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PALLADIUM INDUCED OXIDATIVE STRESS AND CELL DEATH IN NORMAL HEPATOCYTES

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ABSTRACT

Palladium (Pd) accumulates in many organs and renders many deleterious effects. Although the Pd toxicity has been documented, the precise mechanism of Pd toxicity still needs to be elucidated. In the current research, a hepatotoxicity mechanism of Pd has been investigated. Our findings clearly indicate that Pd induces reactive oxygen species (ROS) formation and oxidative stress, mitochondrial and lysosomal injury and finally cell death. These effects are reversed by antioxidants and ROS scavengers, mitochondrial permeability transmission [1] pore sealing agent, ATP progenitor, and lysosomotropic agent. Pretreatment of hepatocytes with ROS scavengers and MPT pore sealing agents reduced cell death which explains the role of oxidative stress and mitochondrial pathway of ROS formation in Pd hepatocytes cell toxicity. Overall, the results have distinctly determined the mechanism by which Pd-induced toxicity in the isolated rat hepatocytes.

Keywords: Palladium; rat hepatocytes; reactive oxygen species; mitochondria; lysosome.

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1. INTRODUCTION

Palladium is a lustrous silver-white metal belonging to a group of metals called platinum group metals (PGMs) with diverse uses. It has three oxidation states including 2^+, 3^+ and 4^+[2], and is used in industrial catalyzers, jewelry making, crude oil refining, and jewelry dehydrogenation, telecommunication systems, dentistry, surgical and electronic instruments , etc [2-5]. The highest amount of Pd usage is in catalytic convertors [6]. In dentistry, palladium is used in a very common component of dental casting alloys that can be released in trace amounts in the body [7]. Pd like many other metals accumulates in the body over time. It can readily transfer into a soluble form such as Pd^{2+} ions through environmental process [6].

Palladium that is deposited in the oxidative and metallic forms can be transferred in to environment via soluble forms including pd^{2+}. It can enter the food chain, and subsequently find its way into plants, aquatic creatures and the human body [8-10]. There is a higher risk-potential of this metalloid getting into the environment, because it is often transported on road transport vehicles, and also because of its rate of emission in comparison to other metalloid from same group [11].

The absorption of palladium in human is low, but it is able to enter into the food chain easily; especially plants and pose dangerous toxic effects on some plants including hyacinth which is extremely sensitive even to minute levels of palladium in water that can be ultimately fatal for it [12]. After entering the body, palladium accumulates in some organs including the heart, liver, kidney, thyroid, brain, etc. [13, 14]. One organ that is readily affected by palladium toxicity is liver. Rat hepatocytes collection and the effects of palladium is a suitable way to determine the toxic effects of this metal.

Deleterious effects of palladium include cytotoxic effects, hypersensitivity, cell damage, DNA degradation and damage, exacerbation of hydroxyl radical damage, cell mitochondria damage and enzyme activation and inhibition [14-18].

Although, some investigations on Pd-induced toxicity have been carried out, there is still an obvious lack of data which can clearly explain the toxicity mechanism. The aim of this study is to clarify precise mechanism of Pd-induced toxicity in freshly isolated rat hepatocytes.

2. MATERIAL AND METHODS

2.1. Chemicals

Palladium (Merck, Germany), collagenase type IV (from Clostridium histolyticum), rhodamine 123, bovine serum albumin, N-(2-hydroxyethyl)piperazine-N 0 -(2-ethanesulfonic acid) (HEPES), 2, 7-dichlorofluorescin diacetate (DCFH-DA) and trypan blue were purchased from Sigma-Aldrich Co. (Taufkrichen, Germany). All other chemicals were of the highest commercial grade available.

