Abstract. Cancer development is a multistep process that may be induced by a variety of compounds. Environmental substances, such as pesticides, have been associated with different human diseases. Organophosphorus pesticides (OPs) are among the most commonly used insecticides. Despite the fact that organophosphorus has been associated with an increased risk of cancer, particularly hormone-mediated cancer, few prospective studies have examined the use of individual insecticides. Reported results have demonstrated that OPs and estrogen induce a cascade of events indicative of the transformation of human breast epithelial cells. In vitro studies analyzing an immortalized non-tumorigenic human breast epithelial cell line may provide us with an approach to analyzing cell transformation under the effects of OPs in the presence of estrogen. The results suggested hormone-mediated effects of these insecticides on the risk of cancer among women. It can be concluded that, through experimental models, the initiation of cancer can be studied by analyzing the steps that transform normal breast cells to malignant ones through certain substances, such as pesticides and estrogen. Such substances cause genomic instability, and therefore tumor formation in the animal, and signs of carcinogenesis in vitro. Cancer induction has been associated with an increase in genomic instability, indicated by the inactivation of tumor-suppressor genes and activation of oncogenes in the presence of malathion, parathion, and estrogen. In the present study, a comprehensive summary of the impact of OPs in human and rat breast cancer, specifically their effects on the cell cycle, signaling pathways linked to epidermal growth factor, drug metabolism, and genomic instability in an MCF-10F estrogen receptor-negative breast cell line is provided.

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1. Introduction

Environmental substances appear to be involved in various human diseases, including breast cancer (1,2). Based on epidemiological evidence, several studies have found an association between human cancer and exposure to agricultural pesticides, such as organophosphorus pesticides (OPs) (3-5). Generally, OPs can protect agricultural products for decades and have therefore been widely used, particularly malathion, to control vectors (6). Parathion has also been used as a pesticide in agricultural settings. These pesticides, however, pose a serious threat to multiple organisms, including humans. For instance, certain pesticides have been associated with blood diseases, such as non-Hodgkin's lymphoma (7-9) and leukemia (10,11).

Other pesticides, including organochlorines, creosote, and sulfallate, have been reported to be carcinogenic in vivo studies (12), whereas dichlorodiphenyltrichloroethane, chlordane, and lindane have been found to act as tumor promoters (13-15). However, individual pesticides have only been evaluated in a limited number of human studies. In addition, certain substances in commercial pesticide formulations may pose a carcinogenic risk to humans (15,16). Thus the International Agency for Research on Cancer (IARC) (17) classified parathion as 'possibly carcinogenic' (Group 2B) and malathion as 'probably carcinogenic' to humans (Group 2A). Furthermore, experimental studies have proposed that malathion or its derivatives could be carcinogenic, indicating that impurities found in commercial malathion, such as malaoxon and isomalathion, induce DNA damage (18,19).

The etiology of breast cancer remains unclear, and humans are exposed not only to pesticides but also to a mixture of...
estrogenic agents (20). Estrogens have been implicated in the etiology of breast cancer by epidemiological and experimental evidence (21-24). Moreover, the importance of hormones in mammary cancer (25), as well as the effect of a variety of compounds on this process (26,27), have been demonstrated. The exposure of human populations to these substances renders it necessary to consider the effect of pesticides and estrogens on human health.

Studies using various human epithelial cell lines have been performed to analyze the cellular and biological processes involved in transforming a normal cell into a cell with a malignant phenotype (28,29). Furthermore, the use of experimental animals and cells in the laboratory has allowed us to determine whether these environmental substances induce breast cancer (20,21,30-36). Table I shows the phenotypic characteristics of cell lines.

The use of the MCF-10F immortalized normal human breast epithelial cell line has enabled the detection of the sensitivity to several substances, such as 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(a)pyrene (BP) (37), and physical factors, such as ionizing radiation (38), and the determination of their carcinogenic properties.

The present study aimed to summarize the in vitro signs of transformation induced by environmental substances, such as malathion and parathion, in the presence of an endogenous substance, such as estrogen, through the use of the MCF-10F human immortalized breast cell line. This type of cell line is an important tool in the experimental study of breast carcinogenesis induction by hormones or transfection with a c-Ha-ras, or its prevention by antioxidants, such as curcumin (39-41). Table I shows the effect of malathion, parathion, and estrogen on anchorage independence and the invasive capabilities of treated cells. The MCF-10F cell line treated with malathion or parathion alone and in combination with estrogen induced anchorage-independent growth and invasion; however, the same cell line treated with estrogen alone and the control were negative under the same conditions. A previous study demonstrated that estrogen exerts its effects when combined with pesticides in this model, providing an approach to studying this process (35). A new approach has emerged for analyzing carcinogens by the IARC and previous studies; carcinogens were classified based on 10 common characteristics associated with carcinogenesis (17,42,43).

