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Biophoton Radiations Induced by Hydrogen Peroxide in Mouse Liver Slices and Hepatocyte Nuclei in Relation to the Biophysical Action Mechanism of Reactive Oxygen Species

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ABSTRACT

Background: Although a large number of studies have confirmed that the different levels of reactive oxygen species (ROS) in cytoplasm and nucleus have effects on cell growth, proliferation, differentiation and apoptosis, the exact mechanism of ROS action is unclear. An important reason is that the production and degradation time of ROS in cells is very short, and therefore it's difficult to understand the mechanism of action based on the traditional molecular action process through the ROS diffusion and target binding. Methods: The fresh liver tissue slices were prepared and the nuclei of hepatocytes were separated from Kunming mice according to the reported method. Liver tissue slices and hepatocyte nuclei were perfused with extracellular or intracellular fluids containing different concentrations of hydrogen peroxide (H_2O_2), and real-time imaging monitoring of biophotonic emission was carried out using an ultra-weak biophoton imaging system. Results: The results showed that the continuous perfusion with different concentrations of H_2O_2 (300, 400 and 500 μM, respectively) resulted in significant increase of biophotonic emissions, presenting a concentration-dependent effect in liver tissue slices and achieving the maximum effect at 400 μM, while the significant enhancement was found after 500 μM treatment on the hepatocyte nuclei. Conclusion: This study suggests that ROS generated in cells may achieve its physiological and pathological effects via biophotonic emissions, which provides a new quantum biological mechanism of ROS, while the detailed clarification requires further research.

*Contributed equally to this work.

1. INTRODUCTION

When natural creatures face disease or harsh environment, the amount of reactive oxygen species (ROS) will be increasingly produced by the organism, including superoxide anion (O^{2-}), singlet oxygen molecule ($^1O_2$), hydroxyl radical (−OH), hydrogen peroxide (H_2O_2) and so on [1-3]. A large amount of evidence have demonstrated that abnormal or high concentrations of ROS are harmful to cells and the accumulated ROS form toxins that damage the plasma membrane, proteins and nucleic acids in cells [4-7], resulting in some diseases [8, 9]. However, more and more studies have found that the normal level of ROS produced in cells plays an important role in physiological activities, such as signal transduction [10-12], growth [13], Ca^{2+} signal pathway [14, 15] and regulation of redox sensitive gene expression [16, 17]. Despite its wide range of activities, the exact action mechanism of ROS is not clear and has remained many unanswered questions. For example, the generation and degradation of a ROS molecule is very fast and the half-value period of ROS is 400×10^{-6} ns, while the diffusion rate is not very special (55 - 3000 nm) [18]. Therefore, the question is: what kind of action mechanism does ROS play its functional activities? Here, we assume that ROS may play its role by radiating biophotons to change the quantum energy level of targeted molecules.

Almost all life, including microorganisms, plants, animals and human beings can spontaneously radiate extremely weak photon beam in the normal or pathological conditions. Such a phenomenon is known as biological ultra-weak photon emissions, or UPE (in short biophoton) [19-21]. It has been believed that biophoton, being a coherent electromagnetic field inside the cells, may be the base of cell-to-cell communication [22], which has been demonstrated in plants, bacteria and certain animal cells [23-24]. It has been suggested that biophoton may play a potential role in neural signal transmission, contributing to the understanding of the high functions of nervous system [25-27].

Therefore, in this study, we used mouse hepatocyte slices and isolated hepatocyte nuclei to observe the radiation characteristics of biophotons through direct perfusion with different concentrations of H_2O_2 combined with an ultraweak biophoton imaging system (UBIS), so as to provide direct and new evidence for clarifying the action mechanism of ROS.

2. MATERIAL AND METHODS

2.1. Experimental Animals

Adult Kunming male mice aged 7 - 12 weeks were purchased from Hubei Provincial Laboratory Animal Public Service Center (Wuhan, China) and housed under standard conditions (12 h light/dark cycle, room temperature 18˚C - 25˚C, 40% - 50% humidity) with access to food and water ad libitum. The protocols were approved by the committee on the Ethics of Animal Experiments of South-Central Minzu University.

