RESEARCH ARTICLE

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; Mussels; Toxicity*

Organisms are able to adapt quickly to their surroundings by developing internal control mechanisms for coping with instability (1). Dynamic control mechanisms could manifest as oscillations such as cytosolic calcium oscillations (2), electrical pacemakers in nerve or cardiac cells and glycolysis activity (3,4). This type of physiological control offers the advantage of quick adaptation to environmental changes, but at the same time it is susceptible to various external stressors such as temperature, food availability/fasting and contaminants. Oscillations in glycolysis were first discovered in yeast cells (3) and later in other cells including muscle, liver and pancreatic cells (5).

Mitochondria are responsible for cellular respiration and energy production, which are pivotal for energy metabolism. Located at the convergence of most catabolic and anabolic pathways, mitochondria are central to aerobic life processes in eukaryotes. They are also the main source of reactive oxygen species (ROS) production in cells during formation of the transmembrane proton gradient potential, especially at complex I of the electron transport chain (5). Indeed, mitochondria produce most of the ROS in cells, which can lead to increased oxidative damage during aging (6). The energetic function of mitochondria involves the handling of huge amounts of oxygen, which represents a constant threat to the redox status in cells where xenobiotics are known to exacerbate this process.

Oscillations in mitochondria involve coupling between glycolysis and the maintenance mitochondrial membrane potential, mediated by the ADP/ATP antiporter and the mitochondrial F_0F_1-ATPase (7). Mitochondria suspensions exposed to a pulse of strontium (a calcium analog efficiently transported across mitochondrial membranes) could trigger sustained oscillations with a period of 2-4 min of divalent ions (calcium, magnesium) and NADH (8,9).

These oscillations result from the dynamic control between NADH formation during the tricarboxylic acid (TCA) cycle, NADH oxidation during electron transport for ATP synthesis and the control of ROS production (1,5). In theory, compounds that cause oxidative stress and reduce the capacity to remove the normal production of ROS during respiration could disrupt the oscillatory behaviour of mitochondria. The dampening in sinusoidal oscillations results from the loss of internal balance between NADH production and oxidation in mitochondria and from desynchronization of mitochondria.

Indeed, the maintenance of oscillations was dependent on the abundance of Mn and Cu, Zn superoxide dismutase and the interplay with ROS production in the respiratory chain (10). At low concentrations of superoxide dismutase, there was an increase in the complexity of wave behaviour of the mitochondrial oscillator. This suggests that oxygen radicals (unbound electrons) could disrupt the process, and this process was at the boundary between the normal and altered energetic behaviour of mitochondria, which was closely related to oxidative stress.

Many chemicals could react with ROS and disrupt the electron chain transport system in aquatic organisms (11), raising legitimate concerns as to whether chemicals could disrupt the oscillatory behavior in NADH levels in mitochondria. In ecotoxicology, there has been little attention on biological rhythms as the basis of important physiological processes or the question of whether contaminants could disrupt those cycles.

The influence of contaminants on the wave-like behavior in molecular changes is just beginning to gain attention (12), and its interest resides in the fact that most biological processes are cyclical, especially at the sub-cellular and molecular levels. For example, circadian rhythms are found in every living organism and underlie many physiological processes such as redox homeostasis, signal transduction and xenobiotic metabolism (13). However, little is known about whether chemicals could disrupt these cycles and initiate toxicity in cells.

The purpose of this study was therefore to examine the influence of selected contaminants on NADH oscillations in mitochondria suspensions isolated from the freshwater mussel *Dreissena bugensis*. The compounds were selected based on their capacity to disrupt mitochondria function and redox status. Erbium (Er) is known to suppress mitochondria membrane potential and increase H+ permeability (14). Zinc oxide nanoparticles (nZnO) are known to produce oxidative stress and disrupt mitochondria function in oysters (15). The toxicity of lutetium (Lu) to mitochondria is much less well understood, but Lu is a trivalent metal with similar redox potential to Er (-2.33 and -2.28 mvolts for Er and Lu respectively). In parallel, the rate of NADH oxidation was also measured in order to determine mitochondria function in the presence of ADP and pyruvate to support aerobic metabolism (TCA cycle). An attempt was made to determine the effects of these contaminants on the wave behaviour of NADH changes in mitochondria.

