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A new technique for evaluating reactive oxygen species generation

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ABSTRACT

Reactive oxygen species (ROS) have a very short life span and exist in extremely low concentration. ROS measurement is a challenging subject for researchers. Due to their high reactivity, ROS are claimed to be involved in many toxic events of the body. When ROS are overproduced or when the levels of antioxidants become severely depleted, these reactive species become highly harmful, causing oxidative stress through the oxidation of biomolecules, leading to cellular damage that may become irreversibile and can cause cell death. Fluorescent probes are excellent sensors of ROS due to their high sensitivity and simplicity in data collection. This study describes a method that may be used in future biomedical investigation to measure the rate of intracellular ROS production and not only their concentration at a particular moment in time.

Ke words: *ROS, 2'-7' diclorfloresceină (DCF), 2',7'-Dichlorofluorescein diacetate (DCF-DA), N-acetil-L-cisteina (NAC).*

1. INTRODUCTION

ROS are any oxygen-containing compounds that are particularly reactive. Some ROS are free radicals where an atom has one or more unpaired electrons in its outer orbital, making it particularly reactive. The best known free radicals in the body are oxygen-based (although other atoms can also exist as free radicals) and are generated as by products of oxidative metabolism.

ROS production can be determined with the help of different methods with different specificity and sensibility. The most used methods are the ones based on the interaction of chemicals with reactive oxygen species consecutive fluorescence emission. In this case the signal quantitative assessment is done using a spectrofluorometer. Chemiluminescent and fluorescent probes are the most commonly used for ROS detection probes [2, 3].

These probes show high sensitivity, simplicity, and reproducibility, but they only show ROS concentration in a particular moment in time[1]. This study aims develop a modified protocol to detect the *rate* of ROS production in response to oxidative stress while the classical methods () assess the ROS concentration at a particular moment of time. 2',7'-Dichlorofluorescein (DCF) is currently widely used and is suitable for measuring total ROS in living cells or tissues [4]. 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) is a non-fluorescent lipophilic probe that can cross the cell membrane.

Inside the cell, $DCFH_2$-DA deacetylates to form 2',7'-Dichlorodihydrofluorescein ($DCFH_2$) which is also a non-fluorescent probe but can't diffuse freely across the cell membrane. $DCFH_2$ reacts with intracellular ROS to yield the highly fluorescent DCF. $DCFH_2$-DA is widely used to evaluate 'cellular oxidative stress'. After passing through the plasma membrane, this lipophilic and non-fluorescent compound is de-esterified to a hydrophilic alcohol $DCFH_2$ that may be oxidized to fluorescent DCF by a process usually considered to involve ROS [5]. We have used tert-butyl hydroperoxide (tBHP) for ROS generation.

The oxidative stress induced by tert-butyl hydroperoxide (tBHP) in increasing concentrations was assessed. Tert-butyl hydroperoxide releases free radicals in a slow and steady maner, which allows its use for longer periods of time.

It is well known that the exposition to certain noxious factors, such as infectious agents, pollution, UV light, radiation and cigarette smoke, may lead to the production of ROS [6-8]. tBHP is a powerful agent of lipid peroxidation which affects the stability of the living membranes.

To avoid an excess of free radicals, the cell antioxidants are trying to keep their production and consumption balance. An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Insufficient levels of antioxidants, or inhibition of the antioxidant enzymes, cause oxidative stress and may damage or kill cells. Oxidative stress is a damage to cell structure and function by overly reactive oxygen-containing molecules and chronic excessive inflammation.

Oxidative stress seems to play a significant role in many human diseases, including cancers. The use of antioxidants in pharmacology is intensively studied, particularly as treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases. For this study N-Acetyl-L-cysteine (NAC) was used as antioxidant. Acetyl cysteine is a derivative of cysteine where an acetyl group is attached to the nitrogen atom. This compound is sold as a dietary supplement commonly

claiming to have antioxidant and liver protecting effects. It is used as a cough medicine because it breaks disulphide bonds in mucus and liquefies it, making it easier to cough up [9, 10]. This study is performed in solution and not on cell culture since its purpose is to develop a new technique for evaluating reactive oxygen species to be later applied on cell culture.

