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MCPA (2-methyl-4-chlorophenoxyacetic acid) and sulfosulfuron – pesticides with potential endocrine disrupting compounds properties

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ABSTRACT

Pesticides are commonly used in agriculture and therefore their residues are constantly detected in soil and water. Contrary to the insecticides, which are widely investigated in terms of carcinogenic action, selected herbicides, such as MCPA and sulfosulfuron, are poorly studied in this respect, especially there are few reports regarding their action on the cellular level. The aim of the study was to evaluate the effect of MCPA and sulfosulfuron on estrogen-dependent MCF-7 breast cancer cell line, therefore, cells viability, proliferation and apoptosis were determined. In addition, pesticides impact on selected oxidative stress parameters and the level of reactive oxygen species (ROS) generated in MCF-7 cells were investigated. Both test compounds exhibit stimulatory effects on proliferation and viability of breast cancer cells. The results showed that the tested pesticides do not significantly stimulate apoptosis in the cells under study and these parameters correlate positively with the induction of oxidative stress in MCF-7 breast cancer cells. Investigated pesticides stimulate oxidative stress in cells by the generation of high levels of ROS in tumor cells, which can lead to their adaptation and resistance to the standard treatment regimen. MCPA and sulfosulfuron exhibit potential carcinogenic activity acting as a possible risk factor for human health.

Keywords: Pesticides; Herbicides; MCPA; Sulfosulfuron; MCF-7; Breast cancer

1. Introduction

Nowadays pesticides are considered as one of the main factors that contribute in the environmental contamination in the world. Because of the pest resistance and a huge human need for more food as the world population grows, formulations of new and potent herbicides constantly arise. According to the literature, long-term exposure to pesticides is harmful and disturbing for human health and influence the function of various organs in the body, such as immune, endocrine, reproductive, nervous, renal, cardiovascular and respiratory systems [1,2]. The connection between pesticides and cancer was noticed for the first time around 50 years ago in reports concerning an increased incidence of lung and skin cancers in the farmers using insecticides [3,4]. Since then many studies have been conducted in this area, which has led to a significant progress in understanding the relationship of pesticides with various types of cancer [5]. Based on epidemiological and agricultural health studies conducted among others by the International Agency for Research on Cancer, different types of malignancies have been notified, for example, breast cancer, prostate cancer, lung cancer, brain cancer, colorectal

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cancer, pancreatic cancer, skin cancer, stomach cancer and others [6–8]. A classification has been created, which is based on the information obtained from animal, metabolic, structural and epidemiological studies. This classification regards a list of Chemicals Evaluated for Carcinogenic Potential established by EPA's Pesticide Program and published in 2010. According to the classification, at least 70 pesticides are considered as potential carcinogens [9].

The carcinogenic effect of pesticides on humans is conditioned by many factors, such as sex, age, susceptibility, dose and time of exposure and simultaneous contact with other, hazardous chemicals. However, pesticides mechanisms of action on the cellular level can be considered in two ways. The first one is direct disruption of genetic material through the induction of structural and functional changes in DNA, chromosomes and histones. The second possibility is indirect influence on gene expression through the effect on nuclear receptors, endocrine network and on other factors such as oxidative stress parameters involved in maintenance of cellular homeostasis [10,11].

Some of the pesticides may act as endocrine disrupting compounds (EDCs) by binding to nuclear hormone receptor. According to the Endocrine Society an endocrine-disrupting compound is any substance, which can alter hormonal and homeostatic system [12]. The term "endocrine disruptor" (ED) was introduced for the first time in the year 1991 and it was connected with the chemicals, which interfere with synthesis, secretion, metabolism, transport, binding, action and elimination of hormones in humans [13]. Since then approximately hundreds of pesticides have been recognized as proven and possible ED by the Pesticide Action Network UK [14].

