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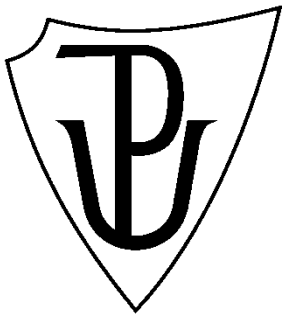

Ultra-weak photon emission from U937 cell culture

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V Olomouci dne…… ……

Abstract

It is a known fact that every living cell produces very weak photon emission, which depends on its biological processes. Due to this fact, ultra-weak photon emission (UPE) is a powerful method to examine cells under in vivo conditions. In this thesis, UPE from human leukemic monocyte lymphoma cell line U937 was detected and combined with other methods as electron paramagnetic resonance (EPR) spin-trapping spectroscopy and high-performance liquid chromatography (HPLC).

Ultra-weak photon emission was used to follow the kinetics of cell oxidative damage induced by addition of hydrogen peroxide (H_2O_2) using highly sensitive photomultiplier tube. To confirm the dependence of ultra-weak photon emission on reactive oxygen species (ROS) various types of antioxidants were used. Data shows significant decrease in UPE in the presence of antioxidants. The addition of ethylendiaminetetraacetic acid (EDTA) into sample containing Fe^{2+} ions enhanced photon emission, whereas deferoxamine mesylate (desferal) suppressed significantly photon emission. These results confirmed that the EDTA-Fe^{2+} complex is an efficient Fenton's reagent.

Evidence that singlet oxygen ($^1O_2$) and hydroxyl radical ($HO^\bullet$) catalyze oxidative damage in U937 cell culture are presented. As confirmed by EPR, the addition of H_2O_2 into RPMI-1640 media resulted in the formation of TEMPO EPR signal, which indicates the formation of $^1O_2$. The addition of reduced transition metals formed $HO^\bullet$ as confirmed by the formation of POBN-$CH(CH_3)OH$ adduct EPR signal. Whereas $^1O_2$ caused a partial cell injury in propidium iodide labelled U937 cell culture, $HO^\bullet$ progressively damaged the cell membranes as confirmed by fluorescence microscopy. Detection of malondialdehyde by isocratic reverse-phase HPLC reveals that $^1O_2$ and $HO^\bullet$ caused lipid peroxidation, the content of lipid peroxides induced by $HO^\bullet$ was double compared to $^1O_2$.

These results reveal that $HO^\bullet$ is the main ROS responsible for oxidative damage and cell injury. The data showed in this thesis represent the first step

towards the development of a new non-invasive and inexpensive technique for the detection of oxidative processes in the human leukemic cells under certain physiological and pathophysiological conditions.

List of Abbreviations

UPE	ultra-weak photon emission
EPR	electron paramagnetic resonance
HPLC	high-performance liquid chromatography
PMT	photomultiplier tube
ROS	reactive oxygen species
$^1O_2$	singlet oxygen
$HO^{\bullet}$	hydroxyl radical
H_2O_2	hydrogen peroxide
CCD	charge-coupled devices
TEMP	2,2,6,6-tetramethylpiperidine
POBN	α-(4-pyridyl-1-oxide)-N-tert-butyl nitrone
MDA	malondialdehyde
DNPH	2,4-dinitrophenylhydrazine
PBS	phosphate buffer saline
EDTA	ethylenediaminetetraacetic acid
DESFERAL	deferoxamine mesylate
PI	Propidium iodide

Content

1. Theoretical part

1.1 Introduction

In 1924, mitotic radiation was described by Alexander Gurwitsch (Gurwitsch 1924). He found out that many organisms emit Gurwitsch mitotic radiation. The name mitotic radiation represented very weak ultraviolet radiation, which was unfortunately impossible to measure by any detector at that time. After quite a big boom in 1920's and 1930's, Gurwitsch theory was forgotten after WW II till mid 1970s, when Popp rediscovered it and changed the name from mitotic radiation to biophoton emission (Bischof 2008), which is a synonym to ultra-weak photon emission (UPE) used by the most of authors. The definition of UPE has changed since Gurwitsch times from UV radiation into radiation in the range from 200 to 800 nm with a magnitude from 1 to 1000 photons per second per square centimetres. Some scientists assume that the radiation originates from an almost perfectly coherent photon field (Popp 2005), but this theory was not fully proved yet and that is why it is not accepted by the whole community.

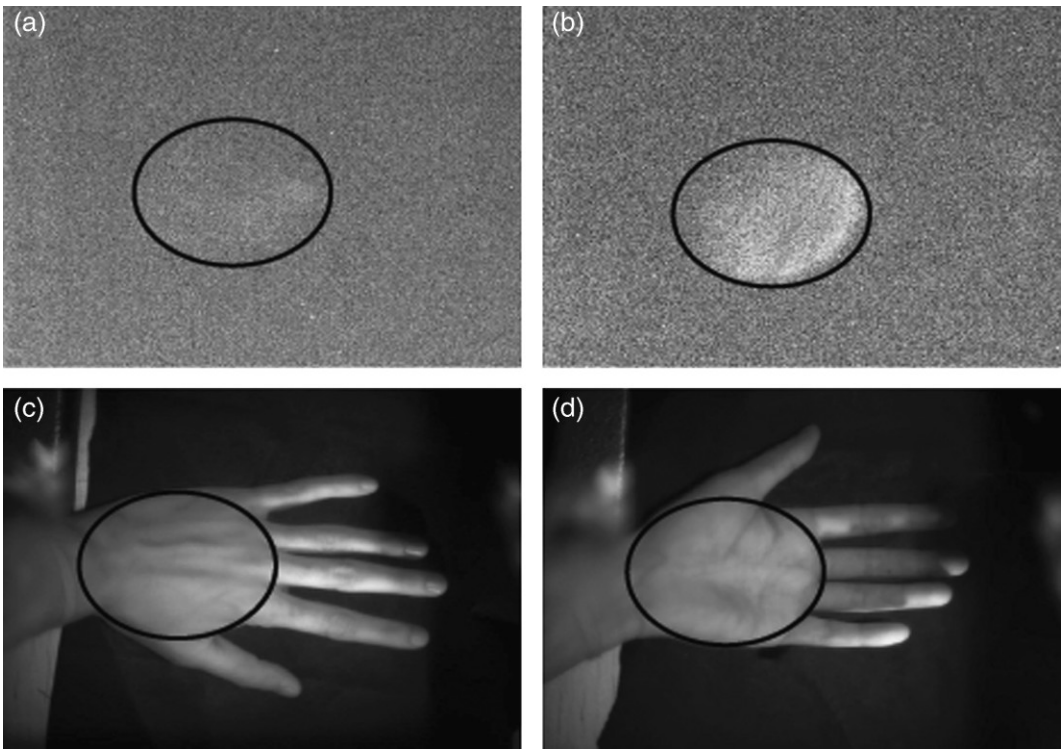
Thanks to development of photomultipliers (PMT) and charge-coupled devices (CCD) it is possible to detect UPE from human skin (Cohen and Popp 1997; van Wijk and van Wijk 2005; Cifra et al. 2007; Rastogi and Pospíšil 2010), plants (Mathew and Roy 1992, Bajpai 2004), animal organs (Kim et al. 2005), brain (Kobayashi et al. 1999), or even cell cultures (Kim et al. 2007) in these days. According to many scientists, these experiments can stand at the beginning of new analytic methods.

One of the basic questions, the origin of UPE is not answered yet. It is known fact that disturbance of system stability increases UPE, but the mechanisms have not been described completely yet. Experiments show that there is not only one source of UPE. It is proved that generation of reactive oxygen species (ROS) (Gurvich 1968, Slawinski 1988, Voeikov 2006, Rastogi and Pospíšil 2010) but also photonic traps created from chiral molecules such as DNA, microfilaments and microtubules (Popp and Li 1992) are inducing UPE.

There is still a lot of work to be done before the origin of UPE will be fully understood.

1.2 Human Skin

All living cells are known to emit UPE, so it can be used for examine skin of living organisms without harming them, which is one of the most important fact of all methods used for human research in vivo. However, before serious diagnostic methods can be developed, fundamental thinks like spatial and temporal variety of UPE from human body under normal, healthy conditions (Cifra et al. 2007).



Img. 1: Two-dimensional image of peroxide-induced ultra-weak photon emission from a human hand measured after topical application of H_2O_2 to the dorsal (a) and the palm (b) side of the hand and corresponding photographs of the dorsal (c) and the palm (d) side of the hand. (a, b) The hand was kept in complete darkness for 20 min before measurements. After dark adaptation, 500 mM H_2O_2 was applied to the fixed area of the right hand and ultra-weak photon emission images were collected for 20 min using a CCD camera. (c, d) Photographs of the hand were taken under weak light using a CCD camera. (taken from Rastogi and Pospíšil 2010)

The most examinated part of human body is hand, because it is the most achievable place at human body. Examining UPE during 24 hours showed that fluctuations are more at the dorsal sides than the palm sides of hand, change in

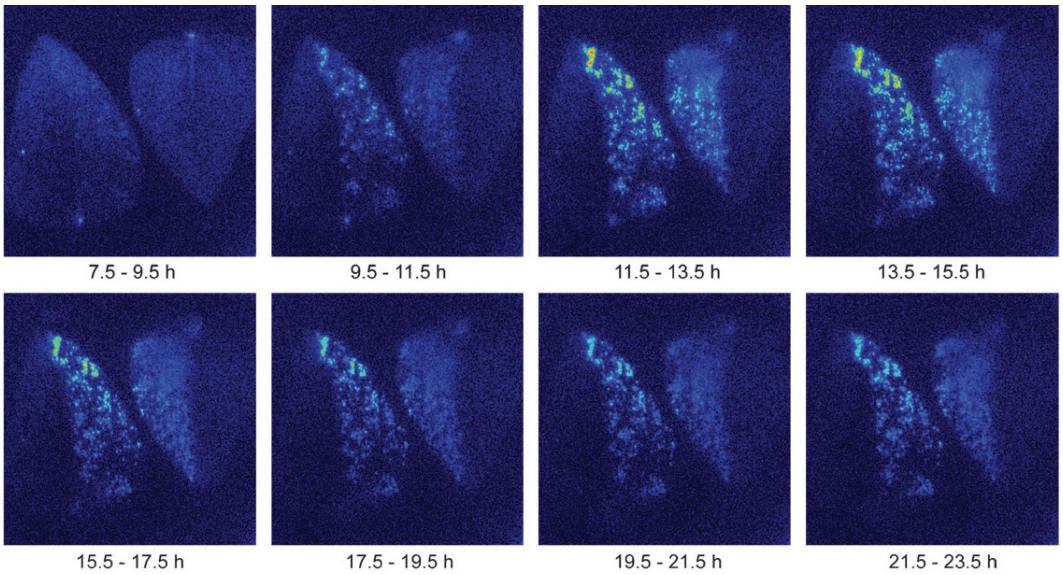
left-right symmetry occurs for the dorsal side but not for the palm side of the hand. Ultra-weak photon emission at the left dorsal location is high at night, while the right dorsal side emits mostly during the day. It was concluded that intensity as well as left-right symmetry vary diurnally, suggesting similar fluctuations in endogenous pro- and anti-oxidative capacities (van Wijk and van Wijk 2007; Cifra et al. 2007). Intensity of UPE under normal physiological conditions may vary to 70% around daily average (Cifra et al. 2008). Despite these differences there was observed no marked dependence on age or tender (Choi et al. 2002). Some experiments implying that muscular activity can increase UPE (Laager et al. 2008) as well as different stressors encompassing exposition to ozone, UV irradiation, or cigarette smoke induce UPE from skin. Critical parameters affecting the quality and quantity of the UPE are also the spectral composition of the exciting UV light, skin temperature, skin humidity, and the O_2 concentration of the surrounding atmosphere (Hagens et al. 2008).

