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光動力治療對白色念珠菌生物膜防治之研究
研究成果報告(精簡版)

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Abstract.

Photodynamic inactivation (PDI) of *Candida albicans* mitochondria by photosensitizers, methylene blue (MB), had been investigated. Mitochondria are subcellular organelles that produce up to 90 % of cellular energy and provide oxidative toxin resistance for eukaryotic cells. Mitochondrial dysfunction would cause serious effect in mammalian and yeast cells. MB inhibition efficiency of yeast was found to cause mitochondrial dysfunction and form petite colonies. TTC-agar detected mitochondrial dysfunction colonies from MB-PDI treated cells and ethidium bromide (EtBr) induced mitochondrial mutation cells. Dyeing the MB-PDI treated injury yeast by JC-1 and observing them by fluorescence microscope, the mitochondrial potential decreased obviously. The mitotracker green FM (MTG) and JC-1 fluorescence intensity were detected by the fluorophotometer, the fluorescence intensity of the damaged cells decreased obviously and no signal of the dead cells. This study shows that MB-PDI is not only killing yeast cells by destroying cellular structure directly, but also inhibiting physiological activity.

1. Introduction.

Mitochondria are cytoplasmic organelles that produce up to 90 % of cellular energy by the process of oxidative phosphorylation and provide oxidative toxin resistance for eukaryotic cells. Mitochondria occupy a unique position among cellular organelles because they possess a separate genome and all the enzymatic machinery for transcribing and translating the genetic information into proteins semi-antunomously. Although mitochondria are also an important source of excited oxygen species [1], oxidative damage of mtDNA has not been assessed.

Mitochondria could be vulnerable to damage by environmental chemical compounds because it contained no introns, had no protective histones or non-histone proteins and was continuously exposed to reactive oxygen species (ROS) [2, 3]. Several factors would be vulnerability to mtDNA include continuous free radical [4], accumulation of lipophilic carcinogens in mitochondria [5] and aromatic compounds, which bound to mtDNA up to 500 times more than nuclear DNA [6]. The hazard of nuclear DNA damages in eukaryotic fungi is furtherly reduced by the presence of a membrane that envelopes the nucleus and might act as a barrier to the penetration of dyes or their high-energy photoproducts [7]

Photodynamic inactivation (PDI) is a new modality of infection therapy based on the dye-sensitized photo-oxidative damage to biological sites. This required the presence of photosensitizers, which are usually aromatic compounds, located in the specific sites to be treated, a light source and molecular oxygen [8]. Excitation of the sensitizers by absorption of light of appropriate wavelength in the presence of oxygen converts the sensitizers to its photoactive triple state, which in turn reacts with either a local substrate (type 1 reaction) to form cytotoxic radicals, or with molecular oxygen (type 2 reaction) to produce cytotoxic singlet oxygen ($^1O_2$) [8]. The reactive oxygen species (ROS) generated then results in cell death.

Our experiments discovered methylene blue (MB)-PDI treated *Candida albicans,* the colonies of survival cells growth on YPD plates would form “petite colonies”.

Ephrussi and his collaborators had reported the yeast “petite colonies” and suggested that these cells may represent damaged or lost mitochondria [9, 10]. In mammalian cells, previous research has shown photodynamic therapy (PDT) will open mitochondrial permeability transition pore (PT pore) and decrease mitochondrial potential [11]. The outside stimulation will affect mitochondrial osmotic pressure, and break the mitochondrial outer membrane [12]. These authors found considerable damage to mammalian cells and evident changes in functions within mitochondria after PDI. Unfortunately, no results for the relationship between PDI and yeast mitochondria were reported. The aim of this present study was to investigate the mitochondrial activity of *C. albicans* treated in MB-PDI and to demonstrate the effect of mitochondria.

2. Materials and Methods.

2.1 Yeast strain and culture medium. *C. albicans* BCRC 20512, purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), was used throughout this study. YPD medium (Difco, Detroit, Mich.) ` used as the liquid medium and PDI on 10^6 CFU planktonic cells.

