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Zobrazení reaktivních forem kyslíku v lidských buňkách v reakci na abiotický stres

BAKALÁŘSKÁ PRÁCE

Autor:	**Alina Ovsii**
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PALACKY UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Biophysics

Imaging of reactive oxygen species in human cells in response to abiotic stress

BACHELOR THESIS

Author:	**Alina Ovsii**
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Abstrakt

U-937 je buněčná linie promonocytární lidské myeloidní leukémie, která byla izolována od pacienta s histiocytárním lymfomem. Tyto buňky se používají ke studiu chování a diferenciace monocytů. Je známo, že pod vlivem abiotického stresu se tyto monocyty diferencují na makrofágy nebo dendritické buňky v závislosti na použitém induktoru diferenciace. Zralé diferencované buňky U-937 se vyznačují plochým nepravidelným tvarem, přítomností pseudopodií a fagocytární aktivitou. Dřívější studie uváděly, že během fagocytózy makrofágů dochází ke vzniku reaktivních forem kyslíku (ROS), nikdy to však nebylo přesně charakterizováno. V současném studiu je diferenciace buněk indukovaná pomocí forbol-12-myristát-13-acetátu (PMA), což je dobře známý aktivátor proteinkinázy C (PKC). Exogenní přídavek PMA může aktivovat nikotinamidadenindinukleotidfosfát (NADPH) oxidázu, což může vést k tvorbě superoxidového aniontového radikálu ($O_2^{\bullet-}$) v buňce. Dismutace $O_2^{\bullet-}$ vede k tvorbě peroxidu vodíku (H_2O_2) a následně může působit jako předchůdce pro tvorbu reaktivnějších forem kyslíku, jako je hydroxylový radikál ($HO^{\bullet}$). Zvláštní morfologie zralých makrofágů byla zkoumána konfokální laserovou skenovací mikroskopií (CLSM) a také bylo vynaloženo úsilí na lokalizaci související tvorby ROS, konkrétně $HO^{\bullet}$. Výsledky byly ověřeny pomocí spektroskopie elektronové paramagnetické rezonance (EPR); modifikace proteinů způsobená $HO^{\bullet}$ byla potvrzena western blotem. Tato práce má za cíl rozšířit naše porozumění ohledně tvorby, lokalizace a obecné charakterizace ROS v buňkách U-937.

Klíčová slova: Buňky U-937; reaktivní formy kyslíku; oxidační stres; monocyty; konfokální laserová skenovací mikroskopie.

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Abstract

The cell line U-937 is a pro-monocytic human myeloid leukaemia cell line which was isolated from histiocytic lymphoma patient. These cells are used to study the behaviour and differentiation of monocytes. Under the effect of abiotic stress, these monocytes are known to differentiate into macrophages or dendritic cells depending on the inducer used. The mature differentiated U-937 cells are characterized by an irregular shape, the presence of pseudopodia, and phagocytic activity. It has also been reported that during macrophage phagocytosis, reactive oxygen species (ROS) are formed, however, it has never been precisely characterized. In the current study, the cell differentiation was induced by using phorbol 12-myristate 13-acetate (PMA) which is a well-known protein kinase C (PKC) activator. The exogenous addition of PMA can activate nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, which can lead to the formation of superoxide anion radical ($O_2^{\bullet -}$) in the cell. Dismutation of $O_2^{\bullet -}$ leads to the formation of hydrogen peroxide (H_2O_2), and subsequently, it can act as a predecessor for the creation of more lethal oxygen species such as hydroxyl radical ($HO^{\bullet}$). In this study, the peculiar morphology of mature macrophages has been investigated by confocal laser scanning microscopy as well as an effort has been made to localize the associated ROS formation, specifically $HO^{\bullet}$. The results were validated using electron paramagnetic resonance (EPR) spectroscopy; protein modification caused by $HO^{\bullet}$ was confirmed by western blot. This thesis intends to broaden our understanding of ROS formation, localization, and its general characterization in U-937 cells.

Keywords: U-937 cells; reactive oxygen species; oxidative stress; monocytes; confocal laser scanning microscopy.

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Contents

Aims of Bachelor thesis

1. Understanding the formation of reactive oxygen species (ROS) in human cells (U-937 cells).
2. Human cell line cultivation and growth procedures optimization.
3. To demonstrate ROS formation and its characterization in U-937 human cells by using confocal laser scanning microscopy (CLSM) and electron paramagnetic resonance (EPR) spectroscopy.
4. To study the protein modification in non-differentiated and differentiated cells.
5. Validate and evaluate the measured results and discuss in relation to the literature.

1. Introduction

Cell differentiation is a process by which less specialized cells undergo maturation and development to become more specific with respect to their form and function. It occurs during the growth and development of a multicellular organism: from embryonic stem cells to any other type of cell. In a mature adult organism, some cells also continue to differentiate during tissue or cell repair. There are many factors and conditions that can activate differentiation. Recently, it was established that U-937 cell differentiation occurs in response to strong chemical compounds such as phorbol 12-myristate 13-acetate (PMA), dimethylsulfoxide (DMSO), retinoic acid, Zn^{2+}, 12-O-tetradecanoylphorbol-13-acetate (TPA), and low glutamine concentration among others (Chun *et al.*, 2001; Pagliara *et al*., 2005; Yamamoto *et al*., 2009; Zamani *et al*., 2013; Mendoza-Coronel and Castanon-Arreola, 2016; Prasad *et al*., 2020).

In our current study, pro-monocytic human myeloid leukaemia cell line U-937 was used. This cell was first isolated from the histiocytic lymphoma of a male patient in the year 1976. This cell line is crucial because it demonstrates many characteristics of monocytes. In suspension culture, undifferentiated U-937 cells have a round shape, short microvilli, and bean-shaped nuclei (Pagliara *et al*., 2005). U-937 has been used as an experimental model to study monocyte-macrophage differentiation. This cell line can be cultivated and stored in an incubator in humidified conditions. They can be frozen for many years and later recovered without obvious changes in monocyte morphology (Sundström & Nilsson, 1976; Chanput *et al*., 2015).

In this study, undifferentiated leukaemia cells went through differentiation in response to abiotic stress caused by exposure to Phorbol 12-myristate 13-acetate (PMA). PMA treated monocytes are characterized as ‘macrophage-like’, which means that their features are not yet fully understood and require more precise examination (Maess *et al*., 2014; Lund *et al*., 2016; Tedesco *et al*., 2018). The purpose of this work is to investigate mature macrophages by confocal laser scanning microscopy as well as *in vivo* imaging of superoxide anion radical ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$) using fluorescent probes at the unicellular level and to understand the consequence of ROS generation.

2. Current state of the topic

2.1. Reactive oxygen species (ROS) formation

Reactive oxygen species (ROS) are highly reactive molecules consisting of one, two, or three oxygen atoms. When a molecule has unpaired electrons, these species are referred to as radical ROS, whereas non-radical ones contain only paired electrons. Reactive species are formed due to electron transport or energy transfer. For the former, molecular oxygen (O_2) acts as an oxidizing agent, gaining one electron. As a result, a $O_2^{\bullet -}$ is formed. That radical oxidizes the next atom or molecule and there is the formation of another ROS. So, by sequential electron transfers, O_2 is reduced to water and ROS are formed as a by-product of these chain reactions. Radical forms formed by electron transport are called type I reactions (Fig. 1). Some radicals can be generated by energy input, for example, singlet oxygen ($^1O_2$), those are called type II reaction (Halliwell & Gutteridge, 2007) (Fig. 2).

