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Biophoton emission of MDCK cell with Hydrogen Peroxide and 60 Hz AC magnetic field

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Abstract: *We studied biophoton characteristics of Madin-Darby canine kidney (MDCK) cells under the influence of H_2O_2 by employing a photomultiplier tube (PMT) and a fluorescence microscope. H_2O_2 was used for producing reactive oxygen species (ROS) in the measurement. Images from a fluorescence microscope show an increase of photon intensity emitted from the sample due to H_2O_2. By using a PMT we measured quantitative change in biophoton emission with application of H_2O_2 to the MDCK cell culture, found that the increase of the biophoton is dependent upon the amount of H_2O_2. The agreement between the results of the PMT and the fluorescence microscope suggests the possibility of quantitative measurement of the influence of ROS on living tissue or cell. In addition we applied a 60 Hz AC magnetic field on the cells to investigate the change in reaction between MDCK cell and ROS. It showed that a decay of chemiluminescence intensity has taken a different path following exposure to the magnetic field. As a result, the PMT measurement might be considered as a useful tool for studying biochemical characteristics in relation to ROS.*

Key words: *Biophoton, MDCK, ROS, PMT, 60 Hz magnetic field*
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Introduction

Photon emission from living organism has been studied extensively since it was introduced by Gurwitsch (Gurwitsch, 1922). Due to the nature of extremely low intensity and poor photon detection technology, however, it has only been intensely studied in the last twenty years (Popp, 1979; Inaba *et al.*, 1979; Quickenden and Que, 1976). It has been known that the intensity of biophoton from living organism is about 10^{-16} W/cm^2 and the spectral range is from UV to NIR of 200 - 800 nm (Popp, 1979; Kobayashi *et al.*, 1999a, b). The origin of the biophoton emission has been debated since its introduction and currently there are two different mechanisms suggested. One mechanism is based on coherent field theory (Popp, 1979; Popp *et al.*, 2002). Biophoton is spontaneous photon emission that could be characterized as extremely low intensity and is stable without external photon or chemical stimulus. The origin of the coherent biophoton is considered to be DNA in the cells (Popp *et al.*, 2002). The other mechanism for the origin of the biophoton emission, suggests that biophoton emission is related to chemical reaction in living organism (Kobayashi *et al.*, 1999a, b). Accordingly biophoton emission from living organism is mostly originated from nothing but biochemical reaction mechanism, and can be categorized as ultra low intensity chemiluminescence (CL). Inaba *et al.* (Inaba *et al.*,1979) claimed that the main source of the CL is reactive oxygen species (ROS) related metabolic activity in living organisms. Photons are emitted by radical reaction with ROS and intracellular substances, implying that biophoton can be an appropriate candidate to study on the role of ROS in living cells (Bagchi *et al.*, 1995). Recently, it has been widely accepted that oxidative stress caused by ROS plays an important role in metabolism of living organism. H_2O_2 has a tendency of attracting additional electron so that it becomes hydroxyl radical and hydroxyl ion, HO^-. HO radical is highly active and initiates chain reactions (Vladimir, 2001). Radical chain reactions are known to damage crucial biological molecules *in vitro* and reactive oxygen species are considered as considerably harmful substances (Fridovich, 1998). It has been believed that ROS is responsible for a primary cause of initiating many diseases.

In spite of the controversy as mentioned above, most researchers agree that the biophotonic characteristics reflect the status of a living organism. Chemical reaction and change in the state of DNAs in the living organism can be studied by detection of biophoton and with the same fashion, the photon counting devices such as photomultiplier tube (PMT) and charge coupled device (CCD) can be utilized for understanding the nature of living organism. And biophoton measurement leads us to have a new tool for investigation of the metabolism pathways (Cadenas and Sies, 1985). Plant stress has been studied for environmental issue and chemiluminescence has been utilized for this study (Tetsuo *et al.*, 2000). Seed quality also can be determined by biophoton assay (Yan *et al.*, 2002).