Preliminary measurements of the UPE and skin temperature from 12 locations on the body show that although correlation of temperature and the emission intensity is high on the hands, but it is usually much lower or unstable on the other parts of the body (Cifra et al. 2008).

There already has been some work done on spatial mapping of UPE intensity and it was found that there exists a typical anatomic pattern of UPE (van Wijk and van Wijk 2005; van Wijk et al. 2006). It is concluded that both patterns and physical properties of UPE hold considerable promise as measure for the oxidative status (Van Wijk et al. 2008).

1.3. Plants

Big part of UPE research utilizes plants thanks to their pigments, which can serve as emitters of UPE. When a plant is placed in darkness, delay luminescence from chlorophyll can be observed for some time. After this luminescence decays, there is UPE present. Published studies indicate that biologic processes increasing oxidative metabolism producing singlet oxygen and other ROS correlate with measured UPE (Hideg 1993). It has been demonstrated that adding the respiratory chain decoupler carbonyl cyanide 3-chlorophenylhydrazone to yeast leads to decrease of UPE (Laager and Soh 2008). During evolution, plants developed a defence system to protect themselves against attack from pathogens such as bacteria, fungi, nematodes and insects. An oxidative burst, increased production of ROS, is a part of this system (Makino et al. 2005), thanks to which detection of UPE has emerged as a non-destructive method to mark the onset of the hypersensitive resistance reaction in Arabidopsis, bean and tomato plants (Mansfield et al. 2005).



Img. 2: Ultra-weak photon emission imaging of the hypersensitive response observed on cowpea leaves after inoculation with cucumber mosaic virus and its comparison among different strains of virus, CMV-Y, CMV-L, and buffer treatment. Time sequence of biophoton emission images of two leaves after inoculation. Images are constructed with time integration over the time range indicated below each image. (Taken from Kobayashi et al. 2007)

In addition, various studies have analysed plant physiological responses to various stresses, including both biotic (Roschger 1991; Makino et al. 1996; Kobayashi et al. 2003; Bennett et al. 2005; Mansfield 2005) and abiotic stress (Kobayashi et al. 1996, 1997; Ohya et al. 2000; Havaux 2003).

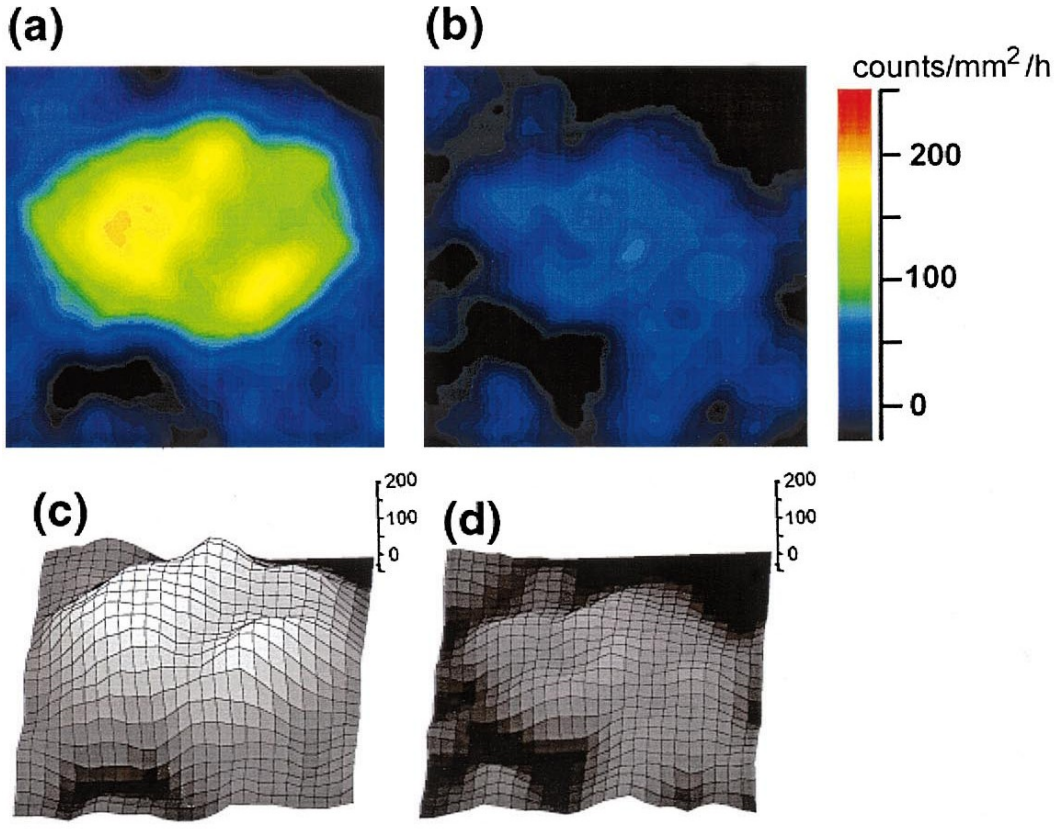
Since cooled CCD cameras are used to detect UPE in two dimensions, these experiments became more popular, because the changes are not only measured but they also can be visualized.

Some practical experiments have been already presented on the field of plant research. The photon-counting tests were used for seed germination and Daphnia under chemical stress. They provide a fast method to evaluate fertilization and lethal conditions for seedlings and point to further explorations of photon-counting applications in toxicology (Siqueira et al. 2007).

The ultra-weak photon emission from a paddy weed (*Scirpus juncoides*) was examinated to assess the availability of UPE for the identification of weed biotypes resistant to sulfonylurea herbicides. The intensity of UPE from the plants increased when treated with a sulfonylurea herbicide in a concentration-dependent manner. The increasement of UPE was higher in the sulfonylurea-resistant biotypes than in the sulfonylurea-susceptible biotypes. The difference between the biotypes was greater in the culms than in the roots and remained so through the vegetative growth stage to the flowering stage. This difference was independent on the seed source or mutations in the acetolactate synthase genes of the resistant biotypes (Inagaki et al. 2008).

1.4. Organs

It has been proposed that oxygen dependent UPE in rat liver nuclei resulted from lipid peroxidation in the nuclear membrane (Devaraj et al. 1991). As a hepatotoxicant, carbon tetrachloride (CCl_4) has been frequently used in models for free radical damage in animal liver. This hepatotoxicant is known to be reductively bio-activated by cytochrome P450 into a trichloromethyl radical ($^{\bullet}CCl_3$), which was subsequently converted into a peroxyl radical ($^{\bullet}OOCCl_3$) in the presence of molecular oxygen. The free radical initiates lipid peroxidation by abstracting a hydrogen atom from the polyunsatured fatty acid of phospholipids (Recknagel 1967). Data shows that UPE is involved in the process of liver tissue repair as well as hepatocellular death after acute exposure to carbon tetrachloride (Kim et al. 2005).



Img. 3: Ultra-weak photon emission image of a normal rat's brain observed outside the skull (a), compared with an image during brain ischemia (b). Integration time is 1 h in both cases. Image size is 25_25 mm. (c) Three-dimensional display of (a). (d) Three-dimensional display of (b). (Taken from Kobajashi et al. 1999)

Development of cooled high sensitive CCD cameras allows scientists to detect UPE in 2D, which can provide more informations that PMT measurement in many cases from basic experiments with human skin to measurement from rat's brain in vivo. It was found that the UPE intensity correlates with the electroencephalographic activity measured on the cortical surface and this intensity is associated with the cerebral blood flow and hyperoxia. It has been proposed that UPE originates from the energy metabolism of the inner mitochondrial respiratory chain through the production of ROS. Imagining of UPE from a brain constitutes a novel method, with the potential to extract pathophysiological information associated with neural metabolism and oxidative dysfunction of the neural cells (Kobayashi et al. 1990).

1.5. Cells

Synchrony between mechanically separated biological systems is well known. Scientific experiments related to the principle of nonchemical non-electrical communication between biological systems can be traced back to 1920 and the work of Gurwitsch. He showed that chemically isolated onion root cells show increases in the number of mitoses when they are near other actively dividing cells. This effect was abolished after using a UV-opaque filter between these cells. Unfortunately, that work, and other similar studies, was not published in English and, therefore, they did not provoke a lot of attention in the western world. Since then, several additional reports have provided evidence of distant, non-chemical, non-electrical cell-to-cell communication. In a series of experiments, cell morphology was observed by staining cells and showed that cytopathic effects induced by radiation, chemical agents, or viral agents could be induced in other, chemically isolated cells. It was also demonstrated that the rate of cellular proliferation in a group of cells would be enhanced if the cells were non-chemically exposed to another group of proliferating cells (Trushin 2004). Farhadi observed the same non-chemical, non-electrical cell-to-cell communication. He put two samples in separate containers. One of them was exposed to hydrogen peroxide (H_2O_2) while the other was not. Changes after 10, 30 and 60 min after exposure were measured and similar changes in the total protein content and structural damage were observed (Farhadi et al. 2007).

Two-dimensional imaging of brain was already described in previous chapter but it is not the only contribution of UPE to studies of the nervous system. The ultra-weak photon emission was detected from cultured rat cerebellar granule neurons. It was discovered that UPE intensity is highly dependent on the concentration of K^+ and Ca^{2+}. This observation indicates dependence on the neuronal activity and likely on the cellular metabolism (Kataoka et al. 2001).

It has already been mentioned for a few times that intensity of UPE and its statistical properties are closely connected to the biochemical processes accuring in a living system, as well as its physiological state (van Wijk and Aken 1992).

The main question to be answered is if tumour tissues can be characterized by a different intensity of UPE compared to the neighbouring tissue. Some experiments suggest, that it will be possible, but so far it is confirmed only that cancerous tissues emit more photons than normal tissues and that there is small variation between emissions from different types of tissues (Kim et al. 2005).

Measurement on cultured HeLa cells proves that there is a close relationship between the UPE intensity and the change rate of HeLa cell population and with rate of viable cells. This data may be useful for analysis of growth activity such as viability and proliferation of cancer cells (Kim et al. 2007).

