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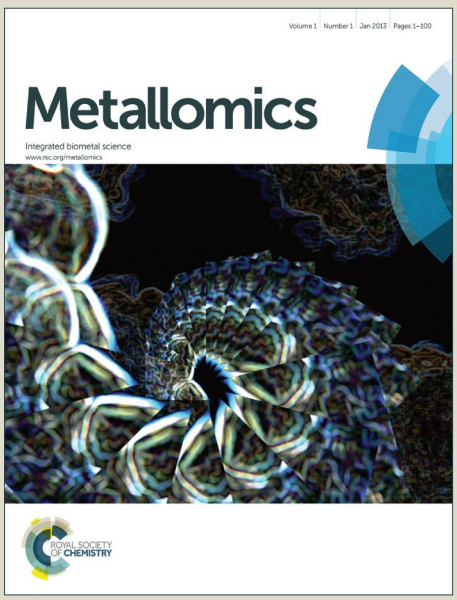

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New Mechanistic approach of inorganic Palladium toxicity: Impairment in mitochondrial electron transfer

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Human activities have increased the levels of palladium (Pd) progressively accumulating in the environment. The growing evidence of Pd toxicity has become the focus of serious concern in the environment, organisms and human with few data on mechanism of Pd toxicity. Recent studies suggested mitochondria have a key role in Pd toxicity via mitochondrial membrane potential collapse and amelioration of cellular glutathione (GSH) level. Therefore, it decided to determine the mechanistic toxicity of Pd on isolated mitochondria via new and reliable methods. Isolated liver and kidney mitochondria were obtained by differential ultracentrifugation and incubated with different concentrations of Pd (100-400μM). Our results showed that Pd induced mitochondrial dysfunction via increase of mitochondrial ROS production, potential membrane collapse which correlated to cytochrome C release. Also, increased disturbance in oxidative phosphorylation was also shown by increasing of ADP/ATP ratio in Pd-treated mitochondria indicates the mitochondrial dysfunction on isolated liver and kidney mitochondria. Our result suggests that Pd-induced toxicity is the result of disruptive effect on mitochondrial respiratory chain predisposing to cell death signaling. Besides, it supposed that kidney is more susceptible to Pd exposure than liver tissue.

Keywords: Palladium (Pd); isolated mitochondria; toxicity; Respiratory complexes

Introduction

Palladium (Pd) is a toxic metal with frequent application such as dental accessory , chemical catalysts, electrical industry, fine jewelry, and also in automotive emission control catalysts as important source in recent years [1]. Several authors have clearly demonstrated the emitting of Pd by catalytic converters in automobiles may be mobile, leached by rainwater and then redistributed in the food chain and emitted with exhaust fumes into the environment in the form of nanometer-sized particles which are deposited on roadside surfaces, so that the metal is now the main component of traffic related pollution [2,3]. Besides, the risk of transfer to the food chain and aquatic environments is real and the toxicity is suspected after exposure with inorganic Pd via inhalation or ingestion from different sources [4]. Therefore, clarify the potential toxic effects of Pd due to increasingly used in modern industry have become important for toxicologist and physicians due to health hazards in human or animals.

A recent study suggested there is some indication of antioxidant activity, anti-cancer agent and cardio-protective effect of organic forms of palladium with powerful therapeutic potential[5].Several studies have indicated inorganic palladium compounds (e.g. $PdCl_2$) are more toxic than organic form which may cause different certain implication for treatment of patient poisoning[6].

Previous study suggested that the highest accumulation of Pd was obtained in kidney, liver, spleen, lung, bone and heart in animal models[7]. Another investigation suggested release of Pd in the environmental samples and after smoking caused accumulation of Pd in the in the respiratory tract of human due to the more soluble nature of Pd and unknown consequences [8,9]. It reported that the inorganic form of Pd could induce oxidative stress [10], inhibition of DNA and protein synthesis and different type of DNA damage in mouse lymphoma cell lines, and deficiency in embryonic cartilage process via inhibition of prolyl hydroxylase, a key enzyme in collagen synthesis, creatin kinase as an important enzyme of energy metabolisms in rats [11,12] and significant depression in heart function via a change in cardiomyocyte membrane potential[13].