Recently, the study of electromagnetic field effect on human has gained importance in scientific society due to its practical aspects (Goodman and Blank, 2002: Adair, 1999; Ghione *et al.*, 2004). The frequency range of most of the regulatory systems in human body is in the vicinity of 1 - 300 Hz. It is interesting to know, that the frequency range of living organism regulatory systems and that of regular electricity, which has AC characteristics of 50 to 60 Hz, are similar (Chacce *et al.*, 1979). The coincidence between the frequency range of ordinary electricity and body's regulatory system makes extremely low frequency (ELF) study crucial due to its significance in environmental safety. In our experiments, we used 60 Hz AC magnetic field for inquiring biological change of the MDCK cell and response with ROS to examine metabolism change induced by the magnetic field (Li *et al.*, 1997).

In this paper, we introduce biophotonic behavior of MDCK cells, detected by a PMT and a fluorescence microscope for the first time. In order to examine the ROS effect on the MDCK cell, we applied H_2O_2 and measured the biophoton from chemiluminescence (CL) as a function of the concentration of H_2O_2 with and without exposure to 60 Hz AC magnetic field.

Materials and Methods

MDCK kidney cell preparation: We used Madin-Darby canine kidney MDCK cells prepared by Department of Molecular Biology, Medical College, KyungHee University, Seoul, Korea (Yoo, 2004; Yoo and Shin, 2004). The cell lasts relatively long and has a characteristic of emitting more photons than other cells so that it is suitable for biophoton measurement. Culture medium for the cell was dulbeccos minimum essential medium (DMEM) (Gibco, Rockville, MD, USA) mixed with 3.7 g/l of $NaHCO_3$, 10% of fetal bovine serum (FBS) and 1% of antibiotics (penicillin + streptomycin). The cells were cultivated in a CO_2 incubator at 37°C. Cell count was done with a counting chamber (Neubauer Improved, Marienfeld, Germany). 10^4 of Kidney cells were planted on 12 well plates respectively. All cells were cultured in DMEM mixed with 10% of FBS and 1% of the antibiotics for 24 hr. They were cleaned with 3 ml of phosphate-buffered saline (PBS) and 1 ml of 2x trypsin were added before starting cultivation for 5 min at 37°C again. After harvested, they were transferred to tubes for centrifugation at 3000 rpm for 10 min at room temperature. Pellets were then suspended in DMEM for cell counting. After a cover slip was placed at the bottom of a well dish, cell culture was done at 37°Cfor 24 hr in the CO_2 incubator. For 2D imaging with a fluorescence microscope, staining process was performed. In order to stain nucleus of the cells selectively, Hoechst 33342 was used for staining. Once the media was changed, 10 μM of the dye was applied and cultivated the cells at 37°C for 15 minutes in the CO_2 incubator for staining. After removing the media from the well dish completely, the cover glass was cleaned 3 times with 1x PBS for 2 minutes. Then the glass was removed from the dish to be placed at a slide glass in mounting solution for observation with a fluorescence microscope (Nikon, Japan).

Optical measurement apparatus: In our experiments, CR120 head-on PMT (Hamamatsu, China) was employed to measure the amount of biophoton emitted from MDCK kidney cell. This detector has its maximum response at 400 nm with the quantum efficiency of 27.4% in a detection range of 300 nm to 650 nm. The rise time of the PMT is about 7 ns. The PMT operates at room temperature without a forced cooling system. Output from the PMT is connected to a signal analyzer which is linked with a PC. We used software and a photon counting board provided by the manufacturer. The dark count at room temperature is about 19 counts per second (cps) at 850 V. The detector is always stored in a dark room to maintain its optimum performance. No additional optic system was used to collect biophoton emitted from samples so that the collection efficiency for our PMT set up is about 20%. A shutter is installed between a sample holder and the PMT to control light irradiation to the PMT. In this way, we can prevent unnecessary exposure of the PMT to any light and execute more reliable experiment. We also put the PMT and sample holder only inside of the dark chamber for dark storage and rest of electronic setup was located outside of the chamber. Dark chamber is designed for maintaining constant humidity and temperature. Atmosphere inside the chamber sets to the same level as ordinary room-air environment. The sample is placed in a 2 mm-thickness quartz cuvette for PMT measurement. The cuvette is mounted on a plate located in a dark chamber. Plate temperature is maintained at 37 ± 0.1°C by a water circulation system connected to a water bath. The reason for not using electrical temperature controller is to prevent unwanted electromagnetic field effects, if any, during the experiment. We employed H_2O_2 (Junsei Chem. 30%, Japan)) to generate reactive oxygen species for the experiments. The concentration of H_2O_2 used was 0 mM, 1 mM, 2 mM, 3 mM and 4 mM respectively. In order to inject H_2O_2 to the sample, a syringe was used while darkroom condition was not interrupted. Cell count was made with 6 ml of cells just before PMT measurement. For magnetic field dependent measurement, we used a laboratory-made Helmholtz coil 60 Hz AC magnetic field generator. 14 G was measured at the sample position. Schematic of our experimental setup is depicted in Fig. 1. For 2D image, the fluorescence microscope

