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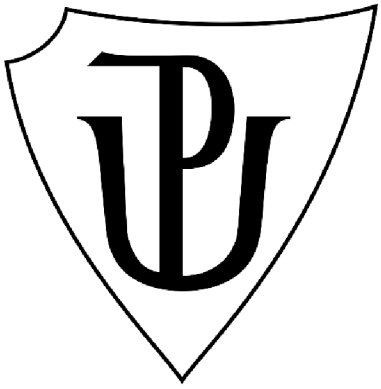

Oxidace proteinů během buněčné diferenciace

BAKALÁŘSKÁ PRÁCE

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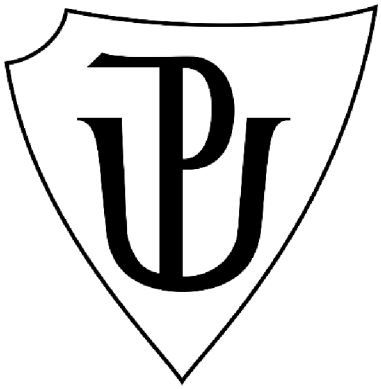

Protein oxidation during cell differentiation

BACHELOR THESIS

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Abstrakt

Současná studie se zabývá premonocytární lidskou buněčnou linií U937, která byla izolovaná z pacientova histiocytického myelomu. V závislosti na prostředí mají monocyty schopnost se diferenciovat na dendritické buňky či makrofágy. Při diferenciaci na makrofágy, která byla v této studii navozena pomocí phorbol 12-myristát-13-acetátu (PMA), dochází ke vzniku reaktivních forem kyslíku.

Během buněčné diferenciace došlo k morfologickým změnám, které byly následně vizualizovány pomocí konfokální laserové skenovací mikroskopie (CLSM). Dále jsem pomocí elektronové paramagnetické rezonance (EPR) byla schopna v buněčné linii detekovat produkci hydroxylového radikálu ($HO^{\bullet}$). Díky western blot analýze bylo zjištěno, že produkce reaktivních forem kyslíku vedla k oxidaci proteinů, které byly vizualizovány pomocí protilátkami proti markeru oxidačního stresu - malondialdehydu (MDA). Hlavním cílem této práce je rozšíření znalostí o tvorbě reaktivních forem kyslíku v lidské leukemické buněčné linii U937.

Klíčové slova	U937 buňky; reaktivní formy kyslíku; buněčná diferenciace; oxidační stres; volné radikály; oxidace proteinů; peroxidace lipidů
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Abstract

The current study focuses on the pro-monocytic human cell line U937, which was isolated from a patient's histiocytic myeloma. Depending on the environment, monocytes can differentiate into dendritic cells or macrophages. The differentiation into macrophages, which in this study was induced by phorbol 12-myristate-13-acetate (PMA), results in the formation of reactive oxygen species.

During cell differentiation, morphological changes occurred, and these were visualized by confocal laser scanning microscopy (CLSM). Furthermore, using electron paramagnetic resonance (EPR), I was able to detect hydroxyl radical ($HO^{\bullet}$) production in the cell line. On the basis of Western blot analysis, it was found that ROS production led to protein oxidation, which was visualized by antibodies against malondialdehyde (MDA), a marker of oxidative stress. The main aim of this work is to extend the knowledge of ROS production in the human leukemia cell line U937.

Keywords	U937 cells; reactive oxygen species; cell differentiation; oxidative stress; free radicals; protein oxidation; lipid peroxidation
No. of pages	45
No. of attachments	1
Language	English

Contents

Aims

1. To learn the procedures for culturing a human cell line and understand the patterns of growth, viability, and mortality.

2. To learn about cell counters, electron paramagnetic resonance spectroscopy, and its control.

3. Develop a brief overview of protein oxidation in human cells.

4. To learn the immunoblotting method.

5. Evaluate the measured results and discuss them in the context of the literature.

1 Introduction

The purpose of this study is to understand the generation of reactive oxygen species (ROS), specifically the hydroxyl radical ($HO^{\bullet}$), along the process of differentiation of monocytes into macrophages and its subsequent detection. Additionally, we have focused on ROS-mediated protein oxidation along this cellular process.

Reactive oxygen species are a natural part of the body's metabolism, serving, for example, in pathogen fighting, when they are produced by phagocytosing cells during respiratory bursts or as signaling molecules (Ray et al., 2012). Their low concentration is maintained in the body either by various forms of antioxidants or by enzymatic means using enzymes found in the body such as superoxide dismutase (SOD) and catalase. In addition, low levels are maintained by nonenzymatic means using carotenoids, vitamin A, vitamin C, etc. However, their excessive production leads to oxidative stress, which results in damage to biomacromolecules (Shields et al., 2021). Since proteins are the basis of all living organisms and their function, whether structural, enzymatic, or protective, ensures the proper functioning of the entire metabolism, damage of these biopolymers often leads to the triggering of various inflammatory and metabolic disorders (Alfadda & Sallam, 2012).

The study utilizes human monocytic cell line U937 as a model system to study cell protein oxidation. U937 exhibiting monocyte morphological features are pro-monocytic cell line isolated from histiocytic lymphoma of a 37-year-old male patient. This human myeloid leukemia cell line is easy to handle, relatively uniform, and serves as an important tool in biomedical research to study the mechanism involved in monocyte-endothelium attachment and monocytes to macrophage differentiation (Verhoeckx et al., 2015). U937 cells can be differentiated in macrophages with aid of various chemical compounds such as phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO), or retinoic acid (Rzigalinski & Rosenthal, 1994; Liu et al., 1996).

In addition, differentiation of monocytes into macrophages is associated with the production of ROS, especially the superoxide anion radical ($O_2^{\bullet -}$), which is generated by the enzyme complex NADPH oxidase (Holland, 2010). More specifically, the $O_2^{\bullet -}$ is converted to hydrogen peroxide (H_2O_2) by the SOD enzyme, and subsequently, the most reactive free radical, the $HO^{\bullet}$, can be generated by the Fenton's reaction. This extremely

reactive molecule reacts with the closest possible structure as soon as it is formed, often giving rise to toxic by-products. One of these is malondialdehyde (MDA), a lipoperoxidation-derived aldehyde that spontaneously attaches to proteins and causes structure-unfriendly modifications (Traverso et al., 2004). MDA serves as a biomarker of oxidative stress.

Thus, the present study utilizes PMA to induce differentiation of U937, and resulting morphological changes, production of ROS, and its subsequent oxidation of proteins were monitored. Confocal laser scanning microscopy was used to verify cell morphology and integrity throughout the differentiation process. We used electron paramagnetic resonance (EPR) spectroscopy to detect $HO^{\bullet}$ generation in the human monocytic cell line U937 through the spin trap method. Additionally, MDA-oxidized proteins were confirmed by western blotting analysis. This study serves as an overview of ROS generation in a human cell line, where we discuss, among other things, the use the of differentiation agent PMA to induce differentiation and its relation to protein oxidation.

2 Current state of the topic

2.1 Free radicals and their formation

Free radicals are molecules containing an unpaired electron in outer orbit. This property makes them very unstable and reactive (Dröge, 2002). The most significant free radicals for humans are undoubtedly oxygen free radicals, which are also referred to as ROS (Fang et al., 2002). Other free radicals include nitrogen-derived reactive nitrogen species (RNS). This study is focused solely on ROS.

Reactive oxygen species are highly reactive molecules derived from molecular oxygen (O_2) formed during various metabolic pathways in living organisms. According to electron configuration, we can classify them into two groups – radical and non-radical ROS. The most well-known ROS are $O_2^{\bullet -}$, $HO^{\bullet}$, and singlet oxygen ($^1O_2$) (Bayr, 2005). Examples of non-radical ROS are H_2O_2, or hypochlorous acid (HOCl) (Fig. 1). All of these molecules are highly reactive and are involved in the oxidation of biomolecules such as proteins, lipids, and nucleic acids. Even though they are involved in degradative pathways, they are part of normal metabolism, where they participate in various types of enzymatic reactions and are part of the electron transport chain in the inner mitochondrial membrane and activate transcription factors/gene expression. (Bayr, 2005).

Broadly, free radical formation in mammalian cells is mediated by two pathways: enzymatic and non-enzymatic. Free radical formation by the enzymatic pathway involves several metabolic processes, such as the mitochondrial electron transport chain, phagocytosis, the reaction cycle of cytochrome P450 monooxygenase (cytochrome P450, CYP, 1.14.14.1) or xanthine oxidoreductase (xanthine oxidase, XOR, EC 1.17. 3.2) and enzymes, which are part of purine metabolism (Colton et al., 1991; Veith & Moorthy, 2018). For example, the most reactive ROS, $HO^{\bullet}$ is formed by the Fenton‘s reaction, which involves the one-electron reduction of H_2O_2 in the presence of transition metals (Bahoran et al., 2007). Non-enzymatically, free radicals are produced when O_2 reacts with an organic compound. Superoxide anion radical can be generated by a semi-ubiquinone compound, which is the redox reactive unit of the electron transport chain (Valko et al., 2007). The formation of ROS and the source involved within mammalian cells is presented in Fig. 2.