It seems that Pd toxicity is related to thiol interaction groups, phosphate residues and macrochelates formation induction, and inhibition of mitochondrial activity in liver and kidney [14,15]. Additionally, acute and chronic studies with Pd compounds in rodents and rabbits did not show any change in histopathological effects in myocardial with specific effects on heart via interfering with bivalent ions (e.g. Ca^{2+}, Mg^{2+}) in ion channels, which leads to disturbances in mitochondrial membrane potential, decreased entry of calcium in cells and decreased contractility and arrhythmia [10,13]. On the other hand, a

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number of studies have shown that Pd nanoparticles (0.012-12 µg/kg) are able to exert some adverse health effects such as oxidative stress, cell death signaling, release and expression of numerous cytokines as the inflammation factor in Pd toxicity. Moreover, a histopathological study in the kidneys revealed significant alterations in the proximal and distal tubular epithelium, suggesting significant renal dysfunction without effect in glomerular filtration[16].

Published data on Pd toxicity in biological system limited to the oxidative stress induction via ROS formation in human lung adenocarcinoma cell line [17] and GSH depletion in blood components. Based on basic document in Pd toxicity, it supposed that mitochondria are the important sub-cellular organelles for Pd toxicity [13,17]. Also, toxicity of Pd in human is limited to sensitization, asthma and dermatologic disorders such as rhinitis, conjunctivitis and contact urticaria in a high percentage of workers in precious metal refineries[19,20]. Taken into consideration limited data about possible toxicity mechanisms of palladium on in vivo and in vitro studies; we planned to study the role of oxidative stress in palladium toxicity in isolated rat liver and kidney mitochondria precisely by measuring different multi-parametric assay.

Results

The effect of in vitro Pd treatment on mitochondrial ROS production: The sign for increased ROS formation in flowcytograms are shifting the DCF peak to the rightward and increasing of AUC in flowcytograms. As shown in Fig.1, the rate of ROS formation in isolated mitochondria obtained from the liver and kidney significantly increased compared to control groups in a time dependent manner after 30 min of exposure in Pd treated groups (200 and 400 µM). The data provided for drawing bar graphs (fluorescence intensity unit) was directly obtained from flowcytometry printed records. However, in comparison with control mitochondrial suspension, ROS generation did not significantly increase in 100µM of Pd on the isolated mitochondria. The level of ROS formation was highest in Pd (400 µM) in Liver and kidney. Also, a more substantial increase in ROS formation was observed in kidney compared with liver mitochondria (Fig.1 and Table.1).

The effect of in vitro Pd treatment on mitochondrial complex II activity: As shown in Table.1, there was a significant decrease in the mitochondrial complex II activity only in Pd (400 µM) of the liver and kidney mitochondria compared with control groups ($P<0.05$) as mitochondrial dysfunction in electron transfer chain (Table.2).

The effect of in vitro Pd treatment on mitochondrial Glutathione: There was a significant reduction in the mitochondrial GSH levels between the control and Pd treated groups in the liver and kidney in a similar manner ($\varphi P<0.05$; Table.2).

The effect of in vitro Pd treatment on mitochondrial membrane potential: As shown in Fig.2, there was a significant difference in the MMP collapse between the control and Pd treated groups (200 and 400 µM) (***P<0.001). Based on our results the order of sensitivity of mitochondrial isolated from kidney against Pd^{2+} induced MMP collapse is higher than liver tissue ($^{\Phi\Phi\Phi}P< 0.001$) while there was no significant effect of treatment between control and Pd (100µM) groups in the liver and kidney ($P>0.05$) with more substantial increase in MMP collapse in kidney isolated mitochondria compared with liver. The sign MMP collapse in flowcytograms are shifting the Rhodamine123 peak to the rightward and increasing of AUC in flowcytograms (Table.3).

DCF fluorescence intensity (%) after incubation time			
Liver	**5 min**	**15 min**	**30 min**
Control	14±3	21±4	32±6
Pd(100 µM)	61±9***	66±5***	90±7***
Pd(200 µM)	78±8***	83±9***	97±12***
Pd(400 µM)	88±5***	97±12***	123±15***
Kidney			
Control	28±8	34±5	38±6
Pd(100 µM)	81±3***	91±7***	94±12***
Pd(200 µM)	88±4***	88±5***	90±4***
Pd(400 µM)	81±8***	101±7***	187±14***