2.2. Isolation and Incubation of Hepatocytes

Male Wistar rats (280-300 g) were purchased from Zabol University of Medical Sciences Animal Farm (Zabol, Iran), and fed a standard chow diet where water ad libitum was used as hepatocyte source. All experiments were conducted according to ethical standards and protocols approved by the Committee of Animal Experimentation of Zabol University of Medical Sciences, Zabol, Iran. Hepatocytes were isolated by collagenase liver perfusion [19]. Cell viability was determined using trypan blue exclusion test which was always found to be above 90% [20]. Isolated hepatocytes (10 6 cells/ml) (10 ml) were suspended in Krebs-Henseleit buffer (pH 7.4) containing 12.5 mM HEPES in continually rotating 50-ml round-bottom flasks, under an atmosphere pressure of 95% O_2 and 5% CO_2 in a water bath of 37°C [20]. To avoid either non-toxic or severe toxic conditions in this study, EC_{50} concentration was used for trivalent palladium (Pb^{3+}). Stock solutions of all chemicals (×100 concentrated) were prepared fresh prior to use [21]. All inhibitors were added 15 mins prior to addition of Pd^{3+} to hepatocyte medium. To incubate Pd^{3+} and all other treatments with the required concentration, 100ml sample of concentrated stock solution (×100 concentrated) was added to the rotating flask containing 10 ml hepatocyte suspension [22].

Antioxidants and free radical scavengers (GSH, mannitol and dimethyl sulfoxide (DMSO)), mitochondrial permeability transition [1] pore sealing agent (carnitine), ATP generator (L-glutamine) and lysosomotropic agent (chloroquine) were used as protective agents at sub-toxic concentrations in all our experiments [23].

2.3. Hepatocyte viability determination

The trypan blue (0.2% w/v) exclusion test was used to determine the number of viable cells present in the cell suspension [24]. Aliquots of the hepatocyte incubate were taken at different time intervals during the 4-hr incubation period [24]. Subsequently, at least 80% of the control cells were still viable after 4 hr.

2.4. Determination of ROS Formation

Hepatocyte reactive oxygen species (ROS) generation induced by Pd^{3+} was determined by adding DCF- DA to hepatocyte incubates [25]. The cells were allowed to incubate in a

thermostatic bath for 10 min at 37° C while shaking. The fluorescence intensity of ROS product was measured at 490 nm excitation and 520 nm emission wavelengths, using Shimadzu Rf-5301PC fluorescence spectrophotometer [21].

2.5. MMP Assay

The uptake and retention of the cationic fluorescent dye, rhodamine 123, was used to estimate mitochondrial membrane potential (MMP). This assay is based on the fact that rhodamine 123 accumulates selectively in the mitochondria by facilitated diffusion. However, when the MMP is decreased, the amount of rhodamine 123 that enters the mitochondria also decreases as there is no facilitated diffusion. Thus, the amount of rhodamine 123 in the supernatant is increased and the quantity in the pellet is decreased [26]. Samples (500ml) were taken from the cell suspension incubated at 37 °C at different time intervals, and centrifuged at 50×g for 1 min. The cell pellet was then re-suspended in 2 ml fresh incubation medium containing 1.5 mM rhodamine 123 and incubated at 37 °C in a thermostatic bath for 10 min while shaking. Hepatocytes were separated by centrifugation and the amount of rhodamine 123 appearing in the incubation medium was measured fluorimetrically, using Shimadzu Rf-5301PC fluorescence spectrophotometer set at 490 nm excitation and 520 nm emission wavelengths. The capacity of mitochondria to take up the rhodamine 123 was calculated as the difference in fluorescence intensity between control and treated cells [27].

3. RESULTS

In the cytotoxicity assessment studies, the EC_{50} of a chemical is the concentration which increases cell death by 50% at a certain time point following the same incubation period. In order to determine a 2-hr EC_{50} ($EC_{50, 2\ hr}$) of Pd^{3+}, some concentration-response curves were plotted, and then $EC_{50, 2\ hr}$ was determined based on a regression plot of different concentrations of Pd^{3+} [23]. The $EC_{50, 2\ hr}$ found for Pd^{3+} was 100 µM.

