Monitoring of ATP Levels in Red Blood Cells and T Cells of Healthy and Ill Subjects and the Effects of Age on Mitochondrial Potential

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Introduction

Energy metabolism plays a role in health and aging as well as disease. Energy must be available for the work of synthesizing new cellular material, maintaining membranes and organelles, and to fuel movement and active transport. Determination of the concentration of the energy carrier molecule, adenosine-5'-triphosphate (ATP), may assess the energy state in cells. Cellular ATP is an important determinant of cell death by apoptosis or necrosis.[1] Cells stay alive as long as a certain level of ATP is maintained. When ATP falls below this level, apoptosis is activated.[2] A severe drop in cellular ATP will result in cellular necrosis.

Metabolic conditions such as, trauma or stress may increase requirements for ATP or reduce the regeneration of ATP, therefore decreasing the overall ATP available to the body. Several pathological conditions may also decrease ATP production in cells. In addition, age and age-related diseases may be due to a fall in energy metabolism in the mitochondria. For example, patients with chronic fatigue syndrome (CFS) have relatively low intracellular ATP concentrations after exercise and a lower ATP synthesis rate during recovery.[3,4] In type II diabetes, alterations in the ATP synthesis may contribute to the pathogenesis of this disease.[5]

Lower ATP levels, number of hemoglobin and RBC deficiencies are common in cancer patients. The cellular level of ATP in subjects with solid tumors may be 30 % lower than that of normal adults.[6]

In RBCs, the adenine nucleotide content is correlated with red cell viability, hereditary metabolic abnormalities, and functional diseases with increased or decreased ATP. Measurements of the level of ATP in red blood cells may be used for evaluation of the pathobiochemical shift in energy production and for evaluation of the therapeutic effect of treatments.

Measurements of the level of bioenergetics of T cells are important in the assessment of disease management and for estimation of the level of cellular immune functioning. ATP measurements can provide an easy and fast method for estimation of the T cell bioenergy and response of cell activation to a variety of stimuli. The method can be used for monitoring the infectious disease and response to treatments. It may be useful in determining the response to nutritional supplements or the effect of aging on cellular metabolism.

Measurements of mitochondrial potential are important since mitochondria play the primary role in energy production and ATP generation. Mitochondria are the energy furnaces of the body. When mitochondria make insufficient ATP, there is inadequate energy for the body. This study sought to evaluate the energy state of mitochondria and dependence of the level of energy production on age.

There are several techniques for measuring ATP in cells, including enzymatic, fluorometric, and chromatographic methods.[7-11] The more widely used is the bioluminescent method.[12-18] This method uses the firefly luciferase assay and has a high level of sensitivity and specificity.

In previous methods, cells were lysed with cold trichloroacetic acid then

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nucleotides were determined in the lysate after dilution with buffer. This step resulted in a significant loss of ATP in the supernatant.

Materials and methods

The method that we used for ATP measurements complements other ATP assay methods. In our measurements, diluted cells were counted by flow-cytometer. Therefore, an analysis of the level of ATP in a known number of cells was performed without incubation or troublesome extraction. For ATP determination, we used a kit from Promega Company, "CellTiter-Glo Luminescent Cell Viability Assay".

Hemoglobin interferes with the luciferease-luminescence assay. The reaction produces a flash of yellow-green light with peak emission at 560 nm. The major peak of hemoglobin absorption occurs at 408 nm. As hemoglobin exists in several forms, each of which has different absorbance spectra, there are additional absorbance at the peak values of 575 nm and 540nm. These forms of hemoglobin are responsible for the interference with the luciferase spectrum.

We analyzed the effect of hemoglobin presented in supernatant at the measured ATP level in cells. The hemoglobin presented in samples was determined by light absorption at wavelength 408 nm and 560 nm. Our results demonstrated an effect of hemoglobin on the luminescence intensity in samples with concentrations of cells higher than 10-15 million cells per mL.

In addition, the level of absorption did not demonstrate a detectable level of hemolysed cells in solution after cells were washed 2-3 times with PBS (phosphate buffered saline).