Table 1. Palladium-induced ROS formation in isolated liver and kidney mitochondria. Reactive oxygen species were measured in a suspension of isolated mitochondria after exposure with Pd. The membrane permeable fluorescent dye DCFH-DA was added to mitochondria energized with succinate (5mmol/l). The change in fluorescence was determined using the flowcytometer compared to control groups. The values represented as mean±SD (n=3). *P<0.05; **P<0.01; ***P<0.001 compared with control mitochondria (mitochondrial suspension treated with equivalent of buffer instead of Pd). Interpretation of flowcytogram in Figure.1 summarized in Table.1

	Mitochondrial swelling percent (%)				
Liver	**5 min**	**15 min**	**30 min**	**45 min**	**60 min**
Control	0	2±1	6±1	9±4	11±2
Pd(100 µM)	4±1	7±2	12±3	15±5	14±3
Pd(200 µM)	5±2	9±3*	14±3*	18±4	21±4
Pd(400 µM)	17±2***	24±3***	32±4***	36±5***	40±6***
Kidney					
Control	1±1	4±1	9±1	9±2	15±3
Pd(100 µM)	4±3	7±1	9±1	10±1	22±4
Pd(200 µM)	6±4	8±1	13±2	16±4	26±5
Pd(400 µM)	13±3***	27±8***	32±4***	45±5***	53±6***

Table.2. The effect of Pd on the mitochondrial swelling after incubation in different interval time . Mitochondrial swelling was measured by determination of absorbance at 540nm as described in Materials and methods. The values represented as mean±SD (n=3). * P<0.05; ***P*<0.01; ****P*<0.001 compared with control mitochondria

Rhodamine 123 fluorescence intensity (%) after incubation time			
Liver	**5 min**	**15 min**	**30 min**
Control	29±4	34±5	38±6
Pd(100 µM)	47±12	91±7***	92±6***
Pd(200 µM)	107±8***	120±8***	122±9***
Pd(400 µM)	130±5***	153±11***	157±6***
Kidney			
Control	38±9	41±7	46±7
Pd(100 µM)	44±7	51±8	55±9
Pd(200 µM)	83±7***	91±11**	100±9***
Pd(400 µM)	90±6***	105±12***	128±14***

Table.3. Palladium-induced mitochondrial membrane potential MMP collapse (ΔΨ%) in isolated liver and kidney mitochondria. Mitochondrial membrane potential collapse (ΔΨ%) was measured by Rhodamine123 as described in Materials and methods. The effect of Pd on the mitochondrial membrane potential decrease in liver and kidney mitochondria was evaluated by flowcytometer.. The values represented as mean±SD (n=3). *P<0.05; **P<0.01; ***P<0.001 compared with control mitochondria (mitochondrial suspension treated with equivalent of buffer instead of Pd). Interpretation of flowcytogram in Figure.2 summarized in Table.3.

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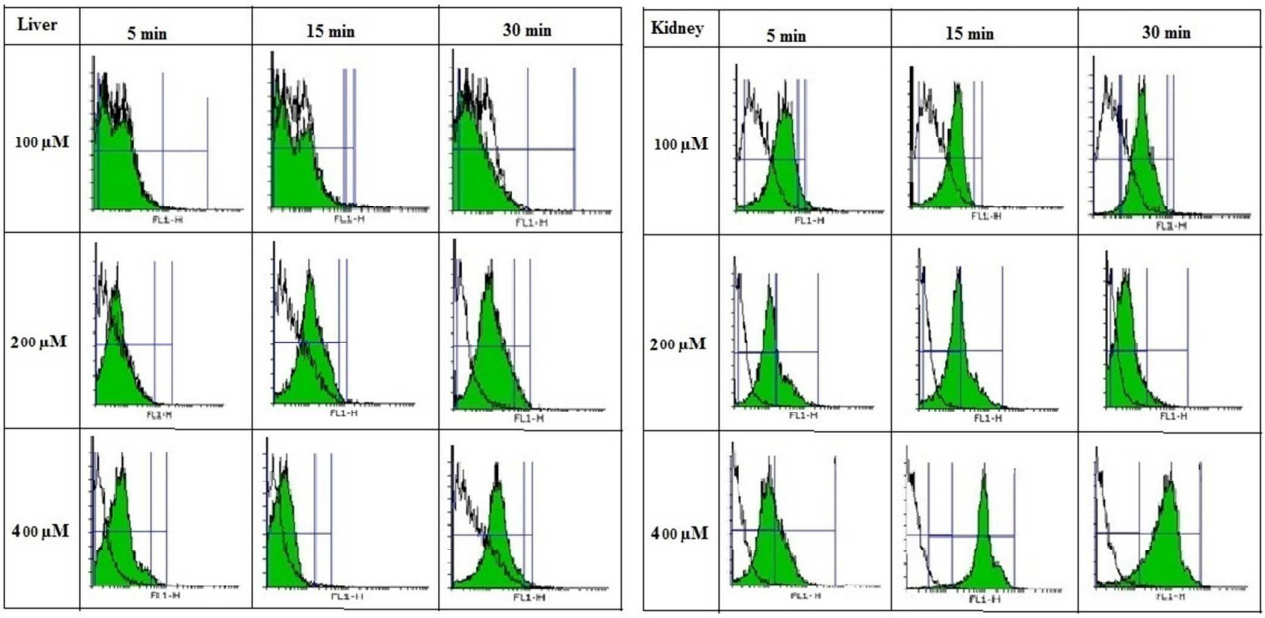

