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Searching for alternative toxicology testing systems: The response of isolated mitochondria from *Saccharomyces cerevisiae*, potato tuber, and mouse liver to a toxic insult

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Abstract

Mitochondria are cellular power plants known as essential organelles for energy (ATP) metabolism. However, today it is evident that various vital compounds are partially or exclusively synthesized in mitochondria. Moreover, this organelle plays a pivotal role in essential processes such as cell death. The isolated mitochondrion is an excellent experimental model for evaluating the role of mitochondria in the pathogenesis of diseases. Various *in vitro* and *in vivo* experimental models have been developed to study mitochondria. On the other hand, some alternative models could also help decrease the use of animal models. In the current study, we compared the response of mitochondria isolated from mouse liver, *Saccharomyces cerevisiae* (*S. cerevisiae*), and potato tuber to various concentrations of calcium (Ca^{2+}) as a robust mitochondrial disturbing agent. The current study found that significant mitochondrial depolarization, decreased ATP levels, mitochondrial permeabilization, and decreased mitochondrial dehydrogenases activity were found in all isolated mitochondrial preparations. No significant difference between mouse liver, *S. cerevisiae*, and potato tuber mitochondria were detected in experiments carried out in the current investigation. We are aware that mitochondria from different species have a huge structural and enzymatic variance. Hence, these models could just estimate the effect of xenobiotics in biological systems. However, the data derived from this study could finally help to decrease the use of experimental animals and provide new approaches for evaluating mitochondrial function.

Keywords: Alternative toxicology models, ATP, Drug development, Mitochondrial disease, Mitochondrial impairment

1. Introduction

Since their identification, a plethora of studies have been carried out on mitochondria, and many physiological roles have been identified for these unique organelles. Mitochondria are critical organelles that play a wide range of pivotal biological actions in eukaryotic cells. Energy (ATP) metabolism is the most crucial function of these magic organelles (1, 2). However, a plethora of other vital processes is entirely or partially occur in mitochondria. For example, heme synthesis, citrate metabolism, folate cycle, and nucleotides synthesis produce several amino acids connected to mitochondria (3-6).

Mitochondrial impairment is crucially involved in the pathogenesis of a wide range of human diseases from cancer, renal diseases, meta-

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bolic disorders, liver diseases, neurodegenerative complications, cardiovascular diseases, or aging (7-14). All these data indicate that investigating mitochondria and finding preventive/pharmacological interventions to target this organelle could considerably change the future of therapeutic strategies for managing various human diseases.

Isolated mitochondria from various sources and investigating the mechanism of xenobiotics on these organelles is a routine process in drug discovery and development (15-27). These studies enhance our understanding of the effects of xenobiotics in biological systems. On the other hand, evaluating the effects of xenobiotics (e.g., very toxic compounds) on these organelles could help find appropriate therapeutic options against these complications. The cases of phosphine or cyanide are well-known examples of these studies. Using experimental animals seems to be inevitable for such investigations to date.

Although mitochondria isolated from other species such as the yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) or potato tuber might estimate the adverse effects of xenobiotics on this organelle, it should be mentioned that extrapolating data from in vitro studies to human cases is a long and restrict process. Therefore, investigating *S. cerevisiae* or potato mitochondria could only estimate the adverse effects of xenobiotics in biological systems. In the current study, we compared the response of mitochondria isolated from mouse liver, *S. cerevisiae*, and potato tuber to calcium (Ca^{2+}) as a robust mitochondrial disturbing agent. The obtained data could help in the development of alternative toxicology testing systems for future investigations.

2. Material and methods

2.1. Chemicals and reagents

Methanol HPLC grade, zymolyase, sucrose, acetonitrile HPLC grade, bovine serum albumin (BSA), rhodamine 123, and trichloroacetic acid (TCA) were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). Tetrabutylammonium hydroxide, 3-diol-hydrochloride (Tris-HCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), calcium anhydride, ethylene glycol-bis(β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA), mannitol, potassium phosphate monobasic (KH_2PO_4), potassium hydroxide (KOH), 3-(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide (MTT), and hexadecyl-trimethyl-ammonium bromide were obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male BALB/c mice (n=60) weighing 25±2 g were obtained from Shiraz University of Medical Sciences, Shiraz, Iran. Animals were maintained at a standard animal house (temperature of 23±1 ºC, ≈40% relative humidity, 12 h dark/light cycle, and adequate ventilation) (28-31). Mice had free access to tap water and a standard commercial rodents diet (RoyanFeed®, Isfahan, Iran). The institutional laboratory animals care and use committee at Shiraz University of Medical Sciences approved all animal experiments IR.SUMS.REC.1398.371/374). *S. cerevisiae* yeast was cultured based on standard protocols in the biotechnology laboratory of Pharmaceutical Sciences Research Center, Shiraz, Iran. The potato was purchased from a retailer in Shiraz, Fars, Iran.