2.2. Preparation of Liver Slices and Resuscitation

After general anesthesia was achieved the peritoneum and the liver were exposed via a midline laparotomy, and then the liver was separated and quickly excised and placed in ice-cold Krebs-Ringer bicarbonate (NaCl 6.92 g, KCl 0.35 g, $NaHCO_3$ 2.1 g, KH_2PO_3 0.16 g, $MgSO_4$ 0.29 g, Glucose 2.1 g, 3 mmol/L $CaCl_2$ 100 mL). The liver was then cut using a surgical blade into a few small pieces (approximately 8 mm × 4 mm × 4 mm), which was quickly placed on the sample support plate of a Vibratome (Leica VT1000S, Germany), and was then placed into a sectioning box filled with 0˚C - 4˚C Krebs-Ringer bicarbonate. The liver slices (700 μm thickness) were continuously cut. The slices were then transferred to Krebs-Ringer bicarbonate and incubated at room temperature (~23˚C) for 40 - 60 min, continuously bubbled with a mixture of 95% O^2 + 5% CO^2.

2.3. Hepatocyte Nuclei Isolation

The isolated liver tissue (1 g) was homogenized in 10 mL hypotonic buffer (10 mmol/L Tris-HCl, pH

7.5, 2 mmol/L $MgCl_2$, 3 mmol/L $CaCl_2$) on ice. The homogenate was filtered with a piece of gauze and sedimented at 1000 g for 10 minutes at 4˚C. The supernatant was completely replaced by 1 mL of the 22% iodixanol solution and the pellet was resuspended by pipetting up and down 15 times to ensure proper dissociation of the material. After that, 43% - 22% iodixanol gradient was prepared in centrifuge tubes. 22% iodixanol was carefully layered onto 1 mL of the 43% iodixanol solution, and the homogenate was added on top. This preparation was centrifuged at 10,000 g for 50 min at 4˚C and the nuclei accumulate between the 22% and the 43% iodixanol layer [28]. The interface of preparation was collected and diluted 1:2 with the intracellular solution ($NaHCO_3$ 10 mM, KCl 10 mM, KH_2PO_3 15 mM, K_2HPO_3 45 mM; pH = 7.2, osmotic pressure: 170 mOsm/L).

2.4. Perfusion of the Liver Slices and Hepatocyte Nuclei and Biophoton Imaging

An ultraweak biophoton imaging system (UBIS) was used to detect the biophotoic emission on liver slice and hepatocyte nuclei induced by differential concentrations of H_2O_2, and UBIS for detecting biophotonic emissions was described in detail in our previously studies [25, 27, 29]. The specific steps and data analysis methods were as follows:

1) Perfusion of the liver slice: The liver slice was transferred to a chamber, where it was submerged beneath continuously superfusing Krebs-Ringer bicarbonate (100 ml). A mixture of 95% O_2 + 5% CO_2 was constantly supplied. Perfusion maintenance was performed as demonstrated in a previous study [25].

2) Perfusion of the hepatocyte nuclei: The liver slice was transferred to a chamber, where it was submerged beneath continuously superfusing Krebs-Ringer bicarbonate (100 ml). A mixture of 95% O_2 + 5% CO_2 was constantly supplied. Perfusion maintenance was performed as demonstrated in a previous study [25].

3) There is no need for oxygenation during the perfusion of the hepatocyte nuclei and imaging. The hepatocyte nuclei precipitation was diluted to 5×10^7 pieces/ ml. 20 μL precipitation containing about 1×10^6 hepatocyte nuclei was placed in three small grooves in the perfusion chamber, and then were perfused with intracellular solution mentioned above and imaged in a similar way.

4) Image acquisition: Real-time imaging of the liver slice and the hepatocyte nuclei precipitation was carried out with EM-CCD in a dark environment. The main setup parameters for the EM-CCD camera during imaging were 1200 × gain, 1 × 1 binning, and photon detection Model 3.

5) Hydrogen peroxide (H_2O_2) treatment: Samples were preperfused with perfusion solutions for 100 min to eliminating the delayed luminescence of EM-CCD and liver slices and hepatocyte nuclei, then they were perfused in turn with 300 μM, 400 μM and 500 μM H_2O_2, of each concentration was maintained for 1 h.

6) Image processing, data extraction and analysis: Image processing was carried out according to our previous study [25]. The data of delayed luminescence period in the first 80 min were removed, and the data of 20 min before the first H_2O_2 perfusion were used as the basic biophoton radiation value. The average gray values (AGVs) of the processed biophoton gray images in the regions of interest (ROIs) and the background gray values (BGVs) from a part of the non-sample region were extracted. Both of these data were exported to Microsoft Excel for further analysis. The relative gray values (RGVs) of the brain slices were calculated and defined as:

$$\text{RGVs} = \text{AGVs (slice area)} - \text{AGV (background area)}.$$

2.5. Data Analysis

Data were analyzed using Graphpad prism v 8.0. For multiple group comparisons and to determine treatment effects, one-way ANOVA post hoc Tukey's multiple comparison tests were used to determine statistical significance. For comparisons between two groups, either unpaired or paired Student's t test (two tailed) was performed to determine statistical significance. $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1. Hydrogen Peroxide Induced Biophoton Emission in Liver Slices

