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Online Publication Date: 01 February 2007

To cite this Article: Mohammadi-Bardbori, Afshin and Ghazi-Khansari, Mahmoud. (2007) 'Comparative Measurement of Cyanide and Paraquat Mitochondrial Toxicity Using Two Different Mitochondrial Toxicity Assays', Toxicology Mechanisms and Methods, 17:2, 87 - 91

To link to this article: DOI: 10.1080/15376510600822664

URL: http://dx.doi.org/10.1080/15376510600822664

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Comparative Measurement of Cyanide and Paraquat Mitochondrial Toxicity Using Two Different Mitochondrial Toxicity Assays

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ABSTRACT Cyanide (KCN) and paraquat (PQ) are very toxic to mitochondria. In this study the toxicity of KCN and PQ in the isolated rat liver mitochondria was determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay and JG-B (Janus green B) assay by multiwell scanning spectrophotometry. JG-B was used not only for the vital staining of mitochondria, but also for the mitochondrial viability assay and was compared to the MTT assay.

The rat liver mitochondria were first isolated by centrifuge in a mixture of 0.25 M saccharose solution and 0.05 M Tris buffer. Various concentrations of paraquat (0.001 to 100 mM) and KCN (0.0001 to 100 M) on the mitochondria isolated from the liver were investigated. The 50% lethal concentration of toxins were found for PQ (4.45 ± 0.02, 4.96 ± 0.01) and KCN (0.22 ± 0.02, 0.49 ± 0.02), as determined by these assays (JG-B and MTT, respectively). Significant correlations were also observed among the two methods with a 95% coefficient interval ($r^2 = 0.84, p < 0.001; r^2 = 0.91, p < 0.001; PQ and KCN, respectively$). These results suggest that both methods are reliable and are comparable for determining the mitochondrial assay. It is concluded that the JG-B assay may be preferable to the MTT assay because of its simplicity, low cost, sensitivity, and objectivity; in addition, this method is not time dependent.

KEYWORDS Rat Liver Mitochondria; Janus Green B; MTT; PQ; KCN

Parkinson’s disease, diabetes mellitus, possibly Alzheimer’s disease, and even aging in general are influenced in time of onset and severity by mitochondrial deficiencies or dysfunction. Mitochondria have been also shown to play a central role in apoptosis, or programmed cell death, cancer research, and death of an organism (Immo and Scheffler 2000).

Many methods have been introduced to detect mitochondrial function such as mitochondrial swelling (Lehninger et al. 1950); enzymes assay, for example, superoxide dismutase activity (Payá et al. 1992), and measurement of surviving mitochondria or living cells can be achieved by the MTT method. Tetrazolium salts (MTT) are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater et al. 1963).
Janus green staining was used by Lewis (1923) in a study on the role of the mitochondria in the development of the visual cells of chick embryos (Elfvin 1953). Janus green B (JG-B) dye is prepared by conjugating diethyl safranine to dimethyl aniline through an azo linkage. Diethyl safranine is a red dye and JG-B is blue. Thus, the conjugation with dimethyl aniline alters the resonating structure of the diethyl safranine and shifts the position of the absorption spectrum maximum toward the longer wavelengths, with the visible absorption spectrum maximum at 605 nm and at pH 7 (Lazarow and Cooperstein 1952). It has been shown that cytochrome C is reduced by leuco safranine and the leuco safranine is in turn oxidized to diethyl safranine. JG-B has been used as a method for studying the oxidation-reduction potential of various tissues and organisms. Vital staining reaction is probably through the cytochrome oxidase system (Cooperstein et al. 1953).

Cyanide compounds are highly toxic, causing harm by interfering with the body’s use of oxygen. Cyanide inhibits the mitochondrial respiratory chain enzyme cytochrome oxidase, causing hypoxia. It is primarily considered as a neurotoxin but its other toxic manifestations are also well documented.

Paraquat (N,N′-dimethyl 4,4′-bipyridium) is very toxic to animals, including humans, with putative toxicity mechanisms associated with mitochondrial redox systems (Lambert and Bondy 1989). Mitochondria are candidate targets of paraquat toxicity in animal tissues and plants (Taylor et al. 2002). In animals and plants, mitochondria are often taken as targets for PQ toxicity (Lambert and Bondy 1989).

In this study we are evaluating the JG-B assay method comparable to the MTT assay method in the mitochondrial toxicity of PQ and cyanide.

### MATERIALS AND METHODS

#### Compounds

Paraquat dichloride salt and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). Janus green was obtained from Merck (Germany). All other chemicals were obtained from Sigma Chemical Co.

#### Animals and Experimental Groups

This study was performed on 36 male Wister albino rats (weighing 180–220 g). They were kept in individual cages in a controlled room (temperature, 20–25°C; humidity, 70–80%; exposed to 12 h of daylight). The rats were fed with standard rat food and tap water until experimentation. Twelve hours before the experiment the rats were stopped feeding but allowed free access to tap water. Limitation of food and water was not applied to the animals that were put into their cages after the experiments.

#### Preparation of Mitochondria

The liver was removed and minced with small scissors in a cold mannitol solution containing 0.225 M D-mannitol, 75 mM sucrose, and 0.2 mM ethylenediaminetetraacetic acid (EDTA). The minced liver (30 g) was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700 × g for 10 min at 4°C to remove nuclei, unbroken cells, and other nonsubcellular tissue. The supernatants were centrifuged at 7000 g for 20 min. These second supernatants were pooled as the crude microsomal fraction and the pale loose upper layer, which was rich in swollen or broken mitochondria, lysosomes, and some microsomes, of sediments was washed away. The dark packed lower layer (heavy mitochondrial fraction) was resuspended in the mannitol solution and recentrifuged twice at 7000 × g for 20 min. The heavy mitochondrial sediments were suspended in Tris solution containing 0.05 M Tris-HCl buffer (pH 7.4), 0.25 M sucrose, 20 mM KCl, 2.0 mM Mg Cl2, and 1.0 mM Na2 H PO4 at 4°C before assay.