2.2 Photosensitizer. Methylene blue is a heterocyclic aromatic compound with chemical formula: $C_{16}H_{18}ClN_3S$. A stock solution containing 10 mM of MB (Sigma Chemical Co, St. Louis, Mo.) per ml was prepared in phosphate-buffered saline (PBS) and filter sterilized (0.22-μm-pore-size Acrodisc; Gelman Sciences, Ann Arbor, Mich.) immediately prior to use. The dye was appropriately diluted with PBS to obtain the desired concentration.

2.3 Light source. A light emitting diode (LED) array with a major wavelength of 630 nm, designed by Industrial Technology Research Institute (Hsinchu, Taiwan), was used to excite photosensitizer. The fluence of light delivered was 100 mW/cm^2.

2.4 PDI on 10^6 CFU planktonic cells. *C. albicans* cells from the overnight culture were harvested by centrifuging at 1395 g for 10 min and resuspended into about 10^6 cells with 200 μl sterile PBS. According our previous studies, the resultant cell suspension was treated with PBS in dark 60 min as normal cells, treated with PBS and irradiated 360 J/cm^2 as control, treated 5 μM MB and irradiated 360 J/cm^2 as damaged cells, treated with 10 μM MB and irradiated 360 J/cm^2 as dead cells.

2.5 2,3,5-triphenyltetrazolium chloride (TTC) -test. Colorless oxidative TTC would be reduced to deep red reduced TTC by yeast colonies with normal mitochondrial succinate dehydrogenase. If mitochondria loss function or mitochondrial copy numbers decreased, the yeast colonies’s color would be pink or white. After MB-PDI, *C. albicans* survival cells growth on YPD plate at 30 ℃for 18 hr. The test was modified from Ogur [13] and performed by pouring 20 ml of TTC agar at 50 ℃ over plates bearing 100 to 200 colonies per plate grown on YPD plates. Red, pink and white colonies were scored routinely 30 min after overlay. We induced yeast mitochondria mutation by EtBr as positive control [14]. 90 μl log phase yeast cells (6×10^5 CFU/ml) mixed with 10 μl EtBr (10 mg/ml) and incubated at 30 ℃for 24 hr. After harvesting yeast cells, we spread yeast cells on YPD plates and the colonies were overlaid by TTC-agar.

2.6 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolcarbocyanine iodide (JC-1) stain. JC-1 monomer emits at 525 nm after excitation at 490 nm in repressive

mitochondria with low negative inner membrane potential. Depending on the membrane potential, JC-1 is able of forming J-aggregates that are associated with a large shift in emission (590 nm) in normal mitochondria with high negative inner membrane potential [15]. MB-PDI treated yeast cells cultured 30 min with JC-1 (final concentration is 5 μg/ml). After removing JC-1 dye, we observed yeast cells with fluorescence microscopy (E600, Nikon), B filter (EX: 450-490 nm; DM: 505 nm; BA:520 nm) for low potential mitochondria; G filter (EX: 510-560 nm; DM: 575 nm; BA: 590 nm) for high potential mitochondria; UV filter (EX: 330-380 nm; DM: 400 nm; BA: 420 nm) for DAPI stain cell morphology.

2.7 MitoTracker Green FM (MTG) stain. MTG is a probe for mitochondrial structure [16]. MTG selective accumulates in the mitochondria matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues. MTG has advantage that it is nonfluorescent in aqueous environment, only becoming fluorescent once it accumulates in the lipid environment of mitochondria. MTG emits at 516 nm after excitation at 490 nm in mitochondria.

2.8 Fluorophotometer. After MB-PDI treated, yeast cells were separated into two parts, one of which was treated with JC-1. After washing with PBS, we excited at a wavelength of 490 nm and the fluorescence intensity was recorded in the wavelength range from 500 to 620 nm by fluorophotometer (MTG was excitated at 490 nm and recorded fluorescence intensity in 516 nm). The other one was treated with 37% formaldehyde for 30 min and stained with DAPI for 10 min. After washing with PBS, we excited at a wavelength of 399 nm and the fluorescence intensity was recorded in the wavelength of 461 nm by fluorophotometer.