2.3. Mitochondria isolation protocols

Mitochondria were isolated from mice livers based on the differential centrifugation protocol (20, 21, 32-38). For this purpose, the liver was excised from deeply anesthetized mice (thiopental, 80 mg/kg, i.p), washed, and minced in an ice-cold solution (220 mM sucrose, 2 mM HEPES, 0.5 mM EGTA, 70 mM mannitol, and 0.1 % BSA, pH=7.4). Then, the minced tissue was transported into a fresh solution (5 mL buffer/1 g of the tissue) and homogenized (15, 20, 25, 39-46). At the first round of the centrifugation (1000 g, 10 min, 4 ºC), unbroken cells and nuclei were first pelleted. Then, the supernatant was further centrifuged at 10000 g for 10 minutes at 4 ºC to pellet the mitochondria fraction. The second centrifugation step was repeated three times (fresh buffer medium each time). The final mitochondrial pellet was suspended in a buffer containing 70 mM mannitol, 220 mM sucrose, 2 mM HEPES, and 0.5 mM EGTA (pH=7.4) (15, 47-57).

For isolating *S. cerevisiae* mitochondria,

the culture medium was centrifuged (3000 g, 10 min, 25 °C), and the yeast pellet was re-suspended in pre-warmed DTT buffer (2 mL/g wet weight cells) and mixed slowly (approx. 80 rpm) at 30 °C for 20 min. Then, samples were re-centrifuged (3000 g, 5 min, 25 °C), and the pellet was re-suspend in zymolyase buffer (about 7 mL/g wet weight) (58). Cells were harvested by centrifugation (3000 g for 5 min), and the pellet was washed with zymolyase buffer (7 mL/g wet weight) again (3000 g for 5 min). Then, the pellet was re-suspend in ice-cold homogenization buffer (5 mL/g wet weight, homogenization buffer components are identical for liver mitochondria isolation) (58). The homogenate was centrifuged (1500 g, 5 min, 4 °C), and the supernatant was collected. The supernatant was further centrifuged (12,000g for 15 min, 4°C) to obtain the mitochondrial pellet. The recent centrifugation step was repeated three times using a fresh isolation buffer medium. Finally, the mitochondrial pellet was re-suspend in the incubation buffer (same for the liver mitochondria) (58).

For isolating mitochondria from potato tuber, peeled potatoes were homogenized using a juice extractor, and the extract pH was adjusted to 7.2 using KOH (2 M) (59). The homogenate was stood for 5 min at room temperature for starch sedimentation (59). Afterward, the supernatant was filtered (cotton and funnel) and centrifuged (3000 g, 5 min, 4 °C). The supernatant was gathered and underwent another set of centrifugation (18000 g, 10 min, 4 °C). The second centrifugation round (18,000 g, 10 min, 4 °C) was repeated three times to purify isolated mitochondria (59). Finally, the mitochondrial pellet was re-suspended in the incubation buffer (as mentioned for liver mitochondria).

2.4. Mitochondrial dehydrogenases activity

The 3-(4, 5-dimethylthiazol-2-yl)-2, the 5-diphenyltetrazolium bromide (MTT) test was used to determine mitochondrial dehydrogenases activity in the current study (60-66). Briefly, a mitochondrial suspension (0.5 mg protein/ml) was incubated with 0.4% of MTT (37 °C, 30 min, in the dark) (25). The product of formazan crystals was dissolved in 1 mL of dimethyl sulfoxide (61, 67-74). Then, samples were centrifuged (5 min, 3000 g), and the absorbance of λ=570 nm was used (EPOCH® plate reader, USA) (23, 65, 75).