Red cell separation

RBCs were separated from other cells and plasma by centrifugation at 500 g for 10 min. The supernatant and "buffy coat" were removed by aspiration. Packed cells were diluted by PBS with 10 mM glucose and 1mg/ml BSA (bovine serum albumin) to preserve the normal shape of RBCs. The RBCs were then washed two times by PBS and centrifuged at 200 g for 2-3 min. The final dilution of cells by PBS was greater than 1:3000. Before ATP measurements, cells were counted by flow-cytometer.

T cells separation

T cells were separated by "PosetteSep" procedure from Stem Cell Technology.[19] In this procedure, 50 uL of RosetteSep cocktail was added to each mL of blood and mixed well. Cells were incubated 20 min at room temperature. After incubation, cells were diluted with an equal volume of PBS containing 2% FBS (fetal bovine serum), mixed well and layered on the top of Ficoll-Paque (Amersham Bioscience). After 20 min of centrifugation at 1200 g, enriched cells were separated, washed in PBS with FBS, counted, and used for analysis.

To remove residual red cells, the solution was lysed with ammonium chloride. Enriched cells were washed one more time after lysis by PBS with 1 mM EDTA. Part of the cell suspension was counted by flow cytometer. Other portions of cells were used for ATP analysis and mitochondrial potential analysis.

ATP measurements

The level of ATP in T cells and RBCs was determined by The CellTiter-GloLuminescent cell Viability Assay kit (Promega). The assay generates a glow type signal produced by luciferase reaction, which is proportional to the amount of ATP present in cells. In this assay, the signal half-life was greater than one hour. For photon count, 50 uL of reaction mixture was mixed with 50 uL of cells and the count was measured by luminometer (BD Biosciences).

During the development of the procedure, the relationship between luminescence output and the number of T cells

and RBCs in culture was determined. The experiments demonstrated significant correlations between the number of cells and luminescent emission (**Figure 1, below**).

There was a linear relationship between the cell number in the range of 0.1-1.5 million cells and the level of output signal (coefficient of correlation R=0.99).

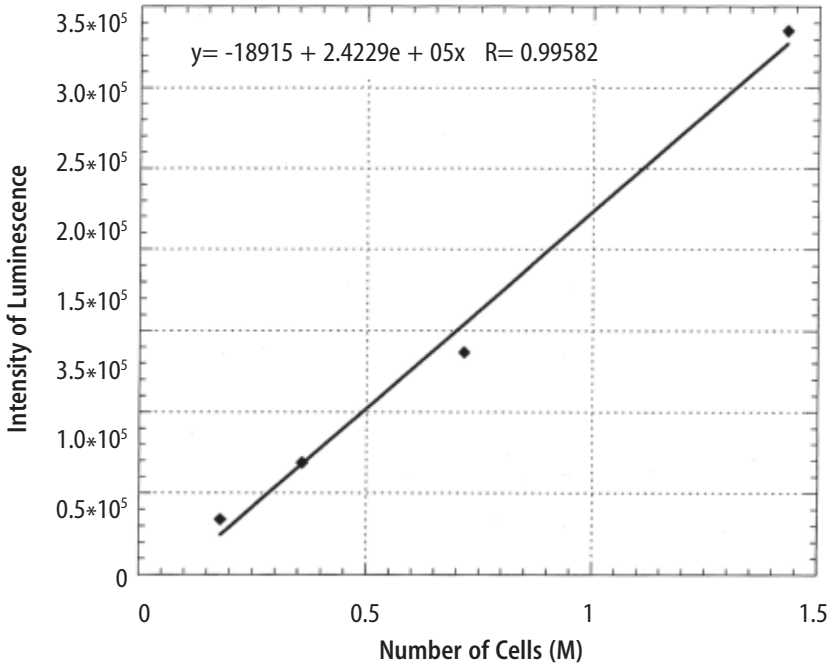
The level of ATP was determined from the standard curve. For determination of the standard curve, ATP (Sigma) was dissolved in PBS to make concentrations of 50-500 nM. The solution was calibrated to an absorbance extinction coefficient equal to 15.4/mM-1cm. The standard was prepared for each day of measurements. The standard curve was linear in the range of ATP at 0-1000 nM. A linear relationship was observed between the number of cells and the amount of ATP in the cells. A high level of reproducibility was demonstrated with triplicate measurements of the samples.