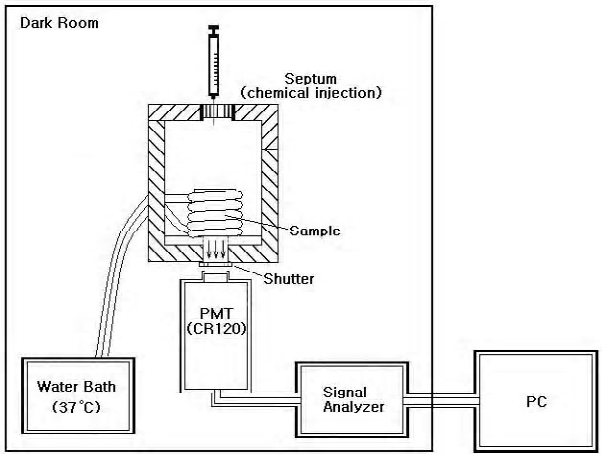

Fig. 1: Schematics of experimental setup

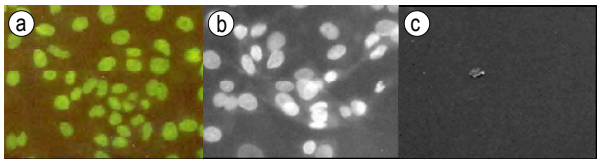

Fig. 2: Obtained from the fluorescence microscope observation, of MDCK cells without, 600 μM, and 800 μM H_2O_2 respectively. For observation, we used Hoechst 33342 as dye to stain nucleus of the cells. Bright cells in Fig. 2(b) seem to radiate more light than grey cells, meaning that chemiluminescence is triggered by ROS and the cells are actually dying

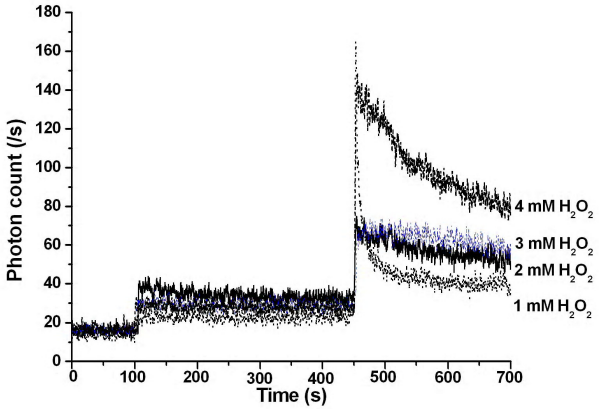

Fig. 3: MDCK cells were treated with various concentration of 1 ml H_2O_2. Three different regions are shown clearly. Dark count is responsible for the first region. The second one could be resulted from spontaneous photon emission or just system background. Interpretation of the data at the third region is mostly due to the chemiluminescence by the reaction between H_2O_2 and the MDCK cells. Magnitude of a peak at the beginning of the region depends on the concentration of H_2O_2 applied

was used. The microscope utilizes the sample characteristic that emits light when it is irradiated with a specific wavelength.

Results and Discussion

We observed the effects of H_2O_2 on MDCK cells from 2D images obtained by a fluorescence microscope. In Fig. 2(a), the cells radiate photons with almost same intensity and there are no apparent bright cells among normal MDCK cells. With an increase in the concentration of H_2O_2 to 600 μM, the total number of the living cells decreased whereas that of bright cells increased as shown in Fig. 2 (b). As the amount of H_2O_2 increased up to 800 μM, there was no biophoton emission recorded from the sample, implying that no cells survived.