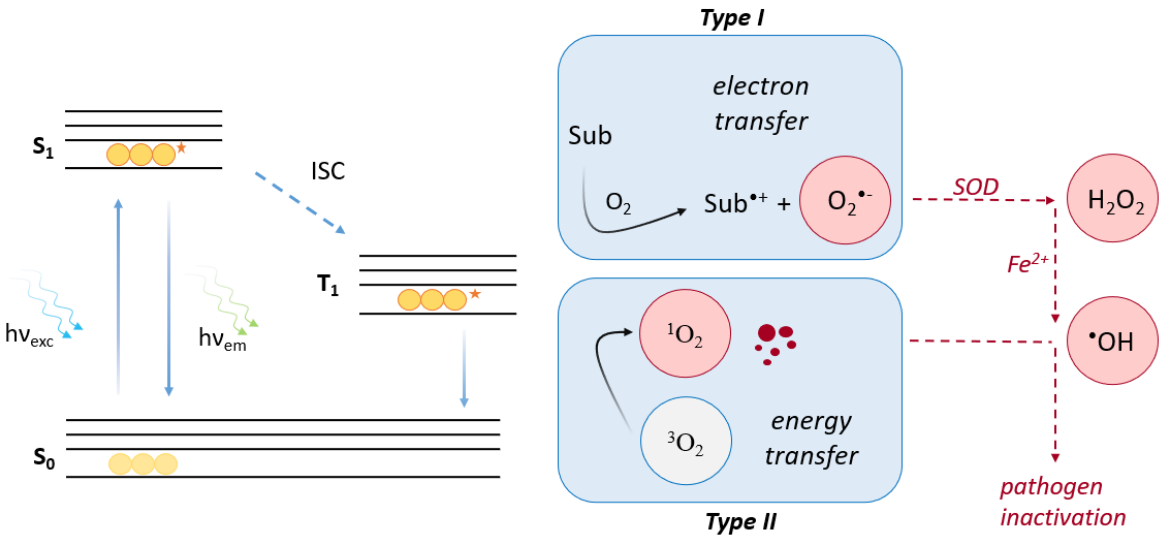

Figure 1: ROS formation by type 1 (electron transfer) and type 2 reaction (energy transfer).

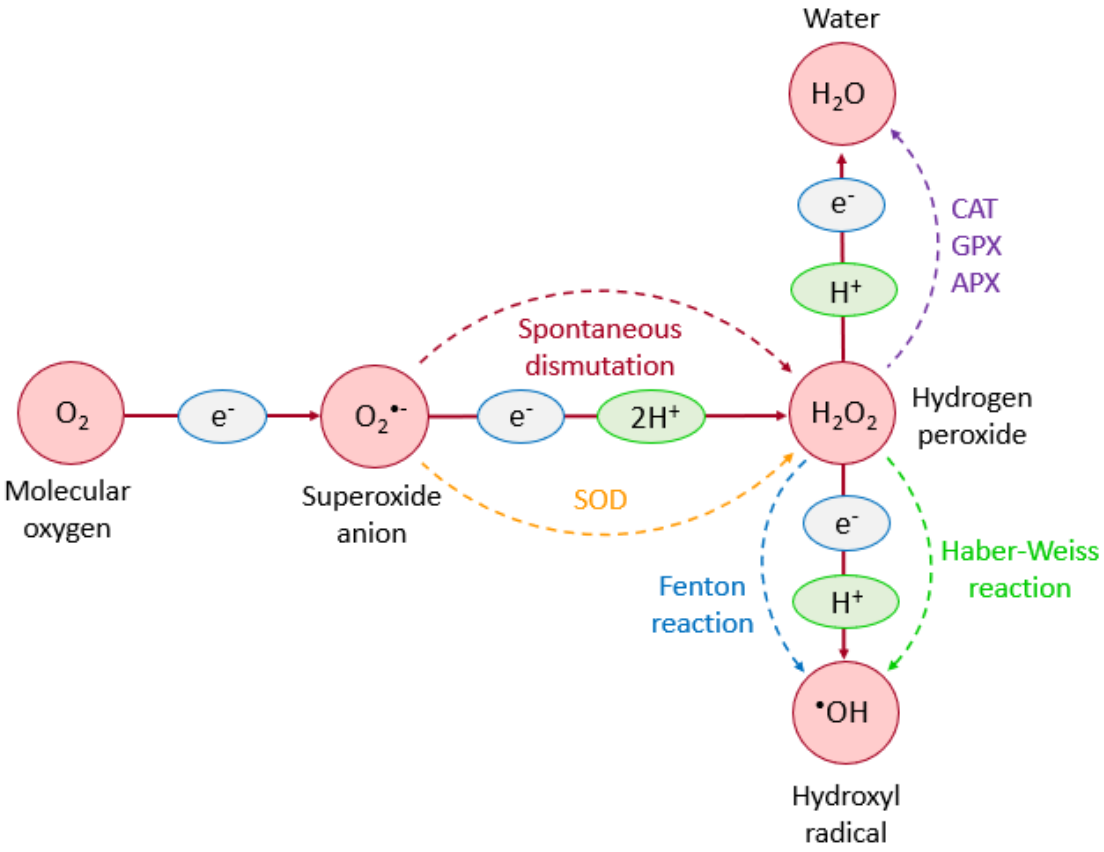

Figure 2: Electron transport showing sequential formation of $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2) and $HO^{\bullet}$ formation from molecular oxygen.

Reactive oxygen species are spontaneously generated in our body during processes such as inflammation, oxidative phosphorylation, respiratory burst, among other processes, however, if the concentration of these species is higher than threshold capacity of scavenging by non-enzymatic or enzymatic antioxidants, they can also have harmful effects on the body. Hydrogen peroxide (H_2O_2), which is a well-known signalling molecule, is known to be generated under stress conditions in many biological systems. Diffusing from one location to another and by oxidizing some molecules, it is known to mediate numerous physiological and biochemical processes (Niu & Liao, 2016).

In general, because ROS molecules are very unstable, they rapidly oxidize biomacromolecules, destroying them or changing their structure. Therefore, this can lead to the destruction of the cell membrane and subsequently to cell death. First, they oxidize the lipids and proteins in the cell membrane. The process, while the lipid is oxidized by free radical and the peroxyl radical is generated as an intermediate, is called lipid peroxidation. The mechanism of lipid peroxidation consists of three main steps: initiation, propagation, and termination (Fig. 3). In the initiation step, free radicals attack the lipid

molecule targeting the double bonds of the polyunsaturated fatty acid molecule (PUFA). As a result, the stable PUFA becomes unstable. In the propagation step, lipid peroxyl radical is formed due to the reaction of fatty acid radical with oxygen molecule. Lipid peroxyl radical reacts with another PUFA, resulting in a formation of lipid peroxide and a generation of another lipid radical, as it was in the initiation step. The cycle of oxidation continues until the termination does not occur (Fig 3). In the last step of the peroxidation reaction, two lipid radicals react and get converted into peroxide (non-radical ROS). It can happen when the concentration of free radicals is high enough to react with each other (Bochkov *et al*., 2010). The end-products of this process are species such as malondialdehyde (MDA) and 4-hydroxy-2-trans-nonenal (HNE) which are known to be acting as reactive species and cause indirect damage to the proteins and DNA (Marnett, 1999; Halliwell & Gutteridge, 2007; Weber *et al*., 2013).

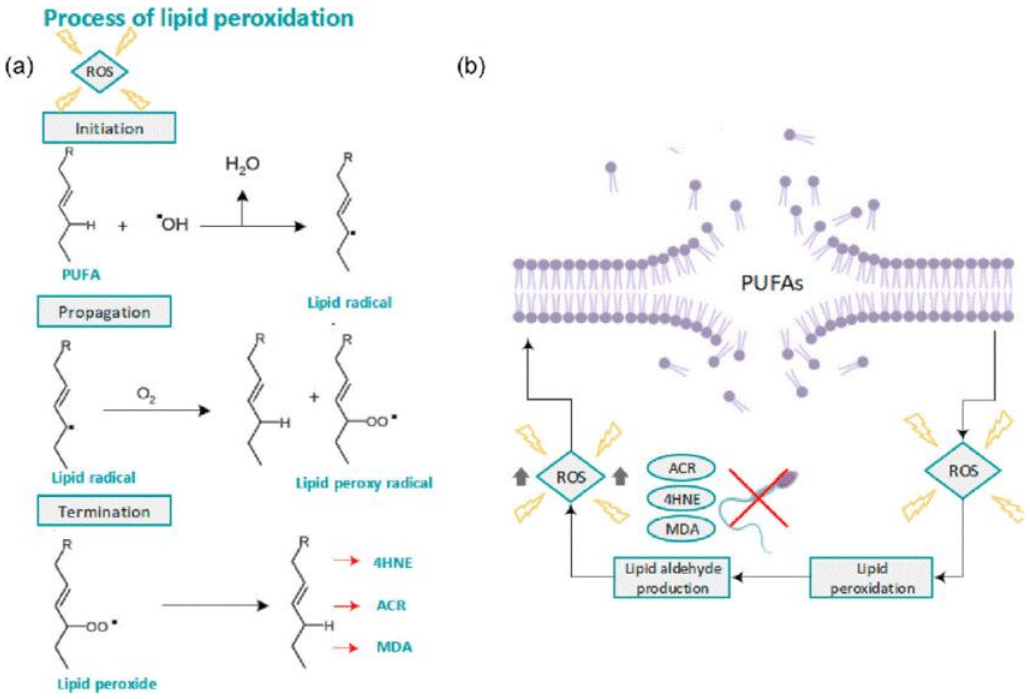

Figure 3: Mechanism of lipid peroxidation (adapted from Evans *et al.*, 2021; doi: 10.1016/j.redox.2021.102071) showing the different reaction steps: initiation, propagation and termination.