The sources of free radicals are divided into endogenous and exogenous. Endogenous free radicals are produced during cancer, infection, aging, or by immune activation of cells. Sources of exogenous oxygen radicals include cigarette smoke, alcohol, drugs, UV radiation, radiation, or heavy- and transition metals. After entering the body, these exogenous compounds are metabolized to form free radicals (Pham-Huy et al., 2008).

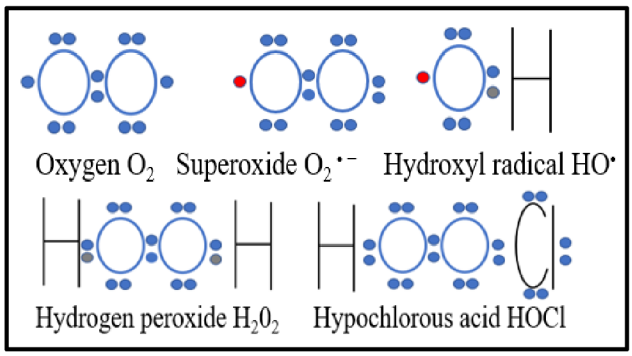

Figure 1. Structural formulas of selected reactive oxygen species

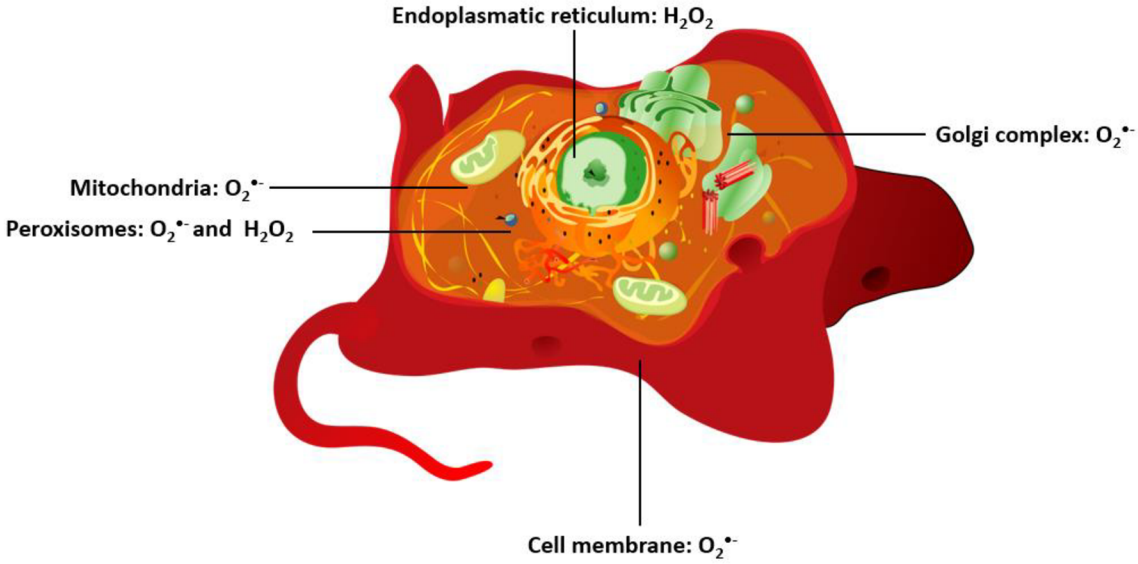

Figure 2. Sites in the mammalian cell responsible for the formation of ROS.

2.2 Oxidative stress and antioxidants

Oxidative stress occurs in cells or tissues when there is an imbalance between the production and accumulation of ROS and the ability of the biological system to reduce these toxic products. Reactive oxygen species such as $O_2^{\bullet-}$ or $HO^{\bullet}$ are present in cells under normal physiological conditions and must be maintained at low concentrations. Being highly reactive, the increased availability of ROS can readily interact and damage macromolecules like proteins, lipids, and DNA or mediate cell death via apoptosis or necrosis. Several studies have confirmed that excess free radicals in the human body generally contribute to the initiation and progression of various types of diseases such as cardiovascular, neurological, autoimmune, and cancer (Pizzino et al., 2017). Since the $HO^{\bullet}$ is the most reactive and therefore the most dangerous radical, it is closely linked to many diseases, such as stroke, where increased $HO^{\bullet}$ production leads to neuronal damage during cerebral ischemia (Zingarelli, 1997) or other diseases mentioned in subchapter 2.3.

However, not only the overproduction of ROS but also its deficiency in the human body can lead to certain types of diseases. Chronic granulomatous disease, manifested by a mutation in the CYBB gene, which is responsible for encoding the NOX2 protein, a component of NAD(P)H: oxygen oxidoreductase (NADPH oxidase, NOX, EC 1.6.3.1) is considered one of the most well known. NADPH oxidase ensures the production of $O_2^{\bullet-}$, which mediates the destruction of phagocytosed microorganisms (Holland, 2010).

To defend against ROS overproduction, cells deploy various mechanisms of enzymatic or non-enzymatic defense mechanisms known as antioxidants. Antioxidants are substances that reduce the activity of free radicals either by converting them to a less active or inactive form or by inhibiting the chain reaction occurring on damaged substrates (Dekkers et al., 1996).

In enzymatic defense mechanisms, superoxide oxidoreductase (superoxide dismutase, SOD, EC 1.15.1.1), which is a naturally occurring enzyme in the body, is capable of converting $O_2^{\bullet-}$ into hydrogen peroxide:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2.$$

Superoxide dismutase is divided into 3 types in eukaryotes, depending on the transition metal ion found in the active site of the enzyme: copper, zinc-dependent SOD,

manganese-dependent SOD, and extracellular SOD (Čolak & Žorić, 2019). Hydrogen-peroxide oxidoreductase (catalase, CAT, EC 1.11.1.6) is an enzyme found in almost all living organisms exposed to oxygen. It catalyzes the reaction that breaks H_2O_2 into water and oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Non-enzymatic free radical scavenging compounds can be water or lipid-soluble and include, for example, carotenoids, vitamin E (tocopherol), vitamin A (retinol), vitamin C (ascorbic acid), and glutathione. Glutathione is a cysteine-containing tripeptide whose thiol group has a reducing function. Its synthesis takes place in erythrocytes, and it protects red blood cells from oxidative damage as ROS are produced during erythrocyte metabolism. Ascorbic acid, also known as vitamin C, is a highly reducing hydrophilic substance. Since the human body cannot synthesize it, it must be taken into the diet. Vitamin C acts as an antioxidant and is involved in several metabolic processes such as proline hydroxylation, immunity support, and iron resorption (Lobo et al., 2010).

However, free radicals (ROS and RNS) can not only hurt the organism, such as aforementioned oxidative stress. They are classified as undesirable only when their amount significantly exceeds the number of antioxidants. At low or medium levels, they are important for the proper functioning of metabolism. For example, they act as a host defense system wherein pathogenic microorganisms are attacked by free radicals produced by phagocytosing cells (Dröge, 2002). One of the main sources of ROS in the human body is NADPH oxidase, an enzyme complex in the cytoplasmic membrane of neutrophils or macrophages that produces $O_2^{\bullet -}$ during respiratory bursts. Patients deficient in NADPH oxidase suffer from a granulomatous disease characterized by recurrent bacterial infections and the formation of inflammatory granulomas (Heyworth et al., 2003). Free radicals also serve as signaling molecules - they are responsible for the induction of the mitogenic response, and nitric oxide (NO), classified as an RNS, serves as a cellular messenger for the regulation of blood flow and central nervous system activity (Pacher et al., 2007). Fig.3 represents cellular damage induced by oxidative stress.