Despite the apparent side effects, pesticides are still widely used in agriculture and therefore their residues and derivatives can be still detected in soil and water, where they can interact with living organisms and may accumulate in the food chain [15]. Especially, commonly used pesticides are from the group of indole analogs, hormonal – auxin-type herbicides, which, as a plant hormone synthetic analogs, stimulate plant cells elongation and cause an increase in protein and nucleic acid biosynthesis [16]. An example of such herbicides is 2-methyl-4-chlorophenoxyacetic acid (MCPA). In contrast to the great scope of carcinogenic studies of structurally similar compounds, a little has been investigated regarding carcinogenicity of MCPA [17]. Bellet et al. [18] revealed that MCPA is non-carcinogenic in rats and mice; however, no studies were performed on the cellular level in established cancer cell lines. Several studies confirmed genotoxic potential of auxin-like herbicides, for example, MCPA [19–21]. However, it should be also mentioned that, according to the literature, MCPA did not reveal any mutagenicity. It has been shown to be clastogenic in vitro in high, cytotoxic concentrations, but it is not structurally alerting for being genotoxic. An available data disclosed that MCPA is not mutagenic or genotoxic in vivo [22].

The other well-known herbicide is sulfosulfuron that helps farmers protect wheat from yield loss by controlling brome, quack/couch, wild oats, gallium and apera [23]. According to the literature, the molecule has a low order of acute toxicity and it is not genotoxic. However, there is a significant lack of information regarding sulfosulfuron activity in human cells on a molecular level. Most of the data available are related to the toxicity of this compound studied in vivo, usually in rat models. Performed genotoxic studies include an Ames *Salmonella* point mutation assay in five test strains, a Chinese hamster ovary (CHO)/hypoxanthine—guanine phosphoribosyltransferase mammalian point mutation assay and a chromosomal aberration test in human lymphocytes [24–26]. Results revealed no genotoxic effects.

The aim of this study was to examine, if there is an influence of two selected pesticides in different concentrations on proliferation and oxidative stress parameters in MCF-7 estrogen-dependent breast cancer cells. Both MCPA and sulfosulfuron are extensively used in crops protection in Poland, especially in cereal plants crops [27]. From the group of herbicides many compounds reveal endocrine disrupting properties and it seemed interesting to investigate a possible effect of MCPA and sulfosulfuron in estrogen-dependent breast cancer cells. There is a lack of data concerning the effect of above-mentioned pesticides in human cancer cells, which is why we chose to examine selected cells proliferation, viability, oxidative stress and apoptosis parameters. Our study focused on the most appropriate research model: estrogen-dependent MCF-7 breast cancer cell line. Concentration range was selected for the experiments on the basis of previously established data. According to the literature, at selected herbicides concentrations of 0.01, 0.1, 1 and 10 μM MCF-7 cells growth was stimulated and an increase in cell viability was observed for some of the tested pesticides [28].

2. Methods

2.1. Reagents

Dulbecco's modified Eagle's phenol red free medium (DMEM), containing glucose at 4.5 mg/mL (25 mM), penicillin, streptomycin, trypsin–EDTA, fetal bovine serum (FBS) and phosphate buffered saline (PBS) (without Ca and Mg) were provided by PAN Biotech, Aidenbach, Germany. GSH/GSSG-Glo™ Assay kit, CellTiter-Glo® 2.0 Assay and ApoTox-Glo™ Triplex Assay were provided by Promega, Madison, WI, USA. Sodium dodecyl sulphate (SDS), trichloric acid (TCA), thiobarbituric acid (TBA), Folin–Ciocalteu reagent were provided by Sigma-Aldrich and DTNB by Serva, Heidelberg Germany. Dichlorodihydrofluorescein diacetate assay (DCFH-DA) was provided by Sigma-Aldrich, St. Louis, MO, USA. Sulfosulfuron and MCPA were obtained from the Laboratory of Pesticide Residues, Institute of Plant Protection – National Research Institute in Białystok, Poland.

2.2. Cell culture

The effect of MCPA and sulfosulfuron was examined in MCF-7 breast cancer cell line, which were obtained from American Type Culture Collection Cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humified atmosphere of 5% CO_2 in air. Adherent cells (2×10^4 cells/mL) in 200 μL of culture medium were incubated with or without the test compounds in tissue culture white and black 96-well plates for fluorescence and luminescence measurements. The cell viability was estimated at MCPA and sulfosulfuron concentration of 0.01, 0.1, 1 and 10 μM. Cytotoxicity,

apoptosis, GSH/GSSG ratio were examined at the same concentrations range. The incubation time was 24 h. For the estimation of TBA reactive species (TBARS) and SH groups, content cells were seeded into six-well plates in 2 mL of culture medium with and without test compounds at above-mentioned concentrations range.