2. Data collection

In the present review, a search on MEDLINE (through PubMed), Web of Science, and SCOPUS was conducted between January 2020 and June 2020 to identify studies examining the in vitro changes of the normal MCF-10F human breast epithelial cell line under the effect of pesticides in the presence of estrogen. The selection was based on cell transformation assays using the MCF-10F cell line to examine the following: i) Cell proliferation by the trypan blue exclusion method; ii) cell growth in a semisolid medium by anchorage-independent assay; iii) cell invasion by cell invasion assay; iv) oncoprotein by immunocytochemistry coupled with confocal microscopy; v) gene expression in several arrays with cell cycle-related key genes; human drug metabolism in gene array including genes that encode important receptors and several enzymes involved in drug transport and phase I and phase II metabolism; and vi) genomic instability in a human cancer oligo array.

3. Parathion and malathion increase cell proliferation

The association between OPs and estrogen was analyzed in relation to mammary carcinogenic capability. Exposure to OPs can be considered an important initiator of breast cancer, as shown by several signs of carcinogenicity detected in vivo and in vitro. The in vivo studies were based on morphological and molecular experiments using Sprague-Dawley rats. Since malathion significantly increased the density of terminal end buds (TEBs), this research allowed us to obtain a model of the initiation of mammary gland cancer. The primary outcome in rats was the increase of mammary cells in TEBs that were then transformed into proliferative ducts by malathion or parathion, eventually resulting in ductal mammary carcinomas morphologically similar to those found in the breast (30,31,44).

When the animal was injected with estrogens, the formed TEBs were transformed into proliferative lobules full of secretions, with a decreased density of alveolar buds, resulting in actively growing tumors (30,31,44); the pathology of these tumors was of the lobular type. Both the ductal and lobular mammary carcinomas were similar to those classified by the World Health Organization. When the animals were exposed to pesticides and estrogen, both types of structures such as ducts and lobules were observed. Mammary gland tumors then metastasized to the bronchi, lungs, and kidneys. The effect of OPs was avoided by atropine demonstrating an association of atropine with the muscarinic receptor. In vivo studies showed signs of carcinogenicity, including cell proliferation leading to tumor formation and genomic instability (32). The mechanisms for mammary carcinogenic potential included acetylcholinesterase inhibition, increased oxidative stress, decreased apoptotic signaling, and endocrine-disrupting capabilities.

Parathion (33) and malathion (20) had been previously found to increase cell proliferation and induce cell transformation affecting protein expression in the MCF-10F cell line (20,33,34,36,45). It was found that malathion alone or in the presence of estrogen, induced anchorage-independent growth, cell invasive capabilities, altered cell cycle regulation, and increased genomic instability in the MCF-10F breast cell line in vitro (20,36). Another study demonstrated that malathion induced changes in gene expression (45). A scheme of exposure to OPs, estrogen, and chemical structures of malathion and parathion is presented in Fig. 1.

When the established model was initially developed, morphological changes were the first observed signs in vitro, which included a changing doubling time, colony agar formation, and invasive capabilities, all indicative of a very aggressive phenotype, as compared to the control cells (20,21,33,36,38,46-49). On the other hand, it was observed that atropine, an antagonist of muscarinic receptors, when combined with any of these pesticides inhibited all aforementioned effects (33).

The same was observed in another cell line, the MCF-7 malignant breast cancer cell line, in which estrogen markedly increased cell proliferation in vitro after 6 days (44).
Table I. Phenotypic characteristics of cell lines (20).

| Treatment                               | Anchorage independent growth assay | Invasion assay |
|-----------------------------------------|------------------------------------|----------------|
| MCF-10F without treatment              | -                                  | -              |
| MCF-10F treated with E2 (10⁻⁸ M)        | -                                  | -              |
| MCF-10F treated with M (0.5 µg/ml)      | +                                  | +              |
| MCF-10F treated with M and E2           | +                                  | +              |
| MCF-10F treated with P (100 ng/ml)      | +                                  | +              |
| MCF-10F treated with P and E2           | +                                  | +              |

E2, 17β estradiol; M, malathion; P, parathion.

Figure 1. Breast carcinogenesis induced by organophosphorus pesticides (OPs), malathion and parathion. (A) Schematic diagram of key carcinogenicity characteristics. (B) Chemical structures of malathion and parathion. (C) Scheme of cell proliferation. (D) In vivo studies: Representative images of protein expression in cross-sections of tissues derived from female Sprague-Dawley rat mammary glands exposed to malathion and atropine. (D-a) Normal duct, (D-b) ductal carcinoma derived from malathion-treated rats immunostained with Rho-A where cells are observed (arrow), and (D-c) ductal carcinoma derived from atropine-treated rats immunostained with c-Ha-Ras where vacuoles instead of cells are observed (arrow). All images were obtained from our own laboratory for this review. Tissues derived from animals were obtained out of a repository of paraffin blocks performed in a previous study (31).
Moreover, it was reported that sumithrin, a pyrethroid pesticide used to control pests in agriculture (50), also induced proliferation in vitro at 10^{-9} M and 10^{-6} M doses after 6 days of treatment (51). In addition, in mammalian cells, the pesticide parathion-methyl increased the number of cells and changes in the MCF-7 cell line at low concentrations, exerting toxicity and altering cell-cell interactions in human intestinal cells; it also had other effects on murine fibroblasts, such as increased DNA synthesis (52). These studies demonstrated not only a cell proliferation effect but also other possible physiological effects with a serious impact on humans.