In general, induced by different concentrations of hydrogen peroxide solution, the biophoton radiation of liver slices gradually increased with the increase of concentration. However, in the action period of each concentration, there is a trend of rapid rise and then slow decline (Figure 1(a), Figure 1(b)). Application 300 μM, there was a significant difference between hydrogen peroxide and biophoton radiation, which increased significantly. Compared with the basic biophoton radiation (BE) without hydrogen peroxide perfusion, the average increase in the first 10 minutes increased by about 41.1% (41.1 ± 6.3, n = 6, P < 0.01), then decreased and stabilized after 30 minutes. Application 400 μM hydrogen peroxide, on the basis of the biophoton radiation at stable period after the action of 300 μM, increased significantly again, showed very obvious in the first 20 minutes, and then decreased slowly and tended to be stable. Different from the previous two concentrations, applied 500 μM after hydrogen peroxide, biophoton radiation only increased slowly in the first 30 minutes, and then decreased to a stable level.

3.2. Hydrogen Peroxide Induced Biophoton Emission in Hepatocyte Nuclei

Application 300 μM hydrogen peroxide, the biophoton radiation of liver nucleus increased slowly until application 400 μM hydrogen peroxide before, but applied 400 μM hydrogen peroxide after, the biophoton

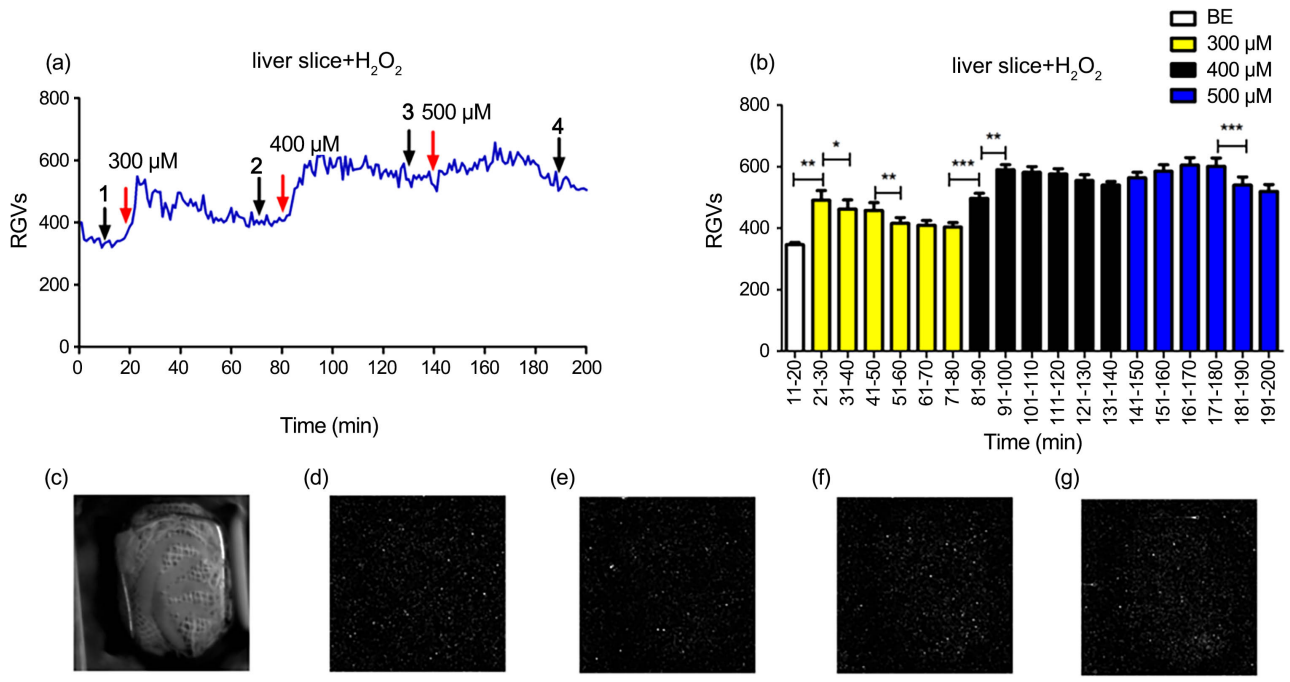

Figure 1. Hydrogen peroxide induced biophoton emission in liver slices. (a) A representative experiment, showing the dynamic changes in biophoton emission in mouse liver slices induced by different concentrations of hydrogen peroxide. The red arrows refer to time point (20, 80 and 140 min, respective) after perfusion of different concentrations of hydrogen peroxide (300, 400 and 500 μM, respectively). (b) Histogram of RGVs, showing average RGVs of every 10 min after perfusion of hydrogen peroxide and 20 min basic emission (be) (n = 6). (c) The location photo of a liver slice. (d)-(g). The representative images at different time points (10, 70, 130 and 190 min, respectively) marked by the black arrow in A. The value is expressed as ± s..e.m. *p < 0.05, **p < 0.01, ***p < 0.001.

radiation of liver nucleus did not increase significantly, and it remained stable to 500 μM hydrogen peroxide before. But application 500 μM hydrogen peroxide after, the biophoton radiation of liver nucleus increased rapidly in the first 20 minutes, and this upward trend did not stabilize until 50 minutes after perfusion (Figure 2).

4. DISCUSSION

ROS play important roles in the cytoplasm and nucleus, involving in regulating multi-level signal pathways from cell membrane to nucleus [30]. A large number of studies have shown that H_2O_2 can increase the concentration of Ca^{2+}. In the cytoplasm of hepatocytes or pulmonary artery endothelial cells, H_2O_2 can induce the accumulation of oxidized glutathione (GSSG) and increase Ca^{2+} concentration [31, 32]. There was evidence that the stimulation of vascular smooth muscle cells with 300 μM H_2O_2 led to the continuous increase of Ca^{2+} concentration in cytosolic [33]. The treatment of cultured rat fetal cortical neurons with 1 mM H_2O_2 can promote the influx of extracellular Ca^{2+} [34] and increase the concentration of intracellular Ca^{2+}. Such a change was observed in rat dentate gyrus granulosa cells treated with 1 - 10 μM H_2O_2 [35]. A small amount of H_2O_2 can induce the activation of proline peptidase (PLD) in mouse embryonic fibroblasts [36], and exogenous H_2O_2 induces tyrosine phosphorylation of T-lymphocyte growth factor receptor [37]. H_2O_2 has a bidirectional regulation effect on protuberance growth for cultured