Figure.1. Flowcytograms obtained in the evaluation of ROS formation in isolated liver and kidney mitochondria. Reactive oxygen species were measured in a suspension of isolated mitochondria after exposure with Pd. The membrane-permeable fluorescent dye DCFH-DA was added to mitochondria energized with succinate (5mmol/l). The change in fluorescence was determined at 488 nm for excitation and at 525 nm for emission after different incubation time , using BD flowcytometry. Interpretation of flowcytogram results summarized in Table 1. The sign for increased ROS formation in flowcytograms are shifting the peak to the rightward and increasing of AUC in color section of flowcytograms. The white peak indicated to Control groups and the green peak is related to treated groups.

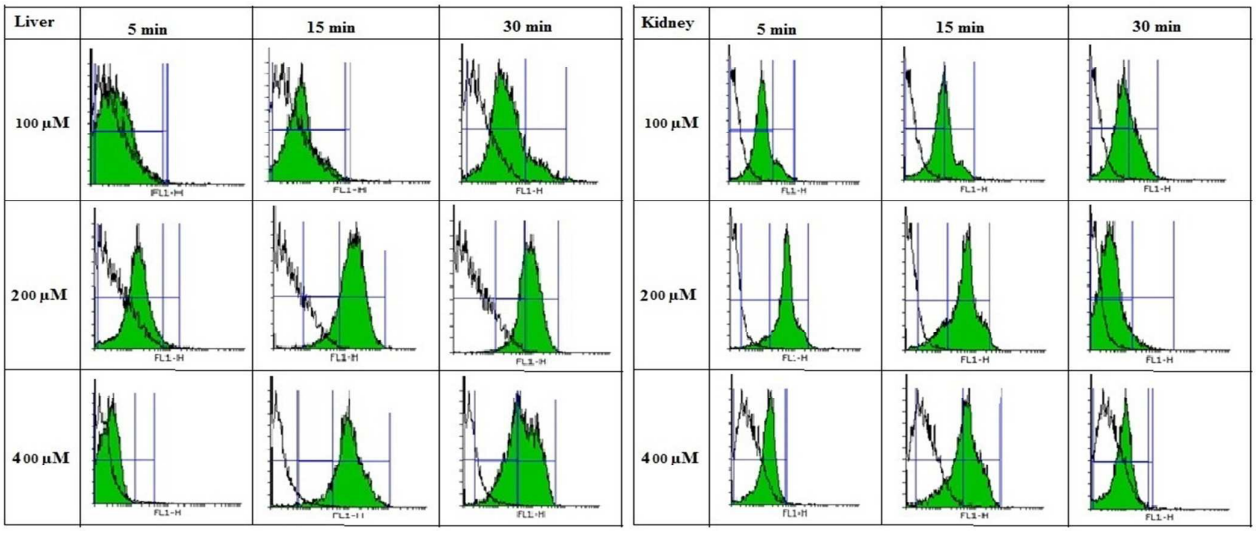

Figure.2. The effect of Pd on the mitochondrial membrane potential (MMP) collapse (ΔΨ (%) on isolated mitochondria.MMP collapse was measured by flowcytometry using Rhodamine123 after addition of Pd at time intervals 5, 15 and 30 min as described in Materials and methods. FL1: the fluorescence intensity of Rh123. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with control mitochondria. The sign for mitochondrial membrane potential (MMP) collapse in flowcytograms is shifting the peak to the rightward and increasing of AUC in color section of flowcytograms. The white peak indicated to control groups and the green peak is related to treated groups.