2.3. Chemical treatment of cells

MCPA and sulfosulfuron were stored in a refrigerator at temperature 4°C. The compounds were added to the cultured cells for a final concentration in the range of 0.01–10 μM. The control cells were incubated without the test compounds.

2.4. Estimation of tested compounds cytotoxicity

Tested compounds cytotoxicity was measured using the CellTiter-Glo® 2.0 Assay (Promega) kit and used according to the manufacturer's instructions. Briefly, the test compounds were added to MCF-7 cells and after the intended incubation period, a 30-min incubation at room temperature followed. CellTiter-Glo solution was then added. Following this, cell lysis was induced for 2 min with shaking followed by a 10-min equilibration at room temperature. Luminescence was read using the GloMax®-Multi Detection System. All the experiments were done in triplicates.

2.5. Estimation of cells viability and apoptosis

The MCF-7 cells were seeded into 96-well plates at a total density of 2×10^4 cells per well. Each well contained DMEM and test compounds where appropriate. ApoTox-Glo™ Triplex Assay (Promega) was used according to the manufacturer's instructions to measure the MCF-cells' viability and apoptosis. After 24 h, the viability reagent, containing the GF-AFC substrate was added to all wells and incubated for 30 min. Caspase-Glo 3/7 was added to the wells and mixed briefly for 30 s, then incubated for 30 min at room temperature. Fluorescence was measured at 380EX/510EM to assess viability, and luminescence was measured to assess apoptosis using the GloMax®-Multi Detection System. All the experiments were done in triplicates.

2.6. Total protein content in cells

Adherent cells (2.5×10^5 cells/mL) in 2 mL of culture medium were incubated with or without the test compounds in tissue culture six-well plates. After the homogenization of MCF-7 cells and extraction in 0.1M NaOH at 4ºC total protein content was calculated. The concentration of proteins was determined spectrophotometrically as per Lowry. Folin phenol reagent with a protein kit calibrated with bovine serum albumin as the standard was used in the experiment. The absorbance of the extracts was measured spectrophotometrically at 750 nm [29]. All the experiments were done in triplicates.

2.7. Determination of TBA reactive species levels

The level of TBARS as membrane lipid peroxidation markers was measured using the method of Jabłońska-Trypuć et al. [29] as described previously. Adherent cells (2.5×10^5 cells/mL) in 2 mL of culture medium were incubated with or without the test compounds in tissue culture six-well plates. The cells were washed with PBS (pH 7.4), scraped from Petri dishes and subsequently resuspended in 1 mL of PBS (between 1 and 5×10^6/plate). TCA (15%, 1 mL) and TBA (0.37%, 1 mL) were added to 1 mL of the cell suspension and mixed. This mixture was submerged in a boiling water bath for 10 min and the concentration of TBARS was assessed spectrophotometrically at 532 nm using the extinction coefficient of 156 mM/cm. All the experiments were done in triplicates.

2.8. Determination of SH groups

SH groups were measured using the method of Jabłońska-Trypuć et al. [29] as described previously. Adherent cells (2.5×10^5 cells/mL) in 2 mL of culture medium were incubated with or without the test compounds in tissue culture six-well plates. Cells were washed twice with PBS (pH = 7.4; temperature = 4°C) and dispersed by scraping. MCF-7 cells were counted, resuspended in 1 mL of PBS and collected by centrifugation at 5,000 × g for 10 min. The pellet was resuspended in 1 mL of 0.5 M phosphate buffer (pH 7.8), containing 0.1% SDS. Subsequently, 25 μL Ellman's reagent (5 mM) was added and the thiol groups were measured spectrophotometrically at 412 nm using the molar extinction coefficient of 13.6 $mM^{-1}\,cm^{-1}$. All the experiments were done in triplicates.

2.9. Determination of GSH/GSSG

Total glutathione and GSH/GSSG ratio were each assayed in triplicate via GSH/GSSG-Glo™ kit (Promega) following manufacturer's instructions. Cells were seeded in white bottom 96-well plates at 10^4 cells/well (Sarstedt, North Rhine-Westphalia, Germany), allowed to attach, and treated with pesticides. Prior to the assay growth media were removed and cells washed with PBS. Assay is based on a luminescence measurement and detect and quantify total glutathione (GSH + GSSG), GSSG and GSH/GSSG ratios in cultured cells. Stable luminescent signals are correlated with either the GSH or GSSG concentration of a sample. In this method, GSH-dependent conversion of a GSH probe, luciferin-NT, to luciferin by a glutathione S-transferase enzyme is coupled to a firefly luciferase reaction. Light from luciferase depends on the amount of luciferin formed, which in turn depends on the amount of GSH present. Thus, the luminescent signal is proportional to the amount of GSH. GSH/GSSG ratios are calculated directly from luminescence measurements. Luminescence was read using the GloMax®-Multi Detection System. All the experiments were done in triplicates.

