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THE EFFECTS OF ALTERNARIOL MYCOTOXIN ON CELL CYCLE AND PROLIFERATION OF PORCINE BLOOD CELLS

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Abstract

Feed contaminants, especially mycotoxins are responsible for important economic loses in swine industry, pigs being very susceptible to contamination with mycotoxins. Alternariol (AOH) is a mycotoxin synthesised by fungi of genus Alternaria and is a common contaminant of different raw materials, as cereal and cereal products that represent important ingredients of swine diets. Recent literature data have shown that in human cell model, AOH can cause DNA damage and induce oxidative stress. However, the effect of AOH in swine it less documented. Consequently, the aim of our research was to investigate how AOH can affect the proliferation, apoptosis, death, and cell cycle using an in vitro model represented by PBMCs (peripheral blood mononuclear cells) isolated from pig`s blood. Cells were stimulated or not with phorbol 12-myristate13-acetate-ionomycin (PMAI), exposed for 4h to different concentrations of AOH (1-100 µg/mL). Our study shows that AOH was able to affect cell proliferation, apoptosis, cell death and cell cycle of porcine PBMCs, with negative effects on the animal health.

Key words*: Alternariol, apoptosis, cell cycle, oxidative stress, pigs.*

INTRODUCTION

Mycotoxin contamination of cereals, fruits, but also of other plant-based products remains a topical issue at the global level, especially in terms of the health of pigs, which mainly consume feed obtained by mixing these types of ingredients. Regarding sensitivity, several studies have shown that pigs are more sensitive than other species to the action of mycotoxins (Oswald et al., 2005). Pigs have also been utilized as models for human nutrition, including metabolic disorders, obesity, bariatric surgery, neurology, brain imaging, food allergies, and alcohol consumption, also they are a suitable model for humans (Roura et al., 2016). Alternariol (AOH) is a secondary metabolite synthesized by *Alternaria alternata* fungi, generally contaminating fruits, but also cereal crops and vegetables (Tournas & Stack, 2001). It is known that AOH, like other mycotoxins produced by *Alternia* molds, have a high thermal stability, contamination can also occur at low temperatures during storage (Asam et al., 2009). Moreover, there are currently no guidelines regarding the concentrations that can be tolerated both in the case of humans and in the case of animals Alexander et al., 2011), although *in vitro* investigations have suggested that AOH can alter the DNA (Pfeiffer et al., 2007), and it can also lead to the appearance of some mutations (Brugger et al., 2006). The AOH mycotoxin can act as a topoisomerase II-a inhibitor (Fehr et al., 2009), this could serve as the primary mechanism by which AOH could break the DNA double strand. There are no data on the toxicity of AOH or other *Alternaria* toxins in other animals, including pigs. *In vitro* studies on RAW 264.7 macrophages have demonstrated that AOH can trigger the oxidative response through the generation of ROS (reactive oxygen species) in addition, AOH could be capable of producing oxidative damage at the level of DNA (Solhaug et al., 2012). Reduced cell proliferation was linked to this effect, which led to an accumulation of cells in the G2/M phase of the cell cycle (Lehmann et al., 2006). Although several studies indicate that AOH causes cell cycle arrest, a more accurate and thorough description of how AOH interferes with cell proliferation, viability, and death is required (Brugger et al., 2006; Wollenhaupt et al., 2008; Schreck et al., 2012).

Our study's objective was to use flow cytometry to assess the effects of AOH on ROS production, cell cycle, cell apoptosis and necrosis in swine

PBMCs (peripheral blood mononuclear cells) newly isolated from peripheral venous blood.

MATERIALS AND METHODS

Cell cultures

The PBMCs were separated using Li-heparin tubes (Vacutest Kima, Arzergrande, Italy), from freshly collected peripheral venous pig blood. Blood collection was carried out in compliance with the current rules and regulations (EU Council Directive 98/58/EC and Law 06/2004 for the care and safeguarding of animals utilized in experiments). The study was performed also following the rules of Ethics Committee of the National Research and Development Institute for Animal Nutrition and Biology, Balotești, Romania (Ethics Committee no. 41/2023). The blood was diluted 1/2 in sterile PBS and placed on Histopaque-1077 medium (Sigma Aldrich, St. Louis, Missouri, US) and centrifuged at 1500 rpm to separate the PBMC from the rest of the blood components. After separation, the white ring of lymphocytes was recovered, the red blood cells were lysed, and the isolated PBMCs were cultivated in culture plates with 24 wells, at a density of 5 x 10^5 cell/mL, in complete RPMI-1640 culture medium (100 µg/mL-streptomycin, 100 U/mL-penicillin, 0.025 µg/mL-amphotericin B, 4 mM-L-Glutamine and 5%-fetal bovine serum) (Sigma Aldrich, St. Louis, Missouri, US). The experiments were carried out for 4 hours in an environment enriched with 5% CO_2 at a temperature of 37°C.