3. Results.

3.1 The color change of the colonies overlaid with TTC agar.

C. albicans suspension was irradiated with red light to activate the photosensitizer. Figure 1 showed the percentage of colonies' colors overlaid with TTC-agar. Yeast cells treated with PBS and irradiated with 480 J/cm^2 or yeast cells treated with 5 μM MB without irradiation. The colonies overlaid by TTC-agar were deep red all. There were 50.42 ± 4.60 % white colonies when we induced yeast mitochondrial mutation by EtBr. And there were 6.66 ± 4.17 and 20.80 ± 8.98 % white colonies when we treated yeast cells with 5 μM MB and irradiated with 120 and 480 J/cm^2.

3.2 The fluorescence micrographs of yeast cells stained with JC-1 (red and green) and DAPI (blue).

C. albicans suspension were irradiated with red light to activate the photosensitizer. After MB-PDI, yeast cells were treated with JC-1 immediately and observed with fluorescence microscopy. Figure 2 showed the fluorescence of normal cells, negative control, damaged cells and dead cells all treated with JC-1. Red and green fluorescence were obviously in normal cells (Fig 2-A. 2-B) and negative control (yeast cells incubated with PBS and irradiated with 360 J/cm^2) was similar to normal cells (Fig 2-D, 2-E). Damaged cell could emit green fluorescence obviously but no red fluorescence (arrows in Fig 2-G, 2-H). And we could not take any photos with green or red fluorescence (Fig 2-J,

2-K) even if the morphology of yeast cells were complete (Fig 2-L contrasted to Fig 2-C, 2-F and 2-I).

3.3 Fluorescence spectrum analysis of yeast cells stained with JC-1.

C. albicans suspension were irradiated with red light to activate the photosensitizer. After MB-PDI, yeast cells were treated with JC-1 or DAPI separately and measured fluorescence spectrum with fluorophotometer immediately. Figure 3 shows the differences of fluorescence spectrum in normal cells, damaged cells and dead cells. Green fluorescence (525 nm) meant low potential mitochondria and red fluorescence fluorescence (590 nm) meant high petential mitochondria. Red fluorescence was obvious than green fluorescence in normal cells. In damaged cells, high potential mitochondria decreased to 4.96 % contrast to normal cells, and low potential mitochondria decreased to 18.88 % contrast to normal cells. The fluorescence intensity of damaged cells decreased obviously and there were no signal of the dead cells.

3.4 Fluorescence photometric analysis of yeast cells stained with JC-1 or MTG.

After MB-PDI, yeast cells were treated with JC-1 or MTG or DAPI separately and measured fluorescence intensity with fluorophotometer immediately. Figure 4 shows the differences of fluorescence intensity ratio in normal cells, damaged cells and dead cells. We set the ratio of normal cells fluorescence intensity as 100 %, and the fluorescence intensity ratio of damaged and dead cells were compared with normal cells. In damaged cells, JC-1 red fluorescence decreased to 7.19 ± 1.15 % and MTG fluorescence decreased to 27.26 ± 18.91 %. In dead cells, we couldn’t detect obvious signal of JC-1 and MTG fluorescence.

4. Discussion.

In this repore, we showed that photodamage by methylene blue leaded to loss of the mitochondrial potential and destroy mitochondrial structure or copy numbers. TTC-agar overlaying test shows that some colonies grown from survival cells with deficient mitochondria (Fig. 1). After MB-PDI, mitochondrial total potential in yeast cells decreased obviously meant mitochondrial total activity decreased, too (Fig. 2 and 3). Because in respiration pathway, mitochondria would store the energy in electrochemistry potential form at mitochondrial inner membrane (250 mV/5-10 nm), terms mitochondrial membrane potential (Dym). And MTG experiment showed mitochondrial membrane structure destroyed or mitochondrial copy numbers decreasded (Fig. 4). These results are interpreted to mean that yeast cells have had sufficient mitochondrial photodamage to destroy mitochondrial and abolish Dym will ultimately affect the cellular physiological activity. A similar phenomenon was reported in murine leukemia p338 cell death [16].

Methylene blue used in this study was known to bind to mitochondria [17]. Although we couldn’t yet exclude the possibility that the agent cause photodamage at additional subcellular loci, like lysosomes [18]. A unique property of PDI was that photodamage didn’t occur until photosensitizers were irradated. When irradiation occurred, yeast mitochondria without photosensitizers wouldn’t be hurt by red light (Fig 2) but ultraviolet would [19]. Mitochondrial activity and structure (Fig. 2,3 and 4) were damaged seriously after irradiation. These result were consistent with Fig. 1 that with deficient mitochondria. Although we got mitochondrial dysfunction yeast colonies on

plates and identified these colonies' would be white or pink. However, these colonies would return to deep red after sub culturing 1-2 times, and we couldn't get stable mitochondrial dysfunction strains (data not show). Perhaps, MB-PDI destroyed some mitochondria in a cell, so destroyed and dysfunctional mitochondria were repaired [20], or regrown and replaced by functional mitochondria [21-23].