Table 2. Effect of Palladium (II) on mitochondrial complex II activity (%), glutathione (µg/mg protein) Mitochondrial ADP/ATP ratio, and cytochrome c release (ng/mg protein) in the rat liver and kidney mitochondria.

Parameters	SDH(complex II) activity (%)		Mitochondrial GSH content (µg/mg protein)		Mitochondrial ATP/ADP ratio		Rate of cytochrome c release (ng/mg protein)	
Groups/ Tissue	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Control	100±4.6	100±5	53.7±1.2	45.9±0.82	0.95±0.06	0.89±0.05	43.4±1.5	37±2.8
100 µM	94±5.6	99±8.6	34.3±1.9 ***	44.1±1.7	1.13±0.05*	1.09±0.08**	48.3±4.3	56.1±4.1
200 µM	96.8±7.7	99.2±3.1	31.3±0.48 ***	31.5±1.3**	1.83±0.08***	1.42±0.01***	53.1±6.3	60.6±6.3
400 µM	34.8±2.7 ***	40.5±3.6***	23.4±0.75 ***	23.5±2.7***	1.96±0.07***	1.94±0.05***	131±17.9 **	148±19.8 ***

Isolated Liver and kidney mitochondria (0.5 mg/ml) were incubated with various concentrations of Pd (0, 100, 200 and 400 µM). Then, each of the parameters was determined as described in Materials and methods; mitochondrial complex II activity based on MTT assay and spectrophotometery, GSH content based on DTNB reagent and spectrophotometery, ATP/ADP ratio using RP-HPLC methods, the amount of expelled cytochrome c from mitochondrial fraction based on Rat/Mouse cytochrome c ELISA kit. Isolated mitochondria (500 µg/ml) were incubated for 1h with various concentrations of Pd (0, 100, 200, and 400µM). The values represented as mean±SD (n=3). **P*<0.05; ***P*<0.01; ****P*<0.001 compared with control mitochondria.

The effect of in vitro Pd treatment on mitochondrial swelling: There was no significant effect of Pd on the mitochondrial swelling between the control and treated groups (100 and 200 µM) groups in both liver and the kidney (P<0.05) .Besides, mitochondrial swelling was seen in Pd (400 µM) group in isolated liver and kidney mitochondria with more substantial increase in kidney (Table.3).

The effect of in vitro Pd treatment on mitochondrial ADP/ATP ratio: There was a significant increased in mitochondrial ADP/ATP levels (P< 0.05) between the control and treated groups with Pd (100- 400 µM) groups in liver and Kidney mitochondria (Table.2) indicating the mitochondrial dysfunction.

The effect of in vitro Pd treatment on cytochrome c release: As shown in Table.2, there was a significant difference between the release of cytochrome c between control mitochondria and Pd-treated mitochondria both liver and kidney (P<0.05), whereas this was not observed on the kidney mitochondria Pd (100µM) (P>0.05). CaCl2 (100µM) a known inducer of mitochondrial permeability transition (MPT) and cytochrome c release was used as a positive control (Data not shown).

Discussion

All the findings presented in our articles were based on significant (P<0.05) toxic effects of Pd induced oxidative stress on rat isolated mitochondria model, a valid method with all the benefits of the in vivo physiological systems and also lack of negative homodynamic and hormonal effect [21,22]. Several authors have clearly demonstrated Pd, belong to the platinum group of element, may cause allergies at very low dose and may be responsible of organell dysfunction including kidney, liver, muscle and mammary gland after single dose administration[23].

The use of Pd as catalysts converters in the automobile causes conversion of the incomplete products of gasoline to N_2, H_2O and CO_2 and increase levels of Pd in environmental matrices such as grass and soil, leached by rainwater and then redistributed in the food chain. It showm that Pd accumulation near roads is up to 90 fold higher than the background levels [24]. Based on limited data ,the Pd concentration after single dose of Pd in milk reported similar with cadmium (Cd) concentration in ranges value from 1.37 to 2.47 µg/day [23, 25]. Also, sub-chronic exposure with Pd in rats showed significantly increased in IL-2, IL-4 and TNF-α as immunotoxicity profile [19].