MATERIALS AND METHODS

Quagga mussels *Dreissena bugensis* were collected on the south shore of the St.

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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 × g for 20 min at 2°C. The supernatant was carefully removed and centrifuged at 10 000 × 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 × 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 KH_2PO_4 and 1 mM $NaHCO_3$ 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 KH_2PO_4, 5 mM $NaHCO_3$, 2 mM $MgCl_2$, 1 mM K-ADP, 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 emission readings with no delay) using the Synergy 4 microplate reader at 360 nm and 460 nm for excitation and emission wavelength respectively. Instrument calibration was achieved with blank solution (composed of the reaction media only) and standard solutions of NADH at 50 µM in the reaction media.

The influence of zinc oxide nanoparticles (nZnO), erbium chloride (Er^{3+}), lutetium chloride (Lu^{3+}) and strontium chloride (Sr^{2+}) 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 ug/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 NAD(P)H was determined as described above. Mitochondrial NADH 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 NAD(P)H 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 NAD(P)H 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 NAD(P)H 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 $g(k) = a_0 + \sum [Ak^* \cos(2\pi(kn/N) + Bk^*\sin(2\pi(kn/N))]$, 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 (Pg) value, which is related to the sum of amplitude variance of the sine and cosine functions at each frequency k: Pg = (Ak2 + Bk2) × N/2, where N is the total number of observations. The Pg value is thus related to the variance of the function at a given frequency. The significance of each Pg value was tested and compared to random "noise" using the Kolmogorov–Smirnov test (exponential adjustment). Correlation analysis between Pg 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 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 NAD(P)H (1.2 fold relative to the controls) 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 NAD(P)H oxidation at a threshold concentration of 10 mg/L reaching a 1.3-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, NAD(P)H levels in control mitochondria oscillate over time during the 40 min incubation period (Figure 2A). Although a general decreasing trend is observed in NAD(P)H levels within the first 20 min, it is accompanied by short oscillation changes in NAD(P)H levels over time. Indeed, NAD(P)H levels oscillate with a period ranging from 1.6 min to 2.2 min (frequencies between 0.24-0.32); 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 NAD(P)H 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 NAD(P)H levels in time is shown in mitochondria exposed to nZnO (Figure 3A). Visual inspection of the figures revealed that the amplitudes of

