Change in metabolic status of glutathione by palladium nitrate in blood components

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Abstract: This piece of research work present the toxicological impact of varied concentrations of Palladium Nitrate [Pd $(NO_3)_2$] by changing the chemical status of glutathione and the way how glutathione plays its role in detoxification and conjugation processes of [Pd $(NO_3)_2$] in whole blood components (plasma and Cytosolic fraction). The impact of different concentration of [Pd $(NO_3)_2$] on reduced glutathione level in whole blood component(Plasma and Cytosolic fraction) were measured spectrophotometrically following Standard Ellman's method. Compared with control sample, significant decrease in the GSH content in whole blood components (plasma and Cytosolic fraction) was obtained with various concentrations (100µM-1000µM) of Palladium Nitrate. Depleted GSH level was more pronounced with time incubation period (0-90) minutes. These finding shows that changes in the GSH status produced by palladium nitrate could either be due to palladium nitrate and glutathione(Pd-SG) complex formation or by conversion of reduce glutathione (2GSH + Pd^{+2} → GSSG). This change in the GSH metabolic status provides information regarding the mechanism of palladium, in blood components.

Keywords: Palladium **N**itrate [Pd $(NO_3)_2$], Glutathione (GSH), Di,thiobis-dinitrobenzoic acid (DTNB), Blood Components, Plasma, Cytosolic Fraction (CF), palladium (Pd^{+2}) palladium Glutathione complex (Pd-SG).

INTRODUCTION

The Glutathione is the most abundant low molecular weight thiol molecule and important antioxidant found in maximum concentration in nearly all body cells. (Meister and Anderson 1983).The sulfhydryl group (SH) of GSH serves as a proton donor and is responsible for the biological activity of GSH.GSH plays important roles including the detoxification of xenobiotics, free radical and peroxides, maintenance of protein structure and function, regulation of protein and DNA biosynthesis and cell growth, and maintenance of the immune function (Meister and Anderson, 1983; Vifia, 1993). Glutathione takes part in the detoxification of heavy metals and forms stable complexes with them (Bartosz, 1993; Haroon Khan *et al.,* 2010, 2011a and 2011b). Palladium compounds showed nephrotoxic effects on the handling of Na^+ and Ca^{+2} ions by inhibiting both the Na^+- Ca^{+2} –anti-porter and the Na- H –exchanger with laxing effects on non voltage-gated Ca- channels at the basolatoral side (Anwar *et al.,* 1995). Palladium compounds showed important antiviral activity against acyclovir-resistant viruses R-100 (HSV1) and PU (HSV2) by suppressing simultaneously virus entry, transactivation of genome, capside assembly and cell to cell spread of infectious HSV progeny (Petia *et al.,* 2004). Some palladium compounds may also acted as a potent neuro-protective agent for victims of transient ischemic attack, cardiac arrest, anesthetic accidents, or drowning (Francis *et al.,* 2004). Palladium complexes show better cytotoxic activity than paclitaxel and cis-platin by inducing apoptosis via the activation of cell death receptor, DR5, against human breast cancer cells lines.(Engin Ulukaya *et al.,* 2001).GSH prevented the enzyme inhibition induced by Pd(II) complex cation. The GSH completely reversed the inhibited activity in the concentration dependent manner, due to the complex formation with [PdCl (dien]$^+$ (Katarina Krinulovic *et al.,* 2006). 4-HNE is metabolized in the body through conjugation with Glutathione (GSH), while most of the metals including Palladium are also widely documented to be detoxified through conjugation with glutathione (GSH). So in terms of conjugating with glutathione (GSH), it is quite understandable that 4-HNE resembles metals including Palladium. 4-HNE is produced in the brain regions particularly in the diseased states. We have investigated the effect of Palladium nitrate on GSH level in the blood samples of healthy human volunteer, so 4-HNE does not seem to affect the results of the present study.

MATERIALS AND METHODS

Materials

Palladium Nitrate (Sigma) L-glutathione (GSH), Potassium di hydrogen phosphate (Sigma), Di-sodium-EDTA (Merck), Di-thiobis. di. Nitro- benzoic Acid

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(DTNB), Chloroform (Sigma), HCl, Rubber gloves (disposable), Glass (disiliconised), Sterile test tubes, Ethanol (Sigma), UV-spectrophotometer, Model 1601 (Shimadzue) centrifuge (H-200 kokusan, Ensik Japan), Eppendolfs tubes (Pyrex-Germany). pH Meter. All chemicals were used for research work without any further purification.