2. MATERIALS AND METHODS

2.1. Chemicals.

This study uses 2',7'-Dichlorofluorescein diacetate (DCF-DA), Dichlorodihydrofluorescein ($DCFH_2$), 2',7'-Dichlorofluorescein (DCF), N-Acetyl-L-cysteine (NAC); they were obtained from Sigma, Aldrich and tert-butyl hydroperoxide (tBHP) was purchased from Sigma Chemical (St. Louis, MO). The chemical formulas of tBHP, DCF, DCF-DA and NAC are presented in Fig. 1, 2, 3 and 4.t-BHP was used in concentration written in table 5 and in volum written in table 4. N-Acetyl-L-cysteine was used in the quantitis presented in table 4, having the concentration of 0.8 mM. DCF hydrolysis has been conducting according to the method presented by Taiji Takanashi and respectively: 0.5 ml DCF-DA was mixed with 2 ml NaOH 0.01N and was kept 30 minutes at room temperature (the solution is kept at 4^0C). The oxidative stress was induced using t-BHP. DCF-H_2 fluorescence spectra were recorded using a Horiba Jobin Yvon Fluorolog-3 spectrofluorometer (λex=495nm and λem=529nm) at a constant temperature of 37^0C. The phosphate buffer solution (PBS) was introduced in a cuvette with glucose and trypsin, using the volumes from table 1. After 1 minute DCF-H_2 is added, followed by t-BHP after 4 minutes and by NAC after another 5 minutes The volumes used are given in table 4.

3. RESULTS AND DISCUSSION

A typical example of DCF-H_2 emission spectra recorded at 37^0C is given in Fig. 6. Due to the fact that in the nearest future the research team intends to extend the study on cell culture trypsin was added to the solution on the study. Trypsin is normally used to detach cells in Petri dish. The latter is used to transfer the detached cells into another recipients.

After many identical experiments, we could conclude that as t-BHP concentration rises, the fluorescence slope increases, as well. When NAC is added into the cuvette, a rapid decrease of the fluorescence's evolution slope is visible, due to the antioxidant effect of NAC. Immediately after, one can observe the intensity of the fluorescence slope increasing again due to the new production of ROS present in solution.

The undertaken experiments offered a firm response to the following question: "*Which is the minimum t-BHP concentration that can prove the presence of ROS using the above presented fluorescence method?*"

The concluding results state that for t-BHP concentration below 11.297 mM, ROS cannot be observed by this fluorescence method but for concentrations between 22 mM and 40 mM, the method is the right one to use due to the high sensitivity to ROS concentration. If we use the method for t-BHP concentrations above the value of 45.188 mM, the decrease in fluorescence slope can still observed but NAC is losing its antioxidant effect.

The average slopes were calculated using the following two fluorescent signal recording areas: (a) from the moment tBHP was added (starting with the 300^{th} second) to the moment when NAC was added (the 600^{th} second); (b) from the moment NAC was added (the 600^{th} second) to the end of the recording (the 900^{th} second).

Several identical experiments have been conducting for the temporal intervals a. and b. and for those two intervals, the results have been interpreted by calculated the average slope. tBHP was used for concentrations in the interval [0-45.188] mM according to Table 5 and NAC at concentration of 0.8 mM was used. The used volumes of tBHP and NAC are specified in Table 4.

In figure 8 one can see the tBHP average slopes evolution with respect to different tBHP concentration using or not NAC. If tBHP concentration is bellow 20 mM than the fluorescent average slopes describe a low sensitivity. For concentrations between 22 mM and 40 mM, the method is the right one to use due to the highly sensitive results of the fluorescence slopes average. When NAC is added the slopes average is significantly decreased, it is way bellow the graph that describe the slopes average without NAC. For concentrations between 22 mM and 40 mM, the method is the right one to use due to the highly sensitive results of the fluorescence slopes average.