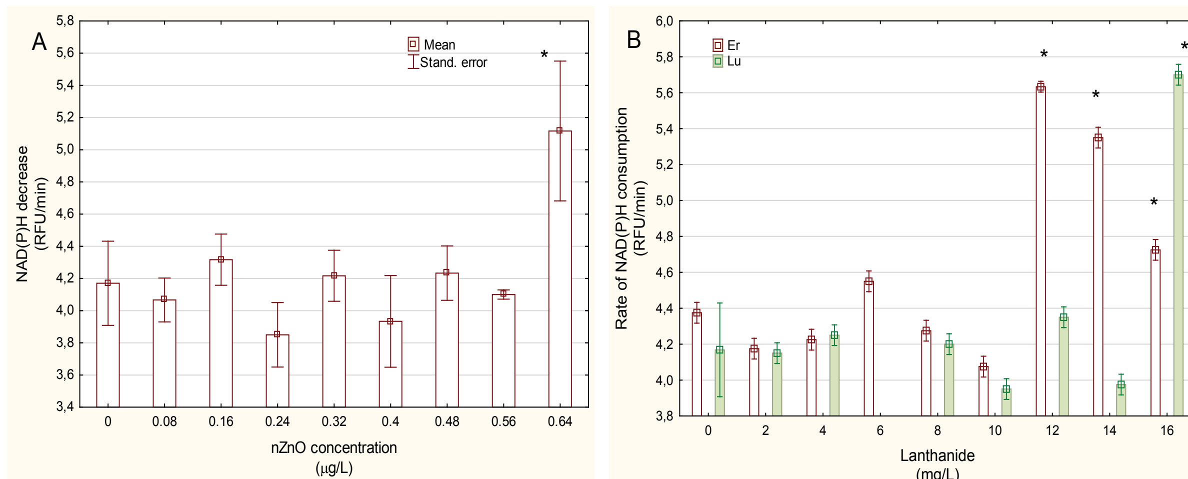


Figure 1) *Rate of NAD(P)H oxidation in mitochondria exposed in vitro to nZnO and lanthanides. (A) Mitochondria suspensions (0.5 mg/mL) were exposed to increasing concentrations of nZnO and (B) lanthanides Er and Lu during incubation with 5 mM ADP and 1 mM pyruvate. The data represent the mean and standard error from n=3 determination.*

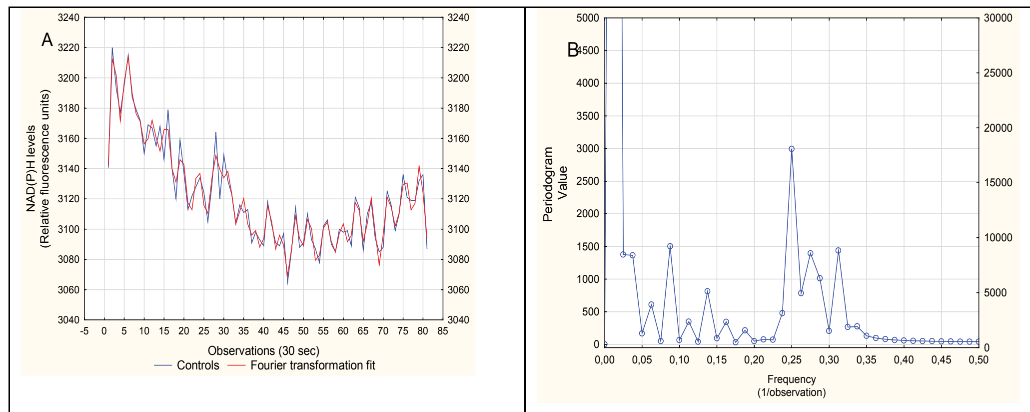
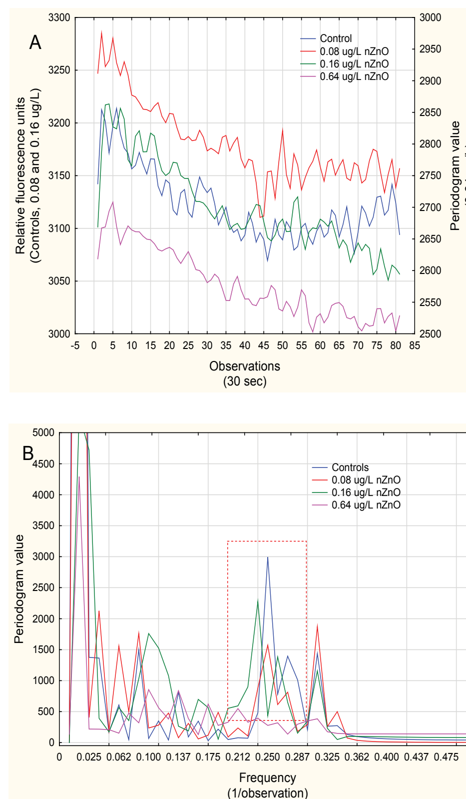


Figure 2) *Oscillatory behaviour of NADH levels in mitochondria.*
(A) Oscillatory changes of NADH levels in mitochondria suspensions over time. The NAD(P)H fluorescence values before and after Fourier transformation fitting (first 30 frequencies) and (B) periodogram analysis are shown.