Fig. 2 (a), (b) and (c) apparently show that application of H_2O_2 to the cells cause the death. It is worthy pointing out that photon radiation increased as the cells and H_2O_2 were reacting. More than a critical amount of ROS such as H_2O_2 seriously delivers oxidative stress to the cells and leads to their death. The reason for emitting more biophoton with H_2O_2 is that radical reaction with H_2O_2 could generate excited species for photon emission (Kobayashi *et al.*, 1999b; Karpinski *et al.*, 1999; Miller *et al.,* 2005). Even though

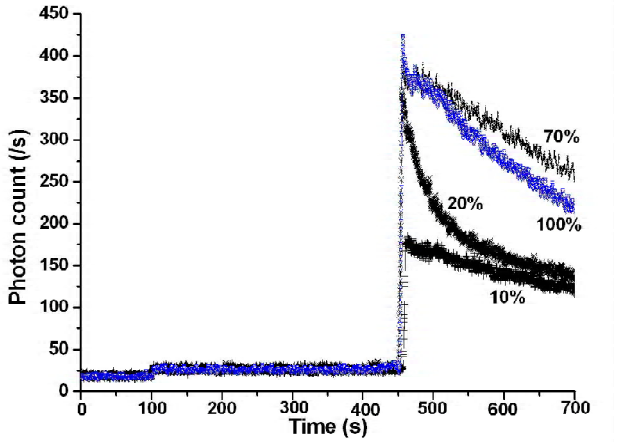

Fig. 4: The concentration dependency of MDCK cell is plotted as a function of time. Fir this measurement, 1 ml. of 4 mM H_2O_2 and the relative cell concentration of 10%, 40%, 70% and 100% were used. The figure clearly depicts the concentration dependency of chemiluminescence intensity. As the concentration of the cells increases, the relative change of the intensity diminishes and intensities from cell concentrations of 70% and 100% are almost overlapped. The chemiluminescence intensity with 10% concentration is almost half of that of 100% concentration and it suggested that results with the concentration lower than 10% could be severely affected by the cell concentration. Therefore, in order to have meaningful results from experiments similar to ours, care chould be taken to use proper MDCK cell concentration

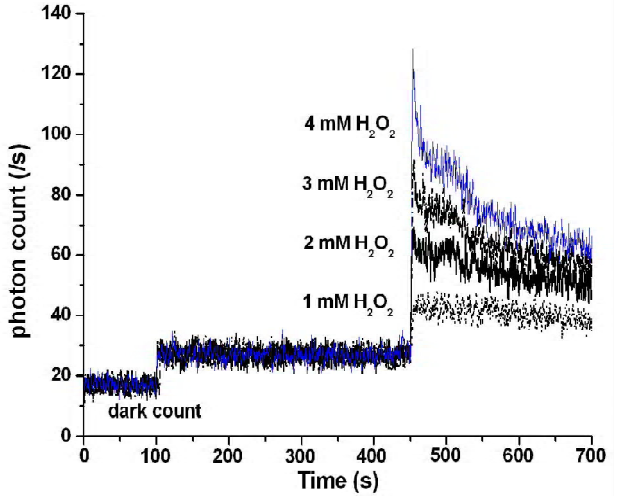

Fig. 5: Exactly same experiment as shown in Fig 3 except that it was exposed with a 60 Hz AC magnetic field. Even though overall decay behavior is similar to the results from the unexposed measurements, the detail of the decay curves is different

the 2D images can support the increase in the biophoton intensity, a quantitative measurement of the biophoton is necessary to elucidate the role of ROS in the cells more clearly.

In order to measure the number of biophoton from the cells, we employed a PMT which is an ultra sensitive device for photon counting. It might be emphasized that the amount of photons from each cell is not directly connected to the intensities of photons measured by the PMT because the staining process was done for the fluorescence measurement only. The intensity of biophoton from

the cells was probed as a function of H_2O_2 applied, similar to the experiment using a fluorescence microscope. The results are shown in Fig. 3 and plotted as a function of time and H_2O_2 concentration. Three different regions are seen in Fig. 3. In the first region, a small value of 10 cps reflects that the dark count of the PMT is low enough to detect biophoton for our experiments. Data in the second region is obtained before exposure of the kidney cells to H_2O_2 after dark count of the PMT system was acquired. The level of the signal is around 30 cps and is 3 times higher than the dark count. It might indicate that intrinsic photon emission from the cells is occurring without external stimulus. However, it is not clear whether the data in the second region exhibits spontaneous photon emission or not. The third region displays the effect of H_2O_2 on the cells. As H_2O_2 was injected into the cells, an abrupt increase in biophoton intensity was observed. It tells that radiation of photons is involved in the reaction between H_2O_2 and the cells.