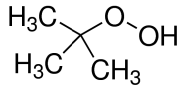

Figure 1. Tert-butyl hydroperoxide (tBHP)

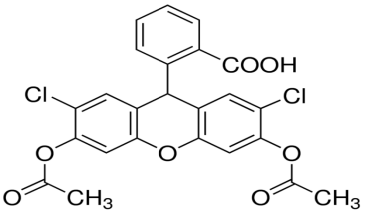

Figure 2. 2',7'-Dichlorofluorescein diacetate (DCF-DA).

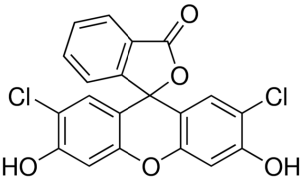

Figure 3. 2',7'-Dichlorofluorescein (DCF).

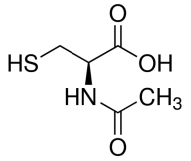

Figure 4. N-Acetyl-L-cysteine (NAC).

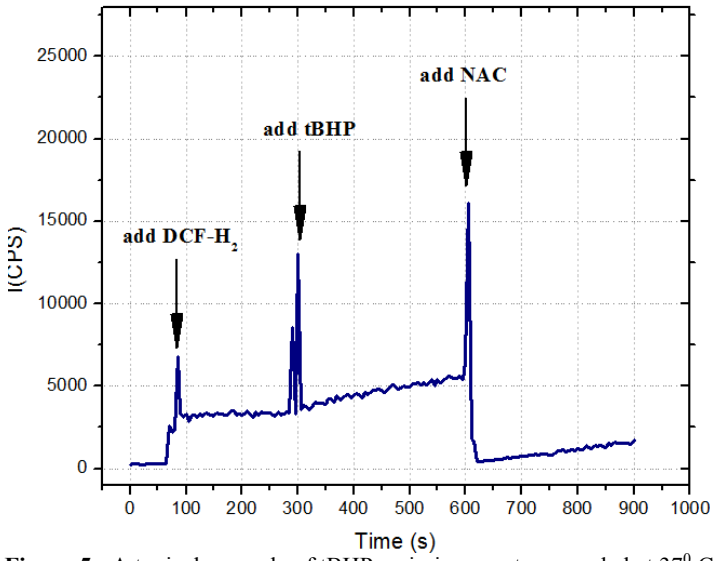

Figure 5. A typical example of tBHP emission spectra recorded at 37^0 C; $DCFH_2$ reacts with tBHP to yield the highly fluorescent DCF and N-Acetyl-L-cysteine (NAC) is used for its antioxidant effects.

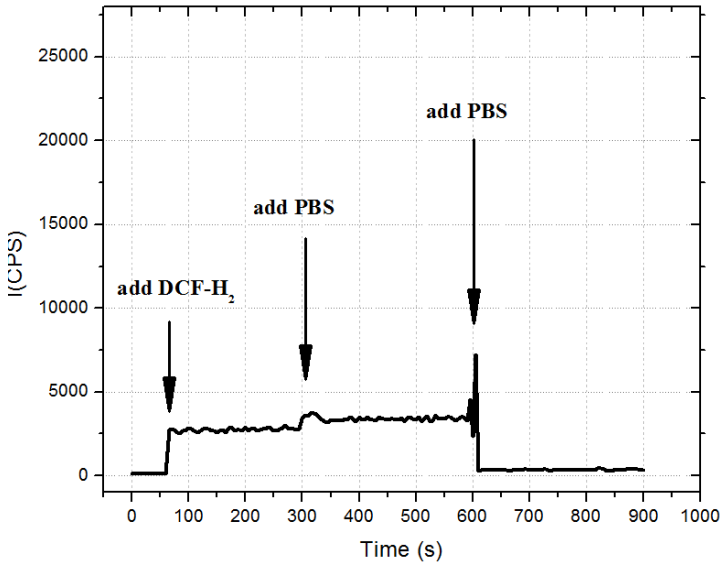

Figure 6. A typical example of $DCFH_2$ emission spectra recorded at 37^0C (PBS replaces tBHP and NAC).