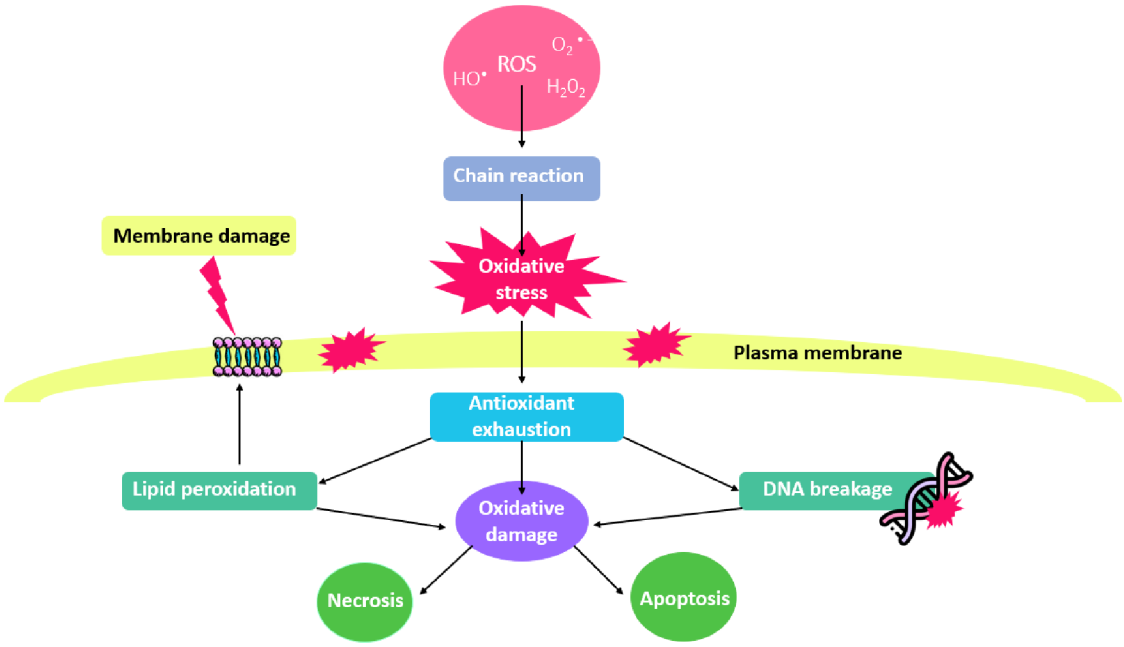

Figure 3. Scheme of oxidative stress formation leading to damage of cellular components. Adopted with modification from the article (Finosh & Jayabalan, 2013).

2.3 Free radicals and human diseases

Excess ROS production leads to oxidative stress, which causes damage to many biomolecules such as proteins, lipids, and nucleic acids. This damage to biomolecules often leads to changes in their function and structure, resulting in the development of various types of diseases (Fig. 4).

Progression of cancer is closely associated with oxidative damage to DNA, where modifications in the structure such as cross-links between DNA and proteins generally occur. For example, tobacco smoke can be considered an exogenous source of oxidative DNA damage. Peroxidation of cell membrane phospholipids produces MDA or acrolein as byproducts. Malondialdehyde can react with the DNA bases dG, dA, and dC to form the adducts M1G, M1A, and M1C, where M1G is considered mutagenic (Valko et al., 2004).

Even the aging process is often associated with free radicals. Reducing their production or increasing the production of antioxidants could significantly delay aging in the future (Ashok & Ali, 1999).

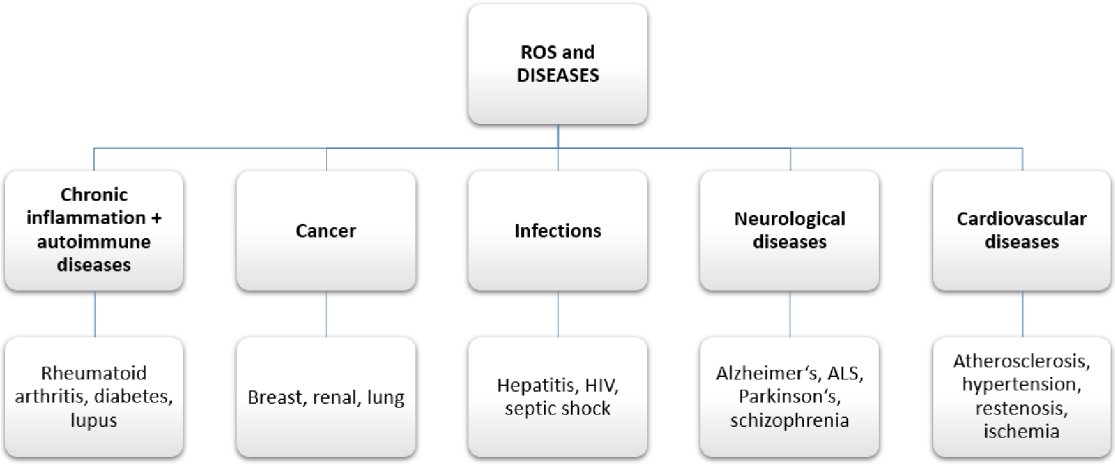

Figure 4. ROS overproduction and related diseases

2.4 Free radicals mediated protein modification

Proteins are high molecular weight natural substances present in all living organisms, where they perform many functions – (a) building (collagen, elastin), (b) transport (hemoglobin), (c) catalytic, control and regulatory (enzymes, hormones, receptors, etc.), (d) protective and defensive (immunoglobins) or (e) functions ensuring movement (actin, myosin). Oxidation of proteins by free radicals is a natural consequence of aerobic life wherein protein function is either impaired or enzyme activity is lost.

There are several ways in which proteins can be oxidized. The most common are (a) oxidation of the protein backbone, (b) formation of protein-protein crosslinks, (c) oxidation of amino acid side chains, and (d) protein fragmentation as presented in Fig.5. Proteins can also be indirectly damaged by either lipid or carbohydrate oxidation.

In this study, we focus mainly on the oxidation of proteins leading to the formation of carbonyls, such as reactive aldehydes and ketones. The formation of peroxyl radicals is caused by the reaction of the radical with the so-called carbon-centered species. However, the oxidation of polyunsaturated fatty acids also results in a chain reaction that leads to the formation of carbonyls. The most well-known, but also the most reactive and toxic to the cell, representatives are α,β-unsaturated aldehydes, dialdehydes (malondialdehyde), and keto-aldehydes (Suzuki et al., 2010). The aforementioned MDA is considered one of the main markers of oxidative stress. This product formed during lipid peroxidation or prostaglandin synthesis interacts with purine nucleotides in DNA and leads to the formation of mutagenic DNA adducts such as M1G (Marnett, 1999). Elevated levels of MDA have been confirmed despite studies in patients suffering from osteoarthritis or keratoconus (Buddi et al., 2002; Tiku et al., 2007).

In order to detect protein carbonyls, 2,4-Dinitrophenylhydrazine (DNPH), an organic compound is also known as Brady's or Borche's reagent, can be used. In this case, Brady's reagent is traditionally prepared by dissolving 3 g of DNPH in 15 mL concentrated sulfuric acid. The mixture is then added to 90 mL of 74% ethanol with stirring (Shriner, 2004). As a positive test result is considered a formation of a yellow, orange or red precipitate - dinitrophenylhydrazone. However, this is not the only imaging method - other spectrophotometric measurements, immunofluorescence imaging, or enzyme-linked immunosorbent assay (ELISA) have also been used (Yan & Forster, 2011).

To be able to detect the presence of protein radicals in various biological systems, Immuno spin trapping techniques can also be employed. For example, spin trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO) traps free radicals modified proteins as nitrone adducts which can be detected by immunoblot techniques using anti-DMPO antibodies. DMPO itself is a nearly nontoxic spin trap with ideal kinetics, used to detect short-lived free radicals by EPR spectroscopy (Anzai et al., 2003). In the course of this reaction, a much more stable compound is formed - the nitroxide radical adduct or the radical adduct. Based on the formation of the nitrone adduct, antibodies can be applied to detect free radicals (Rhodes, 2000). This method is considered very accurate, sensitive, and reliable. It has found regular use in biochemistry, medicine, and toxicology but each technique bears its pros and cons.

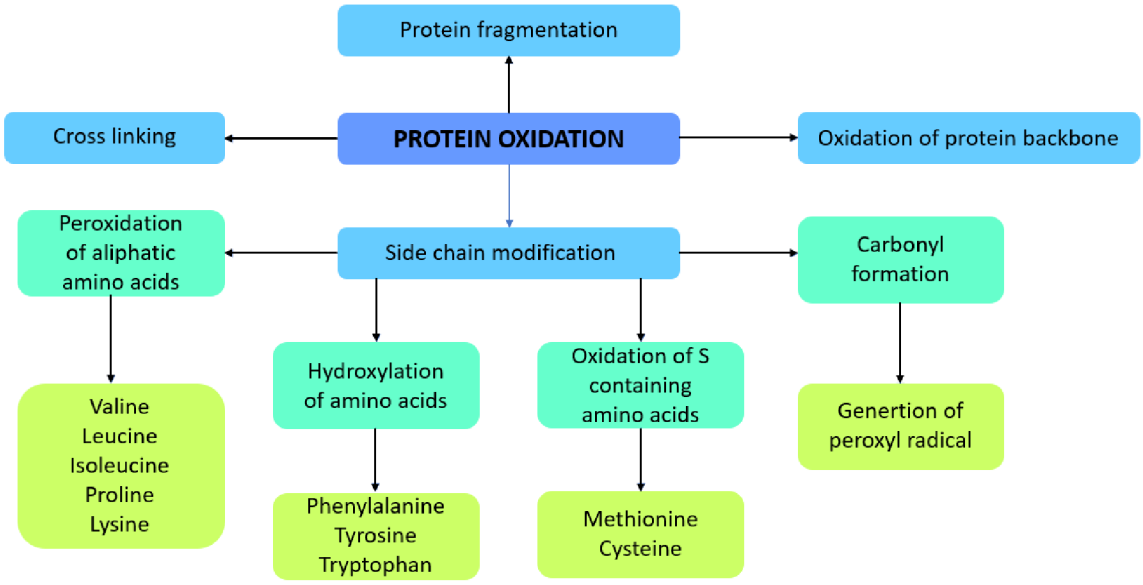

Figure 5. Schematic pathways showing protein oxidation. Adopted with modification from the article (Ahmad et al., 2017).