Isoketal MDA quickly combines with $–NH_2$ groups and form adduct with amino acid residues (especially lysine) on membrane proteins. As a result, there is formation of intra- or inter- molecular cross-links (Halliwell & Gutteridge, 2007; Vieira *et al*., 2017; Prasad *et al*., 2022) (Fig. 4).

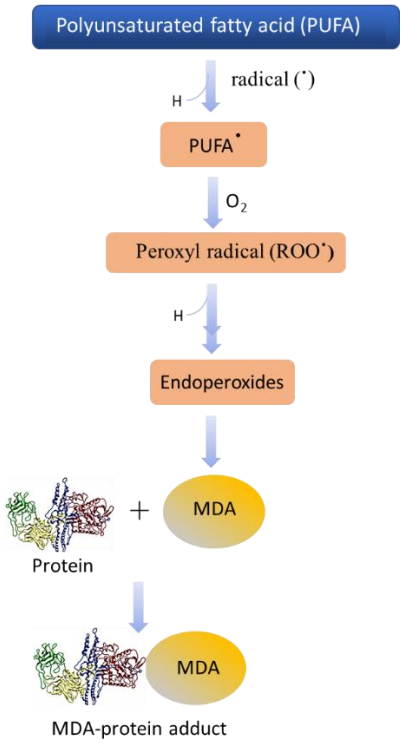

Figure 4: Protein modification caused by MDA (adapted from Prasad *et al*., 2022, doi: 10.3390/ijms23137424).

Oxidative modification of proteins leads to the impairment of active sites of enzymes, disorder the conformation of a structural protein, and proteasomal degradation leading to misfolded or unfolded proteins that are non-functional (Halliwell & Gutteridge, 2007). Once the protein is oxidized, it can also be recognized as a foreign body by immune cells and trigger the production of antibodies. After the free radical outbreak in the protein, it has been reported that more than 20 amino acid residues have been reported to be generated as end products (Halliwell & Gutteridge, 2007). Each of them is capable of forming several unstable oxidation products that can subsequently oxidize other molecules. These are in turn free radicals that set the chain reaction going. Thus, the radicals destroy the cell membrane, cytoplasmic proteins, then the nucleus membrane, and DNA thereby leading to mutation and cell degradation (Halliwell & Gutteridge, 2007).

Fortunately, the human body has a defense mechanism comprising of non-enzymatic and enzymatic antioxidants. Antioxidants prevent or remove oxidative damage (Halliwell & Gutteridge, 2007). They can be divided into two groups by their mechanism/function. Enzymatic antioxidants play a key role in the neutralization of free radicals. One of the most important enzymes that scavenge ROS is superoxide dismutase (SOD). It performs dismutation of very reactive $O_2^{\bullet -}$, which is converted into less reactive

H_2O_2. Glutathione peroxidase, another enzyme scavenger, reduces H_2O_2 to water. When the amount of H_2O_2 is high, then catalase also helps to convert H_2O_2 into water. Non-enzymatic antioxidants break chains of peroxides and form a relatively stable radical.

Among the non-enzymatic scavengers are some vitamins, flavonoids, carotenoids, glutathione, etc. A well-known antioxidant that helps protect our cells from free radicals is vitamin C. Vitamin E is a very effective chain-breaking antioxidant in our tissues. Both enzymatic and non-enzymatic antioxidants in combinations constitute an effective defense mechanism against free radicals in our body (Eddaikra A. & Eddaikra N., 2021).

2.2. U-937 cells

Monocytes are a cluster of cells circulating in the blood, bone marrow, and spleen which make up to about 10% of the total number of leukocytes in humans. Typical morphological features of monocytes are their irregular shape, oval/kidney-shaped nucleus, vesicles in cytoplasm, and a high cytoplasm-to-nucleus ratio. Monocytes can remain in the bloodstream for up to 1-2 days, after which, if they have not been differentiated into macrophages and sent to the tissue in response to inflammation, they expire. Macrophages are a type of immune cells found in the extracellular fluid that are able to consume defective and dead cells or foreign substances including viruses and bacteria. Surrounding the pathogens, they are known to form pseudopodia to engulf the pathogen/foreign substances. The main functions are phagocytoses, antigen presentation, and cytokine production. U-937 used in the current study is a pro-monocytic cell line that can be differentiated into macrophages or dendritic cells in response to abiotic stress (Chanput *et al*., 2015). In 1976, they were isolated from the histiocytic lymphoma of a 37-year-old male patient (Sundström & Nilsson, 1976).

This cell line exhibits several characteristics of monocytes, such as round shape, small microvilli, and bean-shaped nuclei (Pagliara *et al*., 2005). As they are easily cultivated and replicated, they are widely used as the experimental model to study the differentiation process. The great advantage of these cells is that they can be stored for several tens of years and, following the proper protocol, can be recovered without any obvious effect on monocyte-macrophage functions (Chanput *et al*., 2015).

Previously, it was established that U-937 cells differentiate into mature monocytes or macrophages after exposure to PMA (Moreno-Navarrete *et al*., 2009; Zhang *et al*., 2010; Cam & Mejia, 2012; Gillies *et al*., 2012; Chanput *et al*., 2015). The application of PMA activates several processes in the cell which can lead to the formation of free

radicals. So, in this study, we are looking at intracellular ROS generation by U-937 at the cellular level.

2.3. U-937 cells and ROS

During the last few decades, cancer cells have been widely used in ROS studies, because of their ability to produce ROS in response to various abiotic stress, phagocytic activity, simplicity of use/storage, and cost efficiency. In the past, several researchers have reported ROS from cancer cell culture. Liu and co-workers (2016) have used the HeLa cells through UV-VIS spectroscopy and spectrofluorometry to report a new dual emitting fluorescent probe that demonstrates high selectivity for $HO^{\bullet}$ in biological species (Liu *et al*., 2016). Another research has demonstrated the determination of $HO^{\bullet}$ and other ROS in mononuclear macrophage cells by electrochemical impedance spectroscopy, which possesses the advantages of a low detection limit with high selectivity against ROS (Zhu *et al*., 2011). Fluorescence methods are known for their simplicity in sample preparation, high sensitivity, and visible changes of fluorescence and bears potential for examining the spatial distribution. Using a probe that is specific for the radical, it allows localization of the signal and provides real-time monitoring in biological systems without causing significant damage to the sample (Lei *et al*., 2017). Compared to fluorescent techniques, EPR spin-trapping spectroscopy allows one to detect free radicals directly. It is considered to be one of the most precise techniques for the detection/identification of reactive short-lived free radicals by obtaining the electron spin resonance spectrum of the radical addition product. Its selectivity depends on the specific spin trapping agent (Janzen & Blackburn, 1969).

The U-937 cell line, as described in more detail in the previous section, is ideal for studying ROS *in vitro* and better understanding its formation in the cell. They are easily differentiated from monocytes to macrophages. The macrophages in response to stimuli undergoes phagocytosis to kill the pathogen, and ROS are known to be generated during this process (Forman & Torres, 2002). However, the connection between the process of differentiation and ROS production in U-937 is not yet sufficiently understood yet.