1.6. Reactive oxygen species and lipid peroxidation

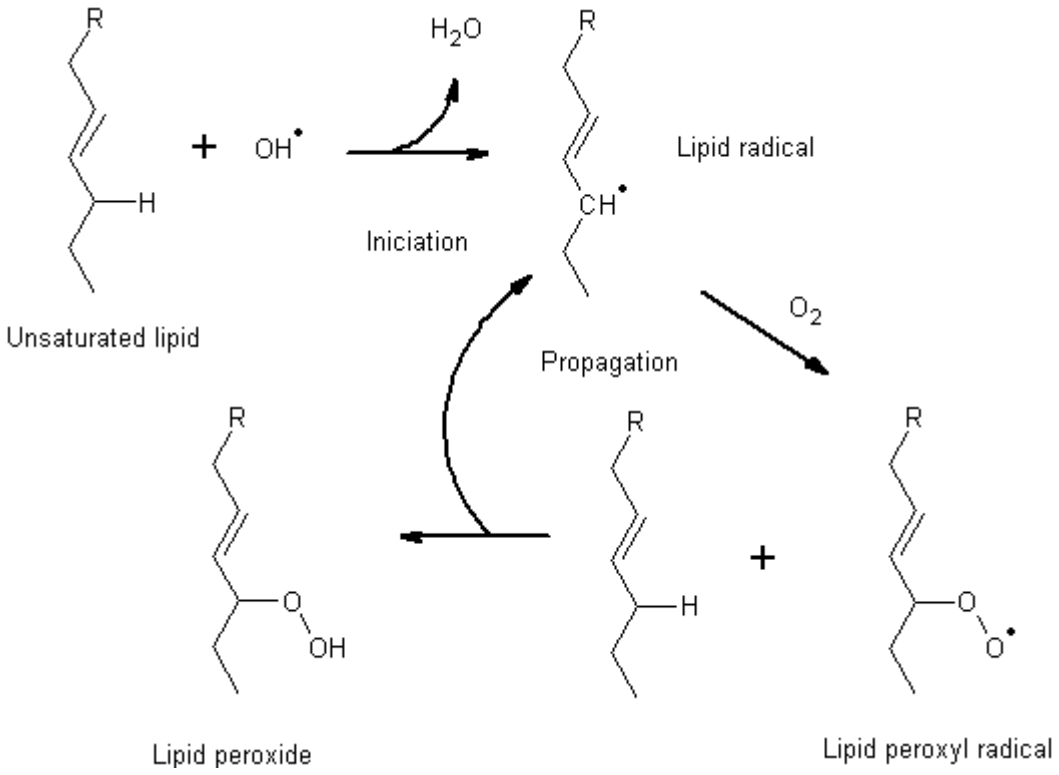
Reactive oxygen species are molecules or ions formed by the incomplete one-electron reduction of molecular oxygen. These reactive oxygen intermediates include singlet oxygen ($^1O_2$), superoxide anion radical, H_2O_2, hydroxyl radical ($HO^{\bullet}$) and hypochlorous acid. Reactive oxygen species are natural by-product of the normal metabolism of molecular oxygen and have important roles in cell signalling. In normal conditions, they are mostly formed in mitochondria, but they can also be produced in white blood cells as a defensive mechanism against pathogens, in cells exposed to environmental stress conditions as UV radiation, ionizing radiation or higher temperature. The increased production of ROS is knows as oxidative stress.

While under normal conditions cells are capable of dealing with ROS using enzymes such as lactoperoxidases, catalyses, superoxide dismutase, glutathione peroxidases, small molecule antioxidants such as ascorbic acid, α-tocopherol, uric acid, during oxidative stress ROS become a serious problem.

One of the best known toxic effects is damage to cellular membranes, which is initiated by a lipid peroxidation. Reactive oxygen species are also responsible for damage of DNA molecules. While nuclear DNA can be repaired, mitochondrial DNA cannot be fixed. Therefore, extensive mitochondrial DNA damage accumulates over time and shuts down mitochondria, causing cells to die and the organism to age (Ozawa 1999).

Lipid peroxidation is a well-established mechanism of cellular injury in all kinds of organisms and it is often used as an indicator of oxidative stress in cells and tissues. The process of lipid peroxidation proceeds by a free radical chain reaction mechanism. It most often affects polyunsatured fatty acids, because they contain multiple double bounds in between which lie methylene –CH2- groups that possess especially reactive hydrogenes. The reaction consists of three steps: initiation, propagation and termination. In the first step, initiation, ROS combine with a hydrogen atom to make water and a fatty radical. The fatty acid

radical is not a very stable molecule, so reaction moves to the next step, propagation, in which radical reacts with molecular oxygen, while peroxyl-fatty acid radical is created. This unstable species reacts with another free fatty acid producing a different fatty acid radical and a lipid peroxide or cyclic peroxide if it had reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way. That is why it is called chain reaction. As far as radical and non-radical react, it always produces another radical. Therefore, the last step, termination, can start only when the concentration of radical species is high enough for radical reactions.



Img. 4: Mechanism of lipid peroxidation

1.7. Fenton's reaction

This is a reaction of ferrous ions and hydrogen peroxide. Reaction products are ferric ion, hydroxyl radical and hydroxy anion. The hydroxyl radical is a very strong oxidizing agent known to attack many organic substrates.

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + HO^{\bullet} + H_2O$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^+$$

The hydroxyl radicals are able to degrade the target contaminants by oxidation. The resulting Fe^{3+} ions may further react with H_2O_2 and a hydroxyl radical, which leads to the regeneration of Fe^{2+} (Prousek and Priesolova 2002; Vyskočilová 2008).

2. Material and methods

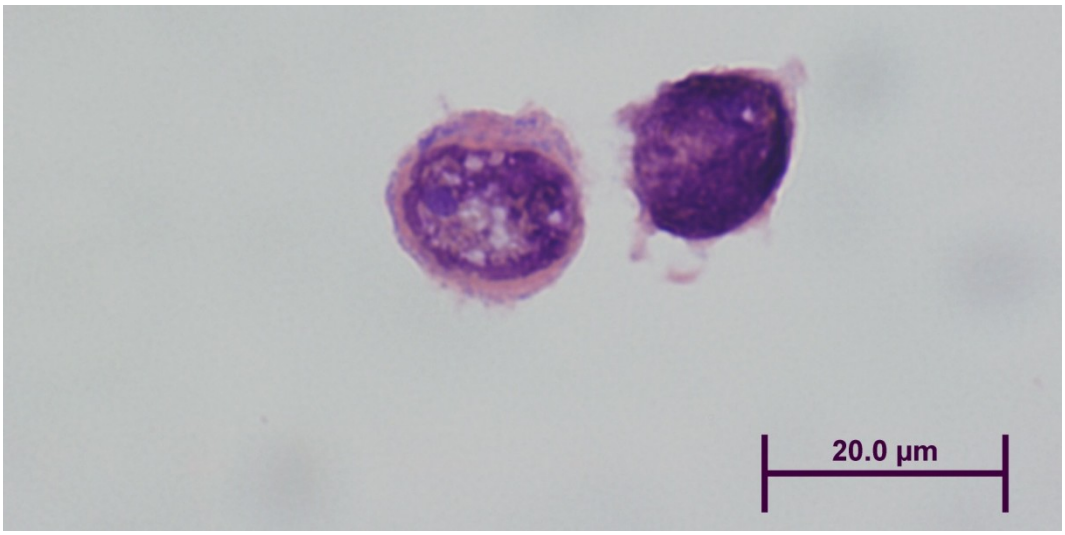
2.1. Cells

2.1.1. Healthy human white blood cells

Healthy white blood cells were isolated from 29 years old briefed male volunteer and they were processed immediately. Hypodermic needle was rinsed out with heparin to prevent the blood from coagulation. The amount of 20 ml of blood was taken by syrette. After the blood was taken, it was placed into cuvette in which the same amount of histopaque was already pored. Cuvette was centrifuged at 850 x g for 10 min at 20 °C. After centrifugation, consistent gradient was created and white cells were extracted by Pasteur pipet. Cells were rinsed for two times in RPMI-1640 medium.

2.1.2. U937 cell culture

Human leukemic monocyte lymphoma cell line U937 was used. The culture was obtained from histocytic lymphoma. A histocityte is a part of the mononuclear phagocytic system known to participate in the immune responses. U937 cell culture were first isolated from a 37-year-old male patient (James and Williams 1999). Cell culture was cultivated in RPMI-1640 medium in incubator at 37 °C in 5% CO_2 atmosphere.



Img. 5: U937 cell culture.

2.2. Medium

RPMI-1640 medium without L-glutamine and sodium bicarbonate was used for cell cultivation, purchased from Sigma-Aldrich (R7755).

Components	g/L
Calcium Nitrate•4 H2O	0.1
Magnesium Sulfate (anhydrous)	0.04884
Potassium Chloride	0.4
Sodium Chloride	6.0
Sodium Phosphate Dibasic (anhydrous)	0.8
Succinic Acid•6H2O•2Na	0.1
Succinic Acid (free acid)	0.075
L-Arginine	0.2
L-Asparagine (anhydrous)	0.05
L-Aspartic Acid	0.02
L-Cystine•2HCl	0.0652
L-Glutamic Acid	0.02
Glycine	0.01
L-Histidine	0.015
Trans-4-Hydroxy-L-proline	0.02
L-Isoleucine	0.05
L-Leucine	0.05
L-Lysine•HCl	0.04
L-Methionine	0.015
L-Phenylalanine	0.015
L-Proline	0.02
L-Serine	0.03
L-Threonine	0.02
L-Tryptophan	0.005
L-Tyrosine	0.02
L-Valine	0.02
D-Biotin	0.0002
Choline Bitartrate	0.00544
Folic Acid	0.001
myo-Inositol	0.035
Niacinamide	0.001
p-Aminobenzoic Acid	0.001
D-Pantothenic Acid (hemicalcium)	0.00025
Pyridoxine•HCl	0.001
Riboflavin	0.0002
Thiamine•HCl	0.001
Vitamin B-12	0.000005
D-Glucose	2.0
Glutathione (reduced)	0.001
Phenol Red•Na	0.00318

2.3. PMT measurement

Hamamatsu photomultiplier tube R7518P (Hamamatsu Photonics K.K., Iwata City, Japan) was used. Spectral response was from 185 to 730 nm with low dark counts 10 cps at normal temperature. Photon counting unit C9744 (Hamamatsu Photonics K.K., Iwata City, Japan) was used for counting. To reduce low dark count, PMT was cooled to -30 °C using thermoelectric cooler C9143. A data equation unit and PC were placed in the dark room. Smaller room was inside this dark room (3x2x2m), painted with black colour, in which PMT and sample were suited. All measurements were done at room temperature.

Before each measurement, samples have been adapted for dark for 10 min. After that, a sample in a plastic Petri dish was placed just under the PMT window. All injections during the measurement were done in the complete dark. The volume of the sample was 5 ml and all concentrations were recounted for this amount.

2.4. EPR spin-trapping spectroscopy

The spin-trapping was accomplished by TEMP (2,2,6,6-tetramethylpiperidine) (Sigma-Aldrich) and POBN(α-(4-pyridyl-1-oxide)-N-tert-butyl nitrone). The samples were prepared for the final volume of 25 µl. Ethanol in the amount of 2.5 µl or 1.25 µl was put into sample with POBN or TEMP, respectively. POBN water solution was used in the final concentration of 20 mM while TEMP was used in ethanol solution in the final concentration of 10 mM. Priori to data collection, the capillary tube was taken away from 4 °C and electron paramagnetic resonance (EPR) spin-trapping data were collected at room temperature. EPR spectra were recorded using EPR spectrometer MiniScope MS200 (Magnettech GmbH, Berlin, Germany). EPR conditions were as follows: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}.