As shown in Fig. 3, the amount of biophoton increases as a function of H_2O_2. This result is in good agreement with the previous results from the microscope observation. After a sudden increase in biophoton, the amount coming from the cells diminishes as a function of time, showing decay. However we did not notice 100% mortality of the cells in our experimental conditions. The reason could be related to different sample treatments for PMT and microscope observation. Since the medium was always employed for MDCK cells experiment, it is quite possible that the results are affected by the presence of the medium as well as the cells. In order to clarify this ambiguity, we measured the concentration dependence of MDCK cells by applying 1 ml of 4 mM H_2O_2. The cell concentration was varied from 10% to 100% relatively and 100% was equivalent to 5 x 10^7 /ml. The amount of DMEM was adjusted for a certain concentration so that total amount of cell and DMEM was 2 3. With an increase in cell concentration, the biophoton emission was augmented as shown in Fig 4. However the relative change in the amount of the biophoton decreased as the concentration increased, showing saturation. The intensities of biophoton emitted from 70% MDCK cell and 100% MDCK cell were of the almost same level. According to this result, we conjecture that the saturation is related to the ratio of H_2O_2 and MDCK cell and the reaction rate between them is limited by the amount of H_2O_2 applied. As a passing remark, we notice that the photon intensity at the first region of Fig. 4 has no apparent concentration dependency. The measured photons came mostly from a quartz cuvette.

For further study, we exposed the MDCK kidney cell to 60 Hz AC magnetic field whose intensity was 14 Gauss. Exposure timewas varied from 2 hr to 3 hr 40 min due to the limitation of the experimental setup. The magnetic field, irrespective of AC or DC, affects the metabolism of a living organism and therefore, changes in the ROS effect on the cells of our experiment are expected (Goodman and Blank, 2002; Kremer *et al.*, 1988). The results are shown in Fig. 5.

The intensity in the first region has no field dependency, which is attributed from the cuvette effect as mentioned earlier. Fig.

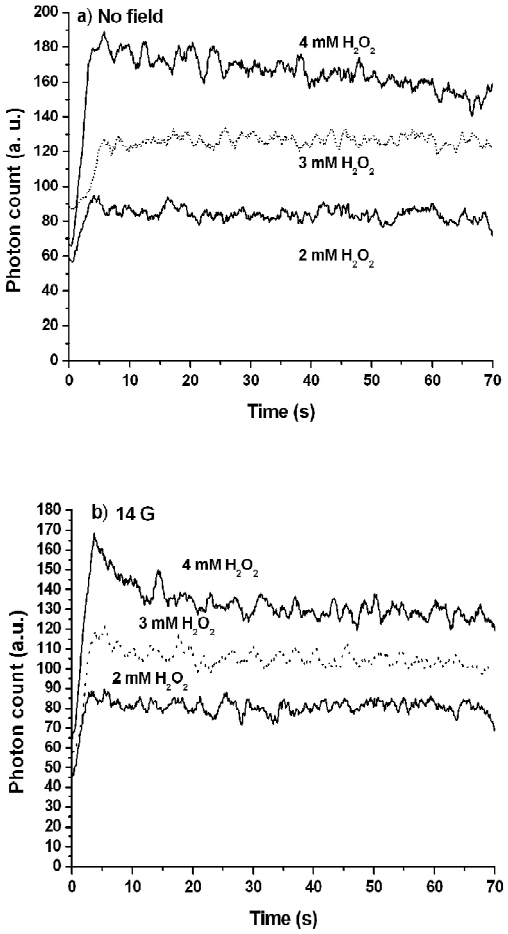

Fig. 6: Decay curve comparison between the unexposed and the exposed MDCK cells to 60 Hz AC magnetic field. Fig. 6(a) represents decay behaviors of MDCK cells in the absence of the magnetic field and Fig. 6(b) does at the presence of 14 G. Curves from the field exposed cells show a peak at the initial stage whereas those from the unexposed one does not. This result implies that the overall CL intensities obtained from two conditions are similar but the actual decay mechanism is affected by the magnetic field. The decay behavior in the time range of few seconds is also important in order to understand the magnetic field effect on ROS mechanism

