# EFFECTS OF THE MITOCHONDRIAL RESPIRATION INHIBITOR, ROTENONE, ON APOPTOSIS, CLONOGENIC SURVIVAL AND DELAYED LUMINESCENCE OF HUMAN LEUKEMIA JURKAT T-CELLS

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*Abstract*

We investigated apoptosis and delayed luminescence (DL) of human leukemia Jurkat cells treated with the mitochondrial respiration inhibitor, rotenone (ROT). ROT decreased clonogenic survival and induced apoptosis in a dose-dependent manner. Apoptosis evolved slowly, with a maximum effect observed at 48 h, when the apoptotic cell fraction was strongly correlated with clonogenic survival. Our data indicate that DL detected at 686 nm in the time range 100 μs - 1 ms can be a good measure of the electron transfer at the level of the N2 center, the closest to the ubiquinone site in Complex I of the mitochondrial respiratory chain, which was characterized by a dominant decay time constant of 132 μs. In addition, our measurements suggest that DL is quantitatively correlated with the level of reduced nicotinamide adenine dinucleotide (NADH), the substrate of mitochondrial respiration at the level of Complex I.

## INTRODUCTION

Rotenone (ROT), a well-investigated inhibitor of mitochondrial respiration [1,2], binds to a specific site on Complex I of the mitochondrial respiratory chain (MRC). The substrate of mitochondrial respiration at the level of Complex I is nicotinamide adenine dinucleotide (NAD) in its reduced form (NADH). In MRC Complex I, the two electrons delivered by NADH to flavine mononucleotide (FMN) are individually transferred between eight consecutive iron-sulfur (Fe/S) clusters and eventually reach the ubiquinone. Rotenone inhibits in a specific manner the transfer of electrons from iron-sulfur centers to ubiquinone. As a direct consequence of the rotenone blockage, the intracellular level of NADH increases due to lack of consumption by mitochondria, and the electrons are deviated from Complex I to the surrounding molecular oxygen, hence producing large amounts of superoxide. At the moment, the current available data on the effects of rotenone on the cell cycle or apoptosis/necrosis in Jurkat T-cells are extremely limited. In addition, we have not found any report regarding their effects on clonogenic survival. Here, we investigated these effects under treatments of different time and dosage and analyzed their correlation with delayed luminescence (DL).

DL, also called delayed fluorescence, represents a very weak, long-time scale light emission following exposure to pulsed light or UV radiation. Its main characteristics are the long-time scale of the process and the peculiar kinetics of the emitted light intensity, which displays a complex, multi-component decay pattern. DL can be generated by direct emitters like flavins, molecular oxygen and its species, the DNA, as well as collective molecular interactions, e.g. charge recombination within the mitochondrial/chloroplast electron transport system [3-10]. Our previous studies [9, 10] supported the notion that FMN can absorb UV radiation thus producing excited singlet states that may either decay to the ground state by prompt fluorescence [11] or undergo intersystem crossing to long-lived triplet states [12] which can further relax to metastable intermediate states [12]. The long lifetime of the triplet- or metastable-state species allows a series of photochemical reactions to occur in MRC Complex I and produce secondary excitations, thus giving rise to delayed luminescence. Hence, our data suggested that charge recombination in the Fe/S sites occurring during successive electron transfer steps in MRC Complex I can modulate the intensity and kinetics of DL on a long-time scale. The progresses achieved in promoting DL spectroscopy as a valuable tool in the diagnosis of mitochondrial disorders or cancer [13-16] encourage further studies aimed at characterizing the exact relation between DL and the mitochondrial respiratory system.

## MATERIALS AND METHODS

*Cell cultures*. Human leukemia Jurkat lymphoblasts were cultured in MegaCell RPMI 1640 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, at 37°C in a humidified incubator with 5% $CO_2$. Rotenone was dissolved in dimethyl sulfoxide (DMSO). DMSO was 0.125% (v/v) in all cultures. After the treatment, cells were washed twice with PBS and resuspended in PBS (for DL samples, ~40 × $10^6$ cells/ml) or in complete medium for apoptosis assessment (~0.2 × $10^6$ cells/ml). DL samples were analyzed immediately by DL spectroscopy. Cell density and viability were examined by microscopy.

