The toxic effects of metallic nanoparticles and rare earth elements on the mitochondrial oscillator

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Mitochondria display non-linear oscillation in NAD(P)H levels as a result of the delicate balance between NAD(P)H production during glycolysis and oxidation during the electron transport chain step. The purpose of this study was to examine the effects of selected elements on the mitochondrial oscillator in freshwater mussels *Dreissena bugensis*. Freshly isolated mitochondria were treated by increasing concentrations of zinc oxide nanoparticles (nZnO), erbium (Er) and lutetium (Lu) for 40 min, and the oscillations in NAD(P)H levels were measured using fluorescence spectroscopy. Under normal conditions, NAD(P)H levels oscillate with a period of 2 min (frequency 0.25) and an initial oxidation rate of NAD(P)H during the first 20 min which stopped for the remainder of the exposure period. Exposure to the selected elements first showed that the NAD(P)H oxidation rate progressed well for over 20 min and the rate was significantly increased for nZnO (0.64 µg/L), Er (12 mg/L) and Lu (16 mg/L). The oscillatory behaviour of NAD(P)H levels (fluorescence data) were examined using Fourier transformation, which revealed that these elements reduced the amplitudes at the normal frequency in a dose-dependent manner, with signals appearing at lower frequency. The decrease in amplitudes at the normal frequency occurred at concentrations 5-10 times less than those that significantly increased the rate of NAD(P)H oxidation. Moreover, the lower frequencies were out of phase with those of the controls, which suggests the appearance of emerging toxic frequency. Oscillations in NAD(P)H could be altered by contaminants and could thus constitute a novel and sensitive biomarker of ecotoxicity.

**Key Words:** Mitochondria; Oscillations; NADH; Musculae; Toxicity

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Lawrence River upstream from the city of Montreal (Quebec, Canada). They were kept at 4°C in the dark during transport to the laboratory and placed in a 60 L aquarium under constant aeration at 15°C. They were fed 3 times a week with commercial suspensions of algae and were kept for 3 months under those conditions. Mitochondria suspensions were prepared from n=10 mussels as follows. Each mussel’s weight and shell length were determined before its soft tissues were removed, weighed and placed in an ice-cold buffer of 200 mM sucrose, 10 mM Hepes- NaOH, 0.1 µg/mL apoprotinin and 1 mM EDTA at 20% w/v ratio. The tissues were homogenized using a Teflon pestle tissue grinder and centrifuged at 1500 x g for 20 min at 2°C. The supernatant was carefully removed and centrifuged at 10 000 x g for 30 min at 2°C. The pellet was removed from the supernatant, resuspended in the homogenization buffer and centrifuged again at 10 000 x g as described above. The pellet was resuspended in 200 mM mannitol, 50 mM sucrose containing 10 mM Hepes-NaOH, pH 7.4, 5 mM KH₂PO₄ and 1 mM NaHCO₃, and stored in that condition at -85°C until analysis. The total levels of protein were determined in the homogenate and mitochondria suspension using protein-dye binding (16). Standard solutions of bovine serum albumin (BSA) were used for calibration.

Oscillations of mitochondria were determined in dark 96 well microplates for fluorescence readings of NAD(P)H. Mitochondria were diluted in dark microplates to 0.1 mg/mL total protein in the reaction media composed of 200 mM Mannitol, 50 mM sucrose, 5 mM KH₂PO₄, 5 mM NaHCO₃, 2 mM MgCl₂, 1 mM KADP, 2 mM pyruvate and 5 mM Hepes-NaOH pH 7.4. Microplate fluorescence readings were taken at 30 sec intervals for 40 min in the sweep mode (excitation using a quick flash burst of 10 msec and 7.4. Microplate fluorescence readings were taken at 30 sec intervals for 40 min of incubation time of mitochondria in the reaction media. The activity of NAD(P)H oxidase activity in mitochondria was determined for the first 15 min of incubation in a rotenone-insensitive manner by NADH cytochrome b5 oxidoreductase activity. The rate of NAD(P)H oxidation was measured in order to determine the general effects of the tested compounds (Figure 1). Mitochondria were suspended in the presence of 2 mM pyruvate and 1 mM ADP as a carbon source for respiratory (glycolysis) activity. Exposure of mitochondria suspension for 40 min to nZnO increased the oxidation rate of NADPH (1.2 fold relative to the control) at the highest concentration tested (0.64 µg/L), giving a toxic threshold of 0.6 µg/L. Exposure to Er also increased the rate of NADPH oxidation at a threshold concentration of 10 mg/L reaching a 1.4-fold relative to controls. For Lu, the rate of oxidation was also increased at a threshold concentration of 15 mg/L, reaching a 1.4-fold increase relative to controls i.e., in naïve mitochondria.