For the evaluation of cell toxicity, hepatocytes homogenates were incubated in buffer with pH 7.4 in 37°C for 2 hr in the presence of Pd^{3+}. As it was shown, Pd^{3+} induced cell death in comparison to the control group (table. 1). Our results indicated that pretreatment of homogenates with antioxidants and free radical scavengers (GSH, mannitol and DMSO), MPT pore sealing agents (carnitine), ATP generator (L-glutamine) and lysosomotropic agent (chloroquine) significantly decreased Pd^{3+}-induced hepatocyte cell death in comparison to cells treated with Pd^{3+}.

Table 1. Effect of antioxidants, ROS scavengers, MPT pore sealing agents, ATP generator and lysosomotropic agent on Pd^{3+} -induced at hepatocytes cytotoxicity

Addition	**Cytotoxicity (%) 3h**
Control rat hepatocytes	21±3
+Pd(III)(100 µM)	77±6[a]
+GSH	42±7[b]
+Mannitol (50mM)	48±3[b]
+DMSO (150µM)	61±8[b]
+Carnitine (2 mM)	31±5[b]
+L- Glutamine (10µM)	54±7b
+Chloroquine (100µM)	64±4b

Hepatocytes (10^6cells/ml) were incubated in Krebs–Henseleit buffer pH 7.4 at 37◦C for 3 hours following the addition of $EC_{50,\ 2h}$ of Pd^{3+}. Cytotoxicity was determined as the percentage of cells that take up trypan blue [28].

Values are expressed as mean±SD of three separate experiments (n=3).

[a] Significant difference in comparison with control hepatocytes ($P < 0.05$).

[b] Significant difference in comparison with Pd^{3+} treated hepatocytes ($P < 0.05$).

Moreover, for determination of ROS levels following the incubation of hepatocytes with Pd^{3+} in a buffer with a pH of 7.4 at 37 °C for 60 min indicated that Pd^{3+} at E $C_{50,\ 2\ hr}$ concentration produced a marked increase in ROS levels in hepatocytes (table 2). Pretreatment of samples with antioxidants, free radical scavengers (GSH, mannitol and DMSO), MPT pore sealing agent (carnitine), ATP generator (L-glutamine) and lysosomotropic agent (chloroquine) significantly diminished ROS levels in hepatocytes in comparison to cells treated with Pd^{3+}.

Table 2. Effect of antioxidants, ROS scavengers, MPT pore sealing agents, ATP generator and lysosomotropic agent on Pd^{3+} induced ROS formation in rat hepatocytes.

Addition	DCF		
	Incubation time		
	15 min	30 min	60 min
Control rat hepatocytes	35±3	62±5	136±9
+Pd(III)(100 µM)	129±9[a]	280±8[a]	312±11[a]
+GSH	101±5[b]	134±9[b]	155±8[b]
+Mannitol (50mM)	88±7[b]	143±2[b]	169±8[b]
+DMSO (150µM)	76±11[b]	123±8[b]	164±9[b]
+Carnitine (2 mM)	71±6[b]	115±7[b]	132±4[b]
+L- Glutamine (10µM)	106±5[b]	227±15[b]	278±10[b]
+Chloroquine (100µM)	125±7	143±6[b]	261±13[b]

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37 °C for 1.0 hr following the addition of $EC_{50,\ 2\ hr}$ of Pd^{3+}. DCF formation was expressed as fluorescent intensity units [21].

Values are expressed as mean ±SD of three separate experiments (n=3).

[a] Significant difference in comparison with control hepatocytes (P<0.05) .

Table 3. Effect of antioxidants, ROS scavengers, MPT pore sealing agents, ATP generator and lysosomotropic agent on Pd^{3+} induced MMP decrease in rat hepatocytes

Addition	%ΔΨm		
	Incubation time		
	15 min	30 min	60 min
Control rat hepatocytes	4±1	7±3	16±3
+Pd(III)(100 µM)	35±3[a]	50±4[a]	74±5[a]
+GSH	32±4	34±5[b]	38±3[b]
+Mannitol (50mM)	32±4	34±2[b]	38±5[b]
+DMSO (150µM)	31±3	36±4[b]	36±6[b]
+Carnitine (2 mM)	30±3	34±5[b]	25±5[b]
+L- Glutamine (10µM)	33±3	35±3[b]	37±4[b]
+Chloroquine (100µM)	30±4	33±5[b]	39±3[b]

[b] Significant difference in comparison with Pd^{3+} treated hepatocytes ($P<0.05$).