*Flow cytometry*. 24 and 48 h after the treatment, samples containing $10^6$ living cells were stained with Annexin-V and 7-AAD (PHARMINGEN) at room temperature in the dark and then analyzed with a Becton Dickinson FACS Calibur flow-cytometer. For data acquisition and analysis, the CellQuest and WinMDI 2.9 software were used. All the data are presented as mean ± standard error of the mean.

*Clonogenic survival assay*. After treatment, the cells were washed twice with PBS and plated in 96 well plates at a density of 3 cells/well in 100 µl of complete medium per well. After 3-4 weeks of incubation, the wells containing colonies with >50 cells were counted by microscopy. The plating efficiency was calculated as ln[96/(no. of negative wells)]/(plating density) × 100. Clonogenic survival was determined as the ratio between the plating efficiency of treated and control cells, respectively.

*Delayed Luminescence Spectroscopy*. We used an improved version of the ARETUSA set-up [17]. The cell samples were excited by a Nitrogen Laser source (Laser Photonics LN 230C; wavelength 337 nm, pulse-width 5 ns, energy 100 ± 5 µJ/pulse). A multi-alkali photomultiplier tube (Hamamatsu R-7602-1/Q) was used as a detector. The detected signals were acquired by a Multi-channel Scaler (Ortec MCS PCI) with a minimum dwell-time of 200 ns. DL measurements were done on at least 3 different drops from each cell sample (drop volume 15-25 µl) at room temperature (20 ± 1°C). PBS luminescence was subtracted from all recordings. DL intensity ($I$) was obtained as the number of photons recorded within a certain time interval divided to that time interval and to the number of living cells in the drop. The quantum yield was calculated in three time-domains of the DL emission: 11-100 µs (DL-I), 100 µs - 1 ms (DL-II) and 1-10 ms (DL-III), as the ratio between the integral of $I$ and the energy of the laser.

*Spectrofluorimetry*. Cells were washed twice in a standard saline solution (SS) containing 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, pH 7.2/NaOH. The cells were then incubated with 5 µM MitoSOX Red for 15 min. in the dark at 37°C, then washed once with SS, resuspended in SS at ~$10^6$ cells/ml and transferred into a 2 ml quartz cuvette maintained at 37°C under continuous stirring. Fluorescence was recorded in a Horiba Jobin Yvon spectrofluorimeter equipped with two monocromators. Every 20 s the cell sample was excited sequentially at 360 and 400 nm and emission was collected at 600 nm and 435 nm. The level of NADH and mitochondrial superoxide is proportional to the fluorescence intensity at Ex. 360/Em. 435 and Ex. 400/Em. 600, respectively.

## RESULTS AND DISCUSSION

Rotenone applied for 1 h decreased clonogenic survival of Jurkat cells in an exponential manner, with a characteristic dose $D_0$ = 184.1 µM (Fig. 1). This effect was associated with a marked dose-dependent induction of apoptosis (Fig. 2), which indicated that ROT induced apoptotic, not necrotic cell death. Apoptosis evolved slowly and the apoptotic cell fraction obtained at 48 h was strongly correlated with clonogenic survival (Fig. 2A). There was a significant fraction of cells in early phases of apoptosis even at 48 h after the treatment, which was closely similar to the fraction of cells in late phases of apoptosis for all doses of ROT except the highest one, 250 µM (Fig. 2B).