Experimental design

To evaluate the effects of AOH on porcine PBMCs cells, two conditions were approached: unstimulated cells, and cells stimulated with para-methoxy-amphetamine (PMA) - 50 ng/mL and ionomycin (I) - 1 µg/mL. Stimulated cells were used as a model of inflammation as inflammatory process is often induced in pigs by various external stimuli such as bacteria, co-contamination with other mycotoxins, etc. AOH was diluted in dimethyl sulfoxide (DMSO), and in the case of both conditions with unstimulated or stimulated cells, the following concentrations of AOH were tested: 1, 5, 10, 50 or 100 µg/mL. When the incubation period ended, the effects of AOH on cell proliferation, apoptosis, necrosis, cell cycle, and oxidative stress were evaluated by flow cytometry.

Effects of AOH on Apoptosis

Through the use of the Muse Annexin & Dead Cell kit, the Muse Cell Analyzer system, and the Muse 1.5 Analysis software all provided by Merck, Darmstadt, Germany, the effects of AOH on PBMCs were assessed in the cases of the four stages of apoptosis (living, early apoptotic, live apoptotic, and death). In summary, flow cytometry analysis was performed after 100 µL of cell suspension (5 x 10^5 cells) was combined with 100 µL of reagent provided by the kit, and the mixture was incubated at room temperature in the dark for 20 minutes. A set of 2000 events was chosen for examination. The percentage of dead, living and apoptotic (early/late apoptosis) cells was the result of the apoptosis analysis; the graphs show the average of four separate tests.

Effects of AOH on Cell cycle

Cell cycle analysis was performed in compliance with the guidelines of Muse Cell Cycle kit (Merck, Darmstadt, Germany) provided by the manufacturer. For the purpose of analysis, 5 x 10^5 cells/mL were fixed in ethanol (70%) for 12 hours, diluted with the reagent from the kit, and then incubated in the dark for 30 minutes at room temperature. A set of 5000 events was chosen for examination. The cell distribution was reported as percentages (%) based on the cell cycle stages (G0/G1, S, or G2/M); the graphs show the average of four separate studies.

Effects of AOH on Oxidative Stress

The effects of AOH on PBMCs at oxidative level were evaluated using Muse Oxidative Stress Kit (Merck, Darmstadt, Germany). Oxidative stress at the level of PBMCs was determined by measuring reactive oxygen species (ROS). In summary, 190 µL of Oxidative Stress Reagent (Merck, Darmstadt, Germany) was combined with 10 µL of cells (1 x 10^5 cells/mL) and incubated for 30 minutes at 37°C. The results were expressed in percentages (%) of cell populations labelled ROS(+) or ROS(-).

Statistical analysis

The software used to assess the statistical significance of the changes between treatments was GraphPad Prism (9.3.0). One-way Anova and Fisher's exact test were performed. The outcomes were shown graphically, with p-values >0.1 indicating a trend and differences between the experimental treatments considered significant at a value of $p<0.05$. The statistical significance was graphically expressed as follows: ***-extremely significant [$p\epsilon(0.0001, 0.001)$], **-very significant [$p\epsilon(0.001, 0.01)$] and *-significant [$p\epsilon(0.01, 0.05)$].

RESULTS AND DISCUSSIONS

Effects of AOH on Apoptosis

Apoptosis is a normal cellular process, important in several biological systems. Inappropriate apoptosis can lead to the appearance of various pathologies such as neurodegenerative, autoimmune diseases or various forms of cancer (Cohen, 1997). It is known that mycotoxins can interfere with apoptotic processes, considering their spread in animal and human food, more studies are needed to identify the health risk to humans and livestock (Wang et al., 1996). Regarding the mycotoxins produced by *Alternaria* fungi, studies on secondary metabolites such as alternariol demonstrated the fact that these mycotoxins inhibit cell proliferation and induce apoptosis (Liu et al., 2007).

In our study, the effects of AOH on cell proliferation were evaluated both in the case of unstimulated PBMC cells and in the case of those stimulated with PMAI. As can be seen in Figure 1, AOH does not significantly influence the percentage of dead cells, but in the case of cell populations in late apoptosis, a significant increase is observed, directly proportional to the increase of AOH concentration. Moreover, once the AOH concentration increased, the population of live cells was significant reduced. It seems that in the case of unstimulated PBMCs, AOH can induce apoptosis, effects accentuated while the concentration of AOH increases, the percentage of total apoptotic cells being 10-30% higher at AOH concentrations above 10 µg/mL. Regarding the cells stimulated with PMAI, as can be seen in Figure 2, significant changes occur only in the case of exposure of stimulated cells to concentrations of AOH higher 50 µg/mL, respectively 100 µg/mL, where the percentage of dead cell populations increases significantly with 15.67% and 18.50%, while that of living cell populations decreases significantly with 14.77% and 18%. However, in the case of stimulated cells, AOH does not induce major modifications in the percentages of early/late/total apoptotic cells. Corroborating the data, AOH in high concentrations induced significant changes on the percentage of living or dead cells in the case of both studied conditions, PBMCs stimulated with PMAI or unstimulated.

