May the variable magnetic field and pulse red light induce synergy effects in respiratory burst of neutrophils in vitro?

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May the variable magnetic field and pulse red light induce synergy effects in respiratory burst of neutrophils in vitro?

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Abstract. We investigated the effect of the red light (R) (630 nm), magnetic field (MF) and magnetic field combined with the red light (MF+R) upon reactive oxygen species (ROS) production by neutrophils in vitro. The object of the research was hydrogen peroxide (H_2O_2) formation during neutrophils respiratory burst or within steady-state. Blood from healthy volunteers was used for the purpose of the study. Flow cytometry method, using transformation of DCFH-DA (2'7'-dichlorofluorescin diacetate) to the fluorescent DCF (2'7'-dichlorofluorescin), was used for estimation of hydrogen peroxide production. The variable magnetic field of ELF range of the mean induction equals 26.7(μT), the red light at the energy density of 1.17(J/cm^2) and their combination were applied for 30 minutes each. The fundamental frequency of pulses was 180÷ 195 Hz. A statistically significant decrease of H_2O_2 production by neutrophils was observed. The level of the decrease was in the range of 10-30% and was dependent on the kind of applied physical factors and whether neutrophils were stimulated or not. The observation showed that the variable magnetic field combined with red light do not induce the synergy effect.

1.Introduction

Neutrophils are highly specialized white blood cells. Their basic function is executing the phagocytosis and successive killing of phagocytized microorganisms by secretion of destructive molecules into the phagosome and by the respiratory burst [1, 2, 3]. The neutrophil oxidative burst can be activated by a number of different soluble and particulate stimuli, including chemoattractants, certain cytokines, phorbol esters, calcium ionophores, different lectins, and opsonized as well as various unopsonized microorganisms [1, 3]. The NADPH oxidase enzymatic complex assembles on the plasma (or phagosome) membrane of granulocytes, macrophages and B cells upon stimulation [4, 5]. The neutrophil NADPH oxidase consists of the catalytic subunit gp91^{phox}, which is located at the phagosome membrane together with p22^{phox}. The assembly of gp91^{phox} and p22^{phox} with the cytosolic subunits p40^{phox}, p47^{phox}, p67^{phox} and small GTP-ase Rac2 results in rapid production of superoxide [1,4, 6, 7]. The phagocyte NADPH oxidase produces superoxide anion ($O^{2\cdot-}$) which is a precursor to other ROS by the electrogenic process of moving electrons across the cell membrane [8, 5, 3]. Most of $O^{2\cdot-}$ formed dismutates to H_2O_2, which is converted to HOCl by myeloperoxidase [1, 2, 3, 7]. Each NADPH molecule donates two electrons, which cross the membrane through a redox pathway contained within gp91^{phox} and reduce two O_2 molecules to $O^{2\cdot-}$. In detail gp91^{phox}, also called Nox2, contains an electron transport chain that comprises NADPH, FAD and two heme groups that pass electrons sequentially to O_2 at an external binding site, to produce $O^{2\cdot-}$ [5]. Electron flux depolarizes the membrane, opening proton channels when the proton flux compensates charge and prevents large

changes in both cytoplasmic and phagosomal pH. This charge translocation must be compensated to prevent self-inhibition of NADPH by extreme membrane depolarization [5, 6, 8]. After about a minute of stimulation, membrane depolarization reaches a steady state, even though superoxide is still being produced, indicating the movement of a compensatory charge into the external media. This is attributed to the delayed release of protons via an associated proton channel, conductance of which is voltage gated [6]. When proton channels are inhibited, pH drops to levels that inhibit NADPH oxidase [5, 9].