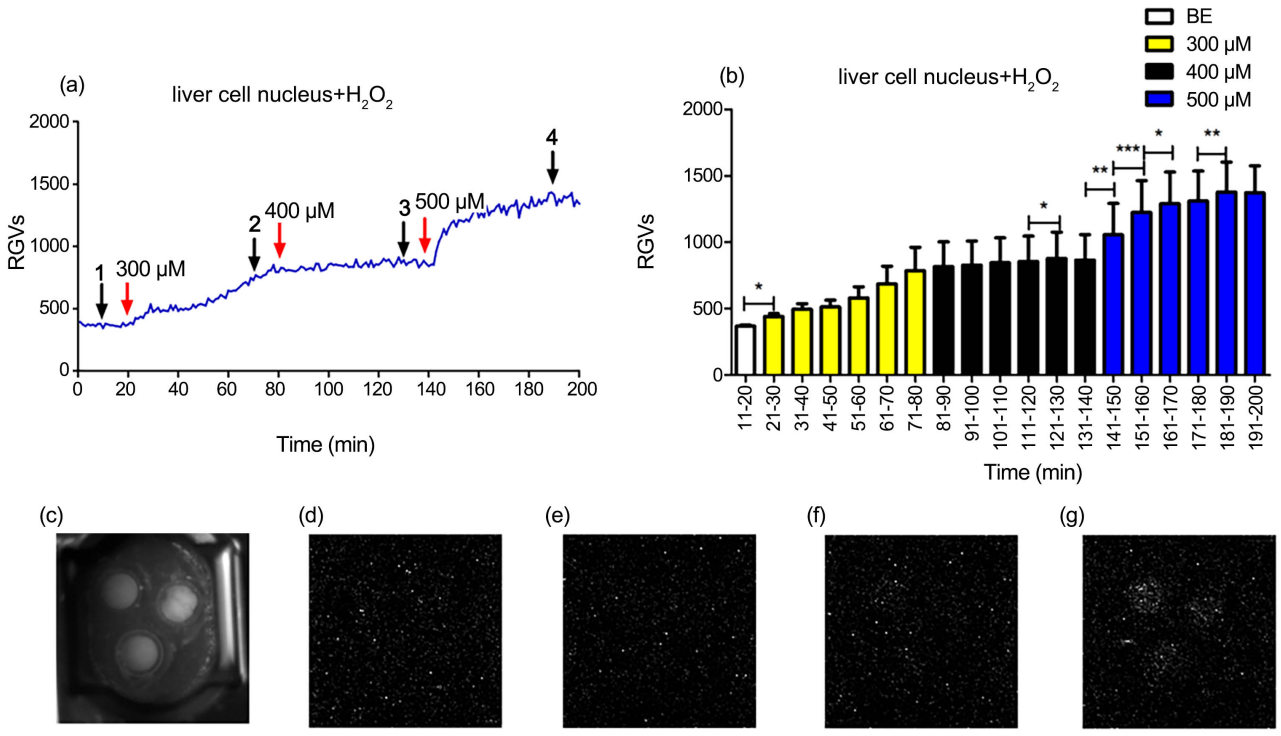

Figure 2. Hydrogen peroxide induced biophoton emission in hepatocyte nuclei. (a) A representative experiment, showing the dynamic changes in biophoton emission in mouse hepatocyte nuclei induced by different concentrations of hydrogen peroxide. The red arrows refer to time point (20, 80 and 140 min, respective) after perfusion of different concentrations of hydrogen peroxide (300, 400 and 500 μM, respectively). (b) Histogram of RGVs, showing average RGVs of every 10 min after perfusion of hydrogen peroxide and 20 min basic emission (be) (n = 6). (c) The location photo of a liver slice. (d)-(g). The representative images at different time points (10, 70, 130 and 190 min, respectively) marked by the black arrow in A. The value is expressed as ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001.

hippocampal neurons [38, 39], which has obvious relationship with the concentrations of H_2O_2. In other studies, it was found that the low level concentration of H_2O_2 can promote the growth of neuronal processes, while the high level concentration of H_2O_2 could produce inhibitory effect and result in significant morphological changes in hippocampal CA1 neurons, such as swelling, disintegration and disappearance of neurites [40]. Many studies have shown that ROS can destroy the nuclear structure such as DNA breakage, which can lead to chromosomal mutations and aberrations related to cell death and cancer [41-43]. During a reaction system of hepatocyte nucleus *in vitro*, H_2O_2 can inhibit the activity of nucleoside triphosphatase (NTPase) on the membrane of liver nucleus in a concentration-dependent manner [44, 45]. In addition, the redox state of the DNA binding domain cys may also directly regulate gene expression [16, 17], and is considered to be the target of ROS.

Although a large number of studies as demonstrated above have shown that ROS affect cell growth, proliferation, differentiation and apoptosis [11, 13, 46] through different levels in cytoplasm or nucleus, the exact mechanism is unclear. In the present study, using different concentrations of H_2O_2 to act on liver tissue slices and hepatocyte nuclei, we found that H_2O_2 can induce biophoton emissions in a concentration-dependent