#### MTT Assay

This assay is a quantitative colorimetric method to determine cell viability. It utilizes the yellow tetrazolium salt (MTT), which is metabolized by mitochondrial dehydrogenase enzyme from viable cells to yield a purple formazan reaction product (Mosmann 1983).

In our experiments, the MTT assay was for rat liver mitochondria suspension in tubes. Mitochondria suspension was mixed with MTT, yielding the final concentration of MTT 0.2 mg/mL and the final concentration of mitochondria 0.2 mg/mL in the sample. In this assay, incubation of mitochondria loaded with MTT lasted 2 h. The first time the mitochondria suspension was mixed with different concentrations of toxicants. Finally, the solution was gently removed. To eliminate the toxicant it is necessary to wash...
mitochondria with the washing solution centrifuged at 7000 g for 20 min and mixed with MTT, and produced formazan crystals were dissolved in 200 µL of DMSO after a few minutes at room temperature to ensure that all crystals were dissolved the plates. Then the plates were read on a microplate reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99.

**JG-B Assay**

This assay is based on specialized mitochondrial staining, using JG-B in two steps. First, the mitochondrial suspension (0.2 mg/mL) is treated with different concentrations of cyanide (0.0001–100 M) and paraquat (0.001–100 mM) at 37°C; 100 µL of this suspension was taken and mixed with JG-B (1:100,000) at 1:1 volume ratio. This mixture was read at a wavelength of 610 nm, a reference wavelength of 540 nm, and multiwell plate reader at 30 and 60 min. Second, after 1 h the suspension was centrifuged at 7000 g for 20 min and pellet was washed out several times with buffer and then incubated again at 37°C. After 2 h 100 µL of this suspension is taken and measured using a micro-ELISA reader at a test wavelength of 610 nm and reference wavelength of 540 nm.

**Viability Percentage Calculation**

Each test sample was calculated according to Heidari et al. (2001) based on the following calculation (Shokri et al. 2000):

\[
\% \text{Mitochondrial toxicity} = \frac{\text{Mean absorbance of toxicant}}{\text{Mean absorbance of negative control}} \times 100
\]

\[
\% \text{Viability} = 100 - \% \text{Mitochondrial toxicity}.
\]

**Experimental Design**

The mitochondria suspension was divided into 14 groups for dose response determination. Group 1 contained control, groups 2–8 contained cyanide (0.0001–100 M), groups 9–14 contained paraquat (0.001–100 mM).

**Statistical Analysis**

The LC50 was determined by probit analysis using the “pharm.pcs” statistical package (Springer-Verlag, New York). Statistical differences were determined by analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test on the Instat package. Differences were regarded as significant at p < 0.05.

**RESULTS**

The mitochondrial toxicity of KCN and PQ was measured at a concentration ranging from 0.0001 to 89

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**FIGURE 1** Determination of KCN toxicity in the isolated rat mitochondria by using two methods: (A) JG-B assay and (B) MTT assay. The results represent the mean ± SD (n = 16).
100 M and 0.001 to 100 mM, respectively. To determine the LC50 of KCN and PQ in each assay, the percentage of mitochondrial toxicity was plotted as probit against log concentrations of the toxins (Fig. 1A, B and Fig. 2A, B).

Accordingly, the LC50 of KCN was found to be 0.22 to 0.49 and that of PQ 4.45 to 4.96 in the JG-B and MTT assays, respectively. With a 95% coefficient interval, a significant correlation was observed for KCN between the two methods ($r^2 = 0.84, p < 0.001$) (Fig. 3), and a very significant correlation was found between the two methods ($r^2 = 0.91, p < 0.001$) for PQ (Fig. 4).

DISCUSSION

We investigated the possibility of using a color reaction as a measure of viable mitochondria. Ideally, a colorimetric assay for cells or mitochondria should utilize a colorless substrate that is modified to a colored product by any living cells or organelles but not by dead cells or mitochondria. The tetrazolium ring is cleaved in active mitochondria and so the reaction occurs only in living mitochondria. Cooperstin and Lazarow (1953) have shown that when a supervitally stained cell is treated with 0.001 M KCN, the JG-B is regenerated, and when the cyanide is removed JG-B is reactive. The cytochrome oxidase system, an enzyme within mitochondria,
prevents the reduction of JG-B. This enzyme system is oxygen dependent and cyanide sensitive.

The main advantage of the colorimetric assay is the speed with which samples can be processed. This allows the assay to be read with no removal or washing steps. This increases the speed of the assay and helps to minimize variability between samples. This colorimetric assay measures the mitochondrial activity at the endpoint of the assay, whereas MTT incorporation measures the activity during the last few hours of the assay. Since the colorimetric assay is so rapid, large amounts of data can be generated.

Two mitochondrial toxicity methods—the MTT assay and JG-B assay—were investigated and compared in this study, despite their differing principles and methodologies. These findings could also be seen in the correlation analysis, in which the two colorimetric assays displayed a statistically higher correlation (p < 0.0001) (see Figs. 3 and 4). It is concluded that the JG-B assay may be preferable to the MTT assay because of its simplicity, low cost, and higher sensitivity and objectivity; in addition, this method is not time dependent (data not shown).

ACKNOWLEDGMENT

This study was supported by a grant from Vice Chancellor of Research of Tehran University of Medical Sciences (132/11737, March 18, 2006)

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