It can be hypothesized that one possible mechanism for breast cancer development is the consequence of excessive estrogenic stimulation that induces cell proliferation of normal breast epithelial tissue (53). More specifically, the malignant phenotype is developed through errors in cell division (DNA copying errors, translocations); furthermore, estrogen is known to control the growth of several carcinomas in experimental animals and humans (53).

Although MCF-10F cells are estrogen receptor (ER)-negative, they were found to be very sensitive to 17β-estradiol (E2) at 10^{-8} M since cell proliferation increased (1.6 fold) after 10 days of culture (53). The addition of E2 and 10^{-7} M tamoxifen to the MCF-10F cells gave similar results to those of the malignant carcinomas in vitro since it was found that E2 increased cell proliferation, as compared with the control. However, tamoxifen alone and tamoxifen plus E2 inhibited cell proliferation more notably when compared to E2 alone. The association between cell proliferation and proteins involved in cell cycle regulation was also investigated (54,55). When cell cycle control is lost, cells are able to continue dividing (55). When the cell cycle becomes deregulated, it can lead to aberrant cell proliferation, eventually resulting in cancer (56). This observation prompted the analysis of gene expression during the cell cycle as well as its regulation and proliferation.

Differential gene expression was studied using the Oligo GEArray® human cancer microarray (cat. no. OHS-802) in estrogen- and pesticide-treated MCF-10F breast epithelial cells. The results indicated that parathion and estrogen alone, or a combination of the two, induced transcriptional alterations in 22/96 genes from a cDNA array. These alterations involved genes associated with the regulation of the cell cycle, such as cyclins A1, A2, C, D3, G1, G2, and H, cyclin-dependent kinases (CDKs), including CDK41, and minichromosome maintenance protein complex (MCM), a 2-7 hexameric helicase, including MCM2 and MCM3 (20,21). Regarding cyclins, this family of proteins, particularly D-type cyclins, form a complex with CDKs, which affects the cell cycle at the G1 phase (57,58), regulating the cell cycle as a whole (59). Pesticides have been shown to have an affinity for CDKs, with OPs exhibiting a particular affinity for CDK2 and CDK4, which affects cell cycle regulation in mammalian cells and other pesticides, such as carbamates and synthetic pyrethroid; these were also evaluated, and a positive interaction was identified between them and CDKs at low doses (60).

Previous studies have indicated that the cyclin A2 gene was downregulated by all the substances under study and, whereas cyclin C and cell division cycle 6 (CDC6) were upregulated 3-fold by parathion, as compared with the control. Cyclin D3 gene was upregulated by both estrogen and parathion. Cyclin-dependent kinase CDKNIA was upregulated 3-fold by parathion alone, and CDKN2C, which is associated with cell cycle checkpoint and cell cycle arrest, was downregulated by both estrogen and parathion (21).

Furthermore, different treatments of pesticides alone or in combination with estradiol were shown to upregulate the cyclin D1 and CDK genes. Of note, the resulting proteins were involved in the phosphorylation of important effectors associated with different stages of the cell cycle (61-64). A previous study revealed upregulation by the effect of E2 in combination with the pesticide compounds, including the upregulation of cyclin family genes, including keratin 18 (20). These results were in agreement with previous findings (33).

The MCM2 family of proteins was upregulated by both malathion and parathion. The MCM family of proteins is known to be involved in the regulation of DNA replication (65,66). It has been reported that the expression of MCM proteins increases during DNA replication (67). The MCM proteins controlled by E2F transcription factors have been shown to promote MCM expression (68). The protein kinase complexes interact with MCM proteins maintaining the post-replication stage and MCM2/MCM4 serve as substrates for CDC2/cyclin B (69-71). MCM3 cleavage can be prevented by caspase inhibitors, resulting in MCM complex inhibition during apoptosis (72). Furthermore, the MCM4, MCM6, and MCM7 complexes have been found to be involved in DNA helicase activity (71,73). In addition, results indicated that parathion and estrogen upregulated the MCM6 labeling index, as compared with the control value (21). Other studies have reported this index to be correlated with cell proliferation and malignant behavior in chondrosarcomas (74).

p53 is another gene involved in the regulation of the cell cycle, serving as a checkpoint for the G1-S phase (75). At the same time, the MDM2 gene regulates p53 and is associated with tumor growth and metastasis (76). It was observed in previous studies that the combination of parathion and estrogen upregulated MDM2 (21) and downregulated p53 (77), thus increasing tumorigenic capabilities (21). Similarly, a study analyzed peripheral lymphocyte DNA obtained from 180 workers with long-term exposure to OPs. That study reported that omethoate, an OP compound, affected the expression of p53, which in turn had an impact on the length of the telomere, suggesting a clear influence of pesticides over the cell cycle and tumor formation (75,78).