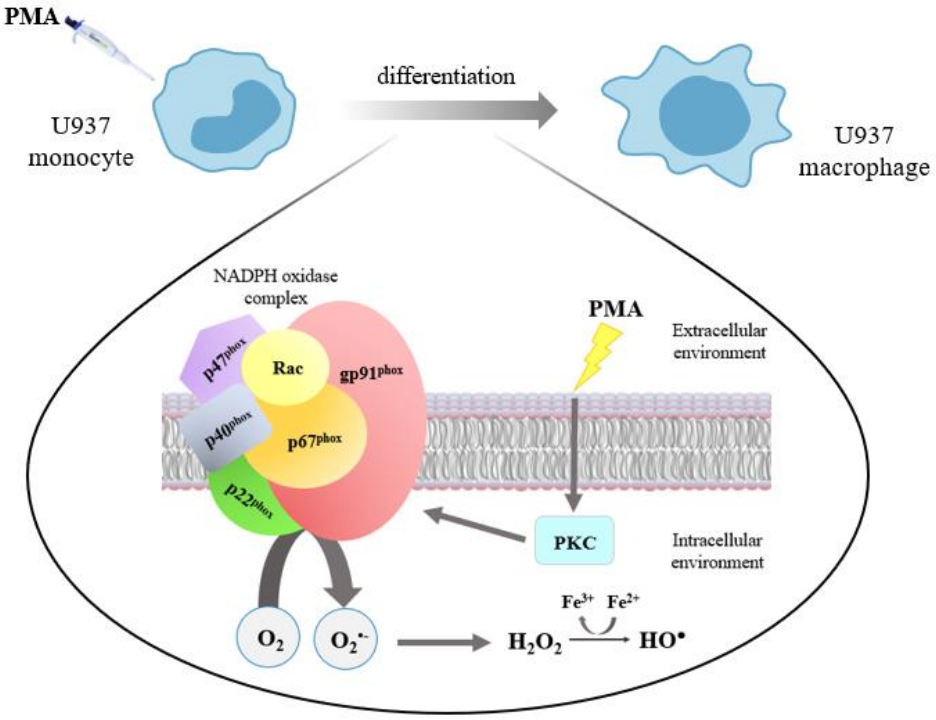

Figure 5: Differentiation initiated by exogenous application of inducer (PMA) and generation of ROS via activation of NADPH oxidase.

2.4. Reactive oxygen species and differentiation

According to Halliwell and Gutteridge (2007), ROS participates in the differentiation of some slime moulds and fungus (Halliwell & Gutteridge, 2007). There is an assumption that an increase in oxidative stress and changes in the redox state could be triggered for differentiation. Several studies established before that the PMA treatment induces the production of ROS in U-937 monocytic cells. Application of the PMA to the cell activates a membrane-bound enzyme complex nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and protein kinase C (PKC), which can lead to the formation of $O_2^{\bullet-}$. Its dismutation leads to the production of more reactive and harmful $HO^{\bullet}$ (Auchère & Rusnak, 2002; Halliwell & Gutteridge, 2007; Prasad *et al*., 2015; Prasad *et al*., 2016; Pospíšil *et al*., 2019) (Fig. 5). It is also known that the process of differentiation in U-937 can be started under the influence of abiotic stress caused by several chemicals such as PMA, DMSO, THA, retinoic acid, glutathione and others (Chun *et al*., 2001; Yamamoto *et al*., 2009; Zamani *et al*., 2013; Mendoza-Coronel & Castanon-Arreola, 2016). Protein kinase C, which was activated by PMA, activates not only NADPH-oxidase and NO-synthase but also the process of differentiation of some mouse and human cells into mature cells (Nishizuka, 1992; Asseffa *et al*., 1993; Tonetti

et al., 1994; Chen *et al*., 1996; Dieter & Schwede, 2000; Mouithys-Mickalad *et al*., 2001; Kasai *et al*., 2005).

Kasai and co-workers (2005) provided real-time monitoring of ROS production during the differentiation of human monocytic leukaemia cell line THP-1 into macrophages by electrochemical and chemiluminescence methods (Kasai *et al*., 2005). According to their report, the ROS response by the PMA stimulation depends on the PMA concentration. The higher is concentration, the faster is the oxidative response and therefore the process of differentiation and/or respiratory burst.

2.5. Oxidative damage due to ROS

Free radicals, as by-products of metabolic processes, certainly play a substantial part in signalling, messenger functions, and inflammatory process, when they oxidize molecules of the foreign body and thereby kill the pathogen, and many others. Still, if their concentration in the body significantly exceeds the concentration of antioxidants, this can lead to oxidative stress which can have a harmful effect on our body. Thus, oxidative stress is alleged to be a precursor of many diseases. Reactive species have an important role in the pathogenesis of some chronic inflammatory diseases such as rheumatoid arthritis (Abbas & Monireh, 2008). Free radical-induced tissue damage appears to be involved in the pathogenesis of chronic ulcerative colitis progressing to chronic glomerular nephritis (Pravda, 2005). These are all the side effects of free radical damage.

Cardiovascular diseases can also be induced by ROS. Free radicals oxidize low-density lipoproteins (LDL) in the body. The human body contains a certain amount of LDL, also known as ‘bad’ cholesterol, which is always present in the blood. These oxidized LDLs contribute to atherosclerosis/blockage of the veins, causing plaque to form within the veins, which can lead to cardiovascular diseases (Moris *et al*., 2017). More recently, free radicals have taken a strong place among the principles that explain the emergence and development of tumours. Cells that are affected by radicals undergo DNA damage, as well as deform or mutate. Mutation is considered one of the reasons for the appearance of carcinogens or cancer in the body (Liou & Storz, 2010). Our lungs are directly exposed to oxygen for long periods; hence there is a greater risk of free radical-mediated damage. They are also responsible for adult respiratory distress syndrome, pulmonary edema, bronchitis, and asthma etc. (Housset, 1994).

In terms of diabetes, when the accumulation of free radicals around the pancreas enhances, it leads to the destruction of islets of the pancreas, which are the organs that produce insulin and maintain our blood glucose levels. Once it happens, this can lead to diabetes, especially type 1 diabetes which is insulin dependent. Free radicals are also known to contribute to cataract formation (Thiagarajan & Manikandan, 2013). They reduce sperm motility and lead to DNA fragmentation in the forms leading to male infertility (Evans *et al*., 2021). Besides these, the role of free radicals in degenerative brain diseases, such as Parkinson's, Alzheimer's disease, dementia, and multiple sclerosis, is indisputable and has been discussed extensively during the past year (Halliwell & Gutteridge, 2007; Kumar *et al*., 2012). Nowadays, the risk of free radical damage is even higher because of air pollution, water pollution, increased radiation, increased use of pesticides and chemical fertilizers in growing food, ultra-processed foods with preservative colors, flavours added, and increased stress levels, decreased activity levels, etc. (Vasudevan *et al*., 2016). Reactive species can cause multiple types of disease, that is why it is essential to study them these days.

3. Materials and methods

3.1. Growth medium and chemicals

The human myeloid leukemia cell line U-937 was grown in suspension. RPMI-1640 medium was supplemented with 2 mM L-glutamine, 10% (v / v) fetal bovine serum (FBS), 1% (v/v) antibiotics (penicillin and streptomycin). To induce abiotic stress in consequence of which the cells are starting to differentiate, phorbol 12-myristate 13-acetate (PMA, 250 nM) was used. The preparation of PMA was performed using an organosulfur compound, polar aprotic solvent dimethyl sulfoxide (DMSO) purchased from Sigma-Aldrich GmbH, Germany. It is important to mention that the final concentration of DMSO was always kept below 1% as it can have a detrimental effect on cells, as evident from our previous study (Prasad *et al*., 2020). POBN [α-(4-Pyridyl N-oxide)-N-tert-butylnitrone] containing 170 mM ethanol was used as a spin trapping agent. The fluorescent reagent, dihydroethidium (DHE, 30 μM) and 3′-p-(hydroxyphenyl) fluorescein (HPF, 1 μM) was used for detection of $O_2^{\bullet-}$ and $HO^{\bullet}$, respectively. 3′-p-(hydroxyphenyl) fluorescein is a probe which is specific for $HO^{\bullet}$ and peroxynitrite. A lipopholic dye FM4-64 (15 μM) and a membrane-permeant stain Hoechst 33342 (2 μM) used to monitor the integrity of the cell membrane and nuclei. α-(4-Pyridyl N-oxide)-N-tert-butylnitrone, HPF, FM4-64 and Hoechst 33342 were purchased from Sigma-Aldrich GmbH, Germany.