Mitochondrial Potential Measurements

To measure mitochondrial potential, cells were harvested from experimental samples at concentrations of $0.5*10^6$cells per mL. Cells were stained in PBS with 2.5ug/ml JC-1 and kept in a dark place at room temperature for 15-20 min. After exposure, cells were washed twice in PBS by centrifuging at 500 g for 5min. For detection of emission by fluorometer, cells were lysed in a solution of PBS with 0.2% Triton X-100 containing 0.5mM dithiothreitol (DTT) (Sigma).

Mitochondrial potential was assessed by using the fluorescent potentiometric dye, JC-1 (5,52,6,62,-tetrachloro-1,12,3,32 tetraethylbenzimidazolylcarbo-cyanine iodide).[20] JC-1 is able to selectively enter the mitochondria of intact cells and form ag-

Figure 1. Correlation of cell number with luminescent output.



gregates that emit at 585 nm (orange-red). If the mitochondrial potential is reduced, JC-1 disaggregates to monomers that fluoresce at 527 nm (green). The ratio between the red and the green signals is indicative of the mitochondrial potential.

Emission was measured by fluorometer (SPEX Instruments, Inc) at excitation 510 nm in the scan range of 520-600 nm. To demonstrate that dye accumulation is due to mitochondrial potential and emission at 595 nm is due to the formation of aggregates, cells were treated by proton ionophore CCCP (carbonyl-cyanide m-chloroprene hydrazone) form Sigma. These molecules carry H+ across the membrane and consequently allow the proton motive force to collapse. As seen in **Figure 2**, (below), there was a dramatic drop of the red fluorescence at 595 nm in cells exposed to the depolarizing agent in a concentration dependent manner. This indicates a loss of mitochondrial potential. In addition, the microscopic observation of cell stained by this dye showed that the dye was indeed taken into the mitochondrial membrane.

We also developed a protocol for cytofluorimetric study of the mitochondrial potential in intact cells. For analysis by flow-cytometer, cells were stained by using the same protocol as for fluorometer. Emission of intact cells was analyzed by detection of green emission at FL1 channel and red emission at FL2 channel.

Results and Discussion

To estimate the normal ranges of ATP in red and T cells, samples from 39 healthy subjects were measured twice at an interval of one month. The distributions for ATP in red blood cells and T cells are presented at **Figures 3** and **4** (p.54),

Figure 2. Effect of proton ionophore CCCP on depolarization of mitochondria.

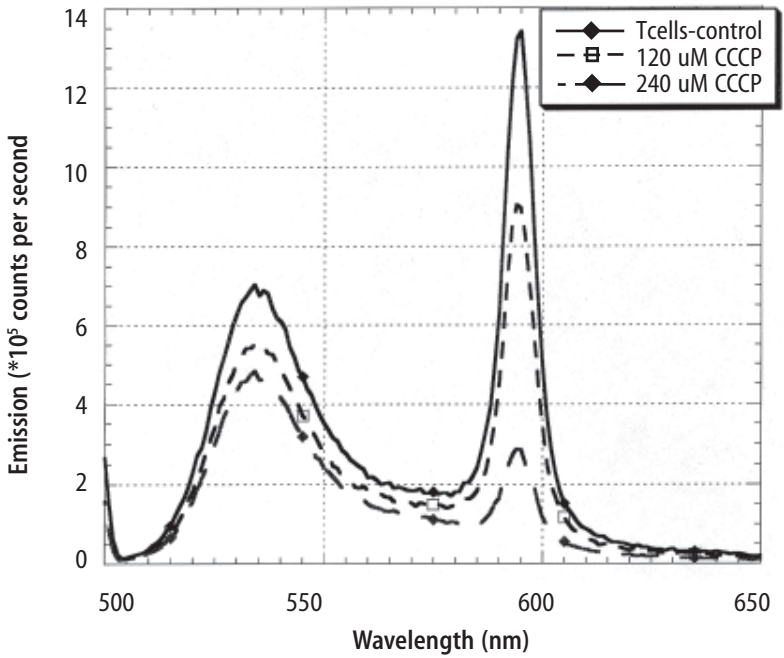


Figure 3. Distributions of the ATP levels in red blood cells of healthy subjects and patients.