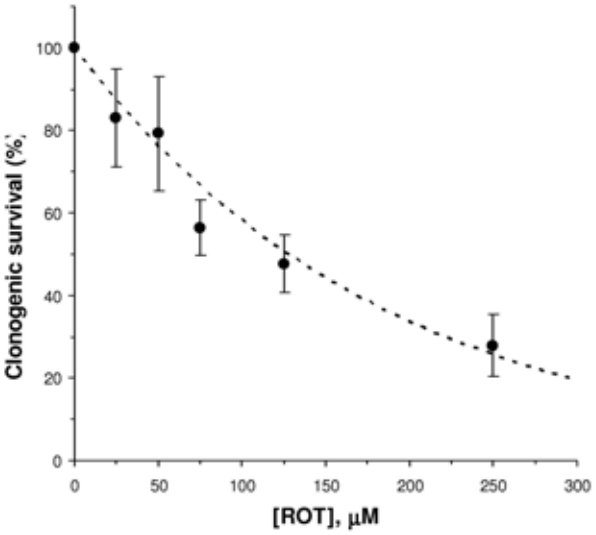


**Figure 1:** Clonogenic survival of Jurkat cells treated with rotenone for 1 h. Average data from 3-5 determinations were fitted to the equation $S(D) = 100 \exp(-D/D_0)$.

ROT produced strong effects on delayed luminescence (Figs. 3, 4). A ~6-fold increase in the DL quantum yield in the visible domain was observed after treatment with 75 µM ROT for 30 min. (Fig. 3). Interestingly, the time-dependence of the ROT effect on DL was biphasic, with a maximum observed for treatments of 60 min. followed by substantial recovery at 90 min. (Fig. 3), indicating that the cells neutralized the rotenone blockage. This idea is also strongly supported by the observed absence of necrosis, which suggests that the cells resumed mitochondrial respiration. The DL augmentation observed at the optimal treatment time (60 min.) increased with the dose of rotenone (Fig. 3).

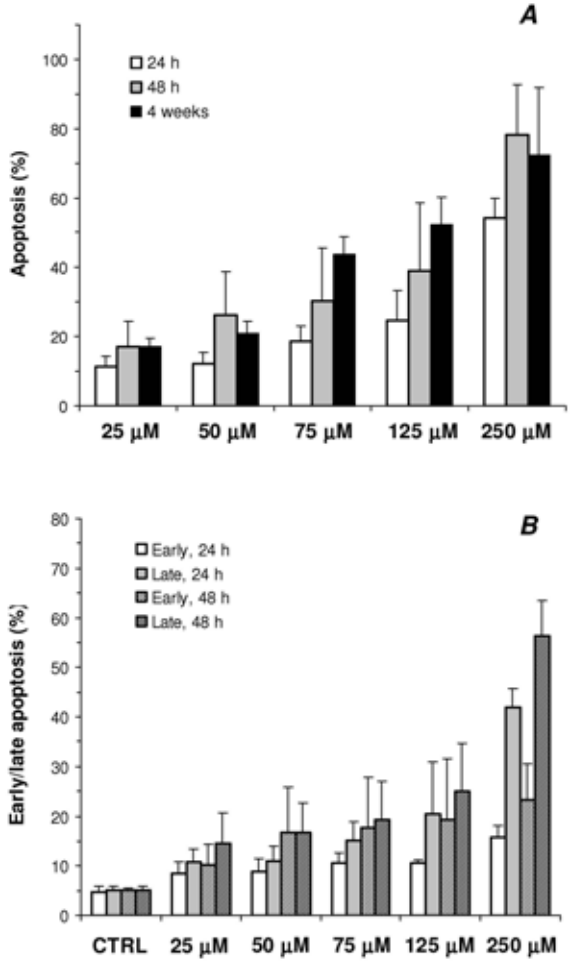


**Figure 2:** Rotenone induces apoptosis in Jurkat cells in a dose-dependent manner. Apoptosis was evaluated 24 h and 48 h after treatment with ROT for 1 h at indicated doses. In B, the death rate calculated from clonogenic survival data obtained after 4 weeks from the treatment (black bars) is compared with the apoptosis rate (white/grey bars) obtained after subtraction of the nonspecific apoptotic rate. Early and late apoptotic cell fractions at 24 h and 48 h after the ROT treatment are shown in C.