2.5. HPLC analysis

The sample preparation and derivation of malondialdehyde (MDA) with 2,4-dinitrophenylhydrazine (DNPH) was performed as described by Pilz et al. (2000) with some modifications. The amounts of 5 ml of samples were incubated for 1 hour in the dark and at the room temperature. After incubation, samples were centrifuged for 20 min at 11000 x g and supernatant was removed. Pellet was stirred up in 200 µm of phosphate buffer saline (PBS). After this, cells were disrupted by sonification for 90 seconds. The sample with disrupted cells was centrifuged at 3500 x g for 15 min. The amount of 125 µm of supernatant was taken into the eppendorf vial and 25 µl of 6 M aqueous sodium hydroxide (NaOH) was added. This mixture was incubated in a 60 °C water bath for 40 min to achieve alkaline hydrolysis of protein bound MDA. Than, protein was precipitated adding 62.5 µl of 35% (v/v) perchloric acid ($HClO_4$). The sample was vortexed and centrifuged at 2800 x g for 10 min. A volume of 125 µl of supernatant was put into the eppendorf vial and mixed with 12.5 µl DNPH prepared as a 5 mM solution in 2 M hydrochloric acid. This mixture was incubated in dark for 30 min at room temperature. An aliquot of 50 µl of this mixture was injected into the high-performance liquid chromatography (HPLC) system.

The samples were analyzed on an HPLC system (Shimadzu LC-20A Prominence, Kyoto, Japan) with UV detection at 310nm. Elution was performed isocraticaly with a mixture of 0.2% (v/v) acetic acid in deionised water and acetonitrile (62:38, v/v) at a flow rate of 1 ml/min at 25 °C.

2.6. Fluorescence microscopy

Cells were mixed with chemicals and they were incubated for 1 hour in the dark. After incubation, samples were centrifuged at 850 x g for 10 min at 20 °C. Pellet was rinsed 2 times with PBS and stained with propidium iodide. Images were taken by Olympus BX51TF (Olympus corporation, Tokio, Japan).

3. Results and discussion

3.1. Spontaneous ultra-weak photon emission from human white blood cells and U937 cell culture

Spontaneous UPE was detected from human white blood cells and U937 cell culture using highly sensitive PMT. When Petri dish with liquid culture medium RPMI-1640 was placed upon the PMT window, the count rate of 1.5 counts s^{-1} was observed (Fig. 1.1).

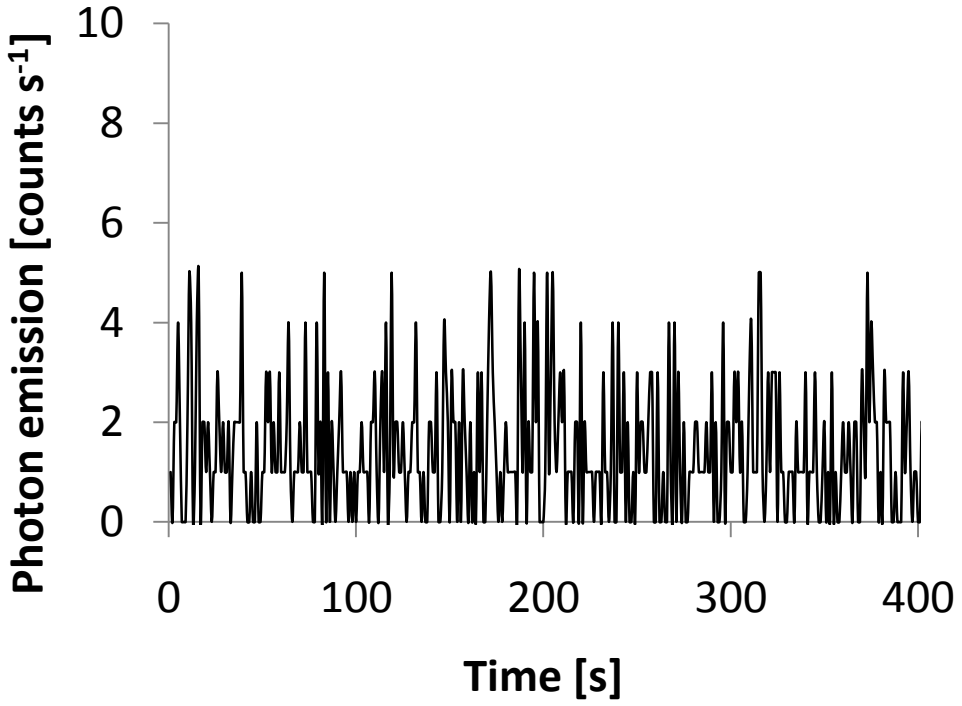


Fig. 1.1: Ultra-weak photon emission from RPMI-1640 medium. Medium in plastic Petri dish was placed under the PMT window. Measurement was taken at room temperature.

After subtraction of the dark count of 1 counts s^{-1}, photon emission from liquid culture medium was determined to be 0.5 counts s^{-1}. The photon emission

from liquid culture medium likely arises from autooxidation of the medium components.

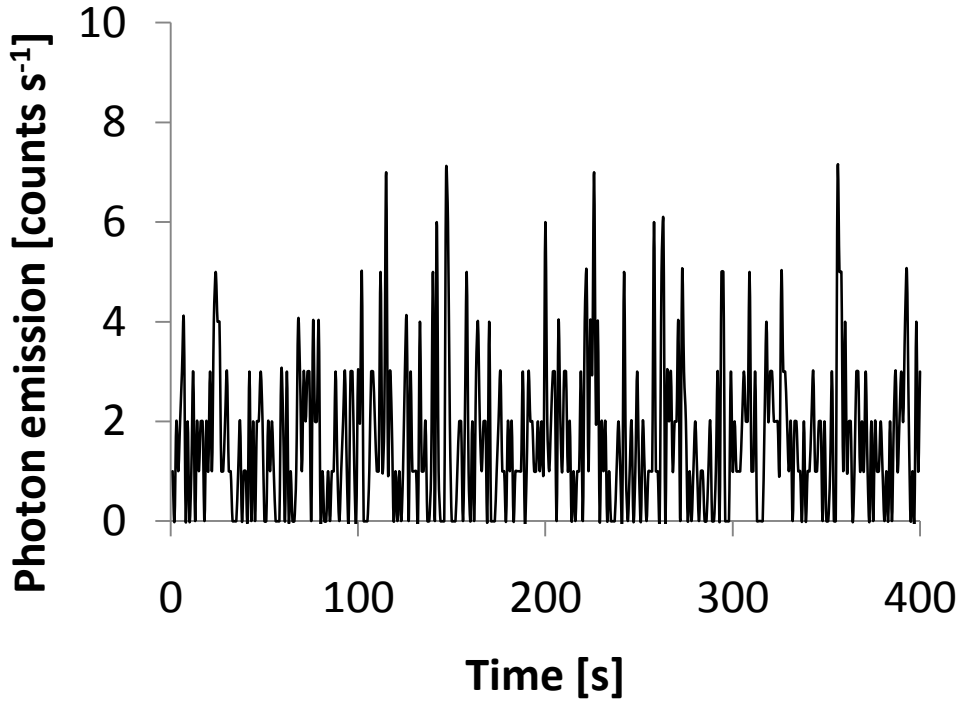


Fig. 1.2: Ultra-weak photon emission from healthy human white blood cells. Cells were placed into plastic Petri dish and placed under the PMT window. Measurement was taken at room temperature.

Spontaneous UPE from healthy human white blood cells was detected (Fig. 1.2). After subtraction of the photon emission from liquid medium, photon emission of healthy human white blood cells was determined to be 0.2 counts s^{-1}.

Interestingly, when U937 cell culture placed on the plastic Petri dish were put under the PMT window, photon emission of 4 counts s^{-1} was observed (Fig. 1.3). After subtraction of the photon emission from liquid culture medium, a photon emission form U937 cell was determined to be 2.5 counts s^{-1}.

When external conditions (temperature, humidity) of the U937 cell culture were unchanged, UPE remains unchanged on the time scale of one hour (data not shown). The decline in UPE observed after one hour was evoked by a decreased vitality of the cells.

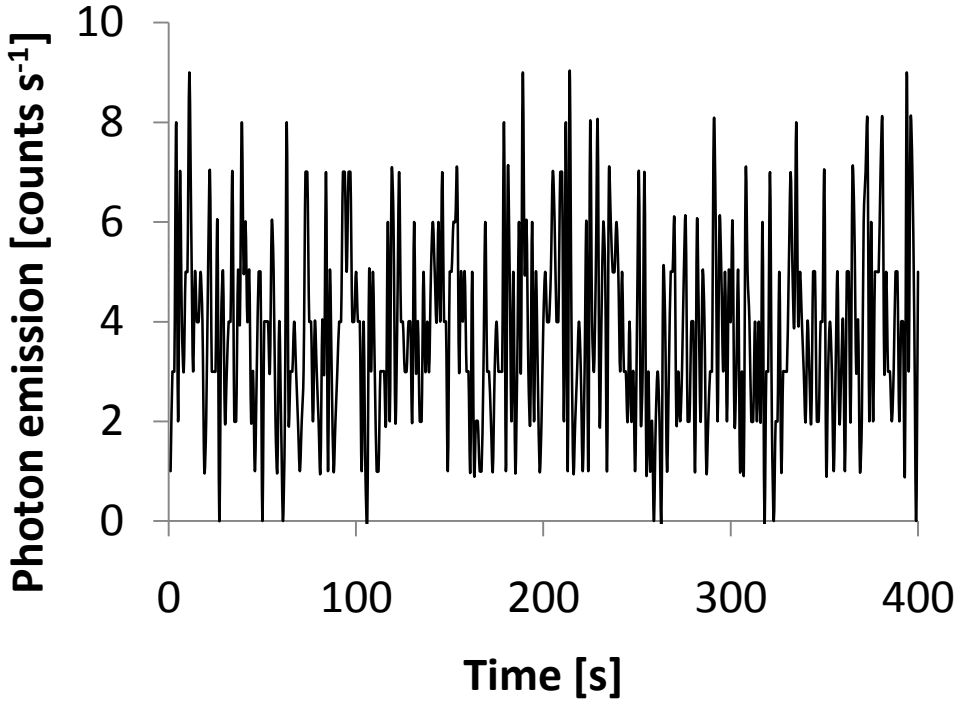


Fig. 1.3: Ultra-weak photon emission from U937 human leukemic cell culture. U937 cell culture was placed into plastic Petri dish and placed under the PMT window. Measurement was taken at room temperature.

These observations indicate that spontaneous UPE from U937 cell culture is higher than spontaneous UPE from healthy human white blood cells. This difference could be caused by differences between human healthy and cancer cell culture. However, it cannot be excluded that the difference in photon emission was caused by different conditions before measurement. While U937 cell culture was in incubator at 37 °C and 5% CO_2 atmosphere, healthy human white blood cells were kept at room temperature after the blood was taken and centrifuged at 20 °C for a few times, which is a standard procedure to extract white blood cells from human blood. The lower temperature in combination with centrifugation could significantly decrease the vitality of the cells and consequently the UPE.