2.5. Mitochondrial depolarization

In the current investigation, mitochondrial uptake of the rhodamine 123 was used to evaluate mitochondrial depolarization (21, 28, 76-78). For this purpose, the mitochondrial fractions (0.5 mg protein/mL) were incubated with 10 µM of rhodamine 123 (15 min, in the dark) (33, 72). Afterward, samples were centrifuged (15000 g, 1 min, 4 ºC), and the fluorescence intensity of the supernatant was measured (FLUOstar Omega® plate reader, $\lambda_{excitation}$=485 nm and $\lambda_{emission}$=525 nm) (79-82).

2.6. Mitochondrial swelling

Analysis of mitochondrial swelling was spectrophotometrically estimated through changes in light scattering as monitored at λ=540 nm (79, 83). Briefly, samples of isolated mitochondria (0.5 mg protein/ml) were added to a 96-well microplate reader, and Ca^{2+} was used as the inducer of mitochondrial swelling (84, 85). Then, the absorbance was monitored at λ=540 nm for 30 min (EPOCH® plate reader, USA). The difference in primary and final absorbance was calculated (55, 79, 85-87).

2.7. Mitochondria ATP levels

Samples (1 mL) of isolated mitochondria (1 mg protein/mL) were treated with 50 µL of the ice-cooled trichloroacetic acid (50% w: v, 4 ºC), incubated on ice for 5 min, and then centrifuged (15000 g, 10 min, 4 °C). The supernatant was neutralized with 15 µL of 4 M KOH (88). Finally, samples were centrifuged (15000 g, 30 min, 4 ºC), and 25 µL of the prepared extract was injected into an HPLC apparatus (89). The HPLC system consisted of a C-18 column and a UV detector (λ=254 nm). An isocratic method was used. The mobile phase was comprised of KH2PO4 (100 mM, pH=7 adjusted with KOH), tetrabutylammonium hydroxide (1 mM), and acetonitrile HPLC grade (2.5% v: v). The flow rate was 1 mL/min (74, 77, 88, 90).

2.8. Statistical analysis

Data are given as mean±SD. The comparison of data sets was carried out by the one-

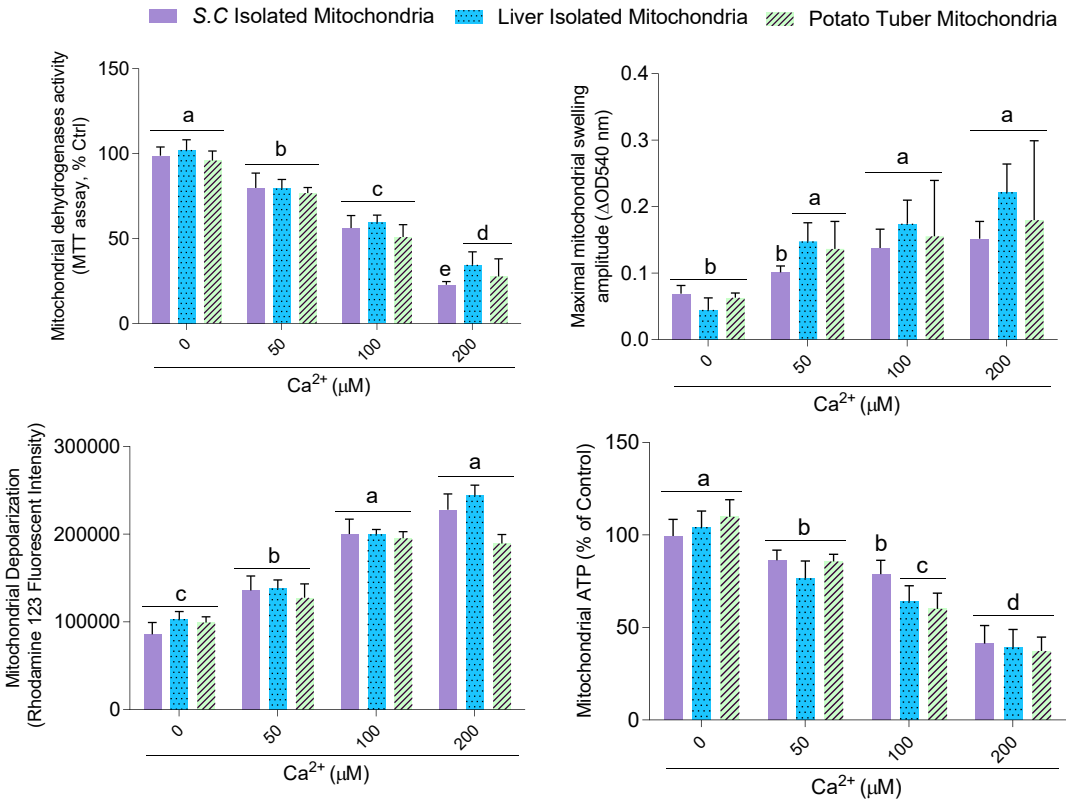

Figure 1. Indices of functionality in mitochondria isolated from *S. cerevisiae*, potato tuber, and mouse liver. Data are represented as mean±SD (n=5).
Columns with different alphabetical superscripts are statistically significantly different (P<0.05).