Qualitatively similar effects were observed when DL was recorded at $\lambda$ = 686 nm (Fig. 3), whereas at $\lambda$ = 460 nm there was no measurable difference from control cells (not shown). DL at $\lambda$ = 686 nm displayed a marked increase in the DL-II domain, and the photoemission kinetics revealed a distinct exponential component with a time constant of 132 µs (not shown). This value is in good agreement with the rate of electron transfer at the two extreme Fe/S centers N1a and N2 in MRC Complex I [18], which suggests that DL-II/686 nm can be a good measure of the electron transfer at the level of the N2 center, the closest to the ubiquinone site in Complex I.

We have found previously that menadione (MD), quercetin (QC) and $H_2O_2$ act as DL inhibitors [9]. Since both MD and QC inhibit mitochondrial respiration at Complex I, we investigated the mechanisms by which these compounds have an effect on DL which opposes to that of rotenone. To this end, we measured the variations in the level of intracellular NADH and mitochondrial superoxide after exposure to various doses of ROT (e.g., Fig. 4). As expected, both levels increased consistently (up to ~8 times). Instead, QC decreased substantially (~3-5 fold) both NADH and superoxide levels (not shown), probably by exercising primarily an antioxidant effect which prevented the accumulation of these species.

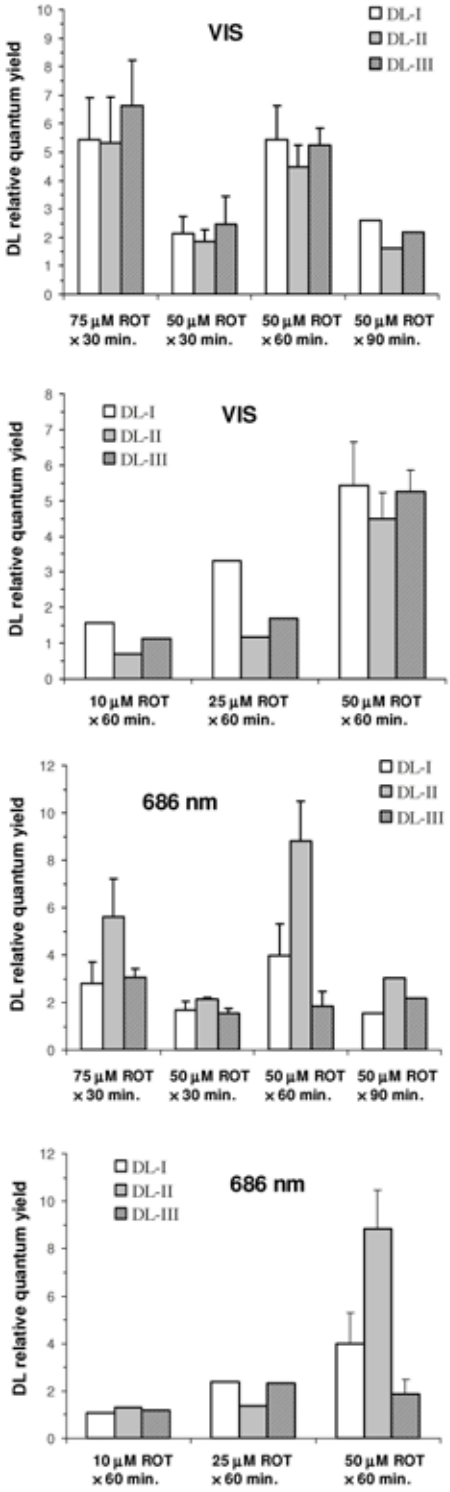


**Figure 3:** Rotenone increases DL emission of Jurkat cells in the three time-regions (DL-I, DL-II and DL-III) in a time- and dose-dependent manner. The DL quantum yield obtained in the visible domain from ROT-treated cell samples was divided to that obtained with not-treated cells.