Protein kinase C (PKC) isoforms are implicated in the oxidative burst as the potential key activators of NADPH oxidase and they are required for phosphorylation of p47phox, resulting in the assembly and activation of phagocytic NADPH oxidase [4]. PKC is also important for gating of the proton channel. Phosphorylation of proton channels enhances the opening of proton channels [9]. PKC-δ is required for full assembly of NADPH oxidase and activation of the respiratory burst. Neutrophils also express PKC-α and -β, which may be involved in adhesion, degranulation and phagocytosis. The conventional PKC (α, β, γ) contains two regulatory domains: C1 and the calcium-binding C2 domain. The PKC-δ has lack of the C2 domain and is therefore calcium-insensitive [4].
Activity of the NADPH complex also requires the involvement of at least two G proteins. These are proteins that act as molecular switches, alternating between binding GDP in the inactive state and GTP in the active state [6].

Binding of respiratory burst activators such as: LTB_4 (leukotriene B4), PAF (platelet activating factor) or FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine) to receptors on the neutrophils plasma membrane releases intracellular calcium ($[Ca^{2+}]_i$) via the phospholipase-inositol triphosphate pathway [10, 11]. This depletion of $[Ca^{2+}]_i$ in turn leads to Ca^{2+} influx via store operated channels resulting in elevated Ca^{2+} levels that are thought to be implicated in several Ca^{2+} dependant pro-inflammatory responses. NADPH oxidase inhibition will also abrogate membrane depolarisation and augments Ca^{2+} entry into the cell. Depolarization alters the electrochemical gradient across the neutrophil plasma membrane and limits Ca^{2+} entry into the cell [10]. Both intra- and extracellular Ca^{2+} Are required for full activation of the respiratory burst of human neutrophils, and the Ca^{2+} influx from extracellular space plays an important role either in generation of reactive oxygen metabolites or in activation of protein kinase C [11].
We have investigated whether the red light combined with variable magnetic field can cause double or higher effect (synergy effect) on hydrogen peroxide formation by neutrophils in vitro. According to the definition, the synergy effect means that the combination of the factors can cause a greater reaction than the sum of the individual effects of each physical factor when used separately.

2.Material and methods

2.1.Material. The blood from healthy volunteers was used for the purpose of the study. As the anticoagulant was the lithium heparin.

2.2.Reagents. DCFH-DA (2'7'-dichlorofluorescin diacetate; Sigma-Aldrich, USA) was used for showing the presence of hydrogen peroxide [12, 13, 14,15, 16]. The 0.0609 g of the substance was dissolved in 5 ml of 96% ethanol. The solution was kept in - 20ºC. Before every measurement DCFH-DA solution was diluted in PBS (phosphates buffered physiological salt solution) in proportion 1µl for 10µl PBS. PMA (phorbol 12-myristate, 13-acetate; Sigma-Aldrich, USA) was used to produce submaximal stimulation of the respiratory burst [3, 12, 16]. The PMA solution was prepared by dissolution 1 mg of PMA in 1 ml of 96% ethanol. The solution was kept in - 20ºC. For the red blood cells lysis the special solution was prepared [13]. The lysis solution was made by dissolution 8.26 g NH_4Cl, 1.0 g K_2CO_3 and 0.037 g four-sodium salt EDTA in 1l of distilled water. pH of the solution was 7.2÷7.4. The lysis solution was kept in 4ºC.

2.3.Method. For one measurement the blood from one person was divided into 4 test-tube containing 50 µl of blood each. 10µl of DCFH-DA diluted solution was added to each test-tube. Two of the examined samples were incubated in the presence of the red light (R) of wavelength 630 nm or variable magnetic field (MF) or combination of those factors (MF+R) for 30 minutes each in the room temperature in dark. The rest of test-tubes as a control was kept also in the same environment conditions. After the physical factor application 2 µl of PMA solution was added to the one of control test-tubes and one which was influenced of R/MF/MF+R. After incubation at room temperature for 15 minute in the dark the lysis solution was added. 700µl of this solution was added to each of test-tube. After 15 minute in the dark at room temperature the flow cytometry measurement was made [12].