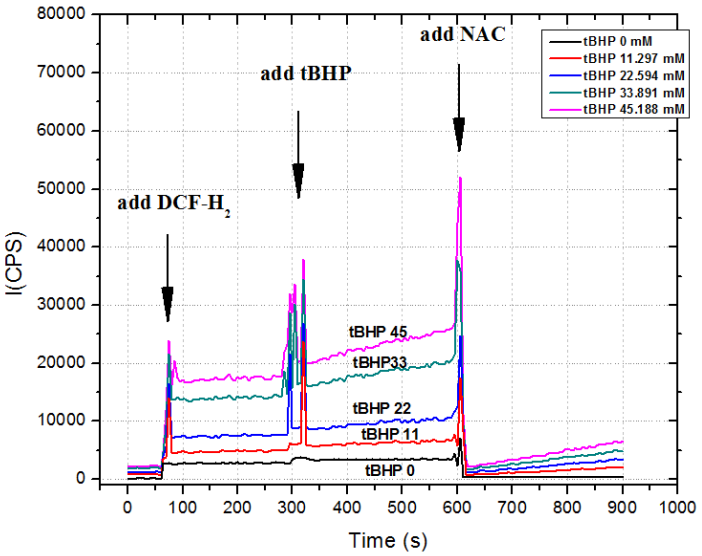

Figure 7. ROS emission spectra recorded at 37^0C; the oxidative stress was induced using t-BHP in different concentrations from 0 mM to 45.188 mM. The generated ROS were neutralized by adding the antioxidant N-Acetyl-L-cysteine (NAC) to the solution.

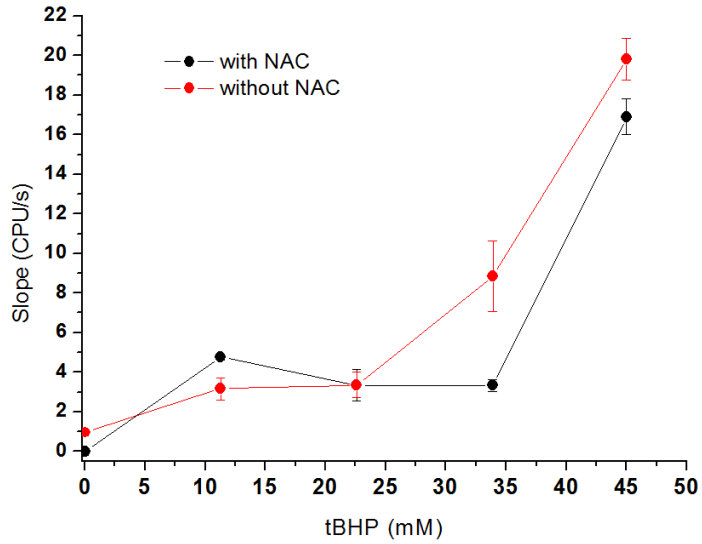

Figure 8. t-BHP slopes average, versus t-BHP concentration, with and without N-Acetyl-L-cysteine (NAC) antioxidant.

Table 1. Volumes.

Blank/each stock solution	
Total 5 stock solutions blank	5x3 mL=15 mL
For blank - 5 cuvettes -> Trypsin	5x0,5ml=2,5ml
For blank - 5 cuvettes -> PBS	5x2,5ml=12,5ml
Conc. TB producer's stock (M)	7,23
MW DCF-DA	487,29g/mol
Conc DCF-DA -Sigma (D6883) (ethanol)- stoc 100%	5mM

Table 2. TB Stock Solutions.

tBHP Stocks	**1**	**2**	**3**	**4**	**5**
tBHP volume (uL)	**0**	**100**	**200**	**300**	**400**
PBS volume (uL)	**400**	**300**	**200**	**100**	**0**
BHP concentration (mM)	**0**	**1807,5**	**3615**	**5422,5**	**7230**

Table 3. NAC Stock Solutions.