Methodology

1mM GSH solution was prepared by dissolving 30.74mg glutathione in 100ml of 0.1M Phosphate buffer (PH 7.6). 10mM or 10^{-2}M of DTNB (di-thiobis di-nitrobenzoic Acid) was prepared by dissolving 390.6mg of DTNB in 100ml of Phosphate buffer solution. For preparing 0.1mM Phosphate buffer solution 21.1 ml of NaOH (0.2M) was mixed with 25ml of (0.2M) potassium-monobasic phosphate solution the final volume of solution was make-up to 200ml with distilled water.PH was adjusted with help of PH-meter (Accumet meter, USA). 50ml of palladium nitrate (2mM) stock solution was prepared by dissolving 26.6mg of palladium nitrate mol.wt. 266 in 50 ml distilled water. 0.9% NaCl solution was prepared by dissolving 9mg of NaCl in 100ml of distilled water.

Preparation and isolation of Blood Components

Isolation of Plasma

12ml fresh venous blood from human healthy volunteer was collected in heparinized tube. 1-ml venous blood was taken and mixed with 1-ml of each concentration of palladium Nitrate (200-2000µM) solution and incubated for 10 minutes. In each tube the final concentration of Palladium Nitrate was from (100-1000µM). Each of this 2ml sample containing Palladium Nitrate solution and whole blood in the 1:1 ratio was then centrifuged at 10000-rpm for 5 minutes. 0.6ml of the supernatant fluid (plasma) was removed by Pasteur pipette. 50µl HCl (0.1N) was added to the plasma sample to allow GSH to be in intact reduced form, transferred to sample tubes and refrigerate till further use. The packed cells were further processed for Cytosolic fraction. The control sample comprised of 1ml of venous blood and 1ml 0.9% NaCl solution was also centrifuged for isolation of plasma.

Isolation of Cytosolic fraction of blood

The packed cells fraction (0.5ml) was collected and washed twice with (0.9% NaCl) solution and centrifuged for 5-minutes. The supernatant was discarded. Distilled water was added in 1:1 ratio to lyse RBC's. 0.6ml of cold chloroform, ethanol mixture (3:5 V/V) was added to precipitate hemoglobin, followed by 0.1ml of distilled water. The resulting mixture was also centrifuged as previously. The pale yellow supernatant (Cytosolic fraction) was collected. 50 µl HCl (0.1N) was added to the Cytosolic fraction to allow GHS to be in reduced form, transferred to sample tubes and refrigerate till further use.

Determination of Inorganic Biological parameters

Inorganic biological parameters i.e. Plasma or Extracellular glutathione and Cytosolic fraction or intracellular glutathione were measured using the following modified standard Ellman's method (Ellman's 1959).

2.3ml buffer (0.1M) was mixed with 0.2ml of sample (plasma/Cytosolic fraction of blood) followed by the addition of 0.5ml of 10^{-2}M DTNB. This mixture was then transferred to spectrophotometer cell. The reference cell comprised of 0.2ml of sample and 2.8ml of buffer. Absorbance (A) for each sample was taken after 5-minutes at 412 nm on UV-visible spectrophotometer of Model-1601 (Shimadzu).

The DTNB blank consisting of 2.5ml of buffer, 0.5ml DTNB was measured against a reference cell containing 3ml of buffer. The glutathione contents were calculated using the standard curve

Standard curve

Standard curve was drawn using 200-2000µM GSH by following the standard Ellman's method prescribed above and as shown in the fig. 1.

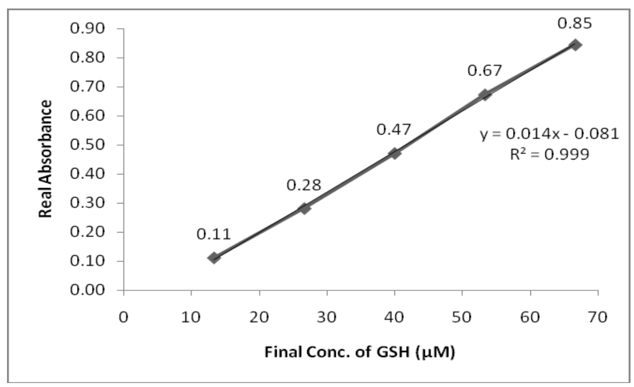

Fig. 1: Standard curve.

RESULTS

Effect of Pd $(NO_3)_2$ on metabolic status of GSH in extracellular plasma fraction of blood

1ml solution of different concentrations (200-2000µM) of Palladium Nitrate was added to equal volume of human venous blood in 10 separate tubes. Final concentration of Palladium Nitrate [Pd $(NO_3)_2$] in each tube was 100-1000µM .Plasma fraction of each tube of the blood was isolated after 10minutes of incubation period and transferred to separate tubes.