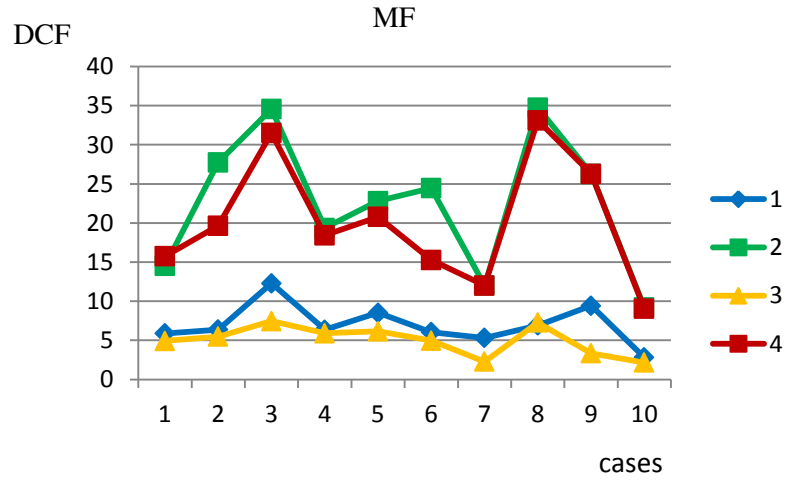
2.4. Viofor JPS. Generator of the red light and variable magnetic field was the device Viofor JPS. The mean induction of the variable magnetic field of ELF range equaled 26.7(µT]), the red light energy density was 1.17(J/cm^2). The distance of 1cm was kept between the source of physical factors and test-tubes to avoid raise of the sample temperature. The level of the magnetic induction or/and the density of light application was growing at 12 seconds interval starting from 0.30(J/cm^2)/4.45(µT) to the selected, then the intensity drops to the initial value, the process is repeated cyclically. The fundamental frequency of pulses was 180 ÷ 195 Hz. The form of impulses is close to the peak shaped. The pulses were administered in the form of packets of pulses (12.5 ÷ 29 Hz), groups of packages (2.8 ÷ 7.6 Hz) and series (0.08 ÷ 0.3 Hz).

2.5. Flow cytometry. The induced respiratory burst was assessed by the intracellular oxidative transformation of DCHF-DA to the fluorescent DCF ($\lambda_{excitation}$=498nm, $\lambda_{emission}$=522nm) via the flow cytometer. The DCF fluorescence is linearly dependent on the quantity of the respiratory burst [12, 16, 17]. Cytofluorymeter FACScan Becton Dickinson equipped with 15 mW argon laser was used for DCF fluorescence measurement [12, 17].

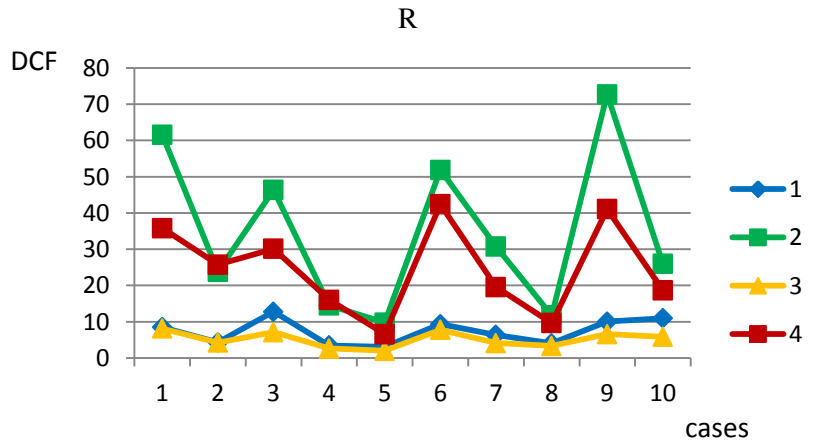
3.Results

We used the following marks of blood samples:
1-contol blood sample unstimulated with PMA, without the influence of MF/R/MF+R
2-neutrophils activated by PMA (control sample for „4")
3-a sample of blood unstimulated with PMA, under the influence of MF/R/MF+R
4-neutrophils activated by PMA, under the influence of MF/R/MF+R
(4-3)-respiratory burst in samples influenced by MF/R/MF+R
(2-1)-respiratory burst in native samples (not exposed to MF/R/MF+R)
We compared samples "1" with "3", and samples "2" with "4" by using Wilcoxon test. We also calculated difference between respiratory burst in native samples and respiratory burst in samples influenced by MF/R/MF+R (Figure 2).
We present our data on graphs (Figure 1, 2), where Y axis presents DCF fluorescence in arbitrary units for each person (case).