Similar results were obtained by Solhaug et al., where the exposure of RAW 264.7 murine macrophages to AOH 60 µM for 24 and 48 hours reduce cell viability and induced the death of cells (Solhaug et al., 2012). Moreover, exposure to AOH was able to induce necrosis, apoptosis, and cell death in the case of Caco-2 human intestinal cells, where concentrations of AOH 15, 30, 60µM induced an increase in the percentage of apoptotic cells between 7.4-27.8% directly proportional to the increase in concentration (Fernández-Blanco et al., 2016). Other studies carried out on murine hepatoma cells (Hepa-1) showed that AOH 40 µM can induce a 3% upregulation in apoptotic cells after 48 hours of treatment (Schreck et al., 2012).

Mycotoxin co-contamination is a ubiquitous issue, and AOH has been shown to potentiate the effects of other mycotoxins. A study carried out on mouse blastocytes showed that even in low concentrations AOH (1 and 2 µM) potentiates the toxic effects of ochratoxin (OTA 8 µM) triggering cell apoptosis, in this case the main mechanism indicated that the accumulation of ROS (reactive oxygen species) is the main factor responsible for triggering the apoptotic process (Huang et al., 2021).

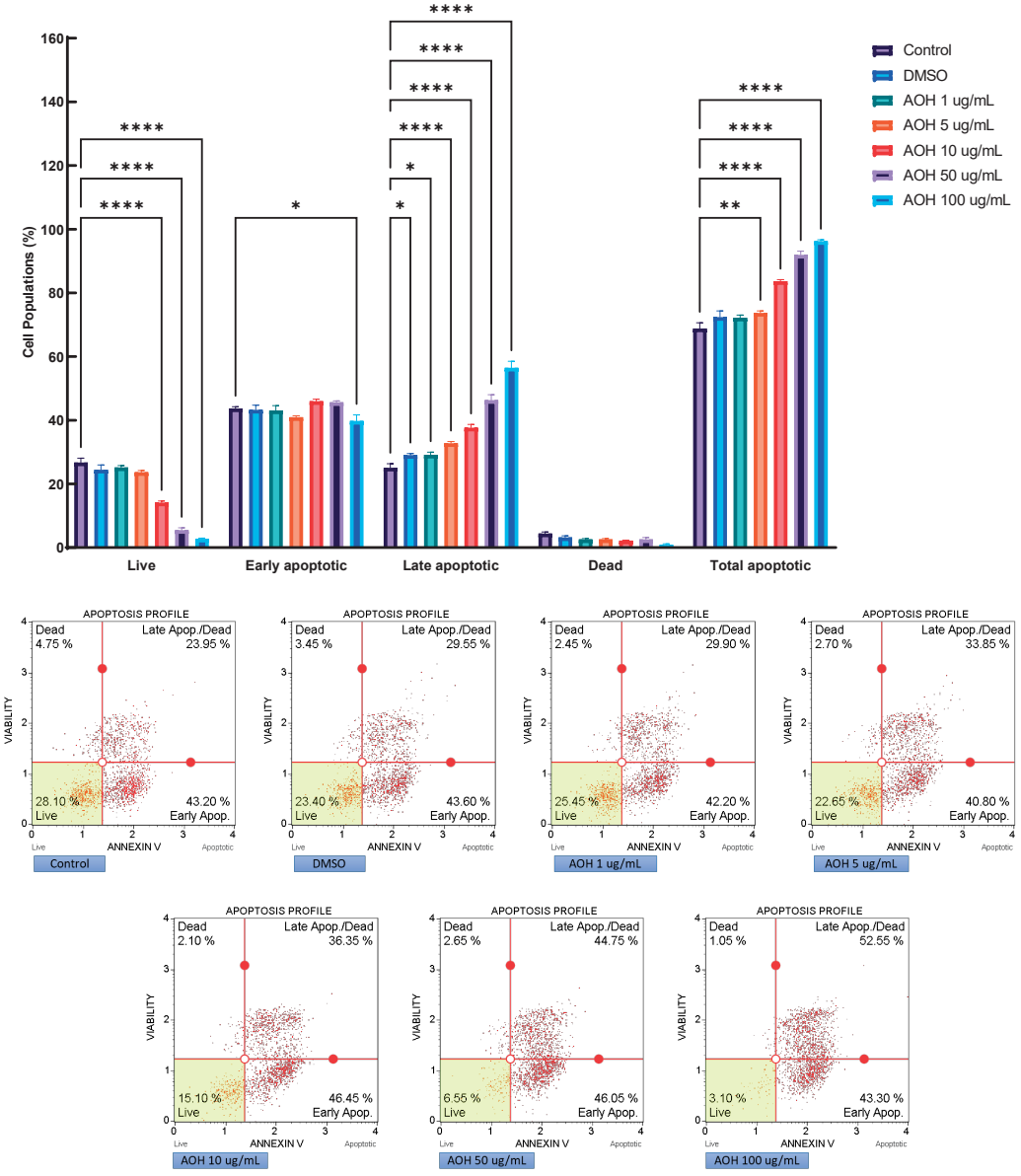

Figure 1. Effects of AOH on cell proliferation, apoptosis, and necrosis in unstimulated PBMCs