$EC_{50,\ 2\ hr}$ concentration produced a decline in MMP (%Δψm) in hepatocytes following 1-hr of incubation. Pretreatment of samples with antioxidants and free radical scavengers (GSH, mannitol and DMSO), MPT pore sealing agent (carnitine), ATP generator (L-glutamine) and lysosomotropic agent (chloroquine) prevented the decline in MMP (%Δψm) in hepatocytes compared to cells treated with Pd^{3+}.

Hepatocytes (10^6 cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37° C for 1.0 hr following the addition of EC 50, 2 hr of Pd^{3+} . Mitochondrial membrane potential was determined as the difference in mitochondrial uptake of the rhodamine 123 between control and treated hepatocytes and expressed as fluorescence intensity unit [27].

Values are expressed as mean±SD of three separate experiments (n=3).

[a] Significant difference in comparison with control hepatocytes (P<0.05).

[b] Significant difference in comparison with Pd^{3+} treated hepatocytes (P<0.05).

When hepatocyte lysosomes were loaded with acridine orange (a lysosomotropic agent), a significant redistribution of acridine orange into the cytosolic fraction ensued within 60 min of incubation with Pd^{3+}, indicating sever oxidative damage to lysosomal membrane (Table 4). Pd^{3+} induced acridine orange release was again prevented by antioxidants and free radical scavengers (GSH, mannitol and DMSO), MPT pore sealing agent (carnitine), ATP generator (L-glutamine) and lysosomotropic agent (chloroquine).

Table 4 Effects of antioxidants, ROS scavengers, MPT pore sealing agents, ATP generator and lysosomotropic agent on Pd^{3+}-induced lysosomal membrane injury.

Addition	**% Acridine orange redistribution**		
	Incubation time		
	15 min	**30 min**	**60 min**
Control rat hepatocytes	1±1	2±2	9±2
+Pd(III)(100 µM)	14±3[a]	19±5[a]	59±6[a]
+GSH	3±1[b]	4±2[b]	46±3[b]
+Mannitol (50mM)	3±2[b]	5±1[b]	44±4[b]
+DMSO (150µM)	4±2[b]	8±3[b]	39±5[b]

+Carnitine (2 mM)	3 ± 2^{b}	7 ± 4^{b}	33 ± 6^{b}
+L- Glutamine (10µM)	3 ± 3^{b}	6 ± 3^{b}	38 ± 4^{b}
+Chloroquine (100µM)	2 ± 1^{b}	6 ± 3^{b}	26 ± 4^{b}

Hepatocytes (10^{6} cells/ml) were incubated in Krebs-Henseleit buffer pH 7.4 at 37 ° C for 4 hr following the addition of EC 50, 2 hr of Pd^{3+}. Lysosomal cell membrane was determined via reemission of fluorescents of acridine orange into the cell cytoplasm and was expressed as percentage of lysosomal injury [29].

Values are expressed as mean±SD of three separate experiments (n=3).

[a] Significant difference in comparison with control hepatocytes ($P<0.05$).

[b] Significant difference in comparison with Pd^{3+} treated hepatocytes ($P<0.05$).

4. DISCUSSION

Pallidum results in bronchial epithelial cell death by increasing the intracellular production of ROS [30], and it is likely to be the most effective mechanism of toxicity. Production of ROS and cells detoxification via ROS degradation is continual process in cell growth cycle that results in the stabilization of the levels of ROS. Either decreasing in the antioxidative cellular capacity defense or increasing the production of ROS leads to cells harmful effects on.

As Pd accumulates in the liver, kidney, lung, muscles and adipose tissues, its deleterious effects are more serious in the aforementioned tissues, and for this reason liver was selected for evaluation of Pd-induced toxicity [31]. Furthermore, Pd is a concern as an occupational hazard as well, and humans are exposed to it through air, water and food.