3.2. Cell growth

The U-937 cells were obtained as a gift from the Immunology department of the Olomouc University Hospital (Olomouc, Czech Republic). During the experiments, the cell culture was grown in RPMI-1640 medium with 1% antibiotics in an incubator at 37°C in 5% CO_2, which refers to the conditions of the human body. On each third or fourth day, the cells were passaged. Half of the cell suspension was discarded and replaced by a new medium. All of this work was performed under sterile conditions in the flow box to avoid cell contamination. To better comprehend how long the cells are in the exponential growth phase and go into the stationary phase, a growth curve was plotted. Cell vitality was counted each day at the same time during the day for two weeks using a 0.05% trypan Blue viability test using an automated cell counter TC20 (Bio-Rad Laboratories, California, USA).

3.3. Cells viability

Cell viability was measured using a 0.05% trypan blue viability test. Trypan blue is a stain used to distinguish viable from non-viable cells. Consequently, 16 µl of the cell suspension was mixed with 4 µl of trypan blue in an eppendorf. Trypan blue stained cells were applied to the microplate and then counted by TC20 Automated Cell Counter (Bio-Rad Laboratories, California, USA). The calculation is based on the principle that dead cells tend to absorb trypan blue opposite to living ones.

3.4. Electron paramagnetic resonance spin-trapping spectroscopy

Electron paramagnetic resonance spectroscopy is a technique which allows one not only to detect species with unpaired electrons (free radicals) but, moreover, to determine specifically what the radical it could be. Because ROS are short-lived, it is necessary to form a more stable spin adduct to detect free radicals using EPR (Suzen *et al*., 2017). In the current study, we used 50 mM POBN for the detection of $HO^{\bullet}$ as a spin trapping agent. The figure below shows different components of an EPR spectrometer (Fig. 6).

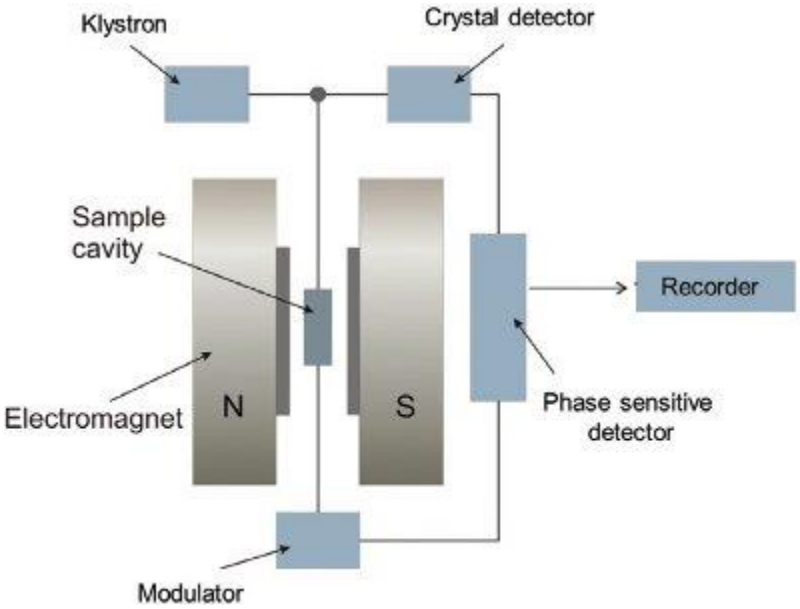

Figure 6: Schematic illustration of EPR spectroscopy (adapted from https://www.americanlaboratory.com/840-AuthorProfile/3842-Hafiz-Muhammad-Shahbaz/).

The sample preparation for EPR spectroscopy can be tricky, as there can be interference due to several components. Thus, first, the medium part from the cell suspension as it could influence the experiment. To increase the concentration of cells in the cell suspension, the samples were centrifuged (5000 rpm) for 2 min, after which the

supernatant was removed. Since abiotic stress could be caused not only by the influence of PMA but also by any other conditional changes such as temperature, pressure, contamination, decreased amount of cultivated medium, etc., control and experiment samples were prepared. After centrifugation, 50 μM PMA was applied to the experiment sample; 50 mM POBN was added to both the control sample and the PMA-treated sample. It is also important to take into account that since the differentiation inducer (PMA) is photosensitive; all the work should be performed under the dark conditions. Additionally, because POBN does not penetrate well through the cell membrane, to speed the permeation of POBN into the cells and to enhance POBN adduct formation, sonication was performed. The samples were sonicated for 30 min at 37°C with an ultrasonic bath sonicator (Elma Schmidbauer GmbH, Singen, Germany).

Electron paramagnetic resonance spectra were recorded on the MiniScope MS400 EPR spectrometer (Magnettech GmbH, Berlin, Germany) under the following EPR conditions: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}; gain, 100.

3.5. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is a technique used for obtaining high-resolution optical images with depth selectivity (Fig. 7). Using fluorescent probes, this technique allows the localization of ROS formation in specific cellular compartments. Visualization of U-937 morphology, as well as cell differentiation, ROS localization and the integrity of cell nuclei and membranes were performed with a Fluorview 1000 confocal unit attached to an IX80 microscope (Olympus Czech Group, Prague, Czech Republic).

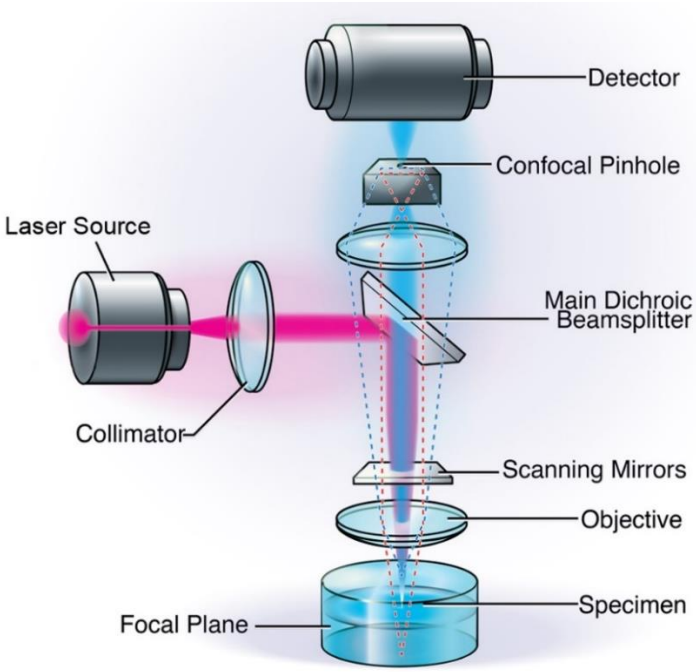

Figure 7: Schematic principle of CLSM (adapted from Riddle *et al*., 2017; doi: 10.5210/jbc.v41i1.7563).

Cell morphology visualization was performed 72 hours after incubation in the presence/absence of 250 nM PMA for the experiment/control sample, using a transmitted light detection module with 405 nm diode laser excitation and differential interference contrast (DIC) filters. Double staining with FM4-64 (15 μM) and Hoechst 33342 (2 μM) was performed to monitor the integrity of the cell membrane and nuclei. 72 hours of grown cells and 250 nM PMA-treated cells were incubated with FM4-64 and Hoechst 33342 and visualized by CLSM. For the excitation of FM4-64 a 543 nm He-Ne laser, and its emission was detected by a 655-755 nm filter. Hoechst 33342 was excited using a 405 nm diode laser, while its emission range was within 430-470 nm.

The intracellular formation of $O_2^{\bullet -}$ was monitored using fluorescent reagent DHE (30 μM) by excitation of 543 nm He–Ne laser. The emission of the oxidized form of 2-hydroxyethidium (DHE oxidase) was recorded using a 655-755 nm filter. Hydroxyl radical imaging and localization in cellular compartments was performed after 12 hours post-incubation (1 μM HPF) of cells treated with U-937 and PMA untreated. In the reaction of $HO^{\bullet}$ with HPF was formed a fluorescent product – hydroxyphenyl fluorescein oxidase (HPFox). As HPF is a fluorescent probe that can be affected by light, both treatment and incubation were performed under dark conditions. HPFox excitation was provided with a 488 nm line of an argon laser and the fluorescent signal was detected by a 505–605 nm emission filter.