2.5 Cell differentiation under chemical stimulation

As a general example of macrophage/monocyte chemical stimulation, specifically by H_2O_2, the high mobility group box 1 (HMGB1) box, also known as amphoterin, is released. Amphoterin is a chromatin protein that interacts with transcription factors secreted by immune cells during inflammation (Tang et al., 2007; Hreggvidsdóttir et al., 2012). In a study in which glucose concentration was observed in biologically stimulated and unstimulated U937 cells using the influenza A virus (H1N1), there was a significant reduction in glucose concentration, which can have major health implications for immune cells (Motawi et al., 2020).

When U937 cells are exposed to a certain type of stimulation, such as physiological type stimulus-interferon-γ (IFN-γ) treatment, or exogenous application of the chemical stimulus like phorbol 12-myristate 13-acetate (PMA) cell differentiation occurs (Naldini et al., 1998; Otte et al., 2011). Depending on the action of the initiators, cells can differentiate into macrophages or dendritic cells (Song et al., 2015). Fully differentiated U937 cells have typical characteristics of macrophages, such as irregular flattened shape, and pseudopodia, and are capable of phagocytosis. The differentiation inducer, PMA is analogous to diacylglycerol, which regulates the enzyme ATP: protein phosphotransferase (protein kinase C, PKC, EC 2.7.11.13); therefore, PMA treatment stimulates the cell signaling cascade. Subsequently, gene expression is altered in U937 cells by transcription factors (Fan et al., 2014). For example, one of the transcription factors subject to alteration is nuclear factor-κB (NFκB), which influences the expression of genes important for immunity and the inflammatory response (Dolcet et al., 2005). Furthermore, it is well known that after appropriate stimulation of the U937 cell line with the PMA reagent, the release of $O_2^{\bullet -}$ by cytochrome *b* occurs, with an increase in the content of this cytochrome during differentiation by PMA (Balsinde & Mollinedo, 1988). At the same time, PMA increases the activity of NADPH oxidase and its associated production of $O_2^{\bullet -}$, which through a cascade pathway can lead to the formation of the more toxic free radical $HO^{\bullet}$ (Prasad et al., 2020).

2.6 Methodologies for oxidative stress detection

In order to detect a certain type of free radical/ ROS with precision, it is necessary to utilize more than one method, mainly due to the instability, short lifetime, and high reactivity of these radicals. The main methods used for detection are presented in Fig. 6.

Spectrophotometric methods are among the reliable methods for ROS detection. This analytical method works on the principle of the redox reaction between the redox species and the free radical, where the subsequent difference in absorbance between the substrates and the products at different wavelengths allows the determination of the amount of free radicals. As an example, we can mention the reduction of cytochrome *c* or the use of nitroblue tetrazolium (NBT) for the detection of $O_2^{\bullet-}$ (Zhang et al., 2018).

Another of the proven method is the use of fluorescent probes. The basic characteristics of these probes include high sensitivity to oxidants and the fact that they do not fluoresce before oxidation. In the case of dihydroethidium (DHE) staining, intracellular $O_2^{\bullet-}$ can be detected, which oxidizes this fluorescent probe, and, subsequently, red fluorescence can be observed (Benov et al., 1998). To detect extracellular H_2O_2, the use of N-acetyl-3,7-dihydroxyphenoxazine, known as Amplex red, is considered a reliable and sensitive method. This non-fluorescent molecule is oxidized to resorufin, whereby light at a wavelength of 590 nm is emitted after excitation at 530 nm (Karakuzu et al., 2019).

Electrochemical biosensors contain electrodes that are capable of converting a chemical signal into an electrical signal and can be used for real-time quantification of $O_2^{\bullet-}$ (Ganesana et al., 2012). Redox probes based on fluorescent proteins, such as roGFP1/2, are a combination of prokaryotic redox-sensitive proteins and fluorescent proteins. The recombinant protein is introduced into the cell using plasmids or adenovirus, targets subcellular organelles, and is able to report on the redox states of the area (Dooley et al., 2004). Chromatographic methods, although fast and sensitive, are not widely used because of the complicated reactions. However, they can still be used for the detection of the $HO^{\bullet}$, most often by a combination of liquid chromatography and mass spectrophotometry. Stabilization of free radicals is provided by benzoic acid, salicylic acid, or dimethyl sulfoxide (DMSO) (Głód et al., 2000). Chemiluminescent probes are generally used to detect $O_2^{\bullet-}$, which reacts with the appropriate probe (lucinigen, luminol) to form a photon that is captured in the photometer (Münzel et al., 2002).

In this study, we decided to primarily use EPR spectroscopy and confocal laser scanning microscopy (CLSM) to detect ROS. Generally, we consider CLSM to be a widely used method providing images of the structure of the sample under investigation. With the development of specific fluorescent probes, this method can allow the detection of ROS. The EPR spin-trapping technology is a method used to detect particles with an unpaired electron. However, these are characterized by a very short lifetime, so spin traps are used to trap the radical, extending its lifetime and allowing us to detect it more easily.

Although today's science provides many methods by which ROS can be detected, we decided to use those that can reliably determine both qualitative and quantitative amounts of ROS.

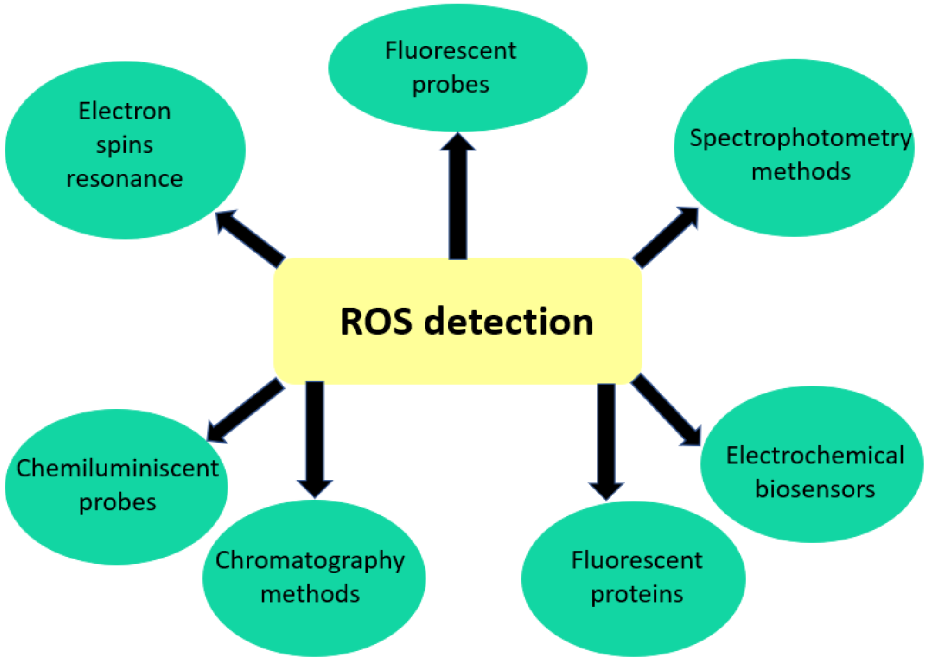

Figure 6. Methods for detection of ROS

3 Materials and Methods

3.1 U937 cell line and growth conditions

The human monocytic U937 cell line used in this study was isolated from a 37-year-old male patient's histiocytic lymphoma (Sundström & Nilsson, 1976). The cell line was grown in RPMI-1640 medium (Roswell Park Memorial Institute 1640 medium), generally employed for suspension cultures of human leukemic cells. This medium is enriched with a glutathione reducing agent and vitamins such as biotin, vitamin B12, or inositol. RPMI-1640 does not contain proteins or lipids, therefore it requires supplementation with fetal bovine serum (FBS). Since the medium is used for the cultivation of a human cell line, it was warmed to 37°C in a water bath before each use. For cell culture, culture flasks (Techno Plastic Products AG, Zollstrasse 7, Switzerland) were utilized and stored in an incubator in a 5 % CO_2 humidified atmosphere at 37°C. This type of environment mimics the physiological conditions of the human body. Passaging of the cells was made every 3 days based on the protocol described by Peters & Wichers (2015). This provides nutrients sufficient enough for cells to achieve proper growth and propagation. All products used for cell line cultivation were obtained from Biosera, Nuaille, France.