A)



B)



C)

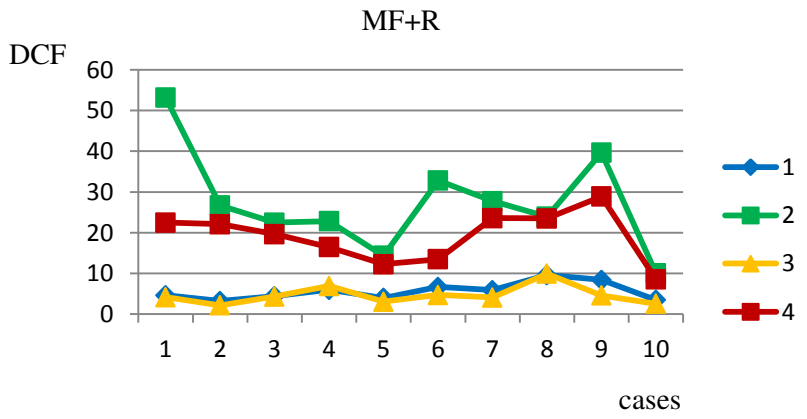


Figure 1. DCF fluorescence after red light (A), variable magnetic field (B), magnetic field combined with red light (C) application.

The magnetic field (MF), the red light (R) and the magnetic field combined with the red light (MF+R) caused statistically significant changes of hydrogen peroxide production by unstimulated (blue and yellow line on each graph) and PMA-stimulated neutrophils (red and green line on each graph) (Figure 1, 2, Table 1).

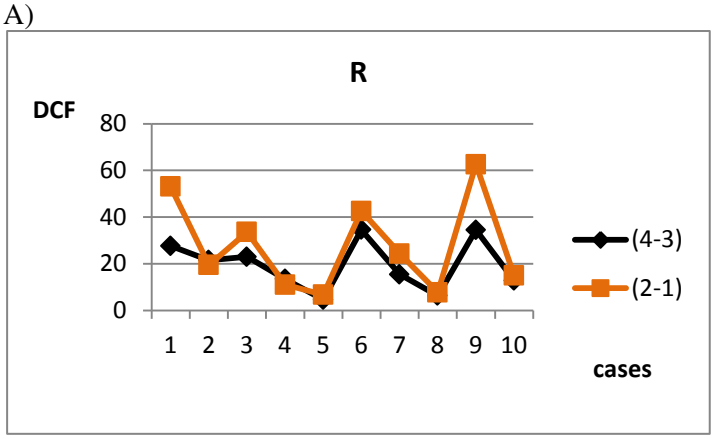
Each of the physical factors applied separately and its combination caused reduction ($p \leq 0.007$) of DCF fluorescence of PMA unstimulated neutrophils average by 30% (Table 1). MF resulted 10.6% DCF fluorescence decrease of stimulated neutrophils ($p=0.028$). The red light caused the largest reduction (29.5%) of hydrogen peroxide formation by stimulated neutrophils ($p=0.01$). MF+R caused 30.3% ($p=0.005$) decrease of DCF fluorescence of those neutrophils.

Table 1. Percentage of DCF fluorescence decrease and "p" value calculated according to the Wilcoxon test.