Discharging of Pd into the environment induces a strongest increasing effect on heat shock proteins (hsp70) expression compared to control groups of zebrafish mussels [26]. On the other hand, there is some evidence about no mutagenic potential or low capable of genotoxicity in mammalian cells with probably without the carcinogenic risk in human [7,27]. Result of kidney perfusion exposure with Pd suggested inhibition of Na+-Ca2+-anti-porter, the Na^+/H^+exchange, Na^+/K^+ ATPase channels and non-voltage-gated Ca^{2+}-channels lead to nephrotoxicity in animals and human [28].

Biological investigation suggested increased of reactive oxygen species (ROS) formation has a key role in toxicity mechanism of metal ions such as Pd and as an early causal role in the terminal differentiation in keratinocytes [29]. Therefore, the objective of this work is the determination of mitochondrial dysfunction as pivotal role in Pd induced oxidative stress. Therefore, the concentrations of Pd were chosen based on our pilot study for selecting the concentration range (0, 10, 25, 50, 100, 200 and 400 µM), but the fact is in lower concentrations applied in our pilot study (0-100 µM) no significant or very little effects observed in isolated mitochondria until mitochondria are exposed to high levels (> 100 µM).

Some studies suggested controversial issue in relation between the level of Pd exposure and serum concentrations in workers with the estimated release in range from a few $ng.km^{-1}$ to several µg km^{-1} in short term exposure [30,31]. The maximum daily intake intake of Pd from food or drinking-water was determined (0.03 µg /person per day). According to a United Kingdom survey, the total daily dietary intake of Pd has been estimated to be up to 2 µg/person /day equal by 100 µg /day in oral administration. There may be a higher intake in some population groups consuming diets with high palladium levels such as some types of mussels [32].

Besides, all in vitro research works in mechanistic toxicology simulated chronic /low concentration, problems with acute/high concentration condition in isolated cell or sub-cellular organelle system which cannot be kept alive or operates more than maximum 3-4 hours following their isolation or preparation. But, most of the mechanisms outcome in the molecular pathology of diseases or

toxicology of environmental pollutants is results of such in vitro studies[33,34]. Therefore, it decided using of relatively high concentration in the designation of an experimental model, regarding this fact that the accumulation possibility of Pd in occupationally exposed workers cannot be neglected. In many countries, the threshold limit value 2000 ng/m^3 has been determined and enforced for Pd because there is still a high incidence of Pd salt allergy[30].

Our data confirmed the involvement of ROS production in Pd induced mitochondrial oxidative stress. Increasing of ROS in mitochondria and neighboring of ROS production site to lipid membrane, makes more susceptible site in mitochondria which leads to oxidative stress and lipid peroxidation. Our results showed no oxidation of lipid membrane in different concentration of Pd on isolated mitochondria versus of my proposed idea (Data not shown). It suggested LPO is a time-consuming process and after induction of highly ROS formation in isolated mitochondria, MDA level may be increased similar to other oxidative stress mechanisms. Present study suggested significant GSH oxidation in liver and kidney of Pd-treated rats in a concentration-dependent manner.

According to many references and previous studies with intact mitochondria and sub-mitochondrial particles has established the production of superoxide anion and hydrogen peroxide increases substantially in the presence of xenobiotics and inhibitors of mitochondrial complexes such as antimycin and rotenone due to an increase in the steady-state levels of the obi-semiquinone pool caused by inhibition of electron transfer [33-38].

ROS comes from electron “leakage” of the mitochondrial respiratory chain, which can be evaluated by the production of DCF, the highly fluorescent oxidized derivative of DCFH-DA. The reliability of the DCF method is proved by using calcium as a positive control, which is well known to induce a ROS increase in isolated mitochondria [37,38].After being loaded with this probe, untreated isolated mitochondria displayed substantial fluorescence, but exposing them to different concentration of Pd caused increasing in ROS formation. These ROS can be originating from superoxide (°O2) production from both complex I and from the ubiquinone/complex III and be caused by partial damage to and/or inhibition of the respiratory chain complexes [34, 39,40].