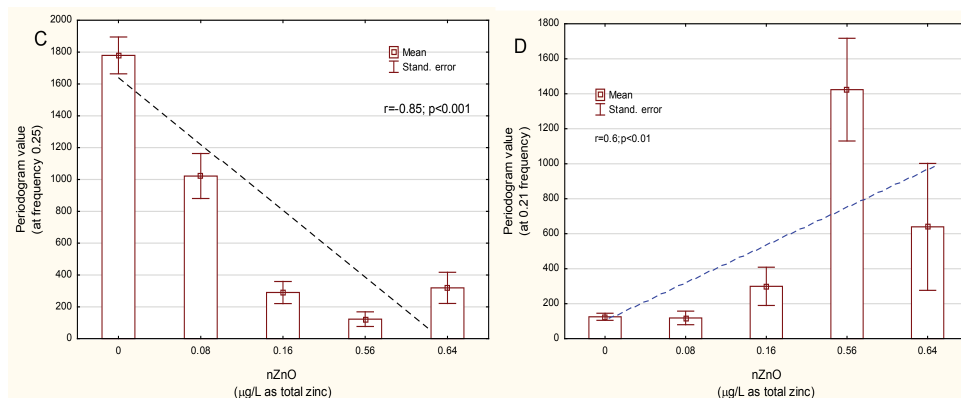


Figure 3) *Frequency profile analysis of NAD(P)H levels in mitochondria exposed to nZnO.*
(A) Mitochondria suspensions were exposed to increasing concentrations of nZnO during oscillations in the presence of 1 mM ADP and 2 mM pyruvate. The levels of NAD(P) H at each 30 sec interval were determined (B) 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.