Dishevelled (DVL) is a gene that regulates the migration and proliferation of endothelial cells present in blood vessels (79,80). Malathion and the combination of parathion and estrogen upregulated the DVL1 gene and increased the protein expression in cells treated with parathion, alone and combined with estrogen, as compared with the controls (20). The mammalian homologs of the Drosophila DVL together with DVL proteins are important molecules in the Wnt signaling pathway (80-82). DVL-2 protein expression was found to be increased by estrogen, malathion, and parathion, regulating the proliferation of the MCF-10F cell line, as compared with the control (20).

Other studies have also reported that genes associated with cell cycle progression, DNA replication, and checkpoint enzymes were affected by malathion (45,83,84).
Cyclin-dependent kinases regulatory subunit 1 is also fundamental in cell cycle progression (85), and associated with genes that particularly affect the G1 phase; G1/M transition was found to be downregulated in estrogen and parathion treatments (21). These results indicated that pesticides affected the regulation of the cell cycle with possible effects on cancer initiation.

E2 at 10⁻⁴ M significantly increased cell proliferation; however, as shown in Table I, E2 did not induce anchorage-independent growth, anchorage independence, or invasiveness in Matrigel®. Other researchers (86) reported the induction of complete transformation of MCF-10F cells by E2, confirming its carcinogenicity; however, E2-treated-MCF-10F cells were trypsinized and seeded in the upper Matrigel-coated invasion chamber, followed by post-seeding of cells that had crossed the Matrigel membrane, giving origin to several MCF-10F cell lines. E2 induced complete transformation of the human breast epithelial MCF-10F cells in vitro, confirming its carcinogenicity and supporting the concept that this hormone could act as an initiator of breast cancer in women.

4. Parathion and malathion modulate epidermal growth factor receptor and estrogen receptor expression

Previous research has indicated that cell growth is affected by the epidermal growth factor (EGF) through its interaction with the EGF receptor (EGFR) (33); since high levels have been found in the surface of different types of cancer cells (87,88) and its association with cancer has been confirmed over the years. The results of a previous study indicated that EGF protein expression was increased in cells treated with the pesticide alone or in combination with the hormone (21). These results are important, considering that growth factors and their receptors are proteins associated with cell growth (21,89). According to previous results, the parathion-treated-MCF-10F cell line induced a higher EGFR/ERBB1 protein expression, as compared with control and parathion plus atropine-treated cell lines (21). EGFR is a receptor tyrosine kinase associated with cancer, the overexpression of which is correlated with poor prognosis, solid tumor growth, cancer metastasis, and lower survival rate (90,91). Such results have indicated that pesticides such as parathion induce EGFR expression.

It has been reported that estradiol increases the risk of breast cancer in women after long-term exposure since estrogens increase cell proliferation by activating ER-mediated transcription; however, this interaction has been shown to induce genomic instability, chromosomal aberrations, and an increase in errors during DNA replication (25,26,92,93).

Since the MCF-10F model lacks ER expression, E2 appears to act through ER-independent mechanisms. The ERs are ligand-inducible transcription factors that belong to the superfamily of nuclear steroid hormone receptors (94,95). The transmission of estrogen signaling includes the activation of ERs and signal transduction, which can be mediated by genomic and non-genomic signaling pathways. Such classification is based on the outcome of cellular events, including the modulation of gene expression or activation of signaling cascades. The classic genomic pathway is the best-characterized ERα signaling pathway, which is initiated by the ligand binding to its receptor. The binding induces a conformational change and dissociation of their chaperones/nuclear matrix-associated binding proteins (96), forming the E-ER complexes that translocate to the nucleus and bind to specific DNA sequences; these are called estrogen response elements (EREs) and are located in or near the promoters of target genes (97). An ERE-independent signaling pathway has been reported, where E2-ER complexes can mediate gene expression through functional interactions with transcription factors on the DNA (98,99). ERs may interact with many other proteins, including adaptor proteins, G-proteins, GFRs (EGFR, IGF1, and HER2), cytoplasmic kinases [mitogen-activated protein kinases (MAPKs), PI3K and AKT], and signaling enzymes, which can eventually lead to indirect changes in gene expression (100).

On the other hand, the influence of ERα-signaling pathways on epithelial-to-mesenchymal transition-related transcriptional factors, which are fundamental in the development of breast cancer, has been reported (34,35,101,102).

5. Parathion and malathion induce metabolic alterations

It has been reported that pesticides affect the human population, due to their long-term exposure and intensity, and that they alter the detoxification rate by changing the expression of enzymes associated with the transport and metabolism of drugs (103). Briefly, the by-products of drug metabolism are substances that may be pharmacologically active, inactive, or toxic (104). This process is divided into two phases, phase I and phase II; the former is mainly associated with a sophisticated enzymatic complex, known as cytochrome P450 (CYP), whereas the latter is associated with the addition of polar moieties to the substrate, to be eliminated by organisms (105).