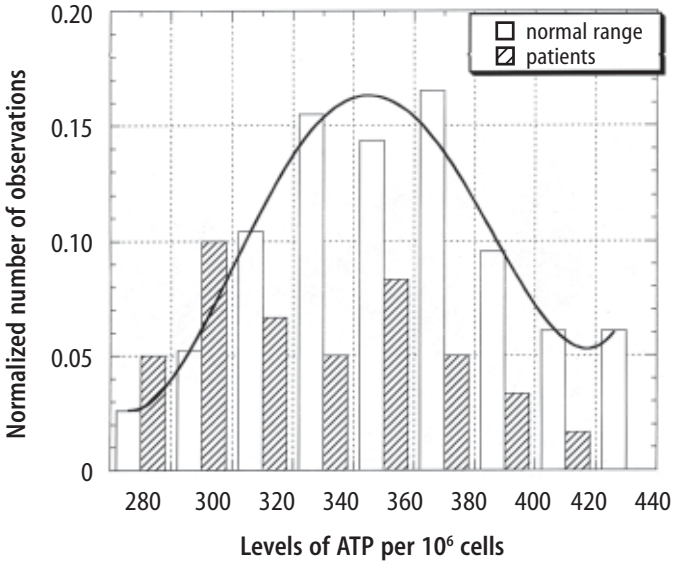
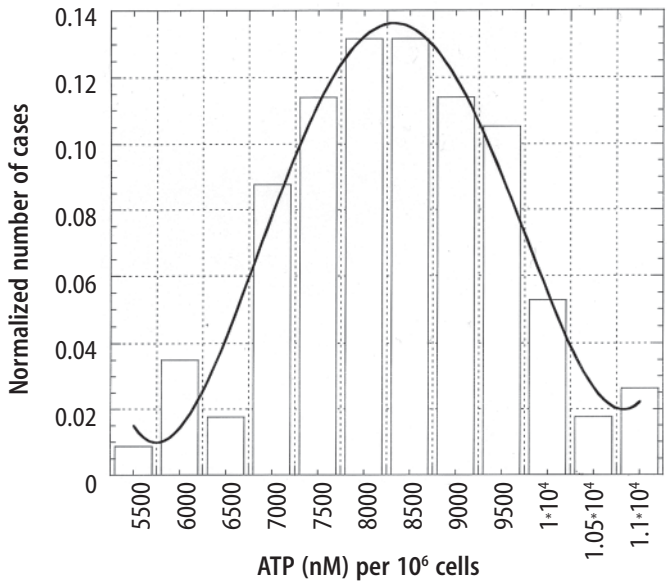


Figure 4. Distribution of ATP in T cells of healthy subjects.



respectively. For red cells, the average level of ATP per 10^6 cells was 360 ± 64 nM. For T cells, the average level of ATP was 8500 ± 1400 nM per 10^6 cells.

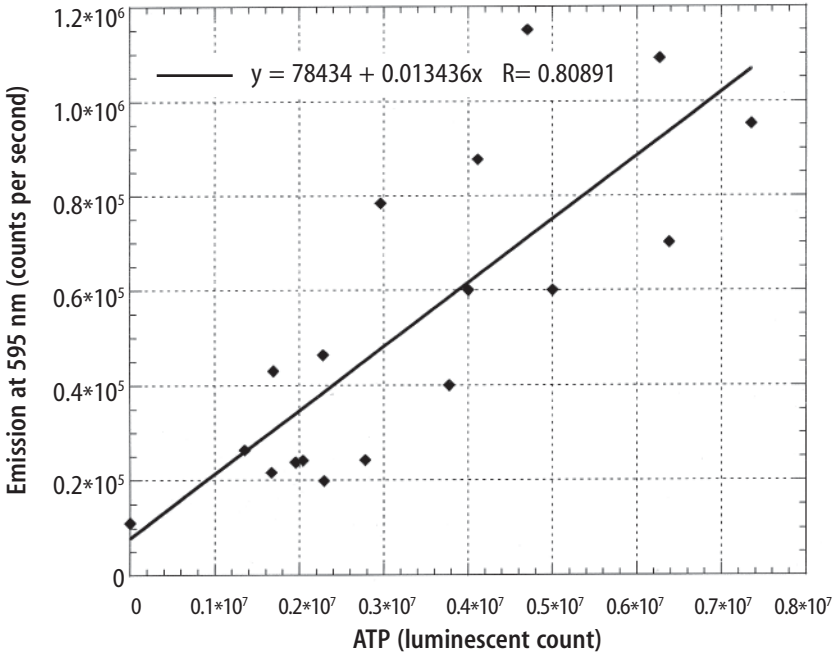
Pathological conditions may have an influence on the ATP content. We compared the distribution of ATP in red blood cells of healthy subjects with the level of ATP in 30 patients. For several patients with the diagnosis of fatigue, arthritis, lung and prostate cancer, the level of ATP was lower than the normal range (**Figure 3**).