manner. H_2O_2 at low and medium levels of concentrations (300 μM and 400 μM) can lead to the rapid increase of biophoton emissions in liver tissue slices, and then maintain stability after a slow decrease. However, such effects were only noticed at high level concentrations (500 μM) in hepatocyte nucleus since the application of the H_2O_2 at low and medium levels of concentrations (300 μM and 400 μM) resulted in a slow increase of biophoton emissions. Because liver tissue slices contain both cytoplasmic and nuclear contents, the pattern of biophoton emissions after application of H_2O_2 on liver tissue slices may be a comprehensive result of the effect on cytoplasm and nucleus, especially at the high concentration level.

Although there are differences in biophoton activity patterns caused by the different concentrations of H_2O_2 on liver slices and hepatocyte nuclei, a common feature is that H_2O_2 can lead to significant biophoton emissions. Combining the effect that different levels of ROS in cytoplasm and nucleus have effects on the state of cell, we assume that ROS change the energy level of targeted molecules by radiating biophotons. The most important reason is that the time of intracellular ROS generation and degradation is very short [18], which ROS's traditional action mode through the diffusion process of ROS molecules and the combination of targets can't explain its mechanism. Therefore, this study provides a new quantum biological mechanism of reactive oxygen species, while detailed clarification requires further scientific research.

Combined with the above-mentioned effects of ROS on cell growth, proliferation, differentiation and apoptosis, we could speculate that ROS may achieve its effects by radiating biophotons. An important reason for such an explanation is that the generation and degradation time of ROS in cells is very short, and the action effect cannot be understood if it is based on the traditional action ways through the molecular diffusion and target binding. Therefore, the findings in this study may provide a new quantum biological mechanism of ROS action, but the detailed elucidation needs more in-depth research.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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