3.2. Effect of hydrogen peroxide on ultra-weak photon emission from U937 cell culture

To test the involvement of H_2O_2 in UPE, the effect of exogenous H_2O_2 on UPE was studied in U937 human leukemic cells. Addition of H_2O_2 into U937 cell culture during the measurements resulted in a pronounced enhancement in UPE (Fig. 2.1).

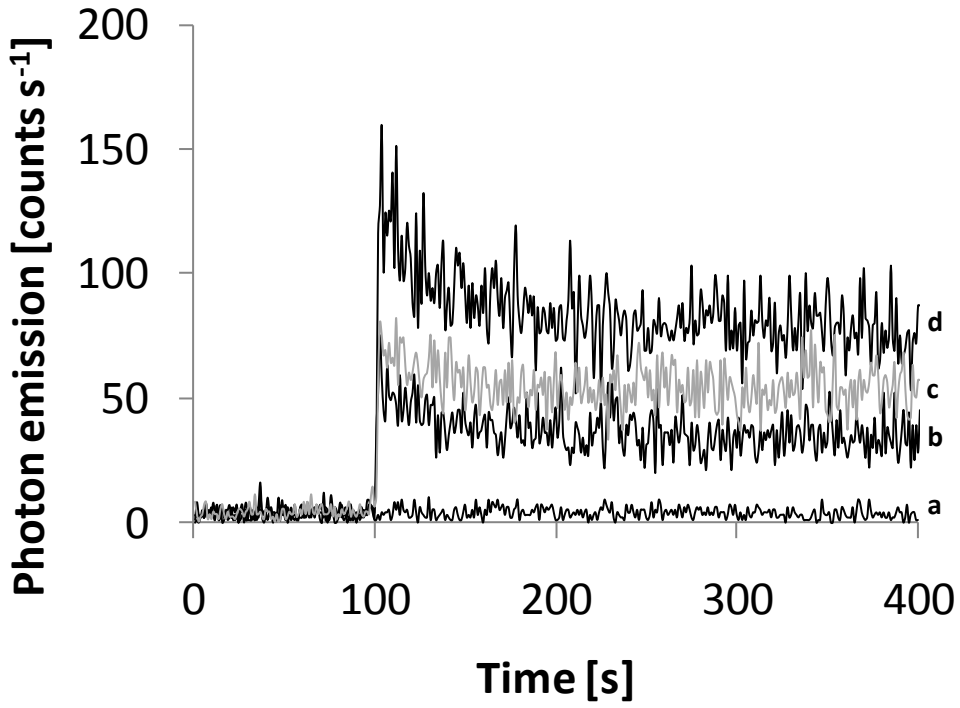


Fig. 2.1: Effect of hydrogen peroxide on ultra-weak photon emission from U937 cell culture. During the measurements, H_2O_2 was injected into U937 cell culture at the concentration of 0 mM (a), 2.5 mM (b), 5 mM (c) and 10 mM (d). The H_2O_2 was added after 100 s.

The sharp increase in photon emission was followed by a gradual decline in photon emission. After achievement of steady-state level of UPE, no further changes in photon rate were observed within the whole measured period. The steady-state level of UPE varied with the concentration of exogenous H_2O_2 applied in U937 cell culture.

Figure 2.2 shows that the ultra-weak photon emission increased with increasing concentration of exogenous H_2O_2.

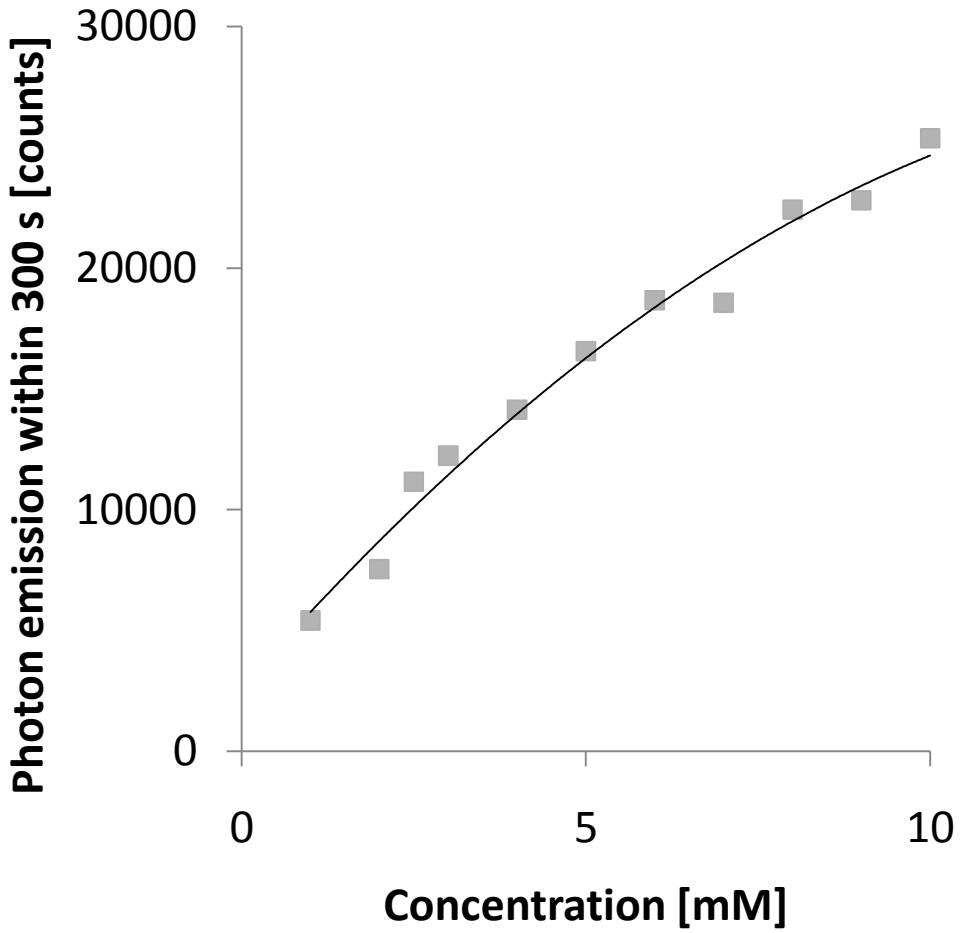


Fig. 2.2: Dependence of ultra-weak photon emission on concentration of H_2O_2 in U937 cell culture. Intensity of ultra-weak photon emission was calculated as the area below the curve from 100 s to 400 s.

These observations indicate that the addition of H_2O_2 in U937 cell culture results in the enhancement in UPE emission. The final concentration of 5 mM H_2O_2, as a concentration corresponds to 50 counts s^{-1}, was chosen for further experiments.

3.3. Effect of hydroxyl radical on ultra-weak photon emission from U937 cell culture

To test the involvement of hydroxyl radical in UPE, the effect of Fenton reagent on UPE was studied in U937 cell culture. When H_2O_2 in the final concentration of 5 mM and ferrous iron in the final concentration of 1 mM were added in U937 cell culture during the measurements, significant enhancement in photon emission was observed. The peak and steady-state value in photon emission observed after simultaneous additions of H_2O_2 and ferrous iron were 2000 and 100 counts s^{-1}, respectively.

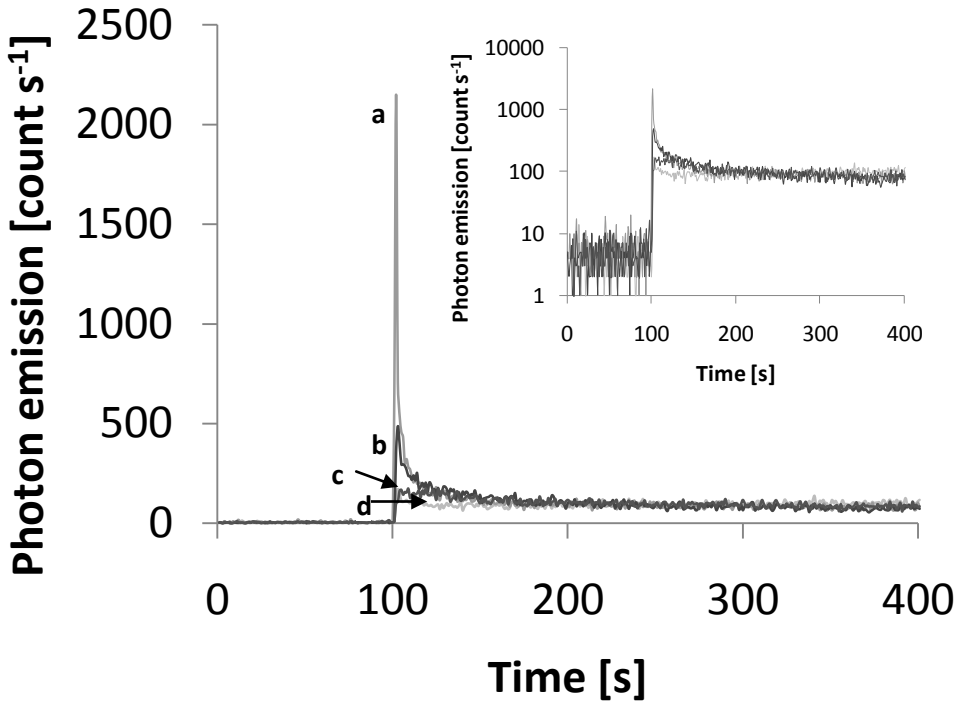


Fig 3: Effect of Fenton reagent on ultra-weak photon emission from U937 cell culture. Final concentration of 1 mM $FeSO_4$ (a), $NiSO_4$ (b), $ZnSO_4$ (c) and $CuSO_4$ (d), were injected in U937 cell culture before measurement. H_2O_2 at the concentration of 5 mM was injected into sample after 100 s. Insert shows the effect of Fenton reagent on ultra-weak photon emission on the logarithmic scale.

Similarly, when ferrous iron was replaced by other reduced metals such as copper, nickel or zinc at the same concentration as ferrous iron, enhancement in the peak and steady-state value in photon emission was observed.

The peak value in photon emission for various metals increased in the following order: $Cu^{+}<Zn^{2+}<Ni^{2+}<Fe^{2+}$, whereas no pronounced changes were observed in the steady-state level of photon emission (Fig. 3, insert). These observations indicate that Fenton reagent added in U937 cell culture caused significant enhancement in ultra-weak photon emission.

3.4. Effect of chelators on ultra-weak photon emission from U937 cell culture

To investigate the role of free metal ions on Fenton's reaction in U937 cell culture, the effect of chelators on UPE was studied. When deferoxamine mesylate (desferal) was added in U937 cell culture before measurements, no significant changes in H_2O_2-induced photon emission were observed (Fig. 4.1). Similarly, the addition of ethylendiaminetetraacetic acid (EDTA) caused no changes in H_2O_2-induced photon emission (data not showed). These observations indicate that free metals are unlikely involved in Fenton's reaction.