Mitochondrial superoxide production had been estimated to be as high as 2 % of all molecular oxygen reduced during respiration [24]. However, it was likely to be an overestimate (maybe less than 0.1 %) [20], and mtDNA could be vulnerable to damage by continuous free radical generation in mitochondria [25]. A long-standing hypothesis suggested high level of mtDNA mutations might increase mitochondrial ROS production leading to cell degeneration or death [26]. Furthermore, characterization of ROS acting was center regulators of yeast apoptosis [27] and ROS generation was essential of the process [28]. Moor's report show PDT would open PT pore in mitochondria, decrease mitochondrial potential, and release Ca^{2+}. Then, cytochrome c released and co-reacted with Apaf-1 and procaspase-9 to activate caspase-9. Caspase-9 activated capase-3 and induced cell apoptosis [11]. Green and Reed show the destroyed mitochondria would release some proteins from intermembrane space to induce apoptosis [12].

In our previous study, we had investigated the relationship between photosensitizers excitation and antimicrobial activity against planktonic cells and biofilms. In this study, we observed that mitochondrial membrane was destroyed (Fig. 4) and mitochondrial potential was decreased by MB-PDI (Fig. 2 and 3). After survival yeast cells growthing on plates, there were some colonies with mitochondrial dysfunction (Fig 1). Based on these experiment, we suggested that yeast mitochondria were hurt by MB-PDI directly and affected mitochondrial activity. Even if we couldn't yet exclude the possibility the agent cause photodamage at additional subcellular loci and decreased mitochondrial activity. Mitochondrial dysfunction by ROS might induce yeast apoptosis. The possibility of MB-PDI is not only killing yeast cells by destroying cellular structure directly, but also leading yeast apoptosis procedure.

The major phenotypes of yeast apoptosis are DNA cleavage, apoptosis-typical chromatin condensation, externalization of phosphatidylserine to the outer leaflet of the plasma membrane [29] and cytochrome c release from mitochondria [30, 31]. The confirmation of these phenotypes is necessary to consist out hypothesis of MB-PDI leading some yeast cells death by apoptosis procedure. And these effects and phenotype of MB-PDI against *Candida albicans* needs further exploration.

References

1. Loschen G, Azzi A, Richter C and Flohe L. Superoxide radicals as precursors of mitochondrial hydrogen peroxide. FEBS Lett 1974;42:68-72
2. Rasmussen L, Singh KK. Genetic integrity of mitochondrial genome. New York: Springer Verlag, 1998 (Singh KK, ed. Mitochondrial DNA Mutations in Aging, Disease, and Cancer.)
3. Singh KK, Sigala B, Sikder HA and Schwimmer C. Inactivation of Saccharomyces cerevisiae OGG1 DNA repair gene leads to an increased frequency of mitochondrial mutants. Nucleic Acids Res 2001;29:1381-8