	1&3	**2&4**	**(2-1)&(4-3)**
MF	28.6 % p=0.007	10.6 % p=0.028	—
R	28.8 % p=0.005	29.5 % p=0.01	29.7 % p=0.037
MF+R	17.8 % p=0.0468	30.3 % p=0.005	33.6 % p=0.005

The production of hydrogen peroxide was statistically significant (Figure 2, Table 1) between the neutrophils under the influence of red light or magnetic fields combined with red light (4-3) and the neutrophils without their influence (2-1).

A)



B)

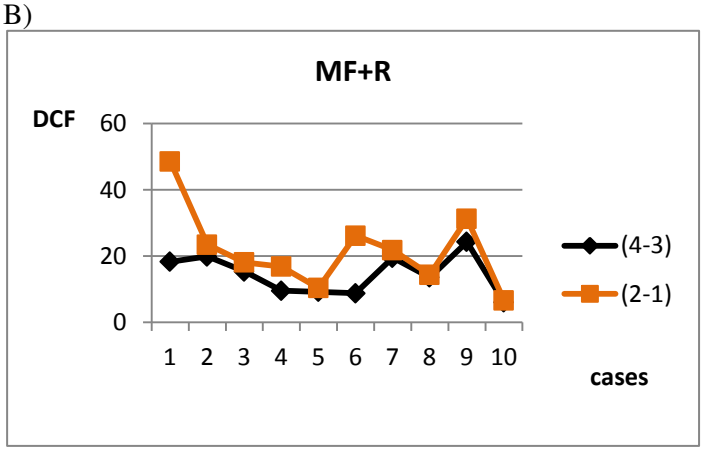


Figure 2. Quantity of respiratory burst after the red light irradiation (A), and the magnetic field combined with red light application (B).

The red light caused the biggest changes of respiratory burst of stimulated and unstimulated neutrophils. Magnetic field made the lowest decrease.
We didn't observe the synergy effect after application of combined factors as variable magnetic field and red light (Table 1).

4.Discussion

The absorption of light by molecules causes electronic excitation. When molecules, such as chromophores, are excited an improvement in kinetics chemical reactions is observed. However, the mechanism of light absorption by chromophores does not explain many of the experimental observations. Light induces a wave-like alternating electric field in a medium that is able to interact with polar structure and produce dipole transitions. This electrical component of electromagnetic energy has an effect on dipolar molecules such as enzymes, ionic pumps, nuclear material and nucleotide molecules [18].

There is evidence indicating that phototherapy has a significant influence on neutrophils [19]. For example: Fujimaki at al. found that Ga-Al-As laser [wavelength: 830 nm, doses: 9.5 and 19 (J/cm^2)] radiation significantly reduced the production of reactive oxygen species by human neutrophils in vitro [19, 20]. Lopes-Martins at co-authors evaluated the effect of the In-Ga-Al-P laser [(wavelength: 650 nm, doses: 3, 7.5 and 15 (J/cm^2)] in vivo on inflammatory cell migration in acute mouse pleurisy. They observed the largest migration reduction for neutrophils after application of 7.5 (J/cm^2) [19,21]. Other studies showed that Ga-Al-As laser [wavelength: 685 nm, dose: 2.5 (J/cm^2)]

irradiation decreased number of neutrophils, the levels of neutrophils anti-apoptotic factors, the IL-1β concentration, and lung myeloperoxidase activity after lipopolysaccharide-induced lung inflammation in rats [19, 22].

According to Panagopoulos at all an oscillating, external electric or magnetic field will exert an oscillating force on every free ion on both sides of the plasma membrane, as well as on the ions within channel proteins, while they pass through them. This external oscillating force will evoke in every ion, a coherent forced-vibration. This vibration of electric charge is able to irregularly gate electrosensitive channels on the plasma membrane and thus cause disruption of the electrochemical balance and function of the cell. The oscillating ions will then represent a periodical displacement of electric charge, able to exert forces on every fixed charge of the membrane, such as the charges on the voltage sensors of voltage-gated channels. This way, the oscillating ions could be able to disrupt the membrane's electrochemical balance by gating such channels [23]. The authors concluded on the base of calculations that the low frequency fields are the most bioactive ones and pulsed electromagnetic fields (PEMF) can be more biologically active than the continuous ones [23]. Balcavage at all stated also that pulsed electromagnetic fields can modify cation flow across biological membranes and alter cell metabolism. PEMF has the potential of regulating the flow through cation channels, changing the steady state concentrations of cellular cations and thus the metabolic processes dependent on cation concentrations [24].