Previously, genes involved in human drug metabolism have been analyzed by cDNA microarrays; CYPs, metallothioneins, and p-glycoproteins were further studied (34). CYPs are an enzymatic complex that belongs to the family of monoxygenases, which are involved in the metabolism of endogenous and xenobiotic compounds (106).

According to cDNA microarray, parathion was found to result in CYP upregulation, whereas estrogen, alone or combined with parathion, induced the downregulation of CYP2F1 and CYP4F3; however, there was no change in the CYP3A7 gene expression following exposure to either substance (34).

Results have shown that catechol formation is a major risk factor for breast cancer (107); since it gives rise to reactive quinones causing DNA damage and redox cycling, which in turn lead to the generation of reactive oxygen species (ROS), which can cause oxidative damage (108).

Other important mechanisms involved in carcinogenic effects, besides the stimulation of cellular proliferation through their receptor-mediated hormonal activity, are the direct genotoxic effects exerted by increasing mutation rates through CYP-mediated metabolic activation. A previous study (86) demonstrated that estrogens are carcinogenic in the human breast by testing the natural E2 or its metabolites, 2-hydroxy, 4-hydroxy and 16-a-hydroxy-estradiol [2-OH-E(2), 4-OH-E(2) and 16-a-OH-E(2), respectively] in an experimental system, and neoplastic transformation of MCF-10F cells was observed, to a degree at least similar to that induced by the BP.
On the other hand, estradiol metabolism may result in quinone derivatives, which directly replace base pairs from DNA through depurination, and can also alter the DNA repairing process (109-111). It has been reported that estrogens are potent mammary tumor promoters influencing post-initiation events through epigenetic mechanisms. The upregulation of the C16α-hydroxylation pathway during E2 biotransformation was associated with mammary cell transformation. The action of E2 metabolites on tumorigenic transformation was studied in a mammary epithelial cell line derived from the C57BL mouse strain, where estrogen or its metabolites were found to function as initiators of mammary cell transformation demonstrated by increased cell proliferation, anchorage-independent growth, and alteration of metabolism (112).

Metallothioneins are proteins with a low molecular weight that are rich in cysteine domains. These proteins play an important role in metal homeostasis, particularly the detoxification of heavy metals (113,114). Their dysregulated expression has been observed in invasive ductal breast carcinoma, and they have been proposed for use as a prognostic biomarker (115). Metallothionein 2A expression has been found to be associated with cell proliferation in breast cancer (113,116). Furthermore, genes associated with metallothioneins have been shown to be altered by pesticides and estrogen; the only functional gene upregulated by parathion alone was metallothionein IX, with estrogen alone and estrogen plus parathion resulting in its downregulation (34).

In this context, epidemiological studies have found an association between metabolic enzymes and the age of onset for sporadic colorectal adenocarcinoma (117,118). Then, variant alleles of phase II, such as GST, uridine 5-diphospho-glucuronosyltransferase (UDP), and glucuronosyltransferase (UGT) can be used as molecular biomarkers of cancer risk (119). For example, GSTM(δ)1 was found to be associated with an increased risk of colorectal, lung, and bladder cancer, and GSTP(σ)1 with prostate cancer (120-123). Furthermore, these enzymes catalyze a large variety of drugs and endogenous compounds, such as molecules with sulfo groups in the case of sulfotransferases (124,125), and are in charge of the biosynthesis of polysaccharides, oligosaccharides, and conjugates, in the case of glucosyltransferases (126,127). Previous studies have indicated that the combination of parathion and estrogen induced the downregulation of all methyltransferase genes, such as TPMT; notably, the CHST5, CHST6, and CHST7 (sulfotransferase) genes were upregulated by parathion and downregulated by estrogen, alone or combined with parathion (34).

The carbohydrate sulfotransferases play a role in oxidative stress and the estradiol signaling pathways in carcinogenesis (128); and have been investigated in breast cancer and glioma patients (119,129). On the other hand, several glycosyltransferases (GSTs) have also been identified; the GSTP1, GSTT2, and microsomal glutathione s-transf erase 1 (MGST1) genes were overexpressed by parathion and downregulated by estrogen, when compared to the control, whereas the combination of estrogen and parathion downregulated MGST1, with no change observed in the GSTP1 and GSTT2 genes (34).

As previously reported, UDP-UGT is another enzyme associated with detoxification (130), which was found to be increased by parathion (UGT1A1 and UGT2B genes) and decreased by estrogen; however, there was no difference in these genes when the substances were used together (34). Clinical studies have shown an increase in the UGT1A1 and UGT2B gene expression in ovarian cancer (119); therefore, it can be a reliable molecular biomarker for the risk of cancer. The carcinogenic activity of 4-hydroxyestradiol was analyzed in a hamster kidney tumor model of DNA damage by steroidal estrogens through catechol estrogen metabolites (24,131-133). It is important to note that the 2-hydroxylation of steroidal estrogens is the major metabolic oxidation of estrogenic hormones in most mammalian species (134,135).