Also, Pd significantly decreased the activity of mitochondrial complex II activity only in higher concentration of Pd in liver which increased the leakage of electrons from the respiratory chain and disturbance in the Krebs cycle in mitochondria leads to more generation of superoxide radicals (Table.2). The similar results was seen in 200 and 400 µM of Pd in kidney, suggesting that succinate dehydrogenase (SDH) enzymes contain a number of cysteine-rich sulfur clusters and can be inhibited by agents that modify sulfhydryl groups. It seems Pd (400µM) in liver and Pd (200 and 400µM) in kidney might directly interact with sulfhydryl groups on succinate dehydrogenase resulting in the decline in its activity. But, in lower concentration of Pd, complex II activity remained unchanged due to the antioxidant potential role of sulfhydryl groups in SDH enzymes.

It supposed that increased in ROS formation and oxidation of membrane protein thiol groups caused mitochondrial permeability transition (MPT) pore opening as event in mitochondrial dysfunction which followed by un-limited proton movement across the inner mitochondrial membrane and mitochondrial swelling induction, MMP disruption and uncoupling of oxidative phosphorylation [22].

Our data confirm significantly increased the MMP collapse as an indicator of MPT pore opening after incubation with different concentration of Pd. Also, we suggested that oxidation of thiol groups in the inner mitochondrial membrane could promote the MPT induction and release of cytochrome c from mitochondria as an endpoint of cell death signaling [41,42].

Our results confirmed the release of cytochrome c from mitochondria in a concentration dependent manner which related to opening large pores in the outer membrane, inhibiting some segments of the respiratory chain in the inner membrane and increasing proton leak without opening the mitochondrial permeability transition (MPT) pore [41]. On the other hand, the release of cytochrome c as important part of electrons transfer between complex III and IV, will impair the mitochondrial respiratory chain activity and uncoupling of oxidative phosphorylation [45].

Previous studies suggested that the Krebs cycle and electron flow in mitochondrial respiratory chain provide the proton-motive force for the transformation of ADP to ATP in F0F1 ATP synthesis complex [45]. Our recent studies showed Pd impaired electron transfer in the complex (V) of mitochondria respiratory chain via dissipation of proton-motive force and failed in ATP production [44]. Besides, ATP is consumed for maintenance of the MMP, which its decline could lead to further reduction of ATP concentration and elevation of ADP levels in mitochondria after ATPase activation and inhibition of ATP synthase in electron transfer chain [45,46].

As seen in Table.1, Pd increased the also ADP/ATP ratio in isolated mitochondria, suggesting induction of apoptosis cell signaling in our desired concentration. It suggested oxidative stress and respiratory failure are common features in the pathology of diseases and toxicity, shifting mitochondria toward consuming rather than synthesizing ATP and application of ADP/ATP have been used to differentiate modes of cell death signaling (apoptosis or necrosis) and viability [47].

It suggested that Pd in all wanted concentration could induce apoptosis (ADP/ATP ratio lower than 5-6) but probably in higher concentrations of 400µM caused higher depletion of ATP level and necrosis (ADP/ATP ratio higher than 5-6). Besides, the consequences of ATP reduction or increased of ADP/ATP ratio is release of cytochrome c into the cytosol and attachment with apoptosis-inducing factor (AIF) results in the activation of caspase 3 and also endonucleases. However, the sequence of events beginning with a reduction in ΔΨ, followed by cytochrome c release from mitochondria and commitment to cell death signals.

Conclusions

This study addresses that mechanism of hepato- and nephrotoxicity of Pd on isolated mitochondria system. These results open the perspective about the role of

oxidative stress in the Pathophysiology of cellular toxicity of Pd and the role of ADP/ATP. Since Pd is now increasingly used in modern industry, it progressively accumulates in the environment, organisms and human body. This investigation suggested that pet toxicity relates to electron transfer chain impairment at complexes II, III and V leading to increased ROS formation and induction of oxidative stress condition in cells, including a decline in ATP/ADP ratio, mitochondrial SDH activity, and rapid increase in mitochondrial membrane lipid peroxidation, and finally the release of cytochrome c from mitochondria which can then promote apoptosis signaling in liver and kidney.

Experimental

Materials: Palladium (II) Chloride and other chemicals were purchased from the company Merck (Darmstadt, Germany) with the best analytical grade available.