4. Bandy B, Davison AJ. Mitochondrial mutations may increase oxidative stress: implications for carcinogenesis and aging? Free Radic Biol Med 1990;8:523-39
5. Grossman LI. Mitochondrial mutations and human disease. Environ Mol Mutagen 1995;25 Suppl 26:30-7
6. Allen JA, Coombs MM. Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. Nature 1980;287:244-5
7. Zeina B, Greenman J, Purcell WM and Das B. Killing of cutaneous microbial species by photodynamic therapy. Br J Dermatol 2001;144:274-8
8. Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 1998;42:13-28
9. Ephrussi B, Hottinguer H. Direct demonstration of the mutagenic action of euflavine on baker's yeast. Nature 1950;166:956
10. Ephrussi B, L'Heretier P and Hottinguer H. Action de l'acriflavine surles levures. VI. Analyses quantitative de la transformation des populations. Ann Inst Pasteur 1949;77:64-83
11. Moor AC. Signaling pathways in cell death and survival after photodynamic therapy. J Photochem Photobiol B 2000;57:1-13
12. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-12
13. Ogur M, St. John R and Nagai S. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 1957;125:928-9
14. Wiseman A, Attardi G. Reversible tenfod reduction in mitochondria DNA content of human cells treated with ethidium bromide. Mol Gen Genet 1978;167:51-63
15. Cossarizza A, Baccarani-Contri M, Kalashnikova G and Franceschi C. A new method for the cytofluorimetric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). Biochem Biophys Res Commun 1993;197:40-5
16. Kessel D, Luo Y. Photodynamic therapy: a mitochondrial inducer of apoptosis. Cell Death Differ 1999;6:28-35
17. Gabrielli D, Belisle E, Severino D, Kowaltowski AJ and Baptista MS. Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. Photochem Photobiol 2004;79:227-32
18. Zhang GJ, Yao J. The direct cause of photodamage-induced lysosomal destabilization. Biochim Biophys Acta 1997;1326:75-82
19. Conconi A, Jager-Vottero P, Zhang X, Beard BC and Smerdon MJ. Mitotic viability and metabolic competence in UV-irradiated yeast cells. Mutat Res 2000;459:55-64
20. Stuart JA, Brown MF. Mitochondrial DNA maintenance and bioenergetics. Biochim Biophys Acta 2006;1757:79-89
21. Eliseev RA, Gunter KK and Gunter TE. Bcl-2 prevents abnormal mitochondrial proliferation during etoposide-induced apoptosis. Exp Cell Res 2003;289:275-81
22. Lee S-LJ, Warmke HE. Organelle Size and Number in Fertile and T-Cytoplasmic Male-Sterile Corn. Botanical Society of America 1979;66:141-148
23. Mancini M, Anderson BO, Caldwell E, Sedghinasab M, Paty PB and Hockenbery DM. Mitochondrial proliferation and paradoxical membrane depolarization during terminal differentiation and apoptosis in a human colon carcinoma cell line. J Cell Biol 1997;138:449-69

24. Chance B, Sies H and Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev 1979;59:527-605
25. Kang D, Takeshig K, Sekigichi M and Singh KK. Mitochondria in aging, disease and cancer: an intruduction. Spring Verlag, New York, NY., 1998 (Singh KK, ed. Mitochondria in Aging, Disease, and Cancer)
26. Dufour E, Larsson NG. Understanding aging: revealing order out of chaos. Biochim Biophys Acta 2004;1658:122-32
27. Madeo F, Frohlich E, Ligr M, et al. Oxygen stress: a regulator of apoptosis in yeast. J Cell Biol 1999;145:757-67
28. Zhang Q, Chieu HK, Low CP, Zhang S, Heng CK and Yang H. Schizosaccharomyces pombe cells deficient in triacylglycerols synthesis undergo apoptosis upon entry into the stationary phase. J Biol Chem 2003;278:47145-55
29. Madeo F, Frohlich E and Frohlich KU. A yeast mutant showing diagnostic markers of early and late apoptosis. J Cell Biol 1997;139:729-34
30. Ludovico P, Rodrigues F, Almeida A, Silva MT, Barrientos A and Corte-Real M. Cytochrome c release and mitochondria involvement in programmed cell death induced by acetic acid in Saccharomyces cerevisiae. Mol Biol Cell 2002;13:2598-606
31. Manon S, Chaudhuri B and Guerin M. Release of cytochrome c and decrease of cytochrome c oxidase in Bax-expressing yeast cells, and prevention of these effects by coexpression of Bcl-xL. FEBS Lett 1997;415:29-32