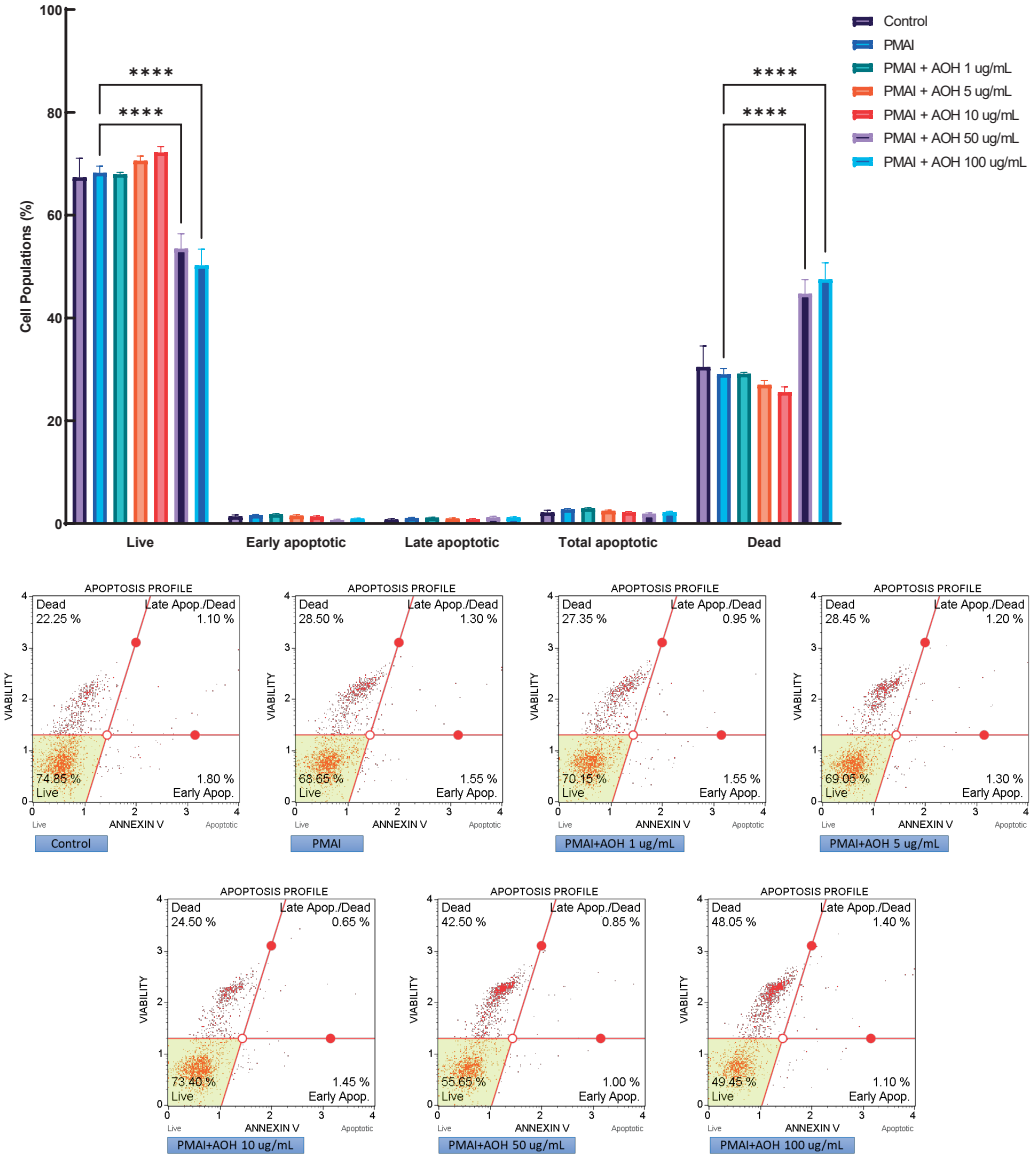

Figure 2. Effects of AOH on cell proliferation, apoptosis, and necrosis in PBMCs stimulated with PMAI