Moreover, there are other enzymes with potential carcinogenic activity in the metabolism of endogenous and exogenous compounds (136,137); for example, the enzymes comprising the aldo-keto reductase (AKR) family involved in redox transformation, with substrates such as glucose and steroids, as well as environmental pollutants, among others (138). AKR1C1 and AKR1C2 were also upregulated by another non-organophosphorus pesticide (45). In combination, these examples indicated an impairment of homeostasis by certain substances, ultimately leading to carcinogenesis. Another example is the estrogen-responsive B box protein (139), also upregulated by malathion (137). This protein belongs to the tripartite motif protein family, and its upregulation has been associated with histone acetylation and the transcription of CYP26A1, which is important in retinoid-resistant cancer cells (139).

In this context, particularly in cell metabolism and metabolic pathways, certain studies reported glucose homeostasis impairment (137) and metabolic disorders, with certain metabolic changes still present for a long time even after discontinuing long-term exposure to malathion (140), which was due to OPs. These disturbances may have occurred through physiological stress, oxidative stress, and other mechanisms (141). These results were confirmed by in vivo studies; for example, malathion induced insulin resistance biomarkers and reduced insulin sensitivity (140). Other studies reported that, in general, OPs increased blood glucose (142-144) and induced glycogen phosphorylase and phosphoenolpyruvate carboxykinase activity following malathion treatment in rats (145). Of note, glucose and lipid metabolism were affected in rats under the influence of malathion (146,147).

Similarly, neonatal parathion exposure in rats was found to alter lipid metabolism and induce an inflammatory response in adipose tissue, parathion alone decreased adiponectin levels and increased tumor necrosis factor-α (TNF-α) (148). Adiponectin is a monomeric protein secreted in the circulation with the main purpose of inducing fatty acid oxidation and inhibiting glucose synthesis in the liver. It has also been recognized as an anti-inflammatory agent (149), whereas TNF-α is a cytokine associated with immune homeostasis, inflammation, apoptosis, angiogenesis, and cell migration (150,151).

These in vivo studies supported the in vitro results and demonstrated that exposure to OPs induces a chronic adipose inflammatory response, leading to the emergence of other diseases, such as diabetes, obesity, and cardiovascular diseases (148).

6. Parathion and malathion cause genomic instability
Genomic instability is known to be induced by uncontrolled cell proliferation, with pesticides and estrogen found to
increased the risk of genetic damage (152), involving changes in the expression of oncogenes and the loss or inactivation of tumor-suppressor genes (153,154); this leads to the accumulation of abnormalities in cells. It was demonstrated by human cancer microarray analysis that endogenous and exogenous agents, including estrogens and OPs, affected 408 genes, 17 of which were involved in human cancer regulation. Among those genes that were altered are those associated with cell cycle progression, cell differentiation, and signal transduction pathways (20). To determine specific genetic changes and their biological consequences is crucial for understanding breast carcinogenesis.

Studies have indicated that mutations in the Ras oncogene observed in cancer cells correspond to the amino acid substitutions at positions 12, 13, and 61, and it is important to consider that the oncogenic Ras proteins act downstream of effector pathways to induce the deregulation of cell proliferation and abnormal functional properties of cells (155). The results of previous studies have indicated that allelic imbalance at different chromosomal levels involved the overexpression of the Ras oncogene; with the marker mapped for chromosome 11p14.1 showing microsatellite instability (MSI) in malathion- and estrogen-treated cells (156,157). A different study showed that malathion or parathion, alone and in combination with estrogen, upregulated H-ras (20). Those findings indicated a loss of heterozygosity (LOH) in parathion and estrogen-treated cells, LOH in codon 12 in either malathion- or estrogen-treated cells, and MSI in codon 61 of malathion- and estrogen-treated cells (36). Furthermore, it was shown that chemical carcinogens induced mutations in codons 12 and 61 of H-ras (158). Another study provided an example of the genomic instability of the Ras gene in MCF-10F cells under the influence of the two pesticides and the presence of estrogen (20). The use of microsatellite markers can be useful to determine the degrees of allelic imbalance, LOH or MSI (159-161). The specific genomic imbalances in microsatellite regions of specific genes appear to be important in determining the risk of cancer since tumor pathogenesis is associated with specific imbalances and disease prognosis (162,163), and they may serve as specific therapeutic targets. Genomic instability was observed in parathion-, malathion- and estrogen-treated MCF-10F cells in the form of LOH and MSI (36). The malignant phenotype was characterized by an increase in H-ras oncogene expression. On the other hand, microsatellite markers helped determine that the malathion- and estrogen-treated cells exhibited MSI with a marker for H-ras mapped in chromosome 11p14.1, and LOH in the presence of malathion or estrogen alone (36). Other studies have reported overexpression of the c-Ha-ras p21 protein in human breast cancer (164-166), indicating that the expression of this protein may serve as a marker of breast cancer progression.