2.10. Intracellular ROS detection

The level of intracellular reactive oxygen species (ROS) was determined using dichlorodihydrofluorescein diacetate (DCFH-DA), (Sigma-Aldrich) [30]. After diffusion through the cell membrane, DCFH-DA is deacetylated by cellular esterases to a non-fluorescent compound, which is later

oxidized by intracellular ROS into a fluorescent 2′,7′-dichlorofluorescein (DCF). The MCF-7 cells (2 × 10^4 cells) were seeded in 200 μL of growth medium in black 96-well plates. After 24 h, the medium was removed, the cells were stained with 10 μM of DCFH-DA in PBS at 37°C, 5% CO_2 incubator, for 45 min. Next, the dye was removed and replaced with pesticides in DMEM, at 0.01, 0.1, 1 and 10 μM concentrations and incubated for 24 h. Then, the DCF fluorescence intensity was measured by using the GloMax®-Multi Detection System at the excitation wavelength of 485 nm and the emission wavelength of 535 nm. The intracellular ROS generation in pesticides-stimulated MCF-7 cells was shown as the intensity of fluorescence of the DCF. All the experiments were done in triplicates.

2.11. Statistical analysis

The influence of the tested compounds was assessed by using analysis of variance with Statistica 13.0 applied. Significant differences between means were estimated by Tukey test at $p \leq 0.05$. Results presented in figures were expressed as mean ± standard deviation (SD).

3. Results

3.1. Cells viability

Obtained results show a significant increase in cells viability, especially at concentrations of 10 and 1 μM for MCPA, and 1 and 0.1 μM for sulfosulfuron (Fig. 1). An increase in cell number of about 40% for 1 μM concentration of MCPA and sulfosulfuron as compared with the untreated control cells was noticed. We did not observe any decrease in cells viability as compared with the untreated control cells.

3.2. MCPA and sulfosulfuron cytotoxicity

Cells were treated with decreasing concentrations of MCPA and sulfosulfuron (0.01–10 μM) for 24 h. Presented range of concentrations caused dose-dependent rise in cell viability (Fig. 2). When compared with control, untreated cells, MCF-7 cells treated with 10 – 0.01 μM MCPA showed the most significant effect on viability, approaching nearly 130% of total viable cells in 10 μM concentration of MCPA.

3.3. Detection of apoptosis

Cells were subjected to different concentrations of MCPA and sulfosulfuron (0.01–10 μM) for 24 h. Non-significant increase in the level of apoptosis was observed in the presence of especially 0.1 and 0.01 μM of sulfosulfuron. Exposure of MCF-7 cells to tested compound did not increase caspase 7 activity and therefore did not significantly induce apoptosis (Fig. 3).

3.4. Determination of lipid peroxidation

Lipid peroxidation in normal, non-cancerous cells is usually connected with a variety of cellular dysfunctions, which result from the structural modifications of lipid–protein complexes. However, in cancer cells lipid peroxidation serves as a source of free radicals, which promote cancer cells proliferation. The results showed a difference between TBARS level in control and sulfosulfuron-treated cells (Fig. 4). The addition of both tested pesticides induced an increase in TBARS

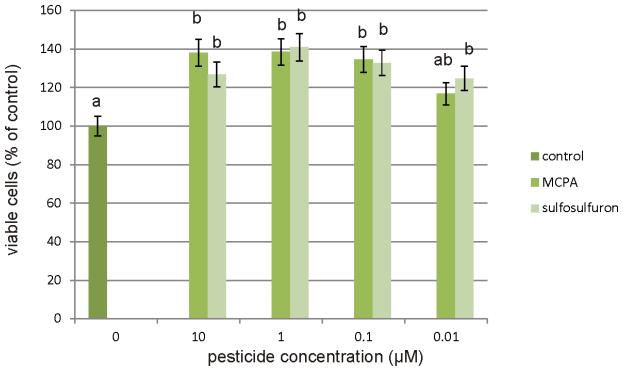

Fig. 2. Viability of MCF-7 cell line treated with different concentrations of MCPA and sulfosulfuron for 24 h. Date are presented as the mean ± SD. Different letters (a and b) indicate statistical differences (≤0.05) estimated by Tukey's test.

