breast cancer cell lines were easy to handle and easy to replace when contaminated due to their ability to grow and self-replicate in infinite quantities. However, one of the disadvantages of using these cell lines is that MCF-7 cells may display genotypic and phenotypic drift after continual culturing. Although, most MCF-7 cells appear morphologically identical, subpopulations and continual culturing may develop variations in cell growth rate, hormone receptors and karyotypes (Bahia *et al*., 2002). Also, MCF-7 cell lines used in this study were derived from pleural effusions and not from the primary breast tumours. Therefore, these cells might have already possessed characteristics of later-stage metastatic tumours as opposed to the primary tumours in the breast. Therefore in order to determine a direct correlation between the level of exposure to organochlorines and the risk of breast cancer, the use of primary cell cultures will also be valuable.

4.6 - Conclusions and Future Directions

Concentrations of endosulfan used in this study were similar to physiological levels found in humans. As previously mentioned, endosulfan is persistent in the environment and its main metabolite, endosulfan sulphate, is bioaccumulative. Endosulfan exhibits estrogenic properties, which may relate to an increase in the risk of breast cancer or may impact the progression of the disease. Our results found endosulfan to be more stimulatory at lower concentrations and toxic at higher concentrations. In fact, a study by Bulayeva & Watson (2004) described that the estrogenic effect of endosulfan showed a bimodal response whereby the estrogenic responses were observed at very low or high levels, but not at medium levels. It would be interesting to measure endosulfan levels present in women with breast cancer and whether the amount of endosulfan present is correlated with the etiology and progression of breast cancer.

In addition, our study tested the effects of endosulfan in the absence of endogenous hormones. It would be intriguing to look at whether the presence of endogenous hormones and growth factors have a synergistic or antagonistic effect on breast epithelial cell proliferation and tumorigenesis. Endosulfan causes activation of key proteins within the MAPKK signaling pathway including ERK1/2, JNK and p38 MAPK that can be connected to mammary epithelium proliferation and tumorigenesis. Further studies may explore other pathways important to cell survival and differentiation. Additionally, how these pathways impact the phosphorylation status of Stat5a will need to be established.

Our study focused on the induction or dissipation of RANK and RANKL gene expression based on semi-quantitative methods using PCR and densitometry. The effects of low concentrations of endosulfan could be further investigated using quantitative real time PCR to measure changes in transcript levels and to compare the statistical differences. Our results showed that endosulfan caused an upregulation of RANKL gene expression while simultaneously causing down regulation of RANK gene expression. Induction of RANKL is correlated with mammary epithelial proliferation, however, there is no current data available on this relationship between RANK expression decrease with the induction of RANKL expression. It would be noteworthy to study the underlying mechanisms of intracellular pathways having opposing effects on RANK and RANKL expression in breast cell lines. This may be of interest in the study of breast cancer progression and metastasis as breast tumours show varying amounts of expression of RANK (Gonselez-Suarez, 2010).

Therefore this research provides important new insight into the regulation of genes known to be important in breast biology and breast cancer through important cellular signalling pathways by a known environmental toxicant.

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