The Trio domains exhibit Rac and Rho activity (167). Rho, another member of the Ras superfamily (168), is present in several cell types and is involved in cell polarity and motility (169,170). Parathion and estrogen, alone and in combination, increased Rho-A protein expression, as compared with the control (21). Kleer et al (170) reported a higher Rho-A protein expression in all breast tumor biopsies, as compared with normal tissues, which was correlated with histological grade; this suggests a role of this protein in tumor progression and indicates that it may serve as a prognostic marker in the clinical setting (21). Rac, a GTP-binding protein of the Ras superfamily, controls several processes, including cell proliferation, cell polarity, and cytoskeletal arrangement (171). Rac was found to be overexpressed in parathion-treated cells; Rac 3 was particularly overexpressed in cells treated with parathion combined with estrogen. In addition, an increase in Trio protein expression was observed in cells treated with parathion, alone and combined with estrogen, when compared to the controls (33). The Trio is a multi-domain protein with two DVL-homology/pleckstrin domains (167,172). The c-Kit protein has also been found to be overexpressed in breast cancer (88,173). A previous study demonstrated an increase of c-Kit protein expression in cells treated with parathion, alone and in combination with either atropine or estrogen when compared to their controls (33).

It is known that the activation of tumor-promoting signaling, such as RAS/MAPK signaling, may promote cancer cell proliferation and invasion (174). OPs altered the c-Ha-Ras oncogene and Rho-A, among others, and estrogen affected ER in the MCF-10F cell line (20,21). Based on these findings, it is, therefore, possible to hypothesize a cross-talk between pesticides and estrogen, with the combination of the two inducing morphological and molecular changes indicative of cell transformation, which would be completely different in the absence of estrogen. Therefore, parathion and malathion combined with estrogen have been found to be involved in breast cell carcinogenesis.

Growth factor regulators, such as fibroblast growth factors (FGFs) and their receptors (FGFRs), regulate different cellular processes, including angiogenesis, metastasis, and tumor progression; the deregulation of these factors affects signaling pathways involved in breast cancer (175-178). Acidic FGF (FGF-1), a member of the fibroblast growth factor superfamily, has important functions in DNA synthesis, cell division, and differentiation (179), and it is also critical for the development of different types of cancer (180-182). FGF-2 and its ligand, FGF/2, along with FGF-1, one of the main ligands for FGFR1, have been associated with tumor progression, regulation of tumor angiogenesis, and metastasis (183); this is due to their presence in the tumor microenvironment, which enables them to mediate the effects of several different pathways, such as MAPL and PI3K (177,184).

Insulin-like are growth factor-binding proteins (IGFBPs), among which IGFBP3 and IGFBP5 are important for the regulation of IGF signaling (185-188). Exposure to either parathion or malathion upregulated both IGFBP3 and IGFBP5 gene expression (20). Regarding the interaction of these compounds with breast cell receptors and their association with an endocrine-disrupting connotation, a review reported that malathion induced ER activity and served as a weak ER agonist in the MVLN human breast carcinoma cell line (189).

The function of the cadherin-catenin system in cell adhesion and intracellular signaling appears to be the result of different mechanisms (190,191). Thus, the E-cadherin-catenin complex is the target of numerous growth factors and hormone-dependent signaling pathways that regulate its function and expression (191). In general, β-catenin has been associated with breast cancer progression due to its invasive capabilities (46,192-194), which make it a very sensitive prognostic marker for invasive breast cancer (190,192-194).
Other OPs have also been evaluated in relation to cell adhesion. Specific genes significantly altered by OPs were detected in *Caenorhabditis elegans*; among them, genes associated with cell adhesion were affected, including *C3C12.5, mua-6 (ifa-2)*, and *zig-7* (195). Other studies on these types of organisms have been found to be a good model for investigating the effects of other substances, due to their similarities with mammals (196-199). The same study also investigated genes that were associated with metabolism, including *CYTP450* and *UDP-glucosyltransferases* (195).

Similarly, MCF-10F cells treated with parathion or malathion, alone or combined with estrogen, induced changes in cell adhesion molecules such as CD146 (35), a surface protein also known as melanoma cell adhesion molecule (200) that is involved in cell adhesion and other processes, including cell proliferation, migration and progression, and particularly angiogenesis and vascular permeability (201-204). The aforementioned treatments also upregulated keratin 18 expression; another component of epithelial cells that protects them from external forces, serves as a biomarker in epithelial cancers and plays other roles in drug response and tumorogenesis (205-215).

Heat shock proteins (HSPs) are a group of highly conserved, abundantly expressed proteins with diverse functions (216), including the assembly and sequestering of multi-protein complexes, transportation of polypeptide chains across cellular membranes, regulation of protein folding (217-221), and certain functions that protect against stress-induced injury (137). HSPs are known as molecular chaperones and are organized into six general families according to their molecular weight and activity: HSP20, HSP40, HSP60, HSP70, HSP90, and HSP100 (222). Typically, they are proteins constitutively expressed in the cytoplasm that co-localize to the nucleus under stress induced by physical and chemical insult. Among them, HSP90 and HSP27 are associated with poor prognosis and likely play an important role in drug-resistant breast cancer (223), as well as serve as a biomarker due to toxicant exposure (224). A previous study showed that the gene expression of *HSP27* and *HSP90* was upregulated by malathion or parathion, alone or combined with estrogen (20). The overexpression of *HSP27* and *HSP90* may suggest an association between them and breast cancer. Other HSPs such as HSP70 were also upregulated by OPs, an upregulation positively correlated with ROS generation and apoptotic cell death, suggesting an association between pesticides and adverse conditions promoting cell and tissue injury (217-221).