3.6. Total protein extraction and western blotting

PMA pre-treated cells (72 h, 250 nM) were collected by centrifugation, washed with PBS (pH 7.4). Cells were sonicated in a lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40] containing 1% protease and phosphatase inhibitor. The homogenate was centrifuged at 16,000 rpm and the collected supernatant fraction was quantified using a Pierce BCA protein estimation kit (Thermo Fisher Scientific, Paisley, UK). After quantification, for anti-MDA blotting, 5x Laemmli sample buffer and 100 mM 2-mercaptoethanol were mixed with cell homogenates and protein samples (5 µg/lane) were isolated on 10% SDS gels. The proteins were then transferred to nitrocellulose membranes using a Trans-Blot Turbo transfer system (Bio-Rad, California, USA). The nitrocullulose membranes were then blocked for overnight at 4°C with 5% BSA in tris-buffered saline (TBS) (pH 7.4) and 0.1% Tween 20. The blocked membranes were probed with anti-MDA antibody [Abcam (Cambridge, CB2 0AX, UK), ab27642] (1:5000) for 1h; incubated for 1 hour at room temperature with HRP conjugated anti-rabbit secondary antibody (dilution 1:10000). Immunocomplexes were produced and imaged using the Amersham 600 imager and Immobilon Western Chemiluminescent HRP Substrate (Sigma Aldrich, GmbH, Germany) (GE Healthcare, UK).

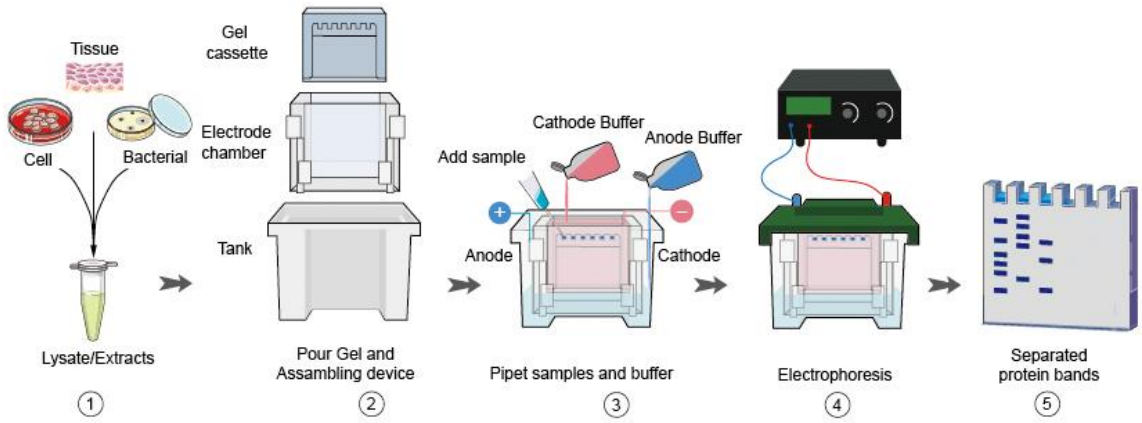

Figure 8: The steps of total protein extraction and western blotting from 1-5 (adapted from https://www.creative-diagnostics.com/Sample-Gel-Preparation.htm).

4. Results and Discussion4.1. Cell growth, its viability and toxicity of solvents

Cell viability was controlled each third or fourth day using the trypan blue viability test using the TC20 Automated Cell Counter. Cultures with live cells close to or above 65% were used for the experiments. To illustrate the rate of growth, reproduction, and death of using cell line, was performed grown curve (Fig. 9). Data were obtained each day at the same time for about 2 weeks. We observed that the lag phase lasted only for 24 hours, while for 7 days the cells rapidly replicated (the exponential growth phase). Within 2-3 days, the number of living cells remained constant (the stationary phase), then the culture had the transition to the decline/death phase. For the experiments, cells from the exponential phase were always used.

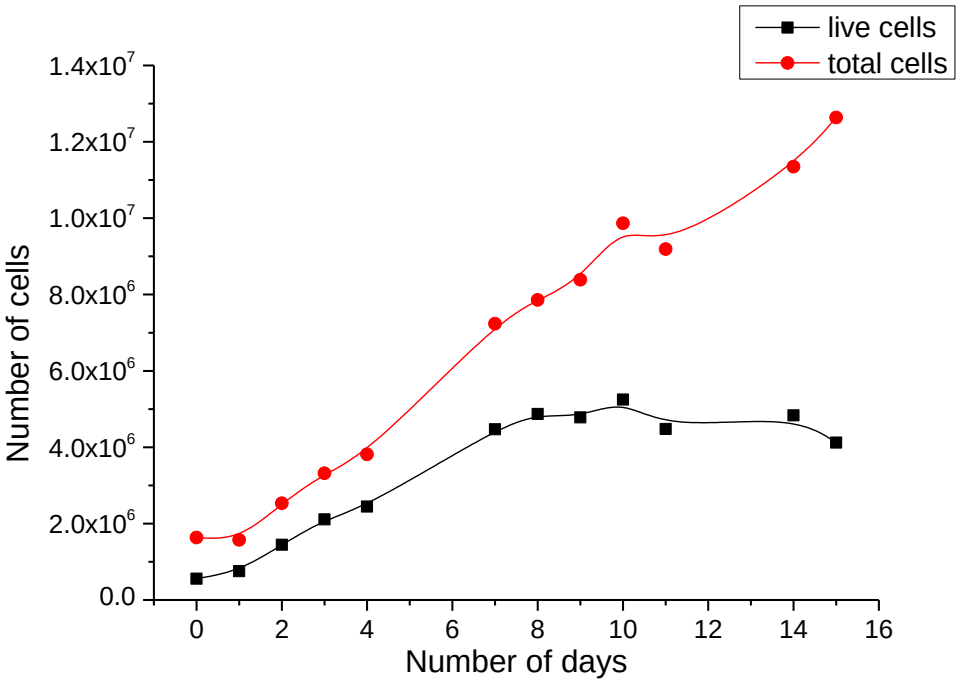

Figure 9: Cell growth curve measured for 2 weeks. The data points were collected each day at the same time.

Before inducing the cell differentiation, we wanted to understand if any occurs additional death solely due to the solvents and/or because of the differentiation inducer (PMA). It was required to check the effect of the chemical on U-937 to optimize the experimental condition to be used. Thus, cells were incubated for 72 hours at 37°C in 5% CO_2 in the presence and absence of PMA with the following concentrations: 150 nM 250 nM, 500 nM, and 1 μM. PMA-treated cells (150 nM) maintained the same viability as non-treated cells, while 250 nM PMA-treated cells showed a decrease of the living cells

about 6% compared to the control and the cells with 500 nM PMA by about 2% (Fig. 10). The higher concentration led to a loss in cell viability of approximately 4%. Therefore, we came to the conclusion that the application of PMA in different concentrations maintained cell vitality equal to or above 61% and comparable to the control, which is why its effect can be considered not significant.

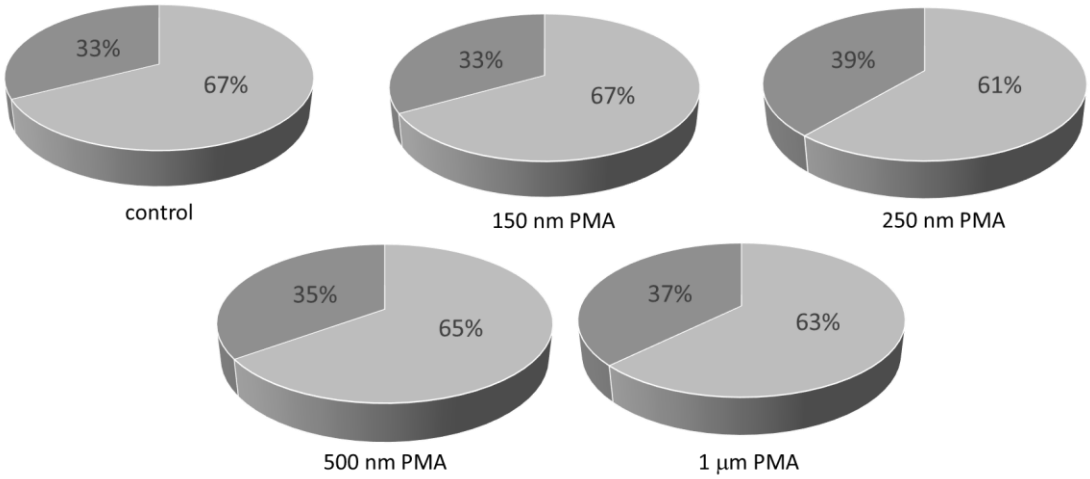

Figure 10: Effect of PMA on the viability of U-937 at concentrations 150 nM to 1μM.