The concentration of zinc oxide nanoparticles (nZnO), erbium chloride (Er⁺), lutetium chloride (Lu⁺) and strontium chloride (Sr⁺) on the mitochondrial oscillator was also determined. The concentrations were chosen as to elicit a quick effect (min) in mitochondrial suspensions. Increasing concentrations of nZnO (0.08-0.64 µg/L), Er (2-16 mg/L), Lu (2-6 mg/L) and Sr (0.3-5 mg/L) were added in the reaction mixture before the addition of mitochondria. The oscillatory activity in NADPH was determined as described above. Mitochondrial NADPH oxidation may proceed by means of two pathways. First, NADH is oxidized by means of a rotenone-sensitive route by the NADH ubiquinone oxidoreductase. Second, NADH is oxidized in a rotenone-insensitive manner by NADH cytochrome b₅ oxidoreductase which is located in the mitochondrial outer membrane (17). The activity of NADPH oxidase activity in mitochondria was determined for the first 15 min of incubation time of mitochondria in the reaction media. The activity was reported as decrease in NADPH fluorescence/min.

DATA ANALYSIS
The non-linear cyclic changes in biomarkers were analyzed using Fourier transformation analysis (Statistica, Tibco scientific, and version 13, USA). In the present study, the time- related changes in NADPH levels in mitochondria were determined in triplicate per treatment. Control mitochondria were exposed to buffer only. The Fourier transform procedure models the periodic nature of the data by fitting sine and cosine functions at different frequencies to the data at each measurement time (30 sec). In essence, the procedure transforms any functions f (x,t) into functions of frequencies g (k). More precisely, the Fourier series is defined as g (k) = aₖ + ∑

\[\frac{A_k}{2}\cos(2\pi kn/N) + B_k\sin(2\pi kn/N),\]

where n=1 to N observations (time in the present case). The variable n represents the individual observations of the series expressed in time (min), and k is the frequency. The constants Aₖ and Bₖ are used to calculate the periodogram (Pₖ) value, which is related to the sum of amplitude variance of the sine and cosine functions at each frequency k: Pₖ = (A₂ + B₂) × N/2, where N is the total number of observations. The Pₖ value is thus related to the variance of the function at a given frequency. The significance of each Pₖ value was tested and compared to random “noise” using the Kolmogorov-Smirnov test (exponential adjustment). Correlation analysis between Pₖ values and exposure concentrations was also performed using the Pearson-moment procedure. Significance was set at p<0.05 in all cases.

RESULTS
The rate of NADPH oxidation was measured in order to determine the general effects of the tested compounds (Figure 1). Mitochondria were suspended in the presence of 2 mM pyruvate and 1 mM ADP as a carbon source for respiratory (glycolysis) activity. Exposure of mitochondria suspension for 40 min to nZnO increased the oxidation rate of NADPH (1.2 fold relative to the control) at the highest concentration tested (0.64 µg/L), giving a toxic threshold of 0.6 µg/L. Exposure to Er also increased the rate of NADPH oxidation at a threshold concentration of 10 mg/L reaching a 1.4-fold relative to controls. For Lu, the rate of oxidation was also increased at a threshold concentration of 15 mg/L, reaching a 1.4-fold increase relative to controls i.e., in naïve mitochondria.

In the presence of ADP and pyruvate, NADPH levels in control mitochondria oscillate over time during the 40 min incubation period (Figure 2A). Although a general decreasing trend is observed in NADPH levels within the first 20 min, it is accompanied by short oscillation changes in NADPH levels over time. Indeed, NADPH levels oscillate with a period ranging from 1.6 min to 2.2 min (frequencies between 0.240.32), the major period is 2 min or a frequency of 0.25 (Figure 2B). These oscillations persist between 17 min and 40 min where no net change in total NADPH levels is observed. These oscillations are similar to those observed in mitochondria of mammals.