It is well known that the cell cycle involves many steps that can be positively or negatively regulated by several factors. The p53 protein is a negative regulator, and its inactivation by mutation, or its interactions with oncogene products of DNA tumors, may lead to cancer (225-228). Furthermore, it has been reported that malathion or parathion, alone or combined with estrogen, led to the upregulation of the gene expression of inducible protein *TP53*. *TP53* was upregulated and the mutant *p53* gene expression was higher in parathion and estrogen-treated cells, as compared with the controls, while the *TP53* gene in Li-Fraumeni syndrome was upregulated by malathion or estrogen treatment (20). As previously mentioned, molecular disorders, such as MSI and LOH, are associated with genomic stability (229); furthermore, the *TP53* gene is located on chromosome 17p13. It was reported that malathion mixed with estrogen induced MSI at loci 17p13.1 (36). This indicated that the loss of p53 function can cause the genetic instability of these transformed cells. Thus, mutant *p53* can act as a dominant oncogene (86). These findings suggested that OPs and estrogen induced the malignant transformation of the MCF-10F cell line, as shown by the phenotypic characteristics and genomic instability indicated by LOH and MSI, which are considered important events in the process of carcinogenesis.

Studies have shown that breast cancer is associated with alterations in the *p53* gene in humans (230), and that mutant *p53* expression increases as breast cancer progresses from early *in situ* to advanced metastatic lesions, with *p53* gene mutations observed in ~20-50% of human cancers (226,228). On the other
hand, a small number of mutations in p53 have been found in ductal breast carcinomas (231,232). The p53 gene was reported to be altered in 17p13.1 during cell transformation and genotoxic stress (157,233). p53 was found to be overexpressed in the MCF-10F cell line when it was exposed to several carcinogens, including DMBA and BP, and α particle (high LET) radiation in the presence of estrogen, inducing an allelic imbalance at the respective chromosomal loci (33,46,47).

In addition, the extent of DNA damage can be quantitatively measured by tail moment (TM), which has been widely used in a genotoxic study (234). An increased TM was observed in human peripheral blood lymphocytes following malathion and parathion exposure, as determined by a comet assay, this study also suggested a role of oxidative stress induced by pesticides in the cytotoxic and genotoxic process (235). Similar results were observed in other cell types, such as the HepG2 human liver carcinoma cells, in which malathion also increased the extent of DNA damage (236,237).

Summary of important findings. A cellular model was presented herein, which was based on the use of the MCF-10F cell line, a human tissue-derived, immortalized, a non-tumor-igenic cell line that enables a valuable experimental approach that minimizes extrapolation, thereby uniquely facilitating the clinical translation of the data. The cellular and molecular endpoints altered throughout these studies in response to treatment with estrogen, malathion, and parathion represent relevant endpoints for tumorigenic transformation. Of note, this is a unique experimental approach that identifies mechanistic signs that link OPs with human carcinogenesis.

A non-malignant cell line, MCF-10F, was used to construct another model, which showed several signs of carcinogenicity due to malathion and parathion exposure, which increased cell proliferation and induced cell transformation by affecting protein expression, promoted anchorage independence, invasive capabilities, modulation of receptor expression, metabolic alterations and genomic instability, among others. The mechanisms underlying the mammary carcinogenic potential involved acetylcholinesterase inhibition and increased oxidative stress. A simplified scheme of the molecular changes induced by the effects of OPs and estrogen is shown in Fig. 2.

7. Conclusions

Exposure to chemical compounds, such as pesticides, and endogenous substances, such as estrogens, exert a significant effect on normal breast cell processes at different levels. Compounds of natural origin, such as hormones, are closely associated with hormone-dependent types of cancer, including breast cancer. The present study provides a comprehensive summary of the impact of parathion, malathion, and estrogen on breast carcinogenesis and, specifically, their effects on cell cycle, signaling pathways linked to EGF and IGF receptors, drug metabolism, and genomic instability in the ER-negative breast cell line MCF-10F.

Cancer initiation and progression have been correlated with an increase in genomic instability identified by the inactivation of tumor-suppressor genes, and the activation of oncogenes in the presence of malathion, parathion, and estrogen. Moreover, advances in science and medicine have helped further identify and elucidate the functions of useful tumor markers or signaling molecules, further enhancing our understanding of genetic changes that are relevant to tumor initiation. Therefore, the signs of carcinogenicity have been proven to be very useful for analyzing the main factors involved in breast cancer initiation and may be used for determining the origin of other types of cancer and the main contributing factors.

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Authors’ contributions

GMC prepared the original draft. GMC, TCB, and DR reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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