Animals: Male Wistar rats (Pasteur Institute, Tehran, Iran), weighing 250-300 g were housed under standard conditions (temperature 22±2ºC, humidity 50±10%, 12 h light-dark cycle and free access to food and water). The experimental protocols were approved by the Animal Ethics Committee in Zanjan University of Medical Sciences. All efforts were made to minimize the number and the suffering of animals used. After the animals were decapitated, liver and kidney tissue were quickly dissected out and rapidly rinsed using isotonic saline buffer. These samples were used for the isolation of mitochondria as described below.

Preparation of Mitochondria: After decapitation of animals and dissection of liver and kidney, mitochondrial isolation was done at 4ºC and freshly prepared according to previous reports [34]. Normalizing and keep the uniformity of experimental condition of sample was done based on the mitochondrial protein concentration of Bradford test (BSA as a standard) and adjusted to 500 µg protein/ml in all the experiments [48].

Flowcytometry (FCM) analysis: Mitochondrial ROS formation induced Pd on isolated mitochondria obtained from liver and kidney (normalized to 100 µg of mitochondrial protein) was measured by 2', 7'-dichlorofluorescein diacetate (DCFH-DA) (final concentration of 10 µM) as a reagent in modified respiratory buffer, including KCl (130mM), MgCl2 (5mM), NaH2PO4 (20mM), ADP (1.7mm), succinate (5mM), FeCL3 (0.1mm), pH 7.4 [49].

Rhodamine 123 redistribution technique was used for MMP measurement. Isolated mitochondria (normalized to 100 µg of mitochondrial protein) were suspended in 1 ml of analysis buffer, including 220 mM sucrose, 68 mM D-mannitol, 10 mM KCl, 5 mM KH2PO4, 2 mM MgCl2, 50 µM EGTA, 5 mM sodium succinate, 10 mM HEPES, 2µM Rotenone, then Rhodamine 123 (10 µM) was added [50]. Mitochondrial fluorescence and light scattering were analyzed for at least 12000 counts per sample in the flowcytometry using the BD Biosciences FACS CalibureTM flowcytometer. Samples were gated on the forward / side scatter to exclude cell debris and clumps. A flowcytometer with the Flomax software, equipped with a 488-nm argon ion laser was used and fluorescence signals were obtained using a 530-nm band pass filter (FL-1 channel).

Spectrophotometer analysis: Mitochondrial succinate dehydrogenase (complex II) activity was measured by the reduction of MTT to formazan at 570 nm as described with previous studies [22]. Mitochondrial GSH content (µg/mg protein) was assayed using DTNB reagent at 412 nm with minor modification [51]. Mitochondrial swelling was measured as the absorbance change of the mitochondrial suspensions in 540 nm [52]. The amount of releasing cytochrome C to medium from isolated mitochondria was performed at 450 nm according to the instructions provided by the manufacturer of the Quantikine® Rat/Mouse Cytochrome C Immunoassay kit (Minneapolis, MN). All of method analysis was done using an ELISA reader (InfiniteM 200, TECAN) in desired observed in all groups.

Assay of ATP, ADP and ATP/ADP ratio: The effect of Pd on the mitochondrial ATP level and the ATP / ADP ratio was measured based on RP-HPLC with LC-18 column which previously described by Mehrabadi et al., 2015 [53]. Briefly, 0.5 ml aliquot isolated mitochondria homogenate in TCA (6%) were mixed with 0.5 mL of KOH 0.05 M (on ice), then, 1 mL deionized water was added; after 2 min, 650 µL of KH2PO4 (0.05 M) was added, and vortexed. After filtering, this solution was kept in freezer until injection to RP-HPLC with defining condition: Mobile phase 0.05 M ammonium dihydrogen phosphate (pH=6.0), Flow rate =1 mL/min, λ=254 nm.

Statistical Analysis: The results of each series of experiments are expressed as the mean values ± SD. Levels of statistical significance were calculated by the Mann–Whitney U test. A P value <0.05 was considered significant. All tests were performed three times. All statistical analyses were performed using the SPSS software, version 17. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test.

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Abbreviations:

Pd; Palladium, ROS; Reactive oxygen species, GSH; reduced glutathione, DCFH-DA; 2', 7'-dichlorofluorescin diacetate, Rh123; Rhodamine 123, BSA; bovine serum albumin, MPT; mitochondrial permeability transition, MMP; mitochondrial membrane potential, MTT; 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide, DTNB; dithiobis-2-nitrobenzoic acid, DMSO; dimethyl sulfoxide