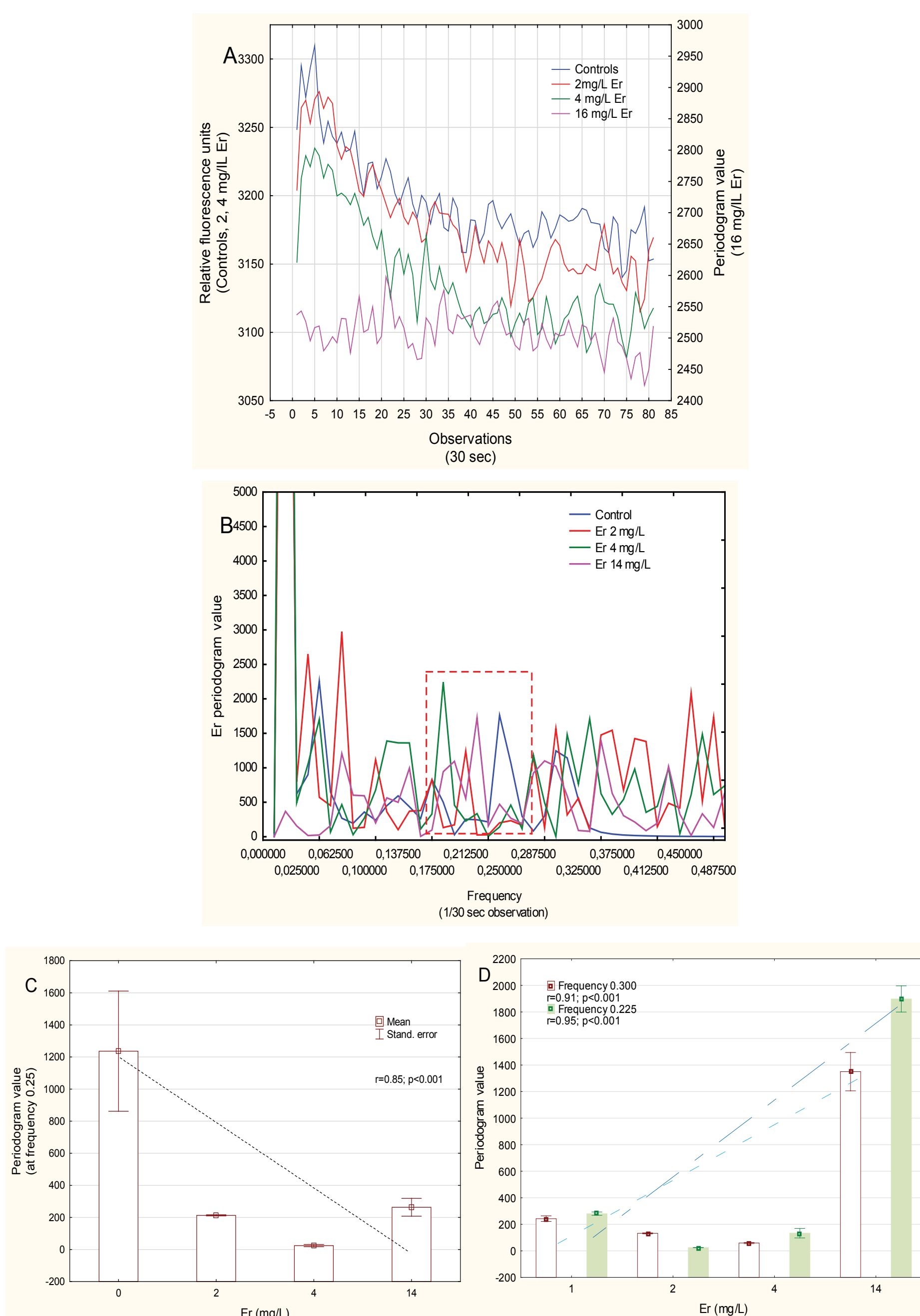


Figure 4) *Frequency profile analysis of NAD(P)H levels in mitochondria exposed to Er.*
(A) Mitochondria suspensions were exposed to increasing concentrations of Er during oscillations in the presence of 1 mM ADP and 2 mM pyruvate. The levels of NAD(P)H at each 30 sec interval were determined (B) Frequency 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