Effects of AOH on Cell cycle

The cell cycle is an intricate process, with three phases, which results in the duplication and transmission of genetic information (Schorl & Sedivy, 2007). In the first phase G0/G1, the cell responds to the stimulation of growth factors and extracellular mitogens, to then synthesize DNA in the S phase, and continue with mitosis (M), which has the final result of cell division (Israels & Israels, 2000). Any toxin or xenobiotic that has the ability to destroy DNA can alter the progress of the cell cycle, this could lead to neoplasia or various forms of cancer (Schafer, 1998). Regarding the changes in the cell cycle under the action of AOH, studies on RAW 264.7 mouse macrophages demonstrated

that after 24 hours, a buildup of cell populations in phase G2/M may be caused by AOH 30 µM, also inducing morphological changes of the nucleus (Solhaug et al., 2012). Additional research revealed that AOH 60 µM significantly decreased cell populations in the phase G1/G0, while those in S and G2/M increased significantly after 48 hours of treatment (Fernández-Blanco et al., 2016). Regarding pigs, after exposure of porcine endometrial cells to AOH 12.5 µM for 24 hours, an arrest in the G0/G1 phase was observed, while the percentage of cells in phase S was reduced by more than 20% compared to the control (Wollenhaupt et al., 2008).

As demonstrated by Figure 3 for unstimulated PBMCs, the exposure to AOH doesn't induce any significant changes regardless of the concentration used. However, PMAI induces a significant decrease of cell populations (8.77%) in phase G0/G1, accompanied by a significant arrest in S phase (5.56%) (Figure 4). In stimulated cells, AOH induced a substantial increase of cell populations in phase G0/G1 in comparison with stimulated control (6.36% for 50 µM AOH) at the same time a notable decline in the quantity of phase S cells is observed for 50 µM AOH (8.33%) and 100 µM AOH (8.60%).