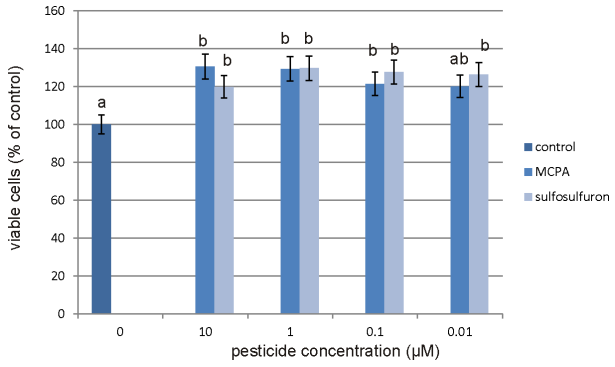

Fig. 1. Effect of MCPA and sulfosulfuron on cell viability of MCF-7 cells. The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuran for 24 h. Data are presented as the mean ± SD. Different letters (a and b) indicate statistical differences (≤0.05) estimated by Tukey's test.

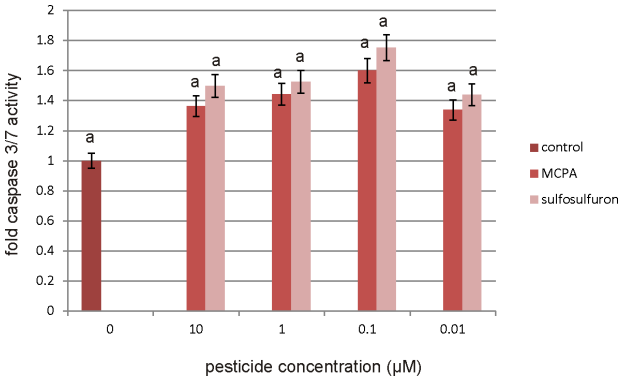

Fig. 3. Effect of MCPA and sulfosulfuron on apoptosis in MCF-7 cells. The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuron for 24 h. Data are presented as the mean ± SD. The same letter (a) indicates no statistical difference (≤0.05) estimated by Tukey's test.

content when compared with the control. Sulfosulfuron in the concentration of 1 μM caused an increase in TBARS content of 134% compared with the untreated cells right after 24 h treatment. However, tested compound was the most efficacious at 0.01 μM concentration, causing an increase of approximately 190% comparing with the control after 24 h treatment. The presented results reveal that both MCPA and sulfosulfuron have pro-oxidative properties and probably acts through the induction of TBARS generation and therefore they stimulate membrane lipid peroxidation.

3.5. Determination of SH group content

Fig. 5 shows the level of SH groups for MCF-7 cells exposed to MCPA and sulfosulfuron for 24 h at 0.01–10 μM. Obtained results revealed the most significant decrease in thiol group content of 84% compared with untreated control cells after 24 h treatment at a sulfosulfuron concentration of 1 μM. Both tested pesticides probably acted as a pro-oxidants in MCF-7, because decreases in all tested concentrations as compared with the control were observed. Presented results revealed an oxidative damage in cellular protein after 24 h treatment under the influence of both MCPA and sulfosulfuron.

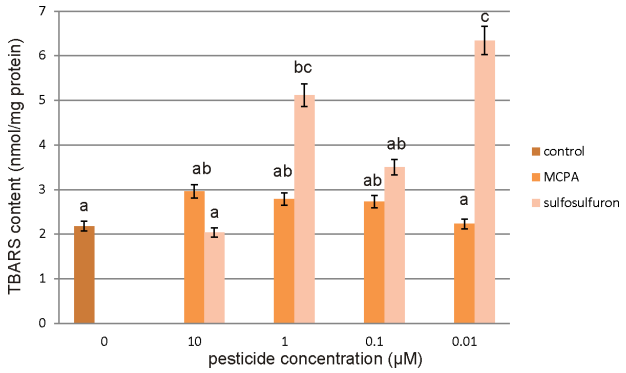

Fig. 4. Effect of selected concentrations of MCPA and sulfosulfuron on TBARS content in MCF-7 cells. The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuron for 24 h. Data are presented as the mean ± SD. Different letters (a, b and c) indicate statistical differences (≤0.05) estimated by Tukey's test.