Levels of ATP in T cells was found to be lower in patients with breast, prostate, colon, and lung cancer, as well as in patients with anemia and thyroid disorder. The lower level of ATP in T cells of these patients indicates a lower host defense capability, as the T lymphocytes are important cellular components of the immune system. T cells can both modulate the function of other immune cells and directly destroy cells infected with intracellular pathogens.

We found a correlation between the level of ATP production in lymphocytes (T cells) and mitochondrial potential. This correlation between level of mitochondrial potential and the level of ATP produced in T cells is presented in **Figure 5 (below)** for measurements by fluorometer. Measurements of potential by flow cytometer are shown in **Figure 6 (p.56)**. The low level of ATP production and low level of mitochondrial potential shown in Figure 5 were observed after induction of oxidative stress in T cells by incubation with hydrogen peroxide for 15 min.

As mitochondria use the respiratory chain to produce a gradient for the synthesis of ATP, this potential becomes key in the

Figure 5. Correlation between ATP level and level of mitochondrial potential (emission intensity at 595 nm) measured by fluorometer

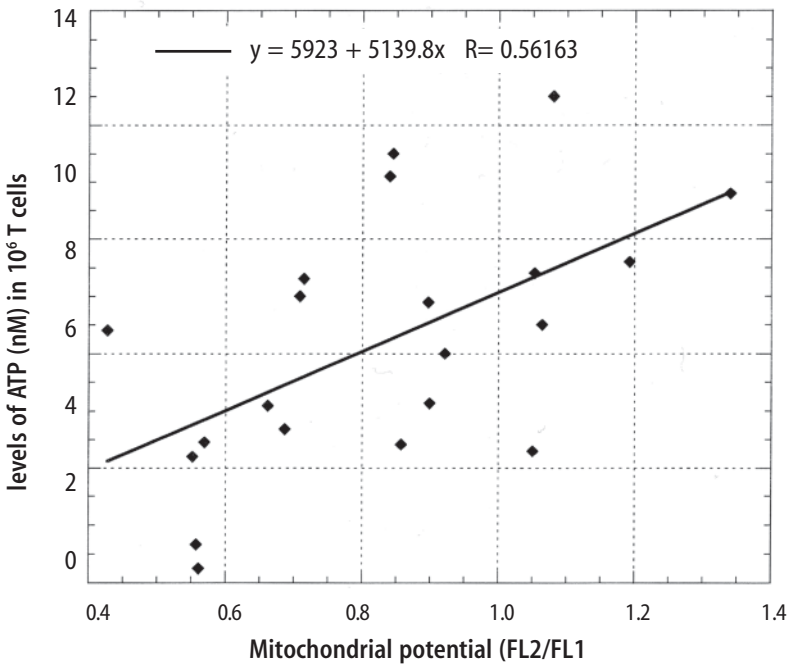


maintenance of energy by driving the synthesis of ATP. According to our results, in T cells the level of ATP production was higher for higher levels of mitochondrial potential.