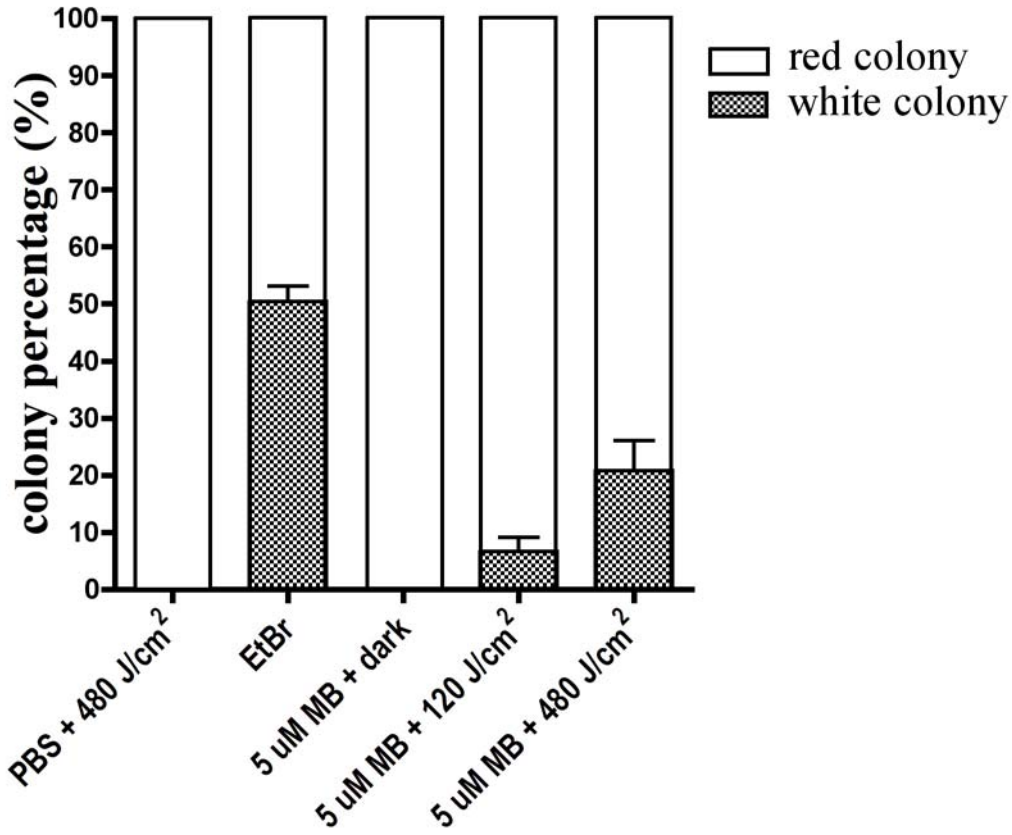

Figure 1. The color change of the colonies overlaid with TTC agar. MB-PDI treated yeast cells grown on YPD plates and the colonies were overlaid with TTC agar. The red colonies meant yeast cells with well function mitochondria. The pink and white colonies meant yeast cells with deficient function mitochondria or mitochondrial copy numbers decreased.