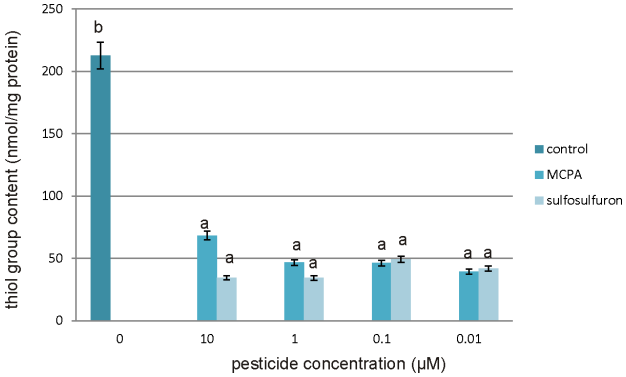

Fig. 5. Effect of selected concentrations of MCPA and sulfosulfuron on SH group content in MCF-7 cells. The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuron for 24 h. Data are presented as the mean ± SD. Different letters (a and b) indicate statistical differences (≤0.05) estimated by Tukey's test.

3.6. Determination of GSH/GSSH ratio

Reduced glutathione is one of the most important low-molecular mass antioxidant and therefore its study is substantial in oxidative stress parameters research. Non-significant decreases in GSH/GSSG ratio were observed in almost every tested concentration of both studied compounds with one exception – 10 μM concentration of MCPA (Fig. 6). In almost every tested concentration, both MCPA and sulfosulfuron caused a decrease in GSH/GSSG ratio as compared with the untreated control cells. Obtained results revealed rather an inhibitory influence of pesticides on GSH amount in MCF-7 cells.

3.7. Determination of intracellular ROS content

Fig. 7 indicates the effect of MCPA and sulfosulfuron on intracellular ROS generation in MCF-7 cells. It shows the fluorescence intensity of 2′,7′-dichlorodihydrofluorescein (DCF) for MCF-7 cells incubated with MCPA and sulfosulfuron for 24 h. Obtained results indicate that both tested compounds in every studied concentration are efficient in the stimulation of the oxidative stress level in MCF-7 cells. The amount of ROS under the influence of both tested compounds was elevated as compared with control, non-treated cells.

4. Discussion

People are potentially exposed to a mixture of environmental pollutants and a number of studies have revealed that some of the industrial compounds, such as pesticides exhibit estrogenic activity [31]. EDCs influence hormone-dependent signaling pathways through different mechanisms of toxicity acting as hormone mimics or antagonists finally leading to hormonal pathways disruption [32–34]. According to the literature, the overall cumulative exposure of women to

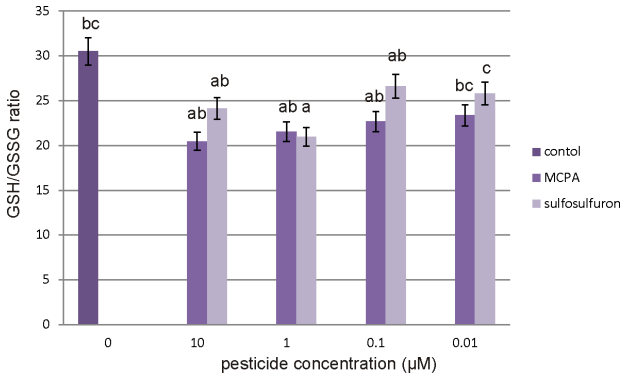

Fig. 6. Effect of selected concentrations of MCPA and sulfosulfuron on GSH/GSSG ratio in MCF-7 cells. The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuron for 24 h. Data are presented as the mean ± SD. Different letters (a, b and c) indicate statistical differences (≤0.05) estimated by Tukey's test.