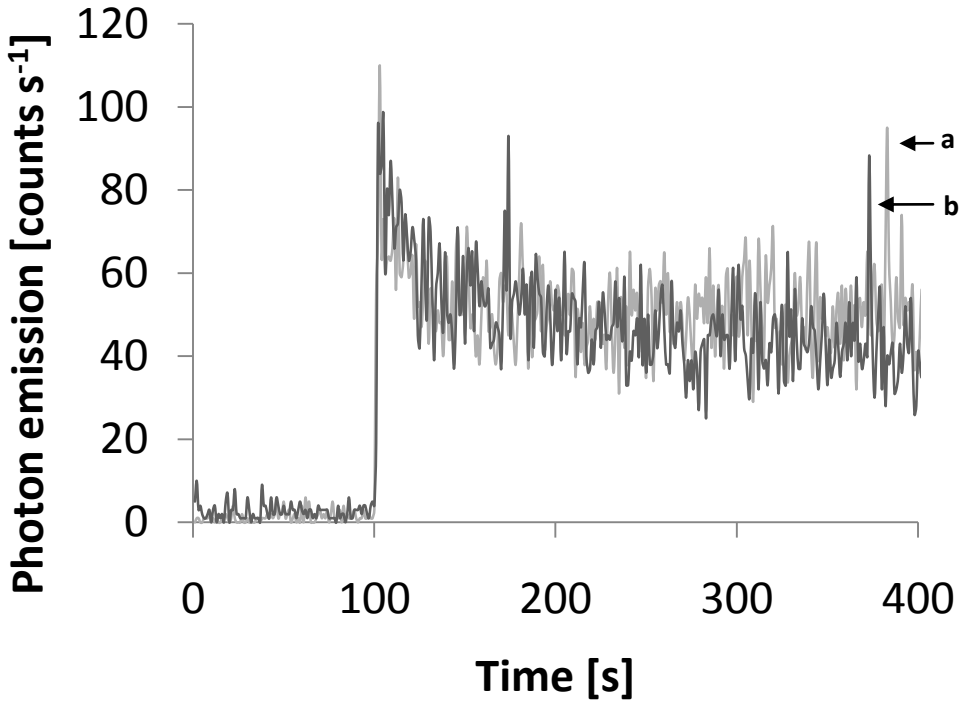


Fig. 4.1: Effect of chelators on hydrogen peroxide-induced ultra-weak photon emission from U937 cell culture. Prior to the measurements, desferal at final concentration of 1 mM was added to the sample. Concentration of H_2O_2 was 5 mM. U937 cell culture (a), desferal (b).

When EDTA was added into U937 cell culture with ferrous iron before measurements, Fenton-induced photon emission was enhanced (Fig. 4.2). Contrary, the addition of desferal into U937 cell culture with ferrous iron before measurements caused partial suppression in UPE. These results indicate that EDTA-Fe^{2+} complex has been shown to be an efficient Fenton's reagent even more effective than ferrous ions by themselves, while desferal-Fe^{2+} complex is suppressor of Fenton's reaction.

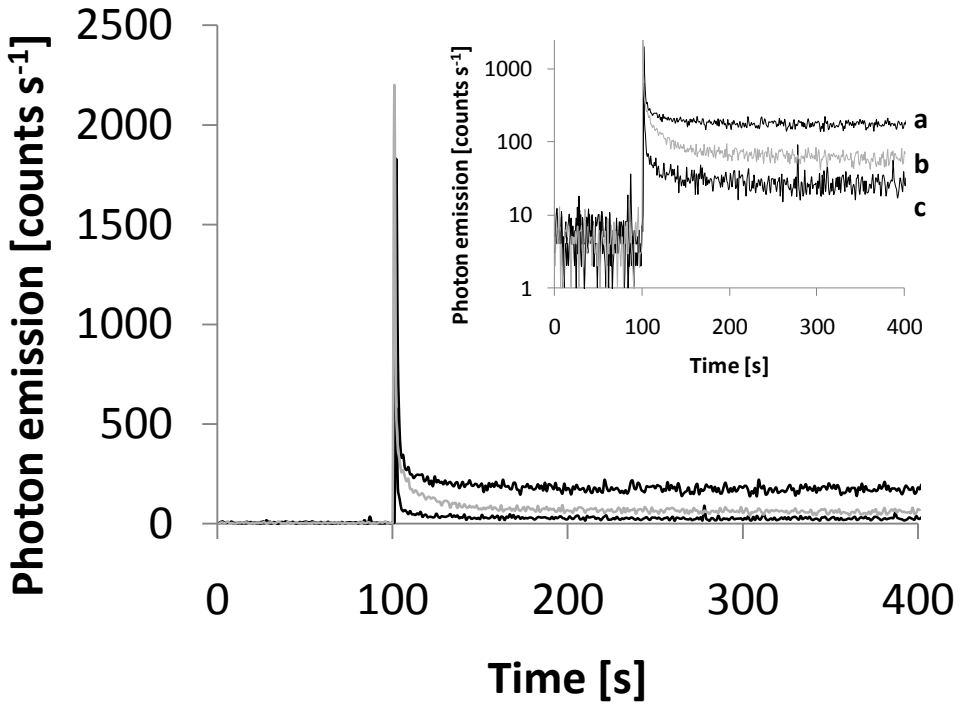


Fig. 4.2: Effect of chelators on hydroxyl radical-induced ultra-weak photon emission from U937 cell culture. Prior to the measurements, $FeSO_4$, EDTA and desferal at the final concentration of 1 mM were added to the sample. Insert shows the same data on the logarithmic scale. Cells with EDTA (1 mM) and $FeSO_4$ (1 mM) (a), cells with $FeSO_4$ (1 mM) (b), cells with desferal (1 mM) and $FeSO_4$ (1 mM) (c).

3.5. Effect of antioxidants on ultra-weak photon emission from U937 cell culture

To investigate the role of ROS in lipid peroxidation, the effect of antioxidant on UPE in U937 cell culture was studied. The additions of mannitol and propyl-gallate significantly suppressed UPE from U937 cell culture induced by hydrogen peroxide, whereas ascorbic acid completely diminished H_2O_2-induced photon emission (Fig. 5.1).

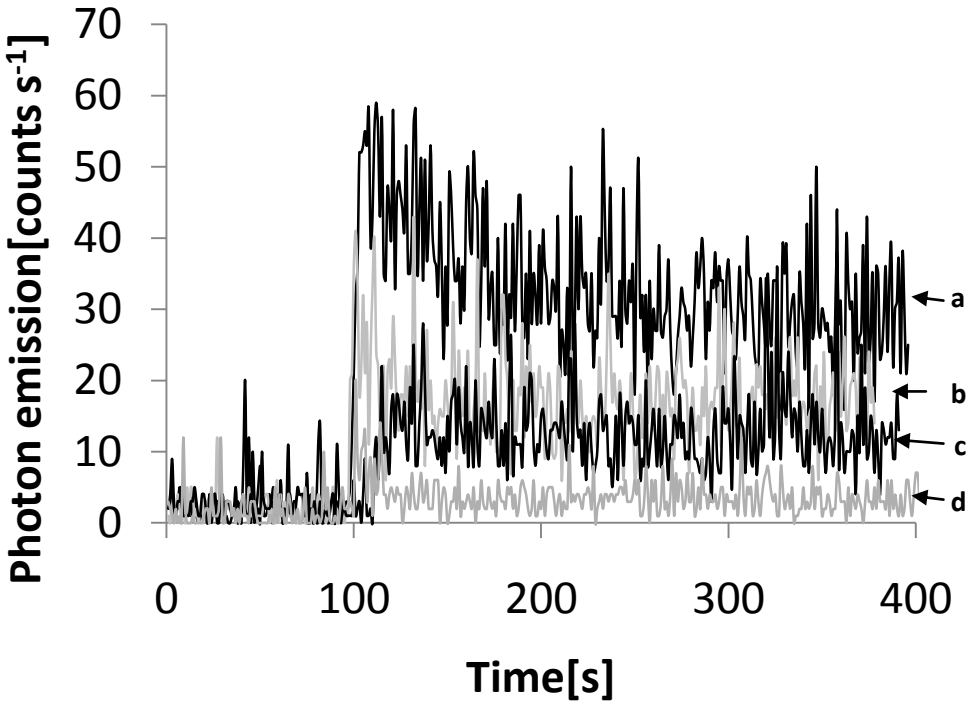


Fig. 5.1: Effect of antioxidant on hydrogen peroxide-induced ultra-weak photon emission from U937 cell culture. Curve (a) represents cells with $FeSO_4$ (1 mM). Mannitol (b) and propyl-gallate (c) were at the concentration of 10 mM, ascorbic acid (d) was at the concentration of 1 mM. H_2O_2 was at the concentration of 5mM.

Propyl-gallate and ascorbic acid significantly suppressed hydroxyl radical-induced photon emission (Fig. 5.2), while mannitol had no effect on steady-state level of UPE, and even raised the peak value.

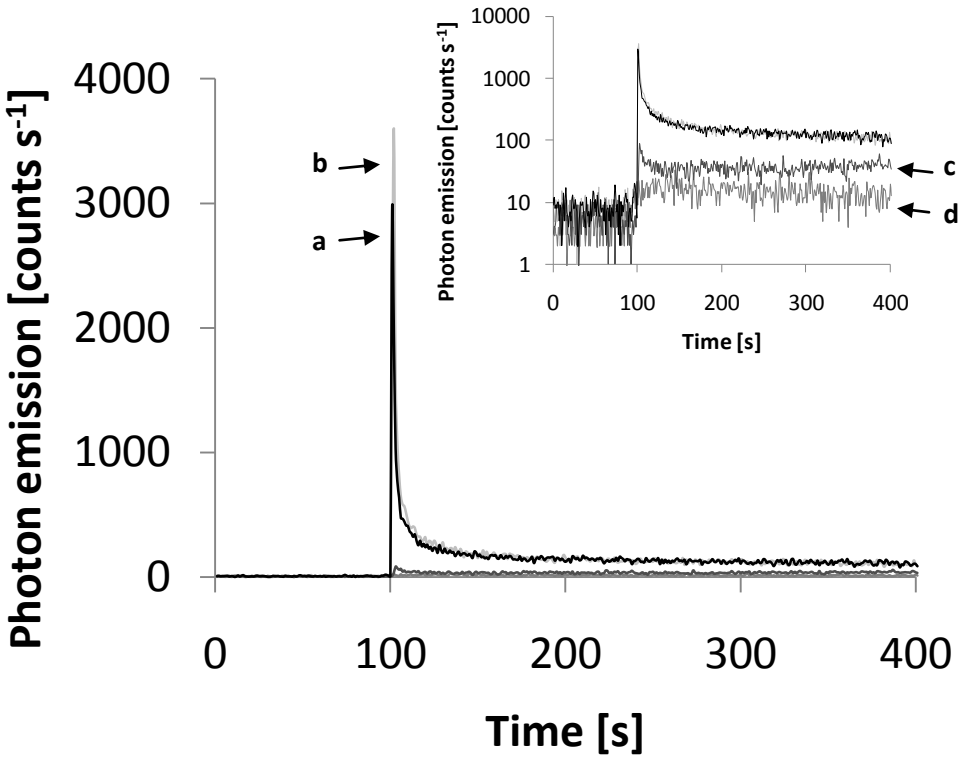


Fig. 5.2: Effect of antioxidant on hydroxyl radical-induced ultra-weak photon emission from U937 cell culture. Mannitol and propyl-gallate were used at the concentration of 10 mM, ascorbic acid was used at the concentration of 1 mM, H_2O_2 was used at the concentration of 5 mM. Insert shows the same effect on logarithmic scale. Cells with $FeSO_4$ (1 mM) (a), mannitol (b), ascorbic acid (c) and propyl-gallate (d).