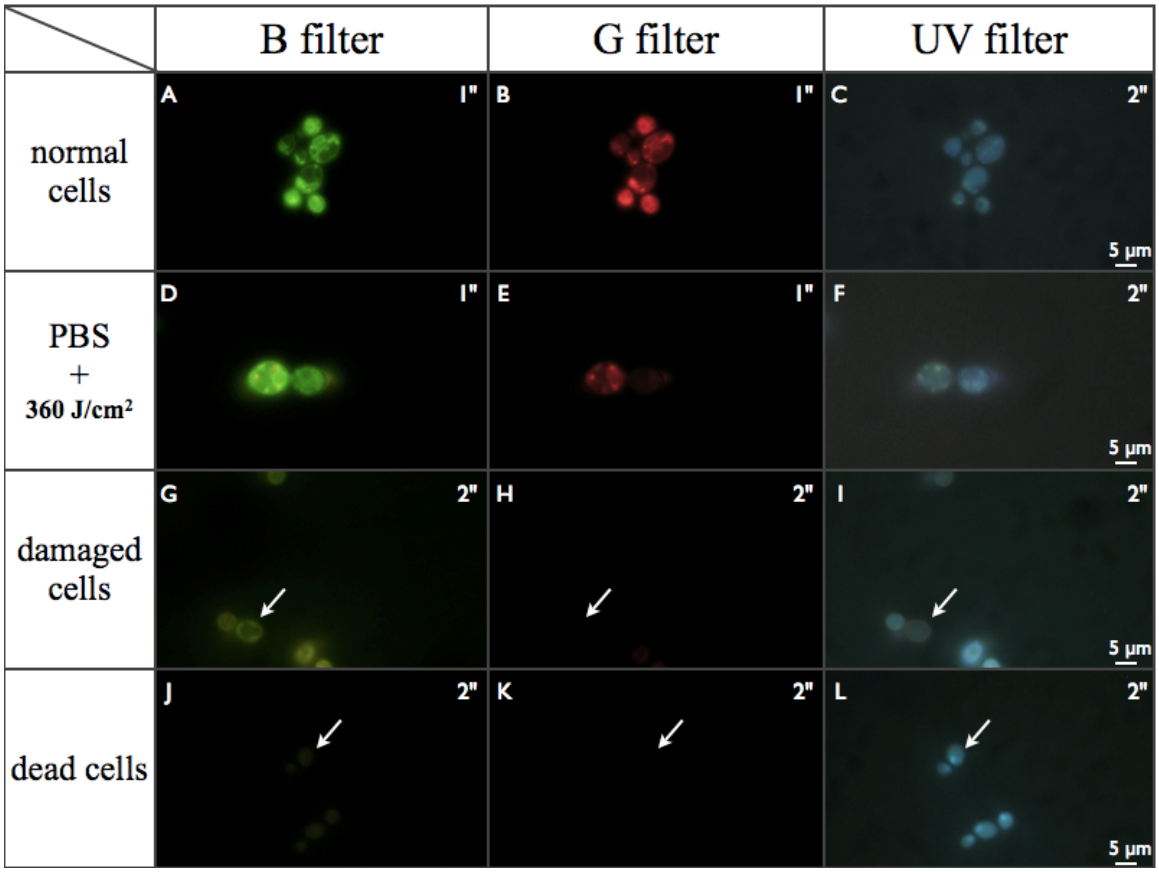

Figure 2. The fluorescence micrographs of yeast cells stained with JC-1 (red and green) and DAPI (blue). Red fluorescence meant high potential mitochondria. Green fluorescence meant low potential mitochondria. DAPI stained cell morphology and emitted blue fluorescence. The number at upper right corner meant the time of exposure (s).