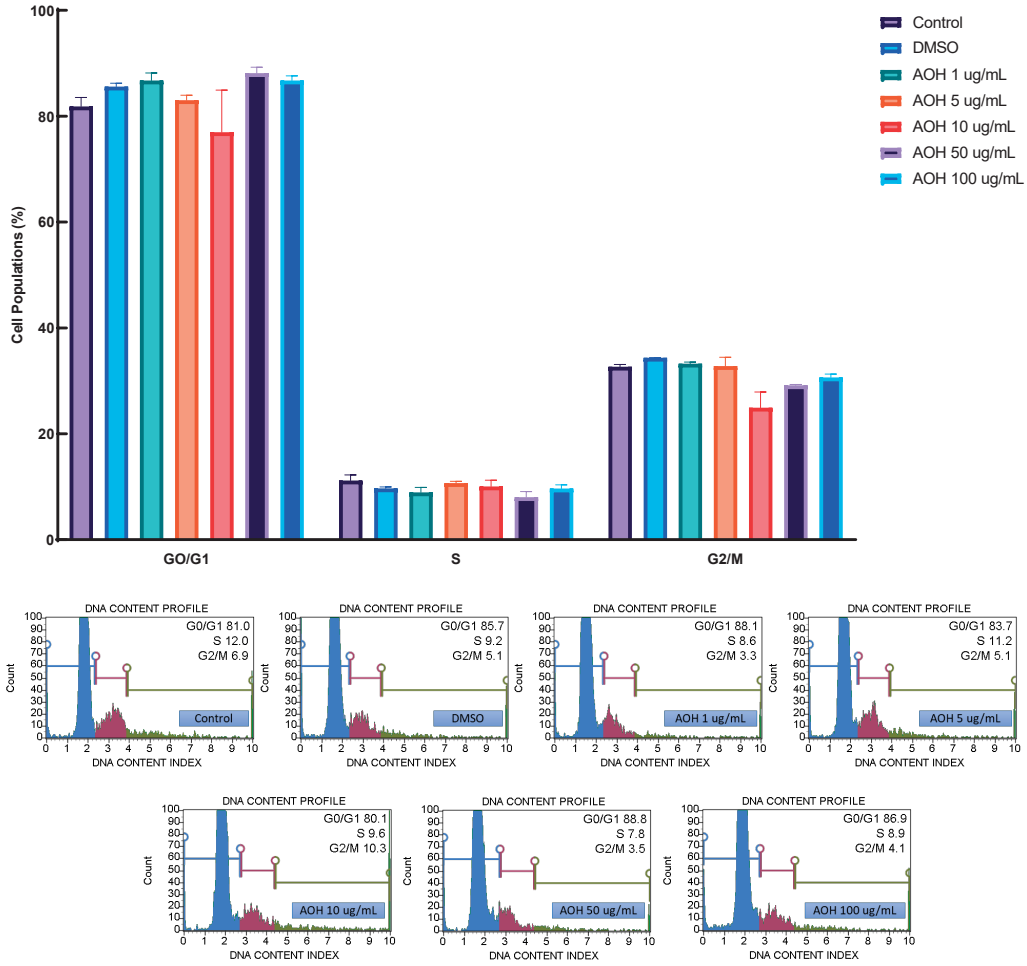

Figure 3. Effects of AOH on cell cycle in unstimulated PBMCs

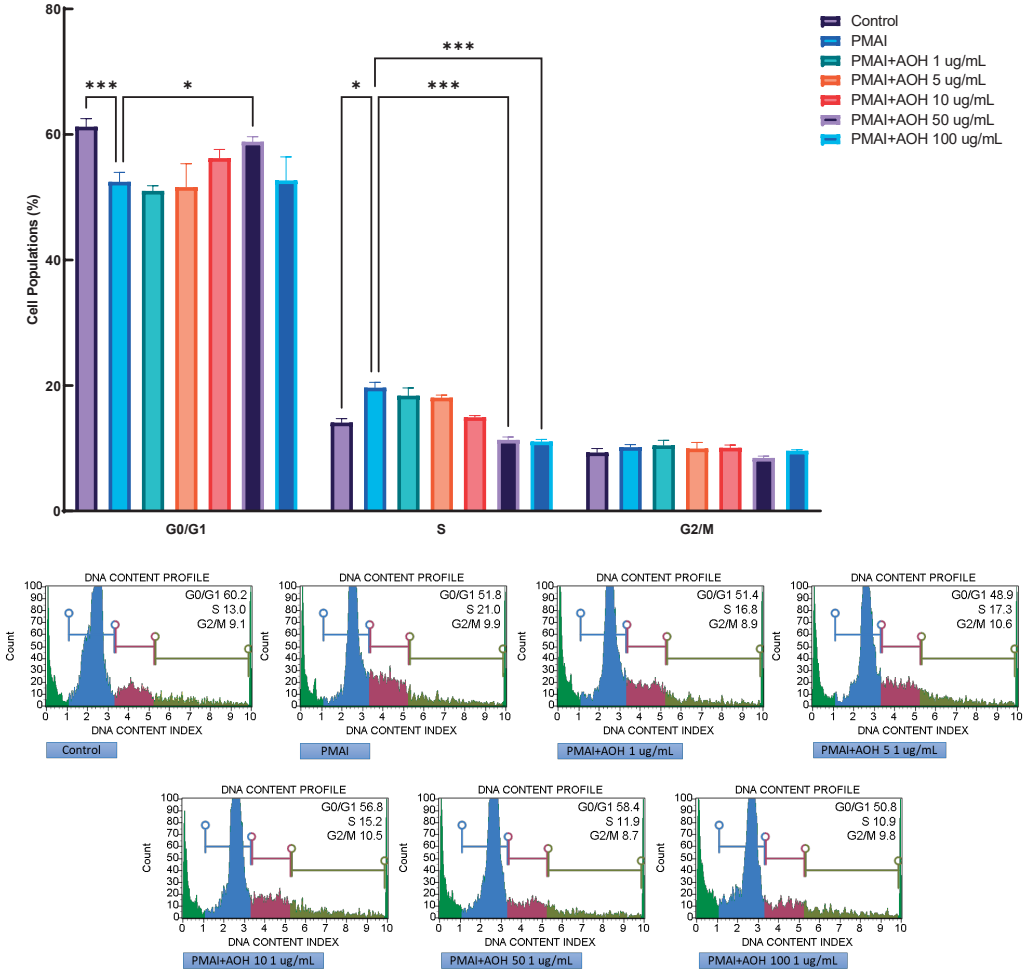

Figure 4. Effects of AOH on cell cycle in PBMCs stimulated with PMAI

Effects of AOH on Oxidative Stress

The role of oxidative stress is significant in many biological processes with important implications for the body, but it can also play a significant part in the etiology of several illnesses (Camhi et al., 1995). Most of the time, oxidative stress is caused by exposure to ROS (reactive oxygen species), such as the $HO^{\bullet}$ (hydroxyl radical), H_2O_2 (hydrogen peroxide) and $O_2^{\bullet-}$(superoxide anion) (Storz & Imlayt, 1999), causing damage to cell membranes, proteins, even nucleic acids (Crawford & Davies, 1994). Studies show that the oxidative response is closely related to apoptosis, the imbalance of redox processes at the cellular level being an important part of the signal transduction pathway(Buttke & Sandstrom, 1994), mediating apoptotic processes (Kannan & Jain, 2000).

Oxidative stress was evaluated under both conditions in unstimulated and PMAI stimulated PBMCs, as in the case of the cell cycle and apoptosis, by detecting changes in reactive oxygen species. For unstimulated cells, as can be seen in Figure 5, AOH was able to generate an increase in the percentage of ROS(+) cell populations in the case of higher concentrations of AOH, respectively 10 µg/mL (10.8%), 50 µg/mL (11.5%) and 100 µg/mL (16.3%). At the same time, a noticeable decrease of ROS(-) cell populations was observed in the case of the same concentrations 10 µg/mL (10.77%), 50 µg/ml (11,39%) and 100 µg/mL (16.49%). Moreover, similar outcomes were obtained in stimulated

PBMCs too. As can be seen in Figure 6, stimulation of cells with PMAI did not produce significant changes regarding the percentage of ROS(+)/ ROS(-) cells populations. However, the exposure to PMAI+AOH 50 µM/100 µM caused a rise in the percentage of ROS (+) cells of 23.66% and 21.77%, simultaneously with a significant decrease in ROS(-) 24.06% and 22.10%. This indicating that AOH can induce oxidative stress through the production of ROS. It seems that both in the case of stimulated cells and those stimulated with PMAI, at the level of oxidative stress, AOH is able to induce an increase in the level of ROS(+).