3.6. Formation of hydroxyl radical in U937 cell culture by decomposition of H_2O_2

To test the formation of $HO^{\bullet}$ after addition of H_2O_2 into U937 cell culture, POBN/ethanol spin-trapping system was used. It has been previously demonstrated that ethanol interacts with $HO^{\bullet}$ to yield α-hydroxyethyl radical ($CH(CH_3)HO^{\bullet}$), which reacts with POBN, forming a stable α-hydroxyethyl radical adduct of POBN (POBN-$CH(CH_3)OH$ adduct) (Pou et al. 1994). The addition of POBN/ethanol spin-trapping system in U937 cell culture caused formation of characteristic POBN-$CH(CH_3)OH$ adduct EPR signal (Fig. 6.1), which reflects $HO^{\bullet}$ formation connected to the enhanced metabolic activity of U937 cell culture.

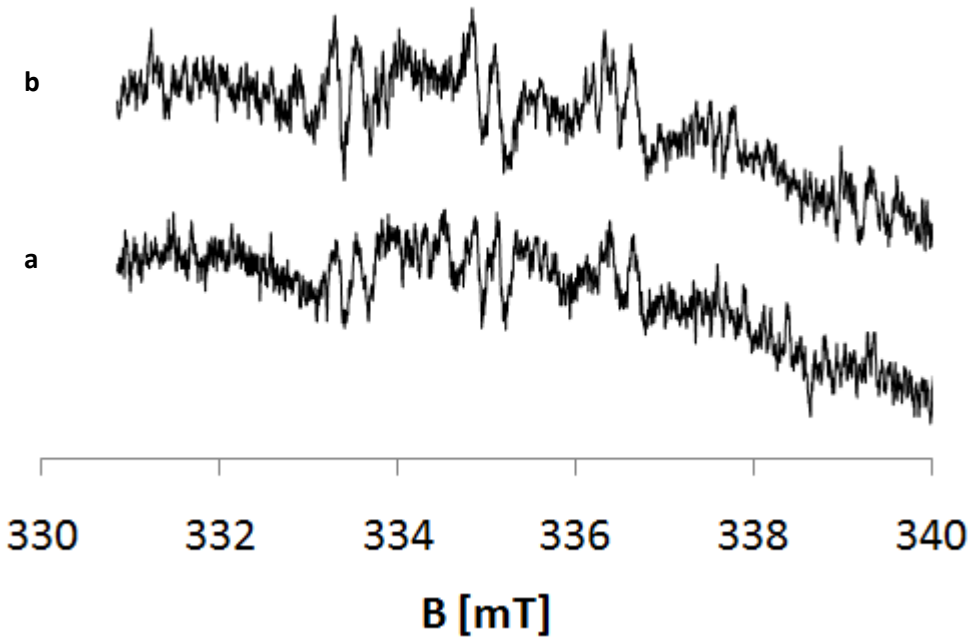


Fig. 6.1: The formation of $HO^{\bullet}$ in U937 cell culture treated with H_2O_2 (5mM). The addition of POBN/ethanol spin-trapping system U937 cell culture caused formation of characteristic POBN-CH(CH3)OH adduct EPR signal (a). Adition of H_2O_2 into U937 cell culture caused no enhancement in POBN-$CH(CH_3)OH$ adduct EPR signal (b).

When H_2O_2 was added in U937 cell culture, no enhancement in POBN-$CH(CH_3)OH$ adduct EPR signal was observed. These observations indicate that addition of H_2O_2 in U937 cell culture does not result in $HO^{\bullet}$ formation.

When POBN/ethanol spin-trapping system was added in U937 cell culture, POBN-$CH(CH_3)OH$ adduct EPR signal was observed (Fig. 6.2). The addition of spin trap compound in U937 cell culture followed by simultaneous addition of H_2O_2 and ferrous iron resulted in the significant enhancement in POBN-$CH(CH_3)OH$ adduct EPR signal. These results reveals that simultaneous addition of H_2O_2 and ferrous iron results in generation of $HO^{\bullet}$ formed by metal-catalyzed reduction of H_2O_2.

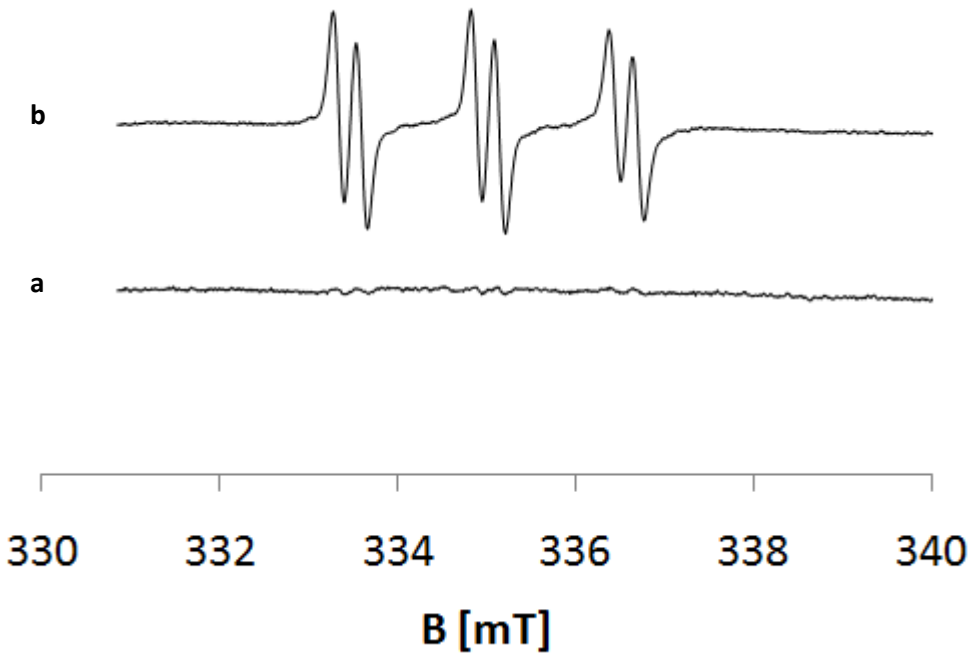


Fig. 6.2: The presence of $HO^{\bullet}$ in U937 cell culture treated with H_2O_2 (5mM) in the presence of ferrous iron. The addition of POBN/ethanol spin-trapping system U937 cell culture caused formation of characteristic POBN-CH(CH3)OH adduct EPR signal (a). The addition of spin trap compound in human U937 leukemia cells followed by simultaneous addition of H_2O_2 and ferrous iron resulted in the significant enhancement in POBN-$CH(CH_3)OH$ adduct EPR signal (b).

3.7. Formation of singlet oxygen in U937 cell culture

Spin trapping was accomplished by utilizing the oxidation of diamagnetic 2,2,6,6-tetramethylpiperidine (TEMP) by $^1O_2$ to yield paramagnetic 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO). Addition of 5 mM H_2O_2 U937 cell culture resulted in the generation of TEMPO EPR spectra (Fig. 7). The residual TEMPO EPR signal observed priori addition of H_2O_2 in U937 cell culture was due to impurity of spin trap compound. When 5 mM H_2O_2 was added in the pure RPMI-1640 medium, TEMPO EPR signal was observed. It was described by Aubry (1985) that H_2O_2 can be decomposited into $^1O_2$ by mineral compounds.

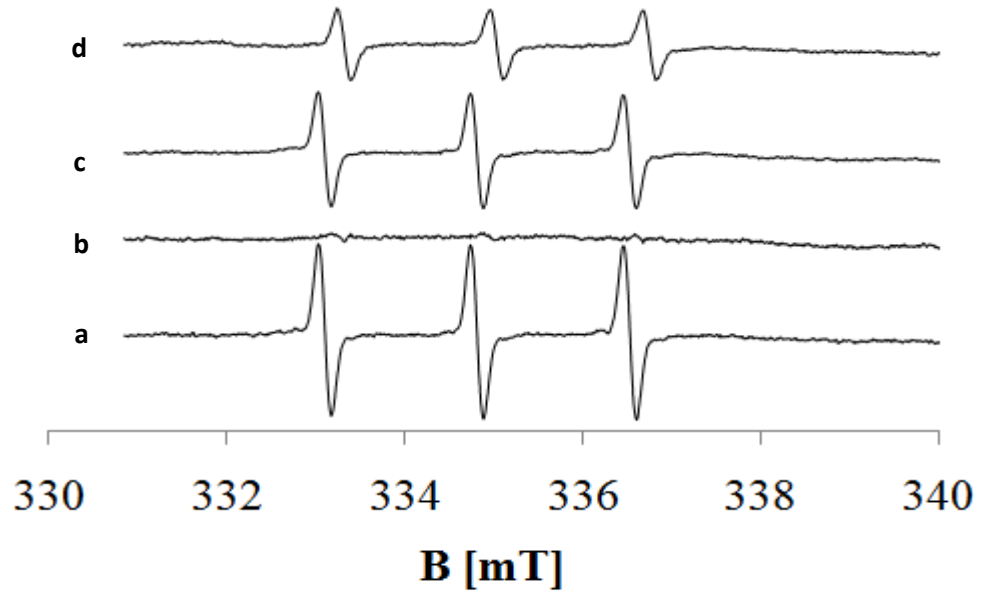


Fig. 7: The formation of singlet oxygen in U937 cell culture. When 5 mM H_2O_2 was added in the pure RPMI-1640 medium, TEMPO EPR signal was observed (a). The residual TEMPO EPR signal observed priori addition of H_2O_2 in U937 cell culture was due to impurity of spin trap compound (b). The adition of H_2O_2 into U937 cell culture (c). The simultaneous addition of H_2O_2 and ferrous iron in human U937 leukemia cells (d).

To test the formation of $^1O_2$ after simultaneous addition of H_2O_2 and ferrous iron in U937 cell culture, TEMP EPR signal was observed. Figure 7 shows that simultaneous addition of H_2O_2 and ferrous iron in U937 cell culture resulted in formation of TEMPO EPR signal. The height of TEMPO EPR signal observed after simultaneous addition of H_2O_2 and ferrous iron is a half of the height of TEMPO EPR signal observed after addition of H_2O_2. According to experiments with POBN and TEMP including cells with $FeSO_4$ and H_2O_2, it could be proclaimed that Fenton's reaction is competitive to formation of $^1O_2$ by mineral compounds and that the rate of Fenton's reaction is much higher.