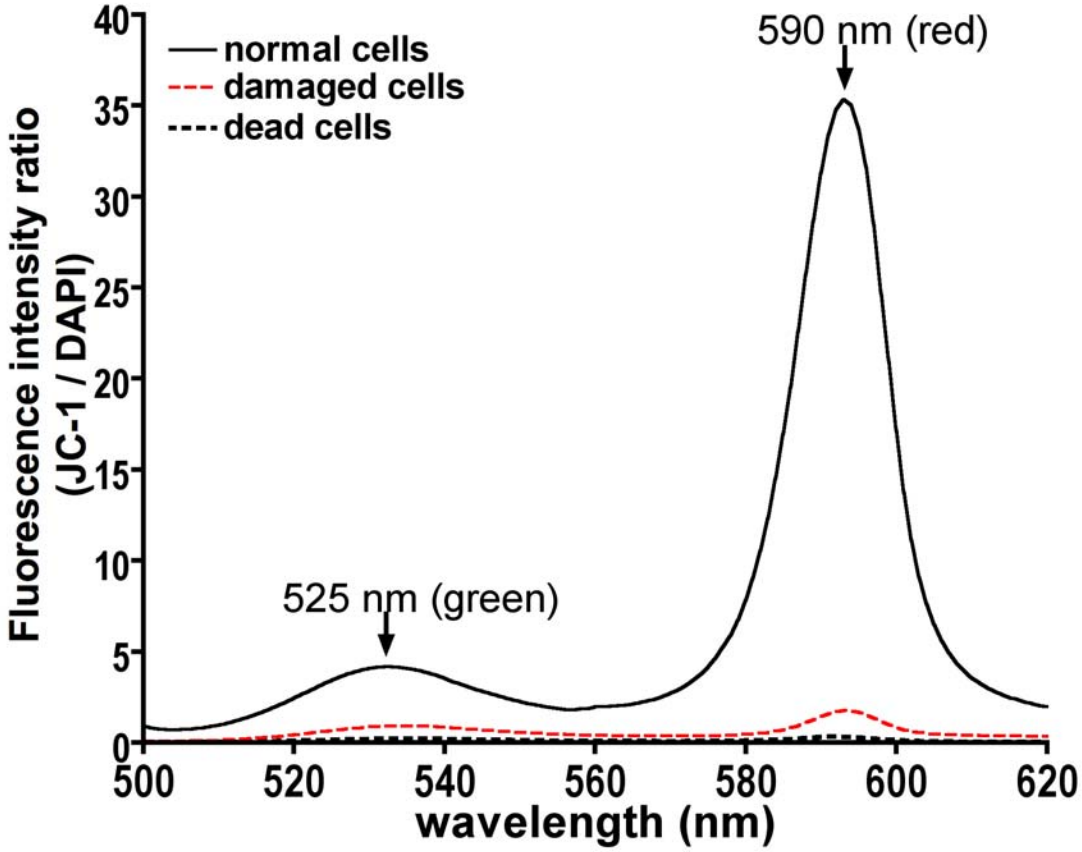

Figure 3. Fluorescence spectrum analysis of yeast cells stained with JC-1. Red fluorescence was obvious in normal yeast cells. The change of spectrum could observe the change of mitochondrial potential. The X-axis was wavelength from 500 to 620 nm. The Y-axis was the (JC-1 spectrum / DAPI fluorescence intensity). DAPI was the dye to calculate the numbers of cells. (JC-1 spectrum/ DAPI fluorescence intensity) was the count measurement normalized by the DAPI stain.

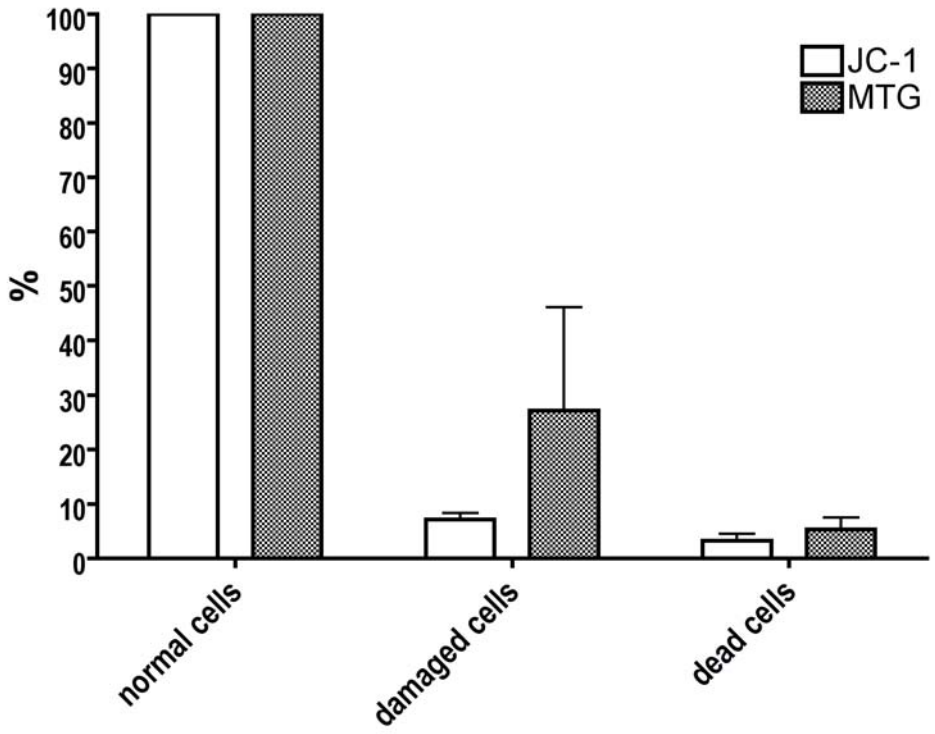

Figure 4. Fluorescence photometric analysis of yeast cells stained with JC-1 or MTG. JC-1 was excited at 490 nm and recorded fluorescence intensity in 620 nm. MTG was excited at 490 nm and recorded fluorescence intensity in 516 nm. The Y-axis was the (JC-1 fluorescence intensity / DAPI fluorescence intensity). DAPI was the dye to calculate the numbers of cells. We set the ratio of normal cells fluorescence intensity as 100 %, and the fluorescence intensity ratio of damaged and dead cells were compared with normal cells. [JC-1 (or MTG) fluorescence intensity / DAPI fluorescence intensity] was the count measurement normalized by the DAPI stain.