Magnetic field can also change cell proliferation, activation of several enzymes, increase of certain protein concentrations [25]. MF can influence the process of ion binding with proteins [26].
There are reports about the influence of magnetic field on neutrophils.
Nody at all presented the results of AC (60 Hz) and DC magnetic fields influence on respiratory burst in neutrophils. MF was in the range of 0.1 to 2 mT. They observed that both 60 Hz and DC magnetic field enhanced the production of DCF fluorescence during the respiratory burst [27].
Sheiko at co-authors evaluated the effects of various physical factors of electromagnetic nature on the activities of neutrophils coming from patients' blood with breast cancer. They found that alternating magnetic field (10 mT induction, 1.5-3-6 Hz frequency, total exposure 15 min), low-intensity laser or photodiode radiation (both: λ=637nm, 7.5 mW/cm^2, duration of exposure up to 5 min), and their combinations induced mobilization of blood neutrophils function [28].

Varani at all has investigated the effect of the magnetic field of the intensity varying from 0.2 to 3.5 mT on human neutrophil A_3 receptors. In human neutrophils A_3 adenosine receptors exert their anti-inflammatory properties by inhibiting degranulation, chemotaxis and superoxide anion production. Authors observed a significant increase of adenosine A_3 density when the magnetic intensity was in the range 1–3.5 mT, not lower [29]. According to the same author in another paper PEMFs evokes an upregulation of the A_{2A} adenosine receptors and alters the response of this receptor subtype in human neutrophils. PEMF treatment causes a reduction of the superoxide anion production as a result of upregulation of the A_{2A} receptors located on the neutrophil surface [30].

Summarizing, the variable magnetic field or the red light affects the cell differently, e.g. the light-induced electric field is able to change the membrane potentials in the mitochondrial membrane by transferring charges from the outer side to the inner side or directly by the polarization of membrane dipoles. Irradiation with light at 633nm increases proton gradient and causes changes in mitochondrial optical properties [31]. A varying magnetic field can cause the normalization of cell's membrane potential or increases the activity of enzymes. Therefore, supposed targets of red light or/and variable magnetic field influence taking into account the mechanism of respiratory burst could be: calcium ions flux/influx (Ca^{2+}), NADPH oxidase complex, protein kinase C (PKC) isoforms, proton channel, the system of antioxidant enzymes: SOD (superoxide dismutase), CAT (catalase) and GSH-Px (glutathione peroxidase). SOD is a metal protein containing zinc and copper, CAT contains hem and GCH-Px selenocysteine. Cieślar at co-authors observed reduced activity of SOD in human blood in vitro study after exposure to the variable magnetic field generated by the device Viofor JPS. The authors concluded that the variable magnetic field indirectly inhibits the influence of reactive oxygen species in living organisms [32].

Our observation did not confirm the synergy effect when the magnetic field and the red light were used together. Probably, the path of the respiratory burst activation prevents evoking of the synergy effect by the variable magnetic field combined with red light.

It is well known that the red light as well as variable magnetic field is used singly as physiotherapy methods [12]. Phototherapy or the variable magnetic field could be applied in autoimmune and inflammatory diseases that are mediated in some part by neutrophils e.g. gout, rheumatoid arthritis, and also in a variety of airway diseases mediated by neutrophils infiltration such as: chronic obstructive pulmonary disease, bronchiectasis, certain forms of asthma etc. [19].

As it has been shown in our experiment, the combination of variable magnetic field and red light is not necessary to obtain higher decrease of hydrogen peroxide production by neutrophils. Both variable magnetic field and red light cause reduction of DCF fluorescence.

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