Automated Cell Counter TC20 (Bio-Rad Laboratories, Hercules, CA, USA) was used to monitor the growth, viability, and density of cells. This device provides us with a total count and live count of cells; values are given in cells/mL. It provides data based on trypan blue exclusion wherein the dye penetrates the membranes of dead cells and stains them blue while living cells with intact membranes remain uncolored. 10 µl of cell suspension was mixed with 10 µl of trypan blue (w/v) (Sigma-Aldrich, GmbH, Germany), and incubated for 2 mins. Samples thus prepared were loaded onto dual-chamber cell counting slides (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to counting. A brief description of the cell viability measurements is given in Fig.7.

Figure 7. Process of cell viability observation (from left to right): A – T25 culture flask used for U937 cell line cultivation, RPMI-1640 medium enriched with L-glutamine, fetal bovine serum, antibiotics [penicilin/streptomycin]; B – sample preparation for cell counting; C– enumeration of cells using Bio-Rad TC20 automated cell counter.

3.2 Growth medium and chemicals

- The RPMI – 1640 medium used for the cultivation of U937 cells was enriched with 1% antibiotics (penicillin and streptomycin) and 10% fetal bovine serum (Biosera, Nuaille, France).
- Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, GmbH Germany), a light-sensitive chemical compound used as a differentiation inducer, was dissolved in 1% dimethyl sulfoxide (DMSO) (Sigma Aldrich, GmbH Germany).
- For detection of ROS, we used cell-permeable lipophilic spin trap α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone (POBN) for experiments (Iannone et al., 1993) (Sigma Aldrich, GmbH Germany).
- As the primary antibody for blotting, rabbit polyclonal anti- malondialdehyde IgG was used to detect LDL and proteins modified with malondialdehyde (Abcam, UK).
- As a secondary antibody, goat polyclonal anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Laboratories, Hercules, CA, USA) was used.
- Hoechst 33342, a dye was used to stain the nucleic acid of living cells (Thermo Fisher Scientific, Paisley, UK).
- FM4-64, a lipophilic dye for the observation of the morphology and dynamics of vacuolar organelles (Sigma Aldrich GmbH, Germany).
- Bovine serum albumin (BSA) was used as a blocking agent (Sigma Aldrich, GmbH, Germany).
- PageRulerTM Plus Prestained Protein Ladder (10-250 kDa) (Thermo Fisher Scientific, Massachusetts, USA) was used as a standard marker to determine the molecular weight of the protein under study.

3.3 Electron paramagnetic resonance spectroscopy

Electron paramagnetic resonance, also known as electron spin resonance (ESR), is a spectroscopic method used to study chemical species with unpaired electrons. In our study, we focused on the detection of HO• because it is considered the most lethal ROS due to its high reactivity and short in vivo lifetime of about 1 ns. Cells were mixed with a 50 mM POBN spin trap (Sigma Aldrich, GmbH Germany) containing 170 mM ethanol.

To improve membrane permeability for the spin trap to penetrate more effectively into the cells, sonication was performed using a UP50H sonicator (50 Watt, 30 kHz) (Hielscher Ultrasonics, Teltow, Germany) for 15 min with the POBN/ethanol mixture. Under the above experimental conditions, the EPR spectra were recorded using MiniScope MS400 (Magnettech GmbH, Berlin, Germany) using the following parameters: microwave power (10 mW), modulation amplitude (1 G), modulation frequency (100 kHz), sweep width (100 G), scan rate (1.62 G s−1) (Prasad et al., 2020b).

3.3 Immunoblotting

Both control and PMA-treated U937 cells were centrifuged and washed with phosphate-buffered saline (PBS), pH 7.4 to remove the rest of the media. Cells were dissolved in RIPA lysis buffer [150mM NaCl, 50 mM Tris (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40] containing 1% (v/v) protease inhibitor and subjected to sonication. The prepared homogenate was then subsequently centrifuged at 14,000 rpm and the fraction of supernatant collected was quantified using BCA Protein Assay Kit.

Following sample preparation and denaturation, protein samples were separated onto 10% SDS PAGE and transferred onto nitrocellulose membrane using the Trans-blot turbo transfer system (Bio-Rad, California, USA). Subsequently, membrane blocking was performed for two hours at room temperature (RT) using 5% BSA in tris-buffered saline (TBS) (pH 7.4) and 0.1% Tween 20. This was followed by incubation with primary antibody-rabbit polyclonal anti-malondialdehyde antibody (Abcam, UK), dilution of 1:5000, for two hours at RT. Subsequently, the membrane was washed in TBST and then incubated with HRP conjugated anti-rabbit polyclonal secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA) for one hour at RT. Immunocomplexes were imaged using an Amersham imager 600 and the chemiluminescent HRP substrate. A brief description of the immunoblotting procedure is shown in Fig. 8.

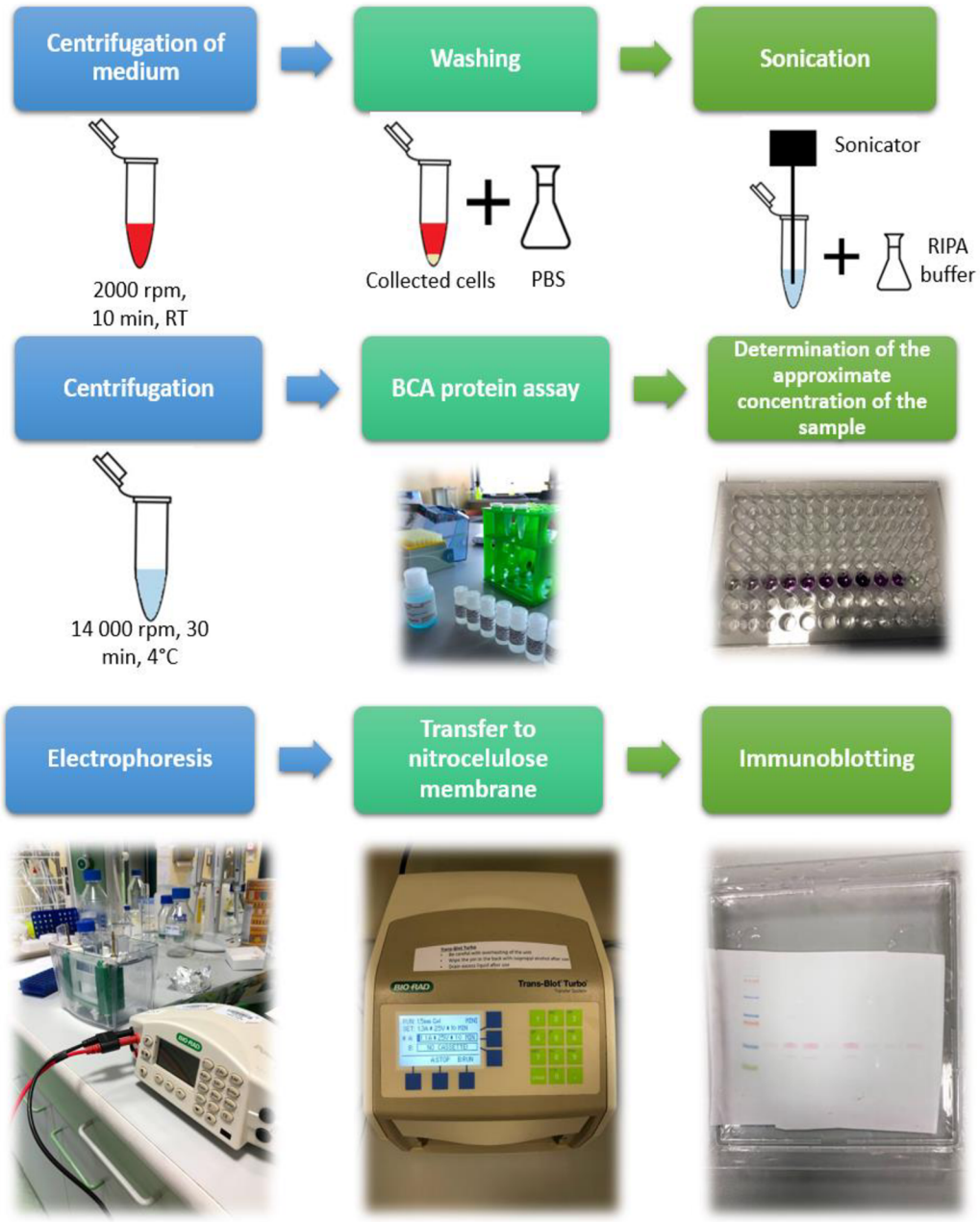

Figure 8. Description of different steps involved in the process of protein sample preparation for electrophoresis and western blot.