NAC Stocks	**1**	**2**	**3**	**4**	**5**
NAC volume (uL)	**0**	**100**	**200**	**300**	**400**
PBS volume (uL)	**400**	**300**	**200**	**100**	**0**
NAC concentration (mM)	**0**	**37,5**	**75**	**112,5**	**150**

Table 4. Experiment

PBS volume (uL)	1737,5	1737,5	1737,5	1737,5	1737,5
DCFH2 volume (uL)	50	50	50	50	50
tBHP volume (uL)	12,5	12,5	12,5	12,5	12,5
NAC volume (uL)	200	200	200	200	200

Table 5. Concentrations

Cuvette concentration	1	2	3	4	5
DCFH2 concentration (mM)	0,005	0,005	0,005	0,005	0,005
tBHP concentration (mM)	0	11,29688	22,59375	33,89063	45,1875

4. CONCLUSIONS

This method shows high sensitivity, simplicity, and reproducibility. It is suitable also for measuring total ROS in living cells or tissues. This method has achieved its purpose: it can monitor and evaluate ROS production. Its goal is to improve current and future management of outputs, outcomes and impact. This study describes a method that may be used in future biomedical investigation to measure intracellular rate ROS production and not only their concentration at a particular moment in time. The highest sensitivity is for ROS concentration between 22 mM-40 mM. For TB concentration lower than 20 mM this method isn't very sensitive. The method presented in this paper generates information with respect to the rate of ROS production. This study is aimed to develop a modified protocol to detect the rate of ROS production in response to oxidative stress not only for the assessment of their concentration at a particular moment in time.

5. REFERENCES

[1] Takanashi T., Ogura Y., Taguchi H., Hashizoe M., Honda Y., Fluorophotometric quantation of oxidative stress in the retina in vivo, *Investigative Ophthalmology & Visual Science,* 38, 13, 2721-8, **1997**.

[2] Gomes A., Fernandes E., Lima J.L., Fluorescence probes used for detection of reactive oxygen species*, J. Biochem. Bioph. Methods*, 65, 45–80, **2005**.

[3] Soh N., Recent advances in fluorescent probes for the detection of reactive oxygen species, *Anal. Bioanal. Chem*., 386, 532–543, **2006**.

[4] Gomes A., Fernandes E., Lima J.L.F.C., Fluorescence probes used for detection of reactive oxygen species, *J. Biochem. Biophys. Methods*, 65, 45–80, **2005**.

[5] Chen X., Zhong Z., Xu Z., Chen L., Wang Y., 2',7 Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of applicationand controversy, *Free Radic Res*., 44, 6, 587-604, **2010**.

[6] Caporaso N., The molecular epidemiology of oxidative damage to DNA and cancer, *J Natl Cancer Inst*, 95, 1263– 5, **2003**.

[7] Taguchi K., Motohashi H., Yamamoto M., Molecular mechanisms of the Keap1–Nrf2 pathway in stress response and cancer evolution, *Genes to Cells*, 16, 2, 123-140, **2011**.

[8] Rösen P., Nawroth P.P., King G., Möller W., Tritschler H.J., Packer L., The role of oxidative stress in the onset and progression of diabetes and its complications: a summary of a Congress Series sponsored by UNESCO-MCBN, the American Diabetes Association and the German Diabetes Society, *Diabetes Metab Res Rev*., 17, 3, 189-212, **2001**.

[9] Moncada S., Palmer R.M., Higgs E.A., Nitric oxide: physiology, pathophysiology, and pharmacology*, Pharmacol Rev*, 43, 109–42, **1991**.

[10] Suzuki Y.J., Forman H.J., Sevanian A., Oxidants as stimulators of signal transduction. *Free Radic Biol Med*, 22, 269– 85, **1997**.

6. ACKNOWLEDGEMENTS

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