4.2. Cell integrity in U-937 cells using a confocal laser scanning microscope

To validate that cell integrity was not influenced under experimental conditions, we performed visualization of cell membrane and nuclei using double staining with 15 μM FM4-64 and 2 μM Hoechst 33342. The demonstration by CLSM was done after 5 min of application of both dyes to the suspension of 72 hours of non-treated and treated cells (250 nM PMA). FM4-64 is a dye used in fluorescent methods to visualize the morphology and dynamics of cell membranes, while Hoechst 33342 is a cell-permeable DNA and nuclei stain. The study of cell morphology under the experimental conditions was done using confocal microscope using a 405-nm diode laser and Nomarski DIC filters (Fig. 11).

U-937 monocytes treated with 250 nM PMA for 72 hours show the characteristics of mature macrophages, such as irregular shape of a cell membrane, formation of pseudopodia, and modified nuclei shape that confirm cell differentiation (Fig. 11, lower panel). Non-treated cells on the contract shows no marked differentiation as they showed the attributes of monocytes: round shape, short microvilli, and bean-shaped nuclei (Fig. 11, upper panel). Additionally, we did not observe visible cell damage in any of the

samples which indicate that our treatment condition has minimal or no morphological effect on U-937 cells.

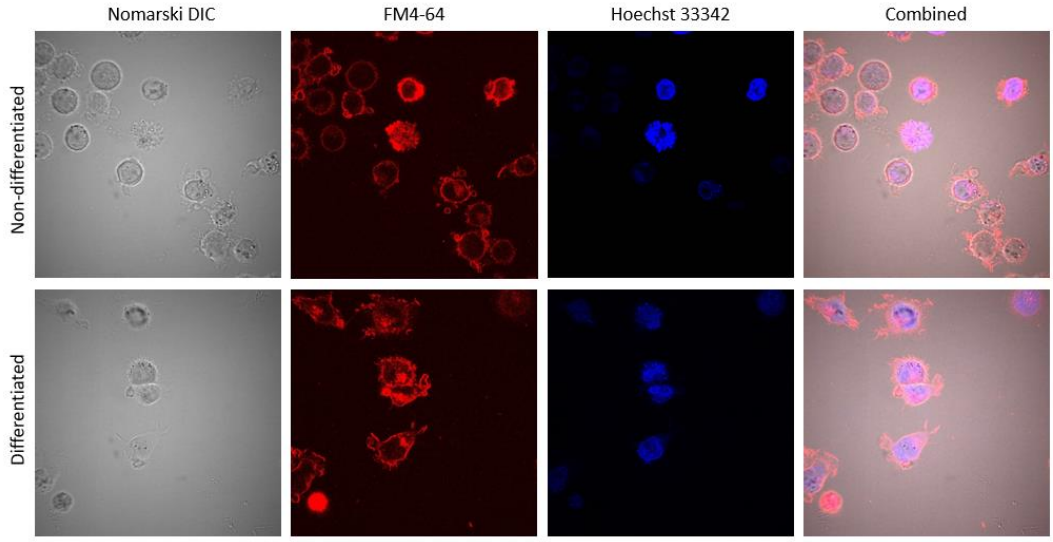

Figure 11: Cell integrity images by confocal microscopy in non-differentiated (upper panel) and differentiated cell (lower panel). Individual channels for DIC, FM4-64 (red signal), Hoechst 33342 (blue signal) and combined are presented as left to right panel, respectively.

4.3. Superoxide anion radical and hydroxyl radical imaging in U-937 cells

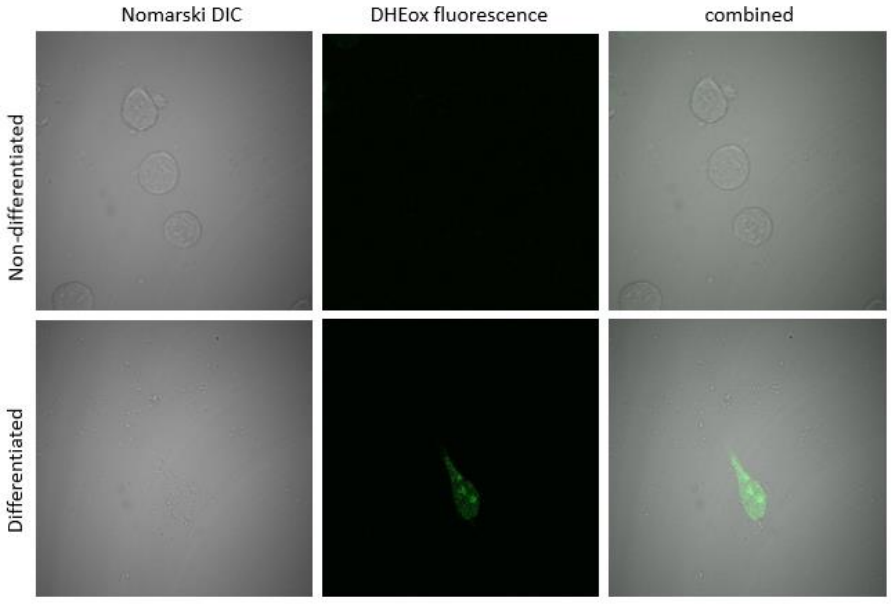

Figure 12: Formation of $O_2^{\bullet -}$ (DHE) in non-differentiated (upper panel) and differentiated cell (lower panel). Individual channels for DIC, DHEox and combined channels are presented from left to right.

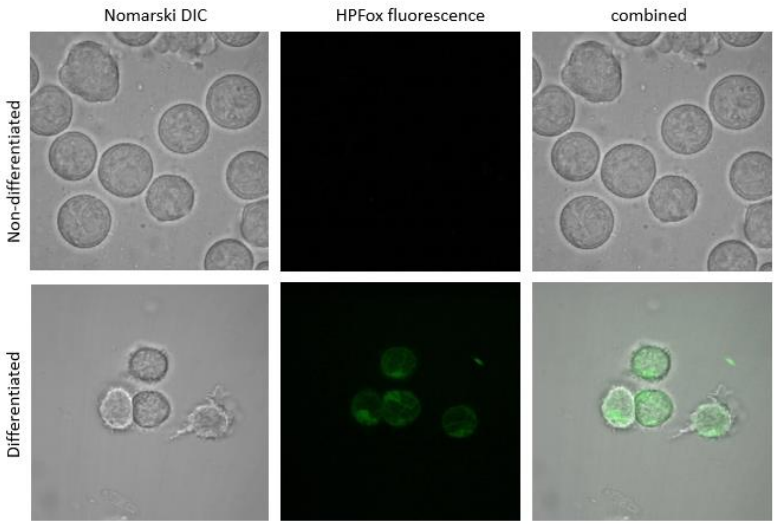

Figure 13: Formation of $HO^{\bullet}$ (HPF) in non-differentiated (upper panel) and differentiated cell (lower panel). Individual channels for DIC, HPFox and combined channels are presented from left to right.

To confirm that $HO^{\bullet}$ is formed from dismutation of $O_2^{\bullet-}$, we performed intracellular $O_2^{\bullet-}$ generation using fluorescent reagent DHE. The reaction of $O_2^{\bullet-}$ and DHE provide a bright fluorescence signal, which was detected using the 655-755 nm filter (green signal shown in the right panel in Fig. 12). Visualization of intracellular $O_2^{\bullet-}$ generation was done under the same experimental conditions as $HO^{\bullet}$ formation imaging using HPF probe except for the incubation time, which in the case of DHE was 4 hours. The morphology of U-937 presented in the left panel in Fig. 12 once again confirms cell differentiation upon 72 h of PMA treatment (250 nM). Differentiated cells compared to non-differentiated showed evident fluorescent signal, which confirms generation of $O_2^{\bullet-}$ during the differentiation. The signal originating from non-differentiated cells can be explained as ROS generation as by-products of normal metabolic processes in human cells. Considering that DHE is a probe that can also react with H_2O_2 and other reactive species, it was necessary to validate our results using another technique.