In the attempt to understand the effects of potentially toxic xenobiotics on the oscillatory behavior of mitochondria, mitochondria were exposed to increasing concentrations of nZnO (0.08-0.64 µg/L), Er (2-16 mg/L) and Lu (2-6 µg/L) during oscillations for 40 min at 30°C. The oscillatory behavior in NADPH levels in time is shown in mitochondria exposed to nZnO (Figure 3A). Visual inspection of the figures revealed that the amplitudes of
Figure 2) Oscillatory behaviour of NADH levels in mitochondria. (A) Oscillatory changes of NADH levels in mitochondria suspensions over time. The NAD(P)H fluorescence values before and after Fourier transformation fitting (first 30 frequencies) and (B) periodogram analysis are shown.

Figure 3) Frequency profile analysis of NAD(P)H levels in mitochondria exposed to nZnO. (A) Mitochondria suspensions were exposed to increasing concentrations of nZnO during oscillations in the presence of 1 mM ADP and 2 mM pyruvate. The levels of NAD(P)H at each 30 sec interval were determined (B) Frequency analysis using Fourier transformation and intensity changes at specific frequencies (periodogram; C, D) are shown.
the changes at the 2 min period are lower in intensity compared to control mitochondria. Moreover, the periods of the changes are longer, from 2 min (frequency 0.25) in controls to periods of 3 to 4 min, i.e., frequencies in the range of 0.125-0.17. Moreover, the steady state phase observed in control mitochondria between 18 min and 40 min was lost in mitochondria exposed to 0.16 and 0.64 µg/L nZnO where oxidation in NAD(P)H continues to drop over time. Frequency analysis was performed using Fourier transformation of the data, and the results are shown in Figure 3B. The intensity of the oscillations at frequency 0.25 was gradually lost with the exposure concentration of nZnO. The intensity at frequency 0.25 (Figure 3C) was significantly correlated with the exposure concentration of nZnO (r=-0.85; p<0.001). The data also showed that nZnO disrupted the oscillatory behaviour of mitochondria at concentrations at least one order of magnitude lower (<0.08 µg/L) than the threshold concentration required to increase the rate of NAD(P)H loss (0.6 µg/L). A close examination of the frequency intensities (periodogram value) revealed that the intensity of frequency 0.21 was significantly and positively correlated with exposure concentration of nZnO (r=0.6; p<0.001), which suggests the formation of lower frequencies as observed in Figure 3A, with intensities related to the exposure concentration. 

In mitochondria exposed to Er, the cyclic changes in NAD(P)H levels were determined over time (Figure 4A). The data revealed that the amplitude change at the normal period of oscillations (2 min; frequency 0.25) decreased in amplitude in the presence of Er. At the highest exposure concentration of Er (16 mg/L), the stabilization in NAD(P)H levels occurred within the first minutes compared to 17-40 min for the controls and the lower Er concentrations. Frequency analysis of the NAD(P)H levels revealed a more complex disruption of the frequency profiles for Er. At the normal frequency of 0.25, the intensities decreased concentration-dependently for Er (r=-0.85; p<0.001); this was accompanied by the appearance of signals and lower and higher frequencies which in some cases were concentration-dependent (Figures 4C and 4D). Concentration-dependence of some of the other frequencies was confirmed at frequencies of 0.225 (r=0.91; p<0.001) and 0.3 (r=0.96; p<0.001), suggesting that Er produced two altered states of the mitochondrial oscillator. Again, these changes occurred at concentrations well below those influencing NAD(P)H oxidation.