way analysis of variance (ANOVA) and Tukey's multiple comparisons. A P<0.05 was considered a statistically significant difference.

3. Results and discussion

Several tests were carried out on mitochondria isolated from mouse liver, *S. cerevisiae*, and potato tuber. First, the activity of mitochondrial dehydrogenases (MTT test) was assessed (Figure 1). This marker reveals the proper action of several dehydrogenases enzymes involved in energy metabolism (91). The complex II of the mitochondrial respiratory chain is a well-known dehydrogenase that uses MTT to produce the purple formazan crystal (91). No significant mitochondrial dehydrogenase activity test changes were detected in the current study when control *S. cerevisiae*, liver, and potato mitochondria were compared (0 µM of Ca^{2+}) (Figure 1). The response of the mitochondrial preparations of these species to various levels of C^{2+} was also the same in the MTT test (Figure 1). At higher doses of Ca^{2+} mitochondrial dehydrogenases, activities were significantly decreased dose-dependently (Figure 1). However, the lowest activity of mitochondrial dehydrogenases was detected in *S. cerevisiae* mitochondria exposed to 200 µM of Ca^{2+} (Figure 1). This finding might indicate that *S. cerevisiae* mitochondria are more susceptible to higher Ca^{2+} concentrations (Figure 1).

Evaluating mitochondrial permeabilization in samples isolated from the species investigated in the current study revealed significant mitochondrial swelling in Ca^{2+}-treated groups (Figure 1). However, the dose of 50 µM of Ca^{2+} caused no significant mitochondrial swelling in *S. cerevisiae* mitochondria than in the control group (Figure 1).

The assessment of mitochondrial depolarization revealed dose-dependent impairment in the rhodamine 123 capturing ability of mitochondria isolated from all species investigated in the current study. The maximum amount of mitochondrial de-

polarization was detected in Ca^{2+} concentration (Figure 1). There was no significant difference in Ca^{2+}-induced mitochondrial depolarization when different mitochondrial preparations were compared in the current study (Figure 1). Therefore, these data could indicate that these mitochondrial preparations could alternatively be used in studies about mitochondrial depolarization.

The current study found that the addition of Ca^{2+} to mitochondrial preparations from different species dose-dependently caused a significant decrease in mitochondrial ATP metabolism (Figure 1). Hence, this toxic insult impaired a fundamental feature of the mitochondrion. Like other measurements carried out in the current study, no significant difference in Ca^{2+}-induced ATP depletion was detected when various mitochondrial preparations were compared (Figure 1).

Evaluating the effects of novel pharmaceuticals on the function of cellular powerplants is a critical process in drug development (92-94). Hence, it is crucial to evaluate these candidates' effects or assess toxins to find therapeutic/preventive options in this field. But we, as members of the scientific community involved in the development of new drugs or the study of the mechanism of damage of these substances in biological environments, must know that different models essentially could give a completely different result. Therefore, finding an answer in a model doesn't simply mean that it could be extrapolated to other models.

To introduce a new drug and ultimately use it in humans, very complex steps must be taken (95, 96). In this order, several factors, including the biocompatibility and safety of these candidates, should be tested. Obviously, isolated mitochondria preparations used in this model have extreme differences in their size, structure, enzymes, and genetic content. However, hopefully, they almost revealed a similar response to a common toxic insult. But, it should be mentioned that molecules such as drugs may not have the same results in such systems. Hence, using other toxicity testing systems is a crucial and inevitable component of the drug development process. Finally, it should be mentioned that all models, despite their differences, could together give reasonable estimates of the effects of a drug on a biological system. To date, these are all available tools we have as scientific methods. In the future, the convergence of these models and other technologies such as artificial intelligence could probably make developing secure pharmaceuticals and therapeutic interventions much more accessible and surely with lower ethical issues (e.g., using experimental animals).

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Conflict of Interest

None declared.

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