For visualization of intracellular $HO^{\bullet}$ generation, fluorescent reagent HPF that allows to measure $HO^{\bullet}$ directly in living cells was used. HPFox that was formed in the reaction of $HO^{\bullet}$ and HPF was excited by 488 nm argon laser, while the emission range was within 505-605 nm. For this experiment differentiated (72 h PMA treatment) and non-differentiated U-937 cells, their morphology is presented on the left panel in Fig. 13. An evident fluorescent signal is observed in differentiated cells (right panel, Fig. 13),

while there is only a slight signal from non-treated cells with PMA that comes from a cell undergoing apoptosis or differentiation which is part of normal metabolic processes.

4.4. Hydroxyl radical formation in U-937 cells detected using EPR spin trapping

The validation of intracellular formation of $HO^{\bullet}$ was done using spin trap POBN (50 mM) by EPR spin trapping spectroscopy. POBN-$CH(CH_3)OH$ adduct EPR spectra were measured after 30 min of sonication in the presence/absence of 50 μM PMA. Taking into account the time of sample preparation and subsequent sonication, the final time of cell incubation with 50 μM PMA was 40 min. Rapid release of the ROS, specifically $HO^{\bullet}$, initiated by PMA was confirmed by EPR spectra performed in the presence of PMA (Fig. 14, PMA and Fig. 15), while non-treated cells (Fig.14, control and Fig. 15) showed only a slight signal compared to the experimental sample, which is explained by the usual metabolic processes occurring in the cell.

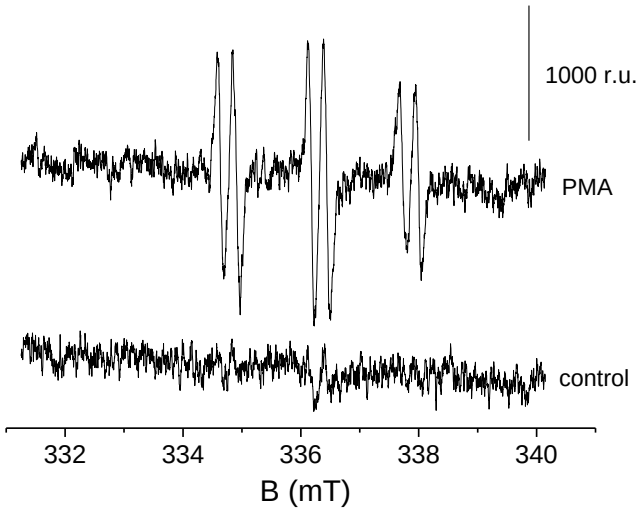

Figure 14: POBN-$CH(CH_3)OH$ adduct EPR spectra measured after 30 min of sonication in the absence (control) and presence (PMA) of 50 μM PMA in dark condition. 50 μM PMA was used to induce respiratory burst.

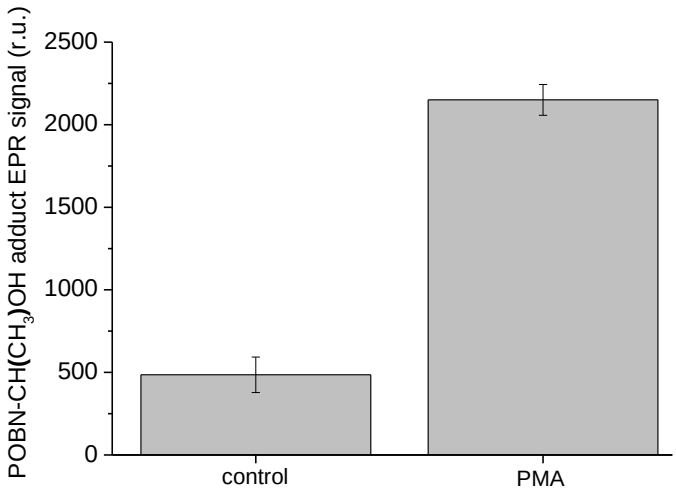

Figure 15: POBN-CH(CH_3)OH adduct EPR spectra measured after 30 min of as described in Fig. 14. The figure shows the relative EPR signal intensity with mean+SD, n=3.

4.5. Characterization of protein radicals in U-937 cells

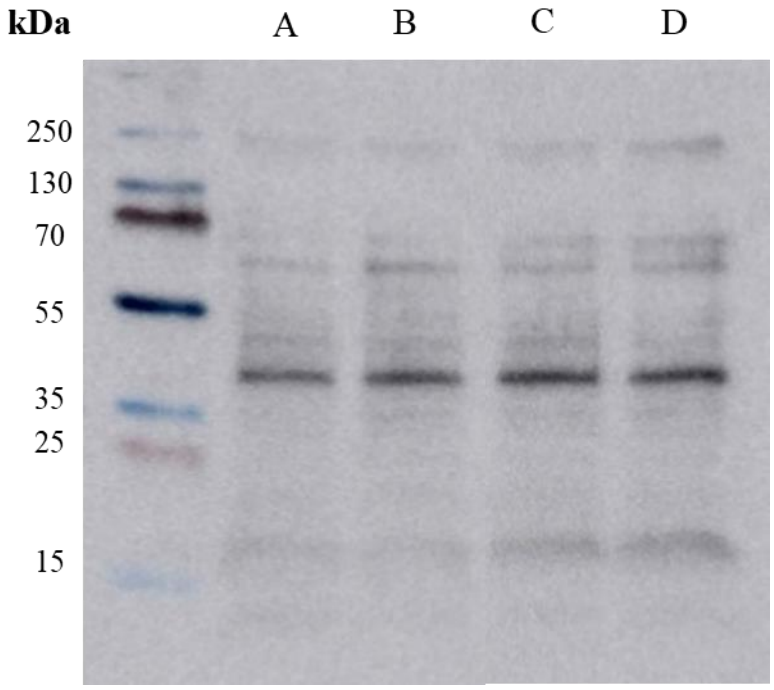

Figure 16: Identification of protein-MDA adducts in whole-cell homogenates of U-937 cells treated with PMA [control (A), 150 nM (B), 500 nM (C) and 1 μM (D)]. Anti-MDA blot represents lipid peroxidation in differentiating cells.

Malondialdehyde is a highly reactive by-product of lipid peroxidation and can easily combine with functional groups and form adducts. In our samples, the MDA protein adducts formed on different proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane. Following this step, it was probed with anti-MDA

antibodies. Western blot analysis of whole cell homogenates with MDA antibody-specific antibody revealed multiple protein bands of which several proteins of approximately of 17 kDa, 40 kDa, 67 kDa and 250 kDa were prominent and were found to be enhanced in differentiated cells (Fig. 16). The results of the anti-MDA immunoblotting study reveal the modification of protein mediated by lipid peroxidation in differentiating macrophages and these proteins can be used as a biomarker for protein modification studies.

5. Conclusion

The current study aims to increase our awareness of ROS formation during the cell differentiation. Previously, ROS have been reported to be generated during macrophages phagocytosis, as they rapidly oxidize biomacromolecules of a foreign body and thereby kill the pathogen. It is also known that ROS are produced during differentiation; however, it has never been precisely characterized, or demonstrated by any of fluorescent techniques at single cell level. The cell line U-937 was used as a demonstration of differentiation, as it easily differentiates from monocytes to macrophages due to the abiotic stress caused by exogenous PMA treatment. Once that compound is applied to the cell suspension, it activates PKC and NADPH oxidase complex, which leads to the formation of ROS. Different concentrations of PMA were used, as well as different incubation times, to investigate the proper conditions for differentiation and subsequent ROS generation. Confocal microscopy was used for demonstration of $O_2^{\bullet -}$ and $HO^{\bullet}$ generation, during cell differentiation, while using EPR spectroscopy we validated the formation of $HO^{\bullet}$ during respiratory burst. Our study showed ROS production and its visualization/ localization at the single cell level. We also attempted to identify the oxidized proteins and found that, in fact, several proteins can be oxidized under differentiation as a result of ROS formation. For further clarification, we must undertake mass spectrometry analysis.

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