Based on previous studies, Pd can increase production of ROS and due to deleterious effects and our results confirmed that palladium can increase production of ROS, and lead to toxicity. Mannitol and DMSO (ROS scavengers) decreased ROS production in the hepatocytes following after cells were treated with Pd (table1, table 2). Increasing ROS production and high rate of hepatocytes cell death confirmed that Pd induces cell death, and ROS scavengers (mannitol and DMSO) reduce cell death in hepatocytes.

Furthermore, our results showed that carnitine (MPT blocking agent), chloroquine (lysosomal protecting agent) and L-glutamine (progenitor of ATP) reduced Pd-induced ROS production. Our results also indicated that Pd poses deleterious effects on mitochondria and the protecting agent including carnitine, chloroquine and L-glutamate reveres those effects (table 3). The results confirmed that the sources of ROS production are mitochondria and lysosomes.

Moreover, our results confirmed that carnitine, chloroquine and L-glutamate in used doses in this experiments, do not show the increasing effects of ROS alone (data not shown).

Decrement in the MMP leads to instability, and the opening of MPT resulting in spontaneous release of free radicals into the mitochondria in the process of energy production, cytochrome C release (induction of apoptosis) and the activation of pro-apoptotic proteins while reduction of ROS production results in mitochondrial stability and reduction of apoptosis. Our results showed that decrement in MMP induced via Pd in rat hepatocytes is inhibited by MPT blocking agent (carnitine), lysosomal protecting agent (chloroquine) and ATP-producing agent (l-glutamate) (table 3).

Interaction of free radicals with electron chain results in free radical formation in the mitochondria [32], and the production of ROS results in the opening of the mitochondrial membrane, and the MPT opening that leads to expansion of ROS production especially H_2O_2. Our results confirmed that Pd leads to lysosomal membrane injury (table 4) in rat hepatocytes, increasing the Pd inhibited lysosomal destruction by MPT blocking agent (carnitine), lysosomal protecting agent (chloroquine) and ATP-producing agent (l-glutamate) (table 4). Furthermore, the results showed that lysosomal membrane protecting agents have preventing effects including lysosomal lysis, and the free radicals produced showed that lysosomal membrane permeability can potentiate and induce cytotoxicity from Pd-induced stress oxidative.

Mitochondria are one of the most important ROS-produced sources. The first radical was produced in the mitochondria by heating electrons from the electron chain with oxygen molecules. Hydrogen peroxide radical in the mitochondria is yielded through the action of SD on superoxide anion. This radical can move freely and cross easily in to the cytosol [33, 34]. Furthermore, mitochondria itself can be the target of free radicals. Mitochondrial injury induced via free radicals can expand in a way that hydrogen peroxide which was produced in mitochondria moves to the cytosol, and then into lysosomes, and is finally changed to Fenton type (Weiss—Haber) reaction which is catalyzed via ferric cations. This reaction produces very active hydroxyl radical that cause lysosomal rupture and disturb lysosomal cell membrane integrity leading to the release of proteolytic enzymes and free radicals into the cytoplasm. One group of enzymes are cathepsins (B, L and D) which lead to MPT opening and release of cytochrome C, triggering streaming that leads to the activation of caspase 3 and apoptosis.

In the rat hepatocytes, lysosomal protective agents prevented mitochondrial cell membrane (Table 3). Furthermore, mitochondrial protective agents prevented lysosomal cell membrane (Table 4). Therefore, it is proposed that there is cross reaction exists between mitochondria and lysosomal injury in the Pd-induced hepatotoxicity.

5. CONCLUSION

Altogether, the results of this study showed that Pd induces hepatotoxicity via induction of stress oxidative. Mitochondrial and lysosomes are two main pathways of ROS production, and that oxidative stress is caused by Pd. Cross talk intention of oxidative stress injury via mitochondria and lysosomes results in the expansion of oxidative stress, and finally hepatocytes death in rat.

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7. CONFLICT OF INTEREST

There is not any conflict of interest.

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