3.4 Confocal laser scanning microscopy

The principle of a confocal microscope is a creation of a point source of light and the rejection of out-of-focus light (Fig. 9). Combining these features gives us the ability to display different types of 3D structures. Confocal laser scanning microscopy is widely used in the biological sciences for imaging the spatial structures of cells.

In our study, we used CLSM to observe cell integrity and PMA-induced morphological changes along with differentiation. We also utilized two different fluorescent dyes, FM4-64 and Hoechst 33342, and cells were observed using the Fluorview 1000 unit attached to an IX80 microscope (Olympus Czech Group, Prague, Czech Republic). The styryl dye FM4-64 (15 µM) with red fluorescence is a lipophilic dye that is used to observe the morphology and dynamics of the vacuolar organelle. Excitation was achieved with a 543 nm He-Ne laser and an emission filter of 655-755 nm was used. Hoechst 33342 (2µM) is a DNA stain that is excited by UV light and emits blue fluorescence 460 - 490 nm. In the case of Hoechst 33342, we used a 405-nm diode laser and the signal was recorded with a 430-470 nm bandpass filter. Nomarski/differential interference contrast (DIC) filter was used in both cases to improve the visibility of low contrast cells.

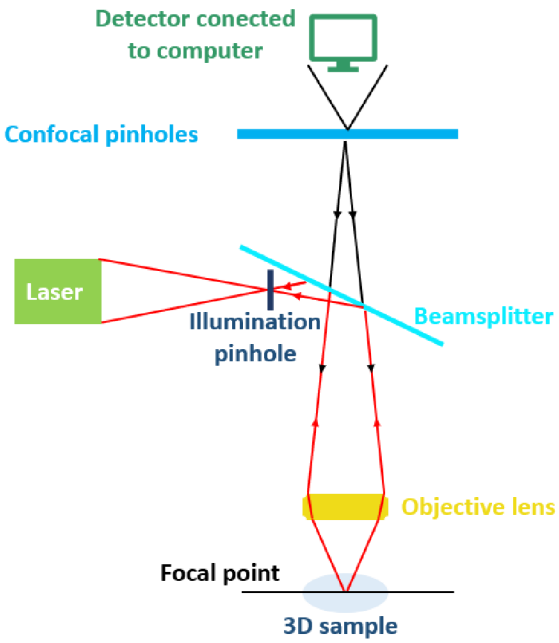

Figure 9. Schematic representation of confocal microscopy

4. Results and discussion

4.1 Growth curve of U937 cells

In order to follow the cell proliferation, cell viability, number, and behavior, the growth curve was plotted (Fig. 10 and Fig. 11). The U937 cell line was monitored for 10 days, where we observed each day whether the cells were developing properly, as any undetected error in their growth could also lead to an error in the experiment. The growth curve has a sigmoidal character: we observed an initial growth phase, an exponential phase, and a stationary phase. Subsequently, there was a phase of cell death. In order for the cell culture to be used in further experiments, its viability had to be at least 65-70 %. Viability was measured with TC20 Automated Cell Counter using trypan blue.

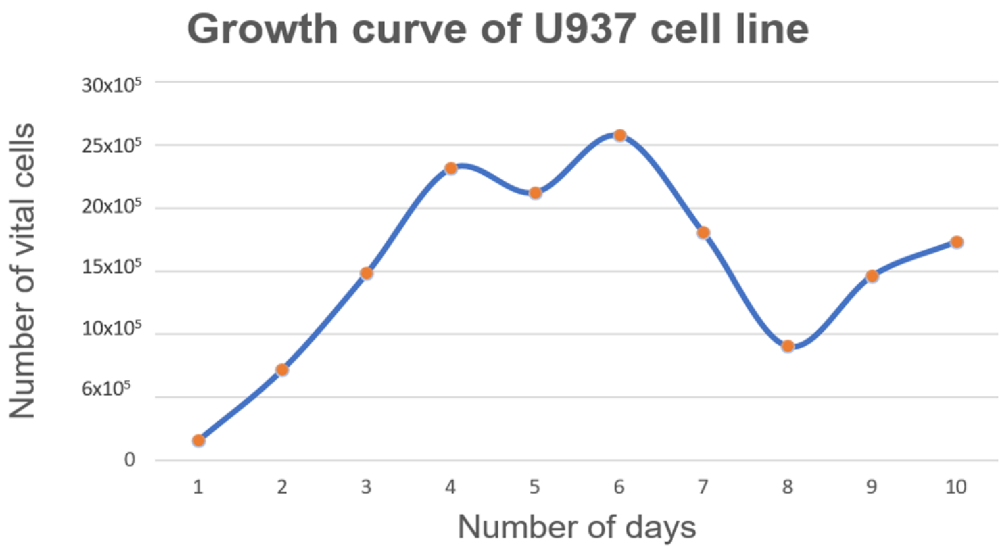

Figure 10. Growth curve of U937 cell line: data measurements were performed for a period of 10 days with the Bio-Rad TC20 Automated Cell Counter using trypan blue in the ratio of 1:1 (cells: trypan blue).

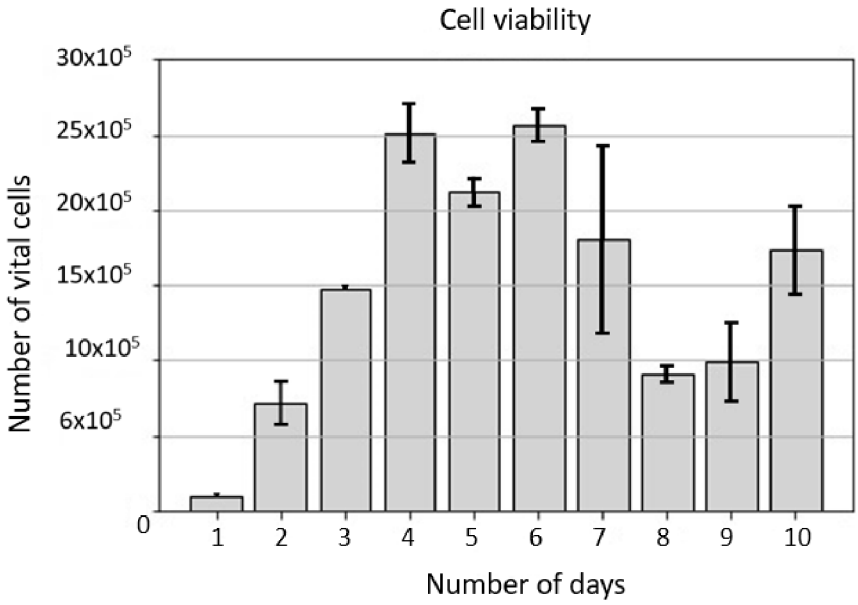

Figure 11. Cell viability of U937 cell line represented by the standard deviation (with n=3). Data was obtained during 10 days of measurements with the Bio-Rad TC20 automated cell counter.

4.2 Effect of solvents on cell line

Due to the use of the aprotic solvent DMSO, which is a toxic organosulfur compound, as the solvent for the PMA differentiation agent, its effect on the U937 cell line was also tested (Fig. 12). Cells were treated with DMSO and PMA for 48 h before measurement. Cell viability was measured by Bio-Rad TC20 Automated Cell Counter using trypan blue in the ratio of 1:1 (cells: trypan blue). The effect of solvents and chemicals on cell culture is investigated primarily in order to avoid various possible measurement errors.

Based on our experiments, we found approximately 14 % dead cells in the negative control (no solvent), which was enhanced by approximately 20 % more in the positive control (sample containing only DMSO). In the case of cells that were incubated with PMA dissolved in DMSO, cell viability was maintained at approximately 70 % (Fig. 12). Therefore, the effect of PMA on U937 cells is significant however at the acceptable level of cell viability.