**Figure 4** Frequency profile analysis of NAD(P)H levels in mitochondria exposed to Er. (A) Mitochondria suspensions were exposed to increasing concentrations of Er during oscillations in the presence of 1 mM ADP and 2 mM pyruvate. The levels of NAD(P)H at each 30 sec interval were determined (B) Frequency analysis using Fourier transformation and intensities of specific frequencies (periodogram; C and D) are shown.
The influence of Lu on the mitochondria oscillator was also examined (Figure 5A). The cyclic changes in NAD(P)H in Lu-treated mitochondria revealed a decrease in the intensity of the normal frequency of 0.25 but at concentrations lower than with Er. The plateau in NAD(P)H was seemingly not affected by Lu, which began around 18-20 min incubation time. Frequency analysis demonstrated that the intensity (periodogram value) at frequency 0.25 decreased with the exposure concentration (Figures 5B and 5C), but a complex pattern of signals at different frequencies appeared. Correlation analysis of the frequency intensities with exposure concentration found the usual significant decrease at the normal frequency 0.25 ($r=-0.81; p<0.001$) and at a lower frequency of 0.21 ($r=0.86; p<0.001$). This suggests that one intermediary state of mitochondria altered by Lu was observed. Mitochondria were also exposed to increasing concentrations of strontium (Sr) ranging from 0.2 to 5 mg/L, and no significant changes were observed in NADH (results not shown). Frequency analysis showed that the frequency of 0.23 was significantly correlated with Sr concentration only ($r=0.69; p<0.001$), suggesting that Sr stimulated NADH oscillations in mitochondria in vitro.

**DISCUSSION**

The oscillations in mitochondrial NAD(P)H are thought to result from the balance between the oxidation of NADH for the electron transport system/production of ROS during electron transport activity in the formation of the transmembrane H+ gradient for ATP production and the production of NAD(P)H during glycolysis of the tricarboxylic acid (TCA) cycle. In the presence of pyruvate and ADP, mitochondria produce NADH through the TCA cycle while NADH donates its electron at complex I of the electron transport chain for the transmembrane proton H+ gradient that drives ATP synthesis.
synthesis. The transfer of high-energy electrons also produces ROS that need to be neutralized by the NADH/ATP-dependent peroxiredoxin/thioredoxin/sulfiredoxin system, superoxide dismutase, catalase and other antioxidants (5). Oscillations arise with mitochondrial flavoprotein redox transients and bursts of mitochondrial membrane potential depolarization from the electron chain transport activity. Complex I is also associated with NADH oxidation, since it is the source of electrons for complex I of the electron chain transport system. NADH oxidase is also involved in the release of electrons from NADH to a donor such as oxygen (superoxide) and other substrates. For example, the apoptosis-inducing factor is a flavoprotein with NADH oxidase activity; it is anchored to the mitochondrial inner membrane and is involved in complex I maintenance (18). Its activity and subsequent release from complex I is dependent on free Ca²⁺ levels, which also regulate NADH oxidase activity in the TCA cycle and mitochondria NADH oscillations (19). After exposure to the selected compounds in the present study, NAD(P)H oxidation activity was increased, which suggests oxidation of NAD(P)H from the electron transport chain activity. Because the intensity of oscillations at the normal frequency did not change during the net decrease in NAD(P)H levels, we cannot determine whether the electron transport chain (complex I) or the decreased NADH production (TCA cycle) was at play during this period of NADP(H) oxidation. It is possible that a desynchronization of NADH production at the normal frequency of 0.25 could contribute to the loss of NADH levels in mitochondria exposed to the compounds. This was corroborated by the significant correlation between the rate of NADP(H) oxidation and the lower frequency of NADP(H) oscillations for nZnO (frequency 0.14; r=0.73; p<0.01), for Er (frequency 0.22; r=0.81; p<0.01) and for Lu (at frequency 0.22; r=0.70; p<0.05). This is consistent with a previous study which showed that the appearance of a complex pattern of frequencies occurred during oxidative stress, leading to mitochondrial dysfunction (10).