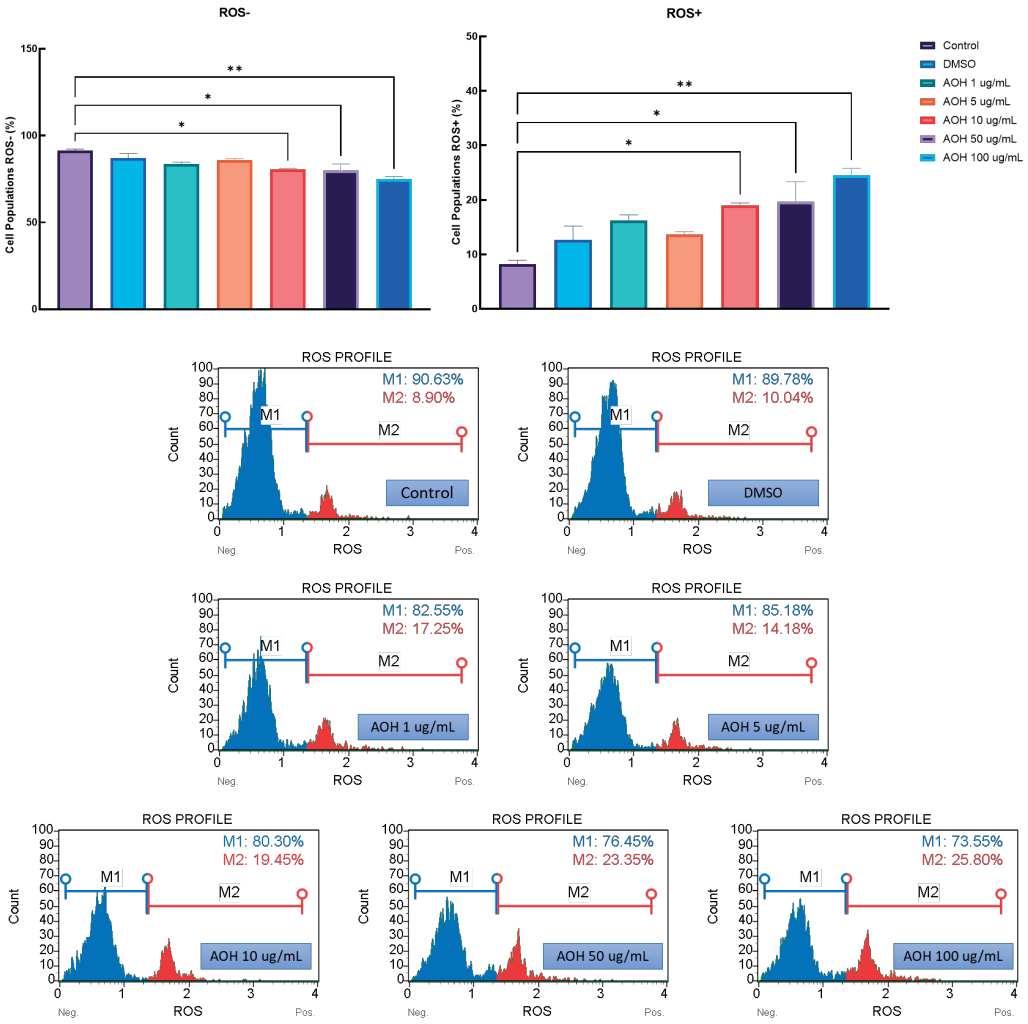

Figure 5. Effects of AOH on Reactive Oxygen Species in unstimulated PBMCs

It has been shown in several research that AOH can cause oxidative stress. In the case of the exposure of CaCO-2 cells to AOH (15, 30 and 60 µM), a reduction in the antioxidant enzymes' GSH, GPx and GR activity, along with DNA damage after was reported 24 hours of treatment (Fernández-Blanco et al., 2015). Additionally, it has been reported that AOH (5 mg/kg/body weight/day - 4 days) administered to pregnant mice was able to induce embryotoxicity and immunotoxicity by triggering apoptosis mediated by the accumulation of ROS (Huang et al., 2021).