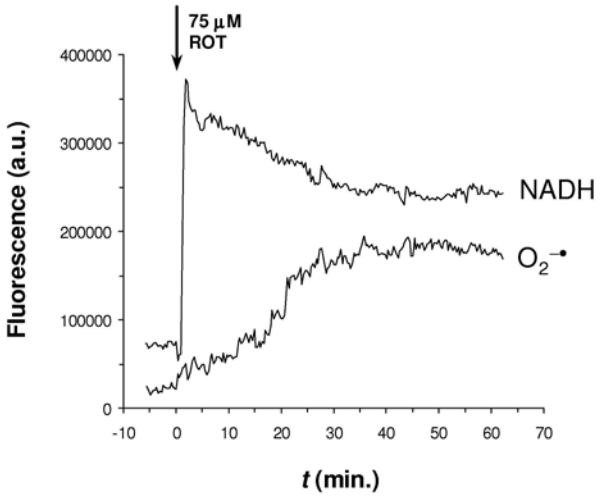


**Figure 4:** Time course of NADH and mitochondrial superoxide levels in suspensions of Jurkat cells exposed to 75 µM ROT.

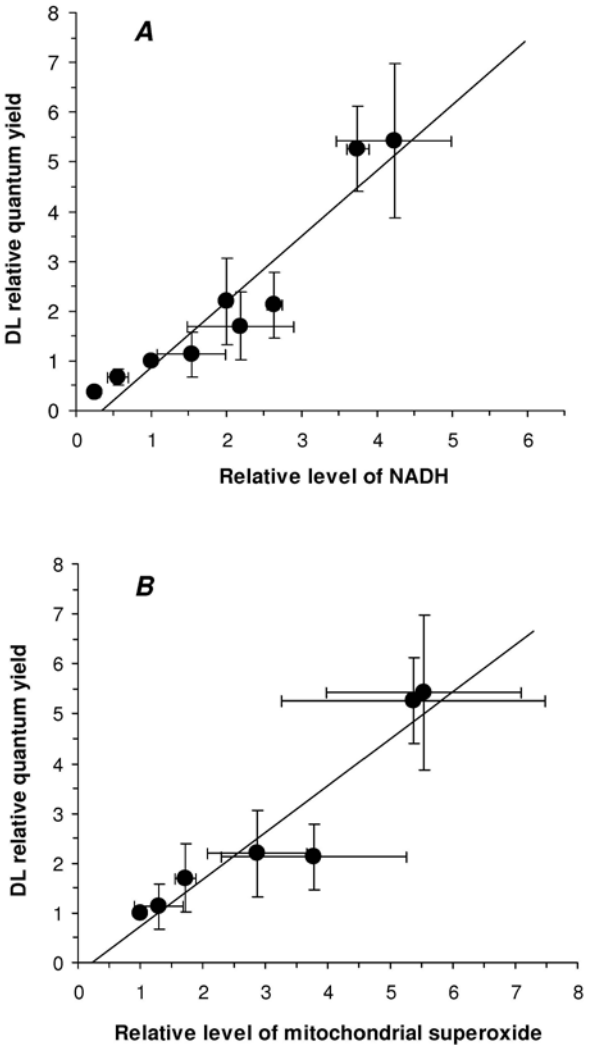


**Figure 5:** Correlation between the DL relative quantum yield in the visible domain and the level of NADH (***A***) and mitochondrial superoxide (***B***) relative to the control.

Importantly, these measurements have indicated for the first time a very strong correlation between the DL quantum yield and the level of cellular NADH and mitochondrial superoxide (Fig. 5), which was characterized by a Pearson correlation coefficient of 0.95 and 0.96 (in the visible domain), and 0.84 and 0.88 (at 686 nm), respectively.

Taken together, these data provide strong evidence supporting the idea that Complex I of the mitochondrial respiratory chain is an important source for delayed luminescence in living cells, and suggest that DL is quantitatively correlated with the level of both NADH and mitochondrial superoxide. In addition, our measurements indicate that charge recombination at the N2 center of Complex I produces delayed luminescence with a characteristic wavelength of 686 nm and a decay time constant of 132 µs.

## ACKNOWLEDGEMENTS

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