It has been demonstrated that metabolites in the TCA cycle (citrate and succinate) also oscillate at the same period and in phase with NADH levels in mitochondria. This suggests that NAD(P)H production and electron transport activity are coupled (19,20). Compounds that would target either the electron transport chain or the TCA cycle could place these processes out of phase with each other. A 2-dimensional Fourier analysis comparing the controls and the nZnO concentration with increased oxidation of NAD(P)H (0.64 µg/L) revealed that frequency 0.14 of the controls was significantly out of phase and less coherent with the 0.64 µg/L group, while at lower concentrations where no change in the rate of NAD(P)H oxidation occurred, the 0.14 frequencies were more in phase and coherent with the controls. We repeated the analysis with Er and Lu at frequency 0.22 and obtained the same observation, i.e., the frequencies of 0.22 in the controls and Er or Lu concentrations that increase the rate of NADP(H) oxidation were less coherent and were out of phase with each other. Hence, the appearance of NAD(P)H changes at frequencies differing from the normal frequency could disrupt this process and favor the oxidation of NADP(H) in mitochondria. It was shown that these complex oscillatory redox dynamics in mitochondria were involved during mitochondrial dysfunction from oxidative stress (10). An equilibrium must be maintained between energy output and the control of ROS within the physiological limits. In the present study, the appearance of low amplitude in NAD(P)H levels at lower frequencies that were significantly correlated with the concentration of the tested elements suggests that this was associated with the oxidation rate of NADP(H) and the decreased amplitudes observed at the normal frequency. Moreover, the magnitude of decrease in the amplitudes at frequency 0.25 was generally higher than the observed increase in NADP(H) amplitudes at lower frequencies for Er, Lu and nZnO, which suggests that the loss of NADP(H) from oxidation depends not only on the intensity of effects but on time-dependent changes in NADP(H) levels.

In a previous study, Er was shown to increase membrane permeability transition in rice mitochondria (14). Er provoked swelling of mitochondria, loss of mitochondrial transmembrane potential and H+ permeability, which is consistent with loss of NAD(H) oscillations before the appearance of NADP(H) oxidation. Lu was shown to strongly inhibit mitochondrial NADPH malic enzyme (21). Malic enzyme leads to the reduction of NADP+ in the presence of malate to form pyruvate and NADP which supply pyruvate for the TCA cycle for NADH production. Bulk and nanoparticles of ZnO were shown to produce oxidative stress in mitochondria (22). An increase in ROS production with alterations in the mitochondrial membrane potential was observed; the associated decrease in levels of lipid peroxidation as determined by the thiobarbituric acid levels in A. cepa root cells. In human keratinocytes, treatment with nZnO resulted in the loss of mitochondrial membrane potential, more energy dissipation, and swelling, depression of respiration and generation of ROS (23). This suggests that loss of NADP(H) oscillations could have resulted from an impaired electron transport chain rather than reduced activity in the TCA cycle. ZnO nanoparticles could also induce apoptosis through ROS accumulation in mitochondria of human keratinocyte and in oysters (15,24). The mitochondrial oscillator is governed by ROS levels (9). When the balance between ROS production in mitochondria (at the electron transport chain) and ROS scavenging is lost, the cell locks to one main low-frequency oscillatory mode. This is consistent with the observed lower frequencies induced by Er, Lu and nZnO. It is noteworthy that these changes at the normal and lower frequencies manifest at concentrations much lower than those required to observe the net increase in oxidation rates. When NADP(H), which could serve as novel and sensitive biomarkers of stress in the environment. It appears that oxidation of glutathione also triggers mitochondrial membrane depolarization and oscillations in heart cells whose levels could also oscillate in time (25-27).

CONCLUSION

In conclusion, the selected toxic compounds in this study were found to alter the oscillation of NADP(H) levels in mitochondria. NADP(H) levels normally oscillate at a frequency of 0.23;0.27, which corresponds to periods of 1.82;2 min under normal conditions, resulting from the balance between NADP(H) production during glycolysis (TCA cycle) and oxidation during the formation of transmembrane potential by the electron transport chain. In the presence of elements such as nZnO, Er and Lu, the amplitude at this frequency is gradually lost, and lower frequencies appear that are less coherent and are out of phase with the normal frequency of NADP(H) oscillations. The change in the intensity of oscillations at these frequencies was related to the exposure concentration. Given that these changes could be detected before the changes in the oxidation rate of NADP(H), the frequency analysis of mitochondria oscillators represents a sensitive endpoint for determining alterations to normal mitochondrial behavior in organisms exposed to environmental pollutants. Further research will be necessary to determine whether these new biomarkers of toxicity (NADP(H) oscillations) are also observed in exposed animals in vivo at environmentally relevant concentrations. The decrease in amplitudes at the normal frequency occurred at concentrations 5-10 times less than those that significantly increased the rate of NADP(H) oxidation. Moreover, the lower frequencies were out of phase with those of the controls, which suggests the appearance of emerging toxic frequency. Oscillations in NADP(H) could be altered by contaminants and could thus constitute a novel and sensitive biomarker of ecotoxicity.

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