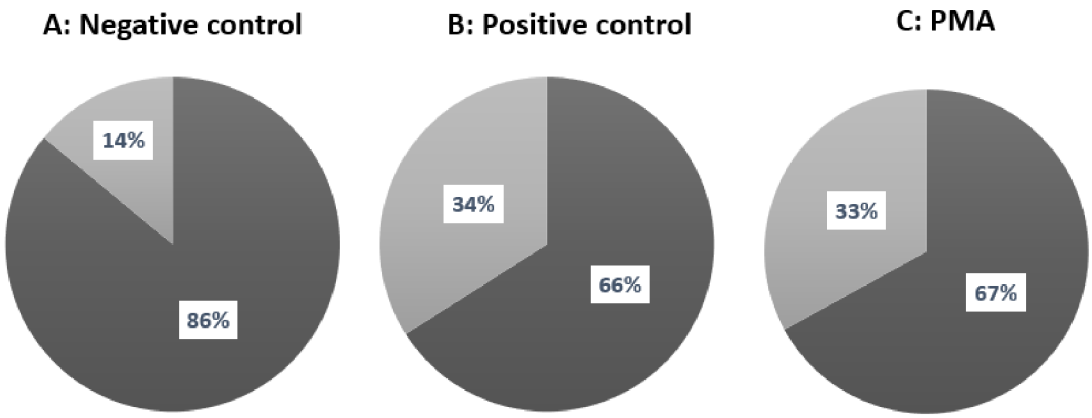

Figure 12. Effect of DMSO and PMA on U937 cell viability. **A:** Control cells; **B:** Cells treated with DMSO; **C:** PMA treated cells.

4.3 U937 cells differentiation using phorbol-12-myristate-13-acetate

The U937 cell line was treated with 150 nM and 250 nM PMA for 48 hours to differentiate monocytes from the promonocytic cell line to macrophage-like cells. Specific morphological changes were observed after 48 hours of incubation with CLSM. As shown in Figure 13, it was evident that the two concentrations used were not lethal to the cell as the integrity of the cell was maintained. We can notice that before the addition of PMA, the pro-monocytic cells were spherical in shape, while after the addition of PMA, we observed oval-shaped cells with visible pseudopodia (Figure 13, middle panel). Cells after 48 hours of incubation also became adhesive and attached to the surface or to each other, which is an important property for the course of many reactions in the immune system, eg, inflammation, healing but also metastasis (Schwarz & Safran, 2013). These morphological changes confirm that they have differentiated into macrophage-like cells.

In previous studies, it has already been shown that exogenous application of PMA results in activation of the NADPH oxidase enzyme complex, whose activity results in $O_2^{\bullet-}$ formation. The decomposition of this free radical results in the formation of H_2O_2 which, when it succumbs to the Fenton‘s reaction, leads to the formation of $HO^{\bullet}$. These radicals can be imaged within a cellular environment with the aid of fluorescent probes like hydroxyphenyl fluorescein (HPF) (Prasad et al., 2020).

Figure 14 describes the formation of $O_2^{\bullet -}$ by NADPH oxidase via activation of protein kinase C. Detailed description of this biochemical pathway can be found in chapter 2.5 of this study.

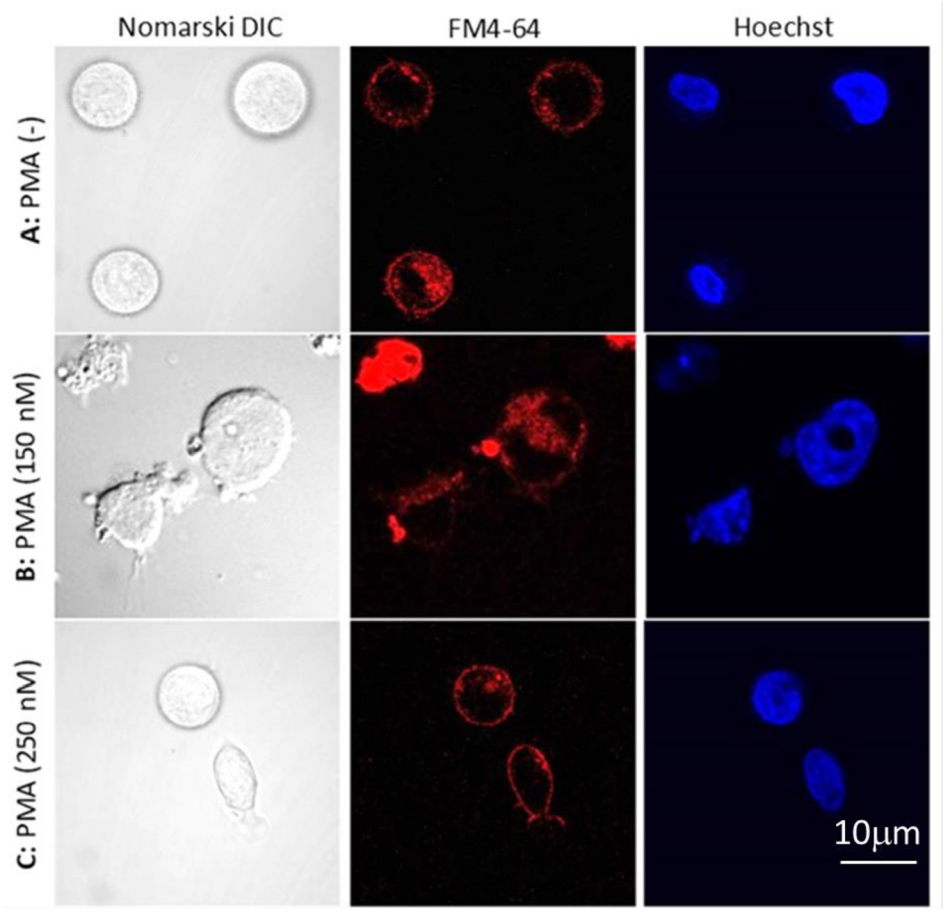

Figure 13. Images of U937 cell morphology were observed by CLSM. **A:** pro-monocytic cells without PMA treatment, **B:** differentiated U937 cells after 48 hours incubation with 150 mM PMA, **C:** differentiated U937 cells after 48 hours incubation with 250 mM PMA. Scale = 10 µm.

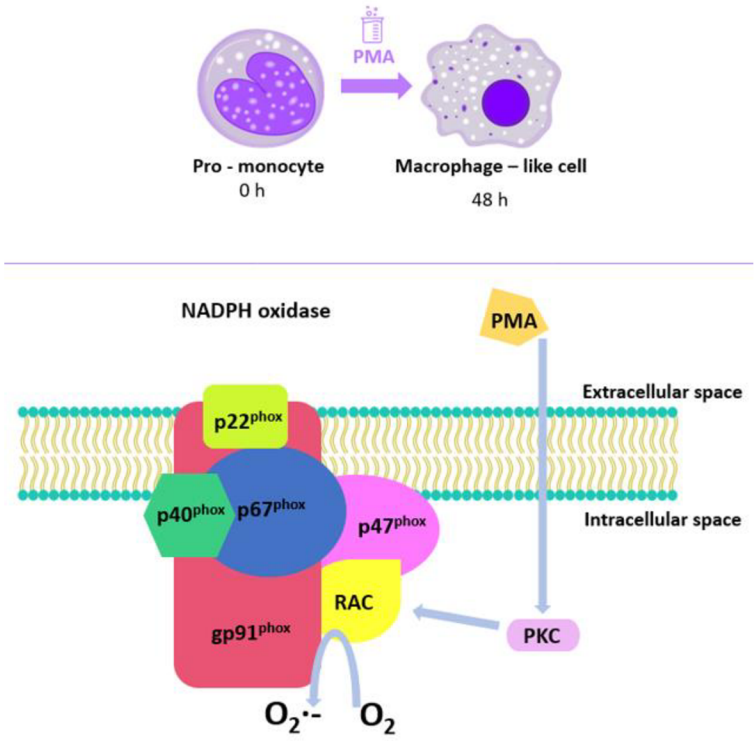

Figure 14. Pathway of $O_2^{\bullet-}$ formation via PMA-activated protein kinase C in the NADPH oxidase enzyme complex. Adopted with modification from (Prasad et al., 2021).

4.4 Hydroxyl radical detection using EPR spectroscopy

In order to detect the generation of $HO^{\bullet}$ in differentiating U937 cells, we used EPR spin-trapping spectroscopy in combination with POBN as a spin-probe. Figure 15A shows control samples where no $HO^{\bullet}$ production occurred and the intensity of the signal is negligible. The application of sonication to this sample did not bring any considerable changes in the EPR signal (Figure 15B). However, when differentiated U937 cells were treated with 250 nM PMA and sonicated, an enhancement was observed in the EPR signal (Fig. 15C). In cases (sonication 15 min), the formation of the α-hydroxyethyl radical adduct of the POBN [POBN-CH(CH_3)OH] adduct was confirmed. This finding verifies that $HO^{\bullet}$ formation occurs in U937 cells in the event of a respiratory burst.