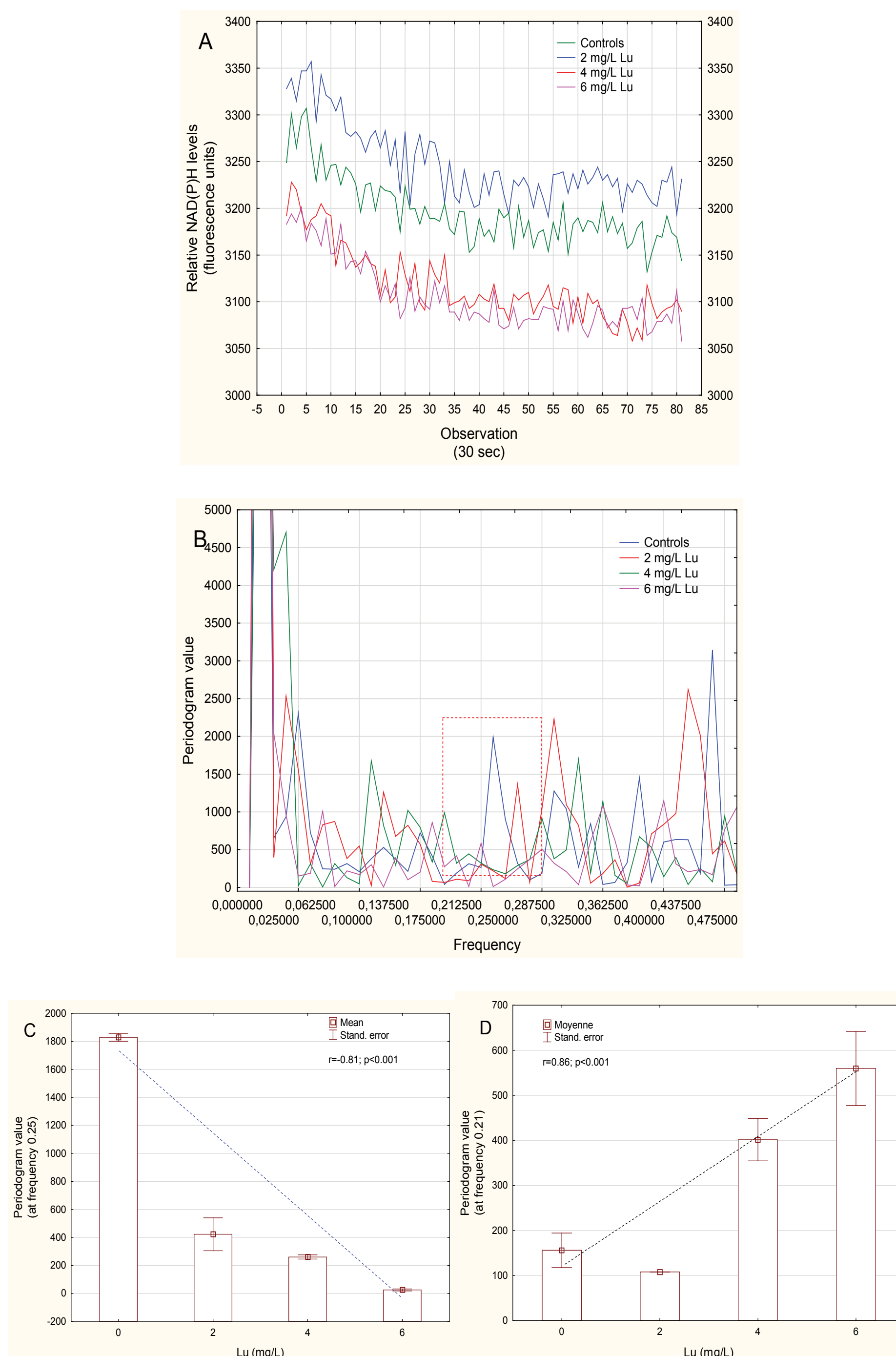


Figure 5) *Frequency profile analysis of NAD(P)H levels in mitochondria exposed to Lu.*
(A) Mitochondria suspensions were exposed to increasing concentrations of Lu during oscillations in the presence of 1 mM ADP and 2 mM pyruvate. The levels of NAD(P)H at each 30 sec interval were determined. (B) Frequency analysis using Fourier transformation and intensities at specific frequencies (periodogram; C and D) are shown.

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 mitochondria is dependent on free Ca^{2+} levels, which also regulate key enzymes 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 chain transport 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 NAD(P)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 NAD(P)H oxidation and the lower frequency of NAD(P)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 NAD(P)H 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 NAD(P)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 NAD(P)H in mitochondria. It was shown that these complex oscillatory redox dynamics in mitochondria were also involved during mitochondria 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 NAD(P)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 NAD(P)H amplitudes at lower frequencies for Er, Lu and nZnO, which suggests that the loss of NAD(P)H from oxidation depends not only on the intensity of effects but on time-dependent changes in NAD(P)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(P)H oscillations before the appearance of NAD(P)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 NADPH 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; this was also associated with increased levels of lipid peroxidation as determined by the thiobarbituric acid levels in A. cepa root cells. In human keratinocytes, treatment of mitochondria 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 NAD(P)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 of NAD(P)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 NAD(P)H levels in mitochondria. NAD(P)H levels normally oscillate at a frequency of 0.23-0.27, which corresponds to periods of 1.8-2.2 min under normal conditions, resulting from the balance between NAD(P)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 NAD(P)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 NAD(P)H, the frequency analysis of mitochondria oscillators represents a sensitive endpoint for determining alterations to normal mitochondria behavior in organisms exposed to environmental pollutants. Further research will be necessary to determine whether these new biomarkers of toxicity (NAD(P)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 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.

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