To study the relationship of mitochondrial potential and age, we analyzed mitochondrial potential in T cells of 39 healthy subjects ranging from 27 to 70 years of age. Over the course of one month, mitochondrial potential was measured three times and averaged for each subject. The within-subject variability of measured mitochondrial potential was less than 35%. A histogram of mean values of mitochondrial potential in different age ranges is presented at Figure 7, (p.57). Statistical significance between groups was determined by analysis of variance. The F ratio indicated that changes had occurred. A Turkey's protected T-test was used to identify individual differences. The difference was significant between mitochondrial potential in groups of subjects aged 27 to 30, 31 to 40, and subjects older than 50 years. The p value was less than 0.05 for the group of 21-30 years vs. all groups older than 40 years. The p value was also less than 0.05 for the group of 31-40 years vs. all groups older than 50, and the group 41-50 vs. 61-70 years.

Results of the analysis allowed us to make important conclusions about the dependence of mitochondrial potential on age and decline in potential with age. This effect may result from age-associated accumulation of mitochondrial defects due to oxidative damage, as these defects are the major contributors to cellular, tissue and organism aging.

Figure 6. Correlation between level of mitochondrial potential, measured by flow cytometer, and level of ATP in T cells..



Conclusion

Rapid, accurate and reliable measurements of nucleotides may be useful in the investigation of normal or abnormal functioning of cells. Measurements of the level of bioenergetics of red cells and T cells may be useful in disease management.

The method of ATP bioluminescence that was developed improved previous methods for ATP assay.

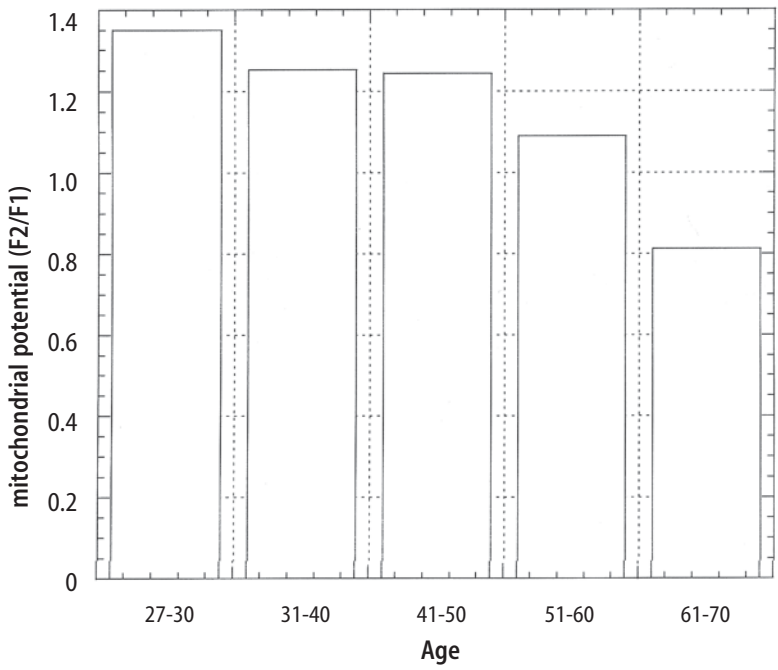
This method was applied for estimation of the normal range of ATP in healthy subjects and for evaluation of disease-induced alterations of ATP in blood cells.

Our measurements showed a positive correlation between the level of ATP production in T cells and the level of mitochondrial potential. According to our results, in T cells the level of ATP was higher for higher levels of mitochondria potential.

We found a decrease in mitochondrial functioning with age and an age-related decrease in mitochondrial protonomotive force in T cells.

Mitochondria are implicated in the aging of cells.[21-24] This is supported by the fact that these organelles are both responsible for most of energy production and for generation of free radicals by the electron transport chain. The age-related alterations in respiration, ATP synthesis, ADP/ATP translocation are found in mitochondria with age. The most important mitochondrial changes are age-related irreversible damage to mitochondrial DNA (mtDNA) by free radicals. The lack of a mtDNA repair mechanism leads to accumulation of errors and increases in gene mutations.[21-22] Continuous accumulation of mutations may lead to deterioration in

Figure 7. Dependence of mitochondrial potential on age.



oxidative phosphorylation and further decline in energy production.

In future, the research in protection of mitochondria by supplementation with nutrients and dietary antioxidants would be important for demonstrating the maintenance of healthy functioning of mitochondria.

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