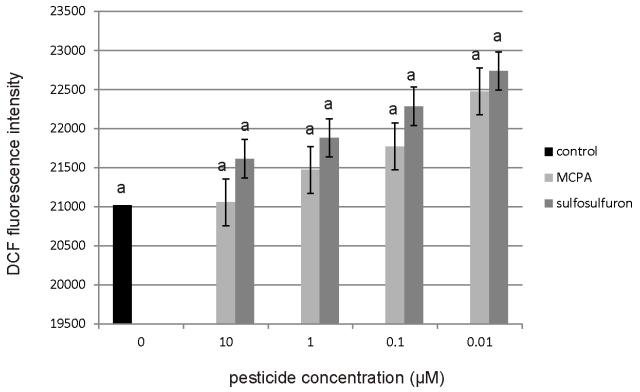

Fig. 7. Intracellular reactive oxygen species (ROS) production in MCF-7 cells under the influence of MCPA and sulfosulfuron as a % of control non-treated cells (control – 100%). The cells were incubated with 0.01, 0.1, 1 and 10 μM of MCPA and 0.01, 0.1, 1 and 10 μM of sulfosulfuron for 24 h. Data are presented as the mean ± SD. The same letter (a) indicates no statistical difference (≤0.05) estimated by Tukey's test.

estrogens and/or compounds that act as estrogens, such as pesticides, results in an increased breast cancer risk [35]. Although many of the commonly used pesticides have not demonstrated carcinogenic properties, it is known that some of them are responsible for the development of certain types of cancer. For example, in reference to MCPA carcinogenicity, one patient case was reported. According to Timonen and Palva [36] an acute leukemia, which was diagnosed, resulted from MCPA exposure.

Permanent changes in the genome of the cells among others caused by an increased level of oxidative stress are the first step of the process characteristic for mutagenesis, carcinogenesis and cells aging. The appearance of DNA mutation is a critical stage in the process of cancerogenesis. In various types of tumor malignancies, an increase in the number of lesions in the DNA has been observed [37]. There is a huge body of literature on a high level of oxidative stress in cancer cells leading to a variety of biological responses, such as cell adaptation, increased proliferation rate and genetic instability resulting in strong resistance to anticancer therapy [38]. Therefore in the present study, we decided to investigate the influence of MCPA and sulfosulfuron on the selected oxidative stress parameters in MCF-7 breast cancer cells. Our aim was to reveal if tested compounds stimulate oxidative stress, subsequently enhancing cancer cells proliferation and possibly cancer progression. We have examined the cytotoxicity and basic oxidative stress parameters under the influence of two selected pesticides commonly used in Poland in agricultural applications. What distinguish this study are two factors: first we chose to examine MCF-7 breast cancer cell line that is commonly used for endocrine-based research, rather than focusing on the other breast cancer cell line or non-human cell line. Discordances in scientific data regarding possible auxin-like herbicides genotoxic and cancerogenic properties may result from the different test models and their differences in the capacities to DNA repair, for example, erythrocytes from bullfrog (*Rana catesbeiana*) tadpoles showed no genotoxicity after being exposed to pesticides from the group of indole analogs, but in CHO cells significantly higher genotoxicity described as a DNA damage tested in comet assay was observed [19,20,39–41]. Therefore, we decided to choose for the experiment human estrogen-dependent breast cancer cell line, which is characterized by the presence of the estrogen receptor (ER+).