3.8. Presence of malondialdehyde in U937 cell culture

Malondialdehyde, a product of lipid peroxidation, is a good marker for development of oxidative stress. An adaptation of a very rapid and simple isocratic reversed-phase HPLC separation of MDA as its DNPH in cell culture (Pilz et al. 2000). Figure 8 shows the amount of MDA after DNPH derivation. Peak in cells without H_2O_2 predicts that there are not only MDA absorbing at the same wavelength in that time. That is why arise of the peak is more important. In correlation with results from PMT, samples containing $FeSO_4$ showed higher amount of MDA as well as higher UPE than cells treated only with H_2O_2.

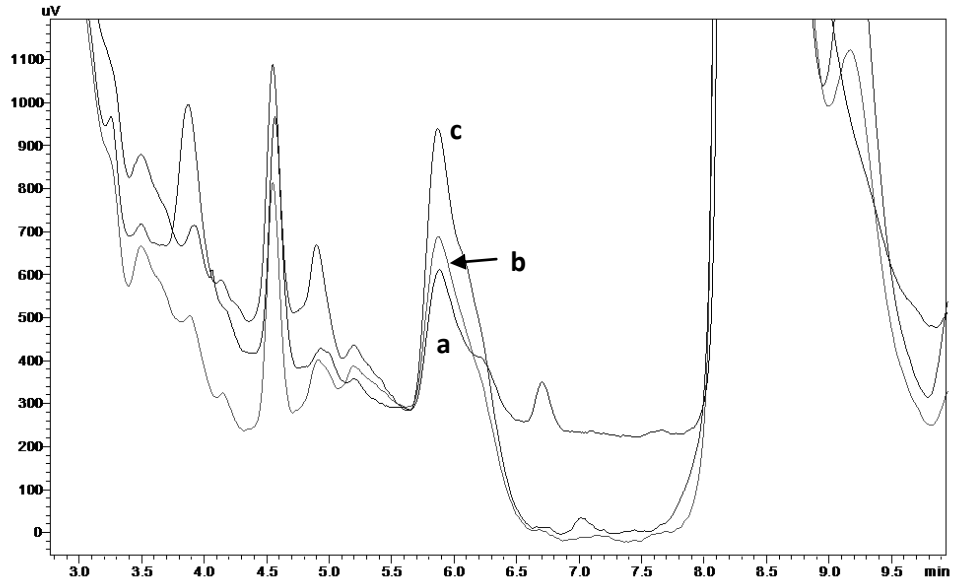


Fig. 8: HPLC chromatograms of cell culture total MDA after DNPH derivatization measured at 310 nm. Pure cell culture (a), cell culture treated with H_2O_2 (5 mM) for 1 hour (b), cell culture with ferrous iron (1 mM) treated with H_2O_2 (5 mM) for 1 hour (c)

3.9. Imaging of U937 cell culture by fluorescenc3 microscopy

To show the damage caused by lipid peroxidation, images from fluorescent microscope were chosen. Samples were cultivated for 1 hour at room temperature. Figure 9.1 shows healthy U937 cell culture. Propidium iodide (PI), used fluorescent molecule, is membrane impairment and fluoresces red. It binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4-5 base pairs of DNA. That is why only dead cells with disrupted membrane are stained red, while vital cells without PI are green. The difference between cells treated with H_2O_2 and cells with $FeSO_4$ is noticeable. While all cell nuclei in the first case still holds its shape, but not the size anymore (Fig. 9.2), in the second case, DNA strings with shape not even close related to original nucleus shape can be found (Fig. 9.3).

Figure 9.3 shows cells with $FeSO_4$ (1 mM) treated with H_2O_2 (5 mM). DNA is no longer holding its original shape.

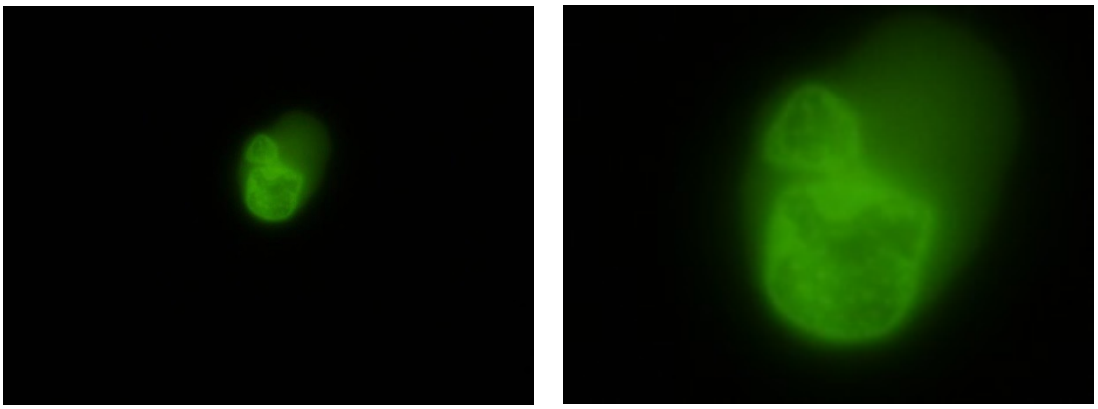


Fig. 9.1: Healthy U937 cell culture. Image taken by fluorescent microscope, PI was used as a dye.

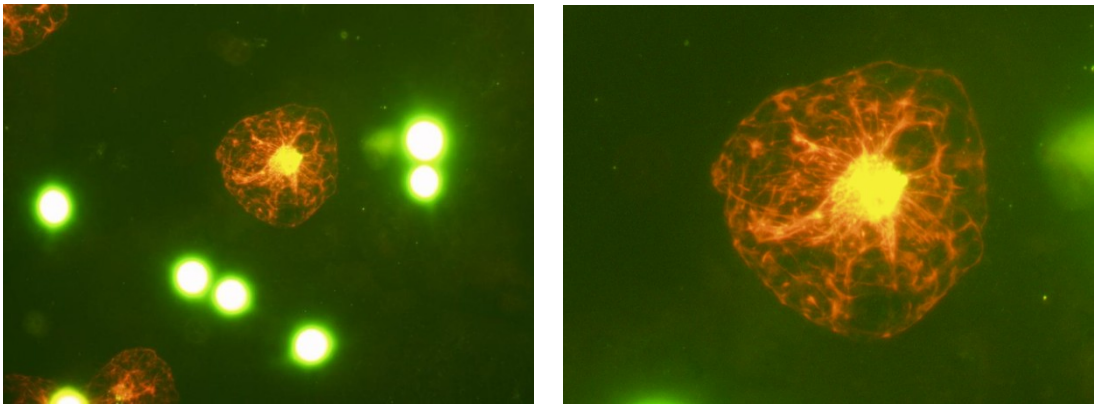


Fig. 9.2: U937 cell culture treated with H_2O_2 (5mM) for 1 hour. Green colour indicates vital cells, red colour indicates dead cells.

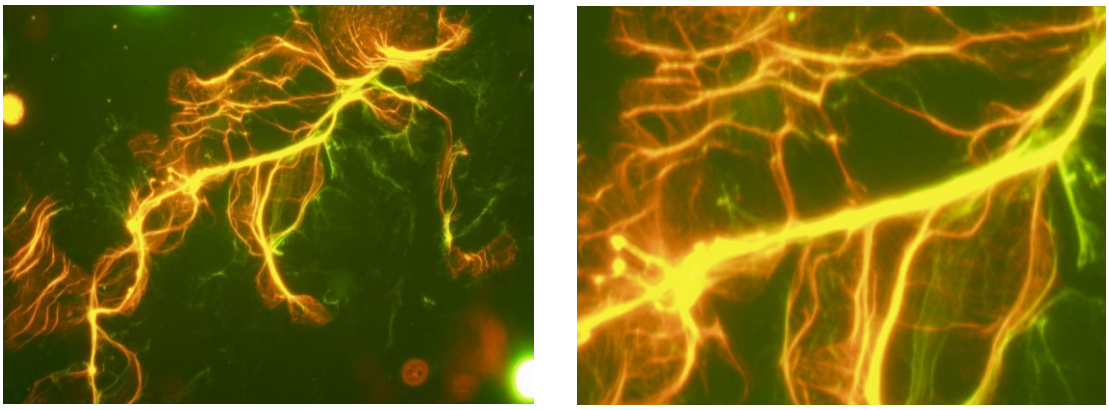


Fig. 9.3: U937 cell culture with ferrous iron (1 mM) treated with H_2O_2 (5 mM) for 1 hour. Green colour indicates vital cells, red colour indicates strings of DNA from dead cells.

4. Conclusion

Ultra-weak photon emission was used to follow the kinetics of cell oxidative damage using highly sensitive PMT. Ultra-weak photon emission from U937 cell culture is 2.5 counts s^{-1} after subtraction of the emission from medium. The addition of H_2O_2 resulted into enhance of UPE to 50 counts s^{-1}. To confirm the dependence of UPE on ROS antioxidants were used. Data shows significant decrease in UPE in the presence of antioxidants. While addition of mannitol and propyl-gallate decreased the UPE to half of the original value, addition of ascorbic acid leads to full suppression of UPE caused by H_2O_2. In the presence of metal ions, the steady-state value of UPE raised to 80 counts s^{-1}. The addition of EDTA into sample containing Fe^{2+} ions enhanced photon emission to double of the original value, whereas desferal suppressed significantly photon emission. These results confirmed that EDTA-Fe^{2+} complex is an efficient Fenton's reagent. Ultra-weak photon emission obtained from U937 cell culture without addition of Fe^{2+} with and without chelators was the same, which confirms that there are no unbounded metal ions presented.

Evidence that $^1O_2$ and $HO^{\bullet}$ catalyze oxidative damage in human U937 leukemic cells is presented. As confirmed by EPR, the addition of H_2O_2 into RPMI-1640 media resulted in the formation of TEMPO EPR signal, which indicates the formation of $^1O_2$, however the exact mechanism is not known yet and further experiments have to be done to enlighten it. The addition of reduced transition metals formed $HO^{\bullet}$ as confirmed by the formation of POBN-$CH(CH_3)OH$ adduct EPR signal. Whereas $^1O_2$ caused a partial cell injury in propidium iodide labelled U937 cell culture, $HO^{\bullet}$ progressively damaged the cell membranes as confirmed by fluorescence microscopy. Detection of malondialdehyde by isocratic reverse-phase HPLC revels that $^1O_2$ and $HO^{\bullet}$ caused lipid peroxidation, the content of lipid peroxides induced by $HO^{\bullet}$ was double compared to $^1O_2$. Those data corresponds with results obtained by PMT when the steady-state value of UPE detected from samples with $^1O_2$ and $HO^{\bullet}$ was 50 counts s^{-1} and 100 counts s^{-1}, respectively.

These results reveal that $HO^{\bullet}$ is the main ROS responsible for oxidative damage and cell injury. Techniques based on UPE takes less time and the cost of apparature is incomparably lower than other methods used in this thesis. Due to those two facts, new non-invasive techniques for the detection of oxidative processes in the human leukemic cells under certain physiological and pathophysiological conditions could be developed.

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