6(a) represents decay behaviors of MDCK cells in the absence of the magnetic field and Fig. 6(b) does at the presence of 14 G. The overall decay characteristics are similar to those without exposure to magnetic field. The intensity levels also seem to be comparable with the previous experiments irrespective of H_2O_2 concentration, indicating that there is no apparent change occurred by the AC magnetic field of 60 Hz. However, the decay patterns in detail are somewhat different and the trend is more apparent as the concentration of H_2O_2 increases.

Fig. 6(a) represents decay behaviors of MDCK cells in the absence of the magnetic field and Fig. 6(b) does at the presence of 14 G. Fig. 6(a) represents no particular structure in the decay

pattern. But magnetic field exposed one as shown in Fig. 6(b) has a peak at the initial stage of the decay and it becomes more apparent with an increase in the H_2O_2 concentration. The result from the highest concentration shows the most eminent sharp peak in Fig. 6(b). Time scale for the reaction responsible for the peak is about few seconds and rest of the reaction occurs gradually, showing a long tail. The decay trend after the initial stage also seems to be different from the previous experiments shown in Fig. 3. From the experiment, we have seen the decay curve that is affected by 60 Hz AC magnetic field of 14 Gauss. However we don't have enough data to present a model to explain the decay behavior. According to various researches done by other groups, the decay might be affected by frequency, intensity, duration, and waveform (Goodman and Blank, 2002; Engstrom, 1997).

MDCK cells are easily treated with chemicals and are sensitive to light. Hence, PMT application to the light radiating cells like MDCK cells could open new approach in biological study because it can directly measure the amount of photon emission at any stage of the biological reaction and easy to interpret. If we can manipulate MDCK cells to change their emission rate of light by chemical treatment, much important information relating cancer or other biological system could be acquired almost in real time. For magnetic field experiment, we have seen a peak at the initial stage of decay, which is more or less meaningful. Further study will be focused on magnetic field dependency of mutation of DNA, brain physiology, transformation of protein under the influence of ROS in order to take advantage of the PMT. As mentioned at the introduction of this article, biophoton characteristic of a living organism reflects its state without any destructive manners. And our method is more effective for light subjecting a microscopic one-celled alga such as *Alexandrium tamarense* that causes toxic red tide. As light condition is crucial for their living, biophoton emission behavior of them after illumination with external light might tell us their environmental behavior. We have executed several preliminary tests on the *Alexandrium tamarense* for this purpose. For plant stress, it is known that plants can be photodamaged by prolonged excess light by generating ROS (Karpinski *et al*., 1999). Therefore our method can be applied to ROS relating stress on plants also.

Conclusively, we studied H_2O_2-initiated biophoton characteristics of Madin-Darby canine kidney (MDCK) cells with a photomultiplier tube and a fluorescence microscope. Results of photon counting by the PMT and optical image by the fluorescence microscope were in good agreement. The biophoton intensity from the cells was dependent upon H_2O_2 applied. With a constant H_2O_2 concentration, biophoton was proportional to the cell concentration but the increase rate in biophoton intensity was decreased with the concentration. We conjectured that this saturation is relevant to the ratio of H_2O_2 and MDCK cells and their reaction was limited by H_2O_2 applied. In addition, we applied a 60 Hz AC magnetic field on the cells to investigate the change in reaction between MDCK cell and ROS. We found a sharp peak at the initial stage of the decay from cells exposed to the magnetic field. It seemed that a decay of chemiluminescence intensity has taken a different path following exposure to the magnetic field. The origin of the sharp peak was not explained. Further study is required for clarifying the mechanism responsible for the decay.

The agreement between the results of the PMT and the fluorescence microscope suggests the possibility of quantitative measurement of the influence of ROS on living tissue or cell. Therefore a PMT can be used as an useful tool to study biochemical characteristic relating ROS. This methodology can be applied to assess environmental effects relating plant and red tide issue.

Acknowledgments

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