Second, we conducted research of the low, environmentally and physiology relevant concentrations of tested compounds, which can be identified in the groundwater system adjacent to the area of the agricultural application. Our results revealed that in all of the tested concentrations both MCPA and sulfosulfuron have caused a significant increase in MCF-7 cells viability. In case of MCPA, proliferation increased with increasing pesticide concentration. However sulfosulfuron in the highest tested concentration caused an increase in cells viability similar to the lowest tested concentration. Obtained results are in agreement with the literature indicating a positive influence of herbicides on MCF-7 cell viability and proliferation. The results obtained by Rollerova et al. [42] support endocrine disrupting effect of selected herbicide – acetochlor, because they demonstrate that acetochlor might interfere with estradiol signaling and thus stimulate MCF-7 proliferation. Whereas in an experiment conducted by Rich et al. [28] a different cytotoxic effect of selected herbicides was observed depending on the cell line used in the research. In the estrogen-dependent MCF-7, mammary epithelial carcinoma cells cytotoxicity caused by atrazine and cyanazine was not observed, but the estrogen independent MDA-MB-231 breast cancer cells and the non-cancerous MCF-10A breast cells showed a significant decrease in cell viability. In our experiment, an increase in cells proliferation was in accordance with other tested parameters, such as oxidative stress parameters and apoptosis. Both tested compounds have stimulated lipid peroxidation and protein oxidative damage and insensitivity of cancer cells to apoptosis. There is much evidence indicating the participation of ROS in promotion of the proliferation of tumor cells, which already passed the stage of initiation caused by the action of mutagenic agents. It has been shown that high levels of oxidative stress is toxic, but a moderate increase of ROS concentration increases cell growth and proliferation, and thus contributes to tumor development [43]. In our experiment, we observed a significant increase in lipid peroxidation products amount. It is in accordance with our other results – such as a decrease in thiol group content and GSH/GSSG ratio and in an increase in ROS content under the influence of MCPA and sulfosulfuron. To assess the effect of herbicides on proteins in this work, we used one of the most popular and widely studied marker of protein oxidation – thiol group content. We have also noticed the oxidative action of sulfosulfuron at lower concentrations (0.1 and 0.01 μM), thus we confirm that tested compound reveal pro-oxidant activity against proteins and that this action is connected with its stimulation of cells proliferation. Both tested pesticides enhanced TBARS production in MCF-7 cells, especially sulfosulfuron in the lowest tested concentration. However MCPA with an increase in its concentration also stimulated lipid peroxidation. According to Duchnowicz and Koter [44], chlorophenoxyacetic herbicides caused lipid bilayer and membrane protein damage by lipid peroxidation and oxidative damage to proteins. Our observations are in agreement with the results of the above-mentioned studies, which indicated that oxidative stress may be induced by the investigated compounds.

The highest increase in ROS content was observed under the influence of both MCPA and sulfosulfuron in their lowest concentration. All tested concentrations caused an increase in ROS content. Obtained results indicate a possible relationship between an increase in ROS content and a decrease in thiol group content. Increased production of free radicals has been demonstrated in many types of cancer as compared with non-cancerous cells, although the mechanism of their formation appears to be similar in both cell types. Oncogenic factors such as c-myc and ras may lead to the stimulation of the production of increased amount of free oxygen radicals. ROS increase in tumor cells leads to induction of various biological responses, including short-term growth retardation and cell adaptation, proliferation rate growth, cell division stimulation, mutation leading to genetic instability, and cell resistance to apoptosis [45–48].

The obtained results have led us to hypothesize that oxidative damage of proteins, high level of lipid peroxidation and an increase in ROS content may be responsible for alterations in regulatory and signaling pathways and be a reason for the observed increase in MCF-7 cancer cells proliferation and their resistance to apoptosis. One of the features of cancer cells is their insensitivity to apoptosis. The entry of cells on the path of apoptosis is conditioned by many factors, and the mechanisms responsible for this process can be divided into sensor and effector. The effector mechanisms of this process include the release of intracellular proteases called caspases [49]. Our results point to the fact, that tested compounds did not cause a significant increase in caspase 7 activity, what may mean that under the influence of selected herbicides MCF-7 cells acquire resistance to apoptosis. MCF-7 cell line does not show detectable caspase-3 level because this cell line has confirmed 47 kb deletion in exon 3 of the CPP32 gene. Nevertheless, MCF-7 cells undergo apoptosis because caspase 7 through the initiator caspase 8 and 9 activity stimulates this process. Caspase 7 reveals the same in vitro substrate activity as the caspase 3 [50]. Our results are in accordance with literature describing the influence of pesticides on the resistance to apoptosis [51]. Cameron and Foster [51] showed that the treatment of human breast carcinoma MDA-MB-231 cells with dieldrin (pesticide) promotes breast cancer by increasing survival of breast cancer cells and their resistance to anoikis (apoptosis triggered by inappropriate anchorage).

5. Conclusion

Although MCPA and sulfosulfuron are widely used herbicides and various toxicities have been described for humans and animals, very few studies were performed at the cellular level to elucidate the mechanisms of their action. Both tested compounds induce cells proliferation and influence oxidative stress parameters. Our results indicate that MCPA and sulfosulfuron exhibit stimulatory effect on ROS generation and lipid and proteins oxidation, which in turns enhances breast cancer cell viability. Therefore we suggest that both the tested substances have positive influence on estrogen-dependent MCF-7 breast cancer cell line, constituting possible hazard for human health.

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Conflict of interest

The authors declare that they have no conflict of interest.

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