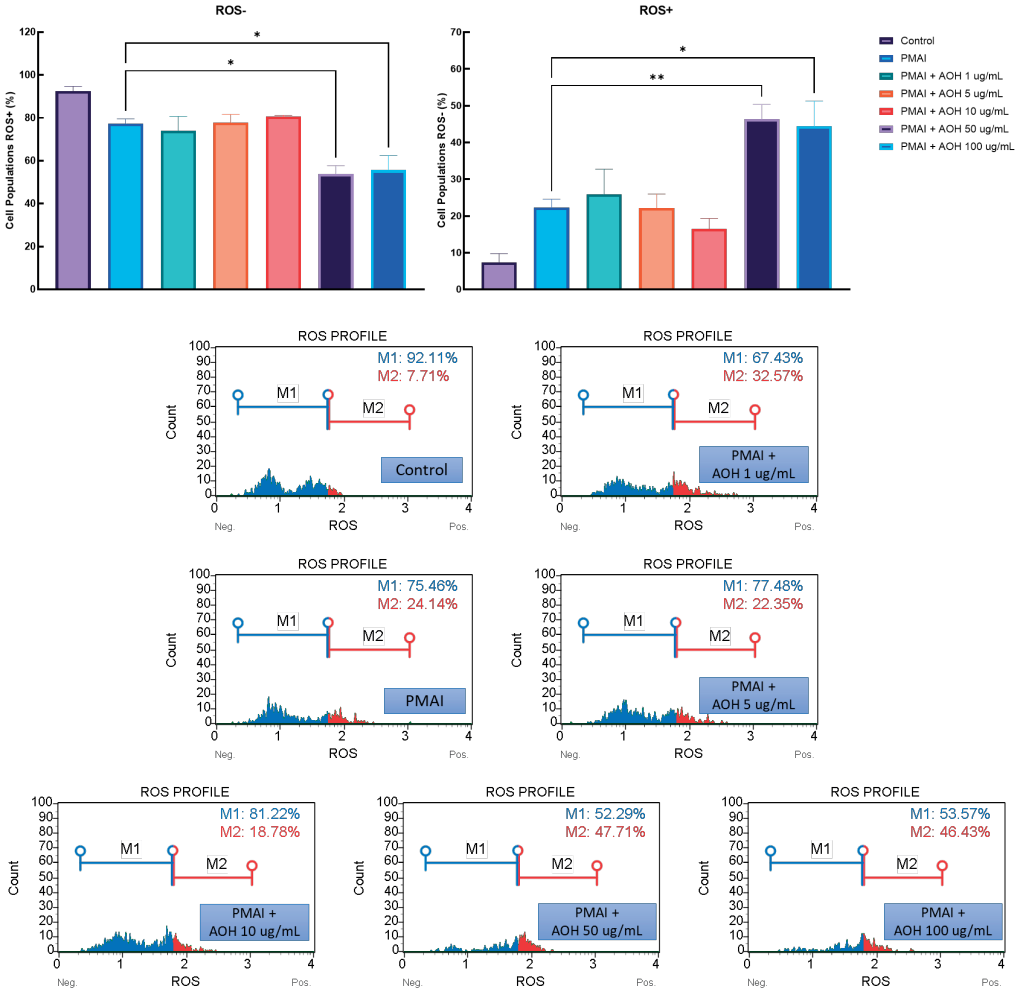

Figure 6. Effects of AOH on Reactive Oxygen Species in PBMCs stimulated with PMAI

CONCLUSIONS

Among animals used in agriculture, pigs are one of the most vulnerable species to mycotoxicosis. This happens mainly due to the diet rich in cereals, fruits, or other plant products, but also due to their innate sensitivity. Our study aimed to evaluate the effects of AOH (1-100 µg/mL) in PBMCs cells freshly isolated from peripheral venous blood from pigs The changes induced by AOH on apoptosis, cell cycle and oxidative stress were monitored under two conditions: unstimulated cells and cells stimulated with PMAI. Our results showed that AOH in high concentrations (50, 100 µg/mL) was able to increase cell death both in the case of stimulated cells and of stimulated ones. Moreover, regarding the unstimulated cells, the exposure to AOH resulted in an increase of the cells in late apoptotic stage for all the studied concentrations. Considering that data from the literature indicate that AOH can induce cycle arrest, the effects of AOH exposure on the cell cycle was also assessed. Thus, in the case of unstimulated cells, no significant changes produced by AOH were observed, but in the case of those stimulated with PMAI, it was observed that the addition of AOH 50 µg/mL and 100 µg/mL led to a rise in cell population counts in phase G0/G1, concurrently, a significant diminish of those in the phase S was also observed, compared to the stimulated control.

One of the mechanisms that could be responsible for the modulation of apoptotic processes is represented by the induction of oxidative stress through the accumulation of ROS. Our results, as in the case of those obtained in the case of apoptosis, shown that the proportion of ROS (+) cell populations could be significantly increased by AOH at 50/100 µg/mL, in the case of unstimulated cells, the increase of ROS(+) percentages starts even from AOH 10 µg/mL. This shows that the main mechanism of induction of apoptosis of AOH could be the production of ROS. Corroborating the obtained data, it can be concluded that in the case of porcine PBMCs cells, AOH was able to induce apoptosis and cell death, the main mechanism that could be responsible being through oxidative stress.

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