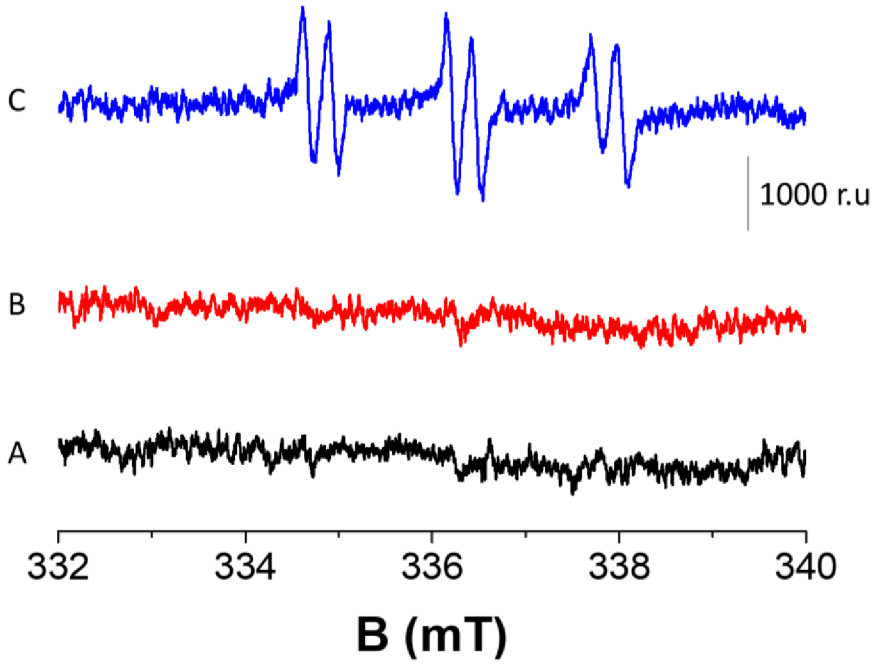

Figure 15. Detection of hydroxyl radical ($HO^{\bullet}$) using EPR spectroscopy in combination with POBN spin trap. **A:** negative control – no sonication, no PMA treatment; **B:** positive control – 15 minutes sonication, no PMA treatment; **C:** 250 mM PMA treated sample, 15 minutes sonication.

4.5 Detection of oxidized proteins by immunoblotting

After protein separation by SDS-PAGE, the immunoblotting method was used to detect malondialdehyde-oxidized proteins with the help of anti-MDA antibodies. It is an aldehyde product of lipid peroxidation that randomly binds to proteins and causes their oxidative damage. Fig. 16 clearly shows that the increased concentration of PMA (250 nM PMA) resulted in the production of more oxidized proteins than in the sample with a lower concentration of the differentiation agent (150 nM PMA). Protein bands are characterized by different molecular weights from the region of approximately 40 kDa to approximately 130 kDa. As the result shows, at least four oxidized proteins in our sample treated with 250 nM PMA were detected.

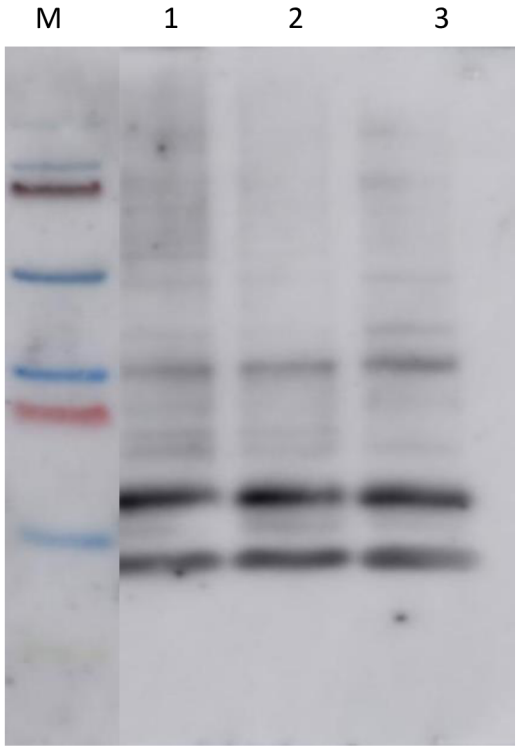

Figure 16. Western blot analysis was used to detect malondialdehyde-oxidized proteins in the U937 cell line. M- Protein marker (Thermo Fisher Scientific, Paisley, UK); Lane 1: control U937 cells; Lane 2: U937 treated with 150 nM PMA; Lane 3: U937 treated with 250 nM PMA

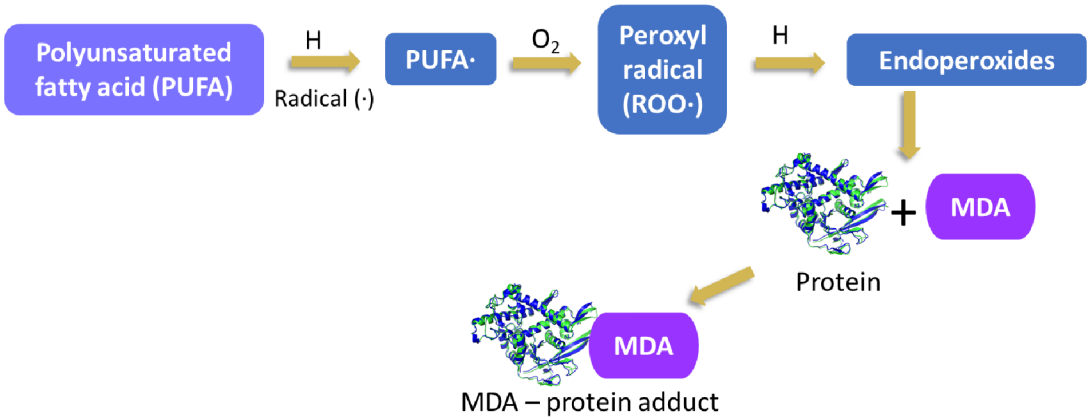

Figure 17. Formation of MDA protein adduct during cell differentiation

The scheme (Fig. 17) describes the formation of the MDA protein adduct in U937 cells during cellular differentiation into macrophage-like cells. In this case, peroxidation of polyunsaturated fatty acids (PUFAs), which are damaged by free radicals, occurs. During initiation, an alkyl radical is formed, and this alkyl radical then reacts with molecular oxygen to form a peroxyl radical upon propagation. The peroxyl radical further interacts with other lipids and a chain reaction occurs. Such peroxidation of PUFAs results in the formation of MDA as a by-product that binds to proteins and causes oxidative damage.

5. Conclusions

The aim of my thesis was to learn the procedures for culturing a cell line to understand the growth and viability, also the damage to cells due to ROS and their subsequent detection. In conclusion, the human cell line U937 serves as a very useful model cell line, either for studying monocyte behaviour and differentiation or for its ease of manipulation. Since the differentiation of the U937 cell line into macrophages has already been shown to be associated with ROS generation, I focused on protein damage in this study. Although the differentiation inducer (PMA) did not show a negative effect on cell structure and integrity according to cytotoxicity assays, CLSM was performed to confirm the preservation of cell membrane integrity using two different concentrations of PMA. Subsequently, EPR was applied to demonstrate the presence of $HO^{\bullet}$, and oxidized proteins were visualized using anti-MDA antibodies by immunoblotting, which showed that more than one protein was oxidized. In our study, the results correlate well with those expected. Understanding and quantifying ROS production in human cells is necessary for evaluating oxidative stress and the current physiological state of either the cell line or the entire organism. However, it is still necessary to develop new innovative methods to facilitate both detection and overall assessment of oxidative stress.

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8. List of Appendices

8.1 List of abbreviations

$^1O_2$	Singlet oxygen
BSA	Bovine serum albumin
CAT	Catalase
CLSM	Confocal laser scanning microscopy
CYP	Cytochrome P450
DHE	Dihydroethidium
DIC	Nomarski/differential interference contrast
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMSO	Dimethyl sulfoxide
DNPH	2,4-Dinitrophenylhydrazine
ELISA	Enzyme-linked immunosorbent assay
EPR	Paramagnetic resonance
ESR	Electron spin resonance
FBS	Fetal Bovine Serum
H_2O_2	Hydrogen peroxide
HMGB1	High mobility group box 1
$HO^{\bullet}$	Hydroxyl radical
HOCl	Hypochlorous acid
HRP	Horseradish peroxidase
HPF	Hydroxyphenyl fluorescein
IFN-γ	Stimulation-interferon-γ

MDA	Malondialdehyde
NBT	Nitroblue tetrazolium
NFκB	Nuclear factor-κB
NO	Nitric oxide
NOX	NADPH oxidase
O_2	Molecular oxygen
$O_2^{\bullet-}$	Superoxide anion radical
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POBN	α-(4-Pyridyl 1-oxide)-N-tert-butylnitrone
PUFAs	Polyunsaturated fatty acids
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640
RT	Room temperature
SOD	Superoxide dismutase
XOR	Xanthine oxidase