Reduced Glutathione (GSH) content was measured in each tube by Ellman's method. The GSH content of plasma fraction of blood decreased with increasing concentration of [Pd $(NO_3)_2$] addition as shown by table 1 and in fig. 2.

Table 1: Effect of different concentrations of palladium nitrate (0.2 to2mM) in the chemical status of glutathione (GSH) with time in Plasma

S. No.	Conc. of. Palladium Nitrate	Avg. ABS at 0 Min.	Escaped Conc. (µM of GSH	Avg. ABS at 30 Min.	Escaped Conc.(µM) of GSH	Avg. ABS at 60 Min.	Escaped Conc.(µM of GSH	Avg. ABS at 90 Min.	Escaped Conc.(µM of GSH
1	100µM	0.523	43.14	0.482	40.21	0.447	37.71	0.417	35.57
2	200µM	0.493	41.00	0.453	38.14	0.421	35.86	0.396	34.07
3	300µM	0.469	39.29	0.432	36.64	0.399	34.29	0.377	32.71
4	400µM	0445	37.57	0.407	34.86	0.379	32.86	0.354	31.07
5	500µM	0.421	35.86	0.386	33.36	0.357	31.29	0.337	29.86
6	600µM	0.403	34.57	0.371	32.29	0.344	30.36	0.321	28.71
7	700µM	0.381	33.00	0.351	30.86	0.327	29.14	0.306	27.64
8	800µM	0.363	31.71	0.332	29.50	0.309	27.86	0.291	26.57
9	900µM	0.347	30.57	0.319	28.57	0.297	27.00	0.279	25.71
10	1000µM	0.329	29.29	0.303	27.43	0.281	25.86	0.263	24.57
GSH Blank		0.61	49.36	0.61	49.36	0.61	49.36	0.61	49.36

Palladium nitrate was injected into whole blood before its separation and incubated for 30 minutes.

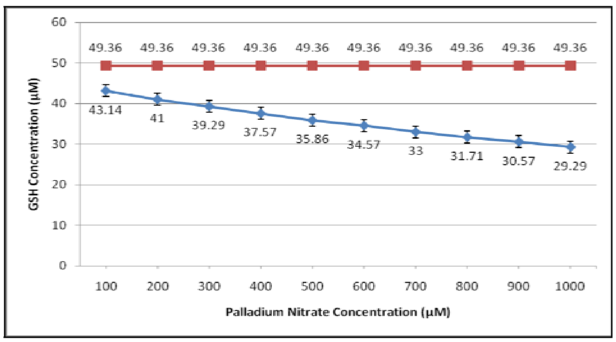

Fig. 2: Effect of (Pd $(NO_3)_2$) concentration on Extra-cellular plasma GSH content.
■Control (1ml 0.9%NaCl/ml of blood) ♦(Pd$(NO_3)_2$)(100-1000µM)
Results are the mean ± SE of 3 experiments of plasma GSH.

A significant change in the content of plasma GSH from control was observed at $p<0.05$. Plasma GSH content at time intervals 0-9minutes was also measured when two different concentration of Pd $(NO_3)_2$ (100, 1000µM) were added. GSH content of extracellular plasma decreased at time intervals (fig. 3). These results show that decrease in plasma GSH content were both Pd $(NO_3)_2$ concentration and time dependent.

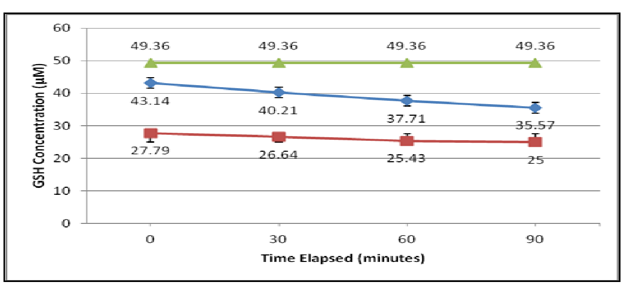

Fig. 3: Effect of (Pd $(NO_3)_2$) concentration on Extracellular plasma GSH content with time of incubation 0-90 minutes.
▲Control (1ml 0.9%NaCl/ml of blood)
■ (Pd $(NO_3)_2$)1(100µM), ♦ (Pd $(NO_3)_2$) (1000µM)
Results are the mean ± SE of 3 experiments of plasma GSH.

Effect of Palladium Nitrate on metabolic status of GSH in intracellular Cytosolic fraction of blood

1ml of Pd $(NO_3)_2$ (100-1000µM) solution was added to 1ml of venous blood to give final concentration of (100-1000µM) Pd $(NO_3)_2$. Upon addition of Pd $(NO_3)_2$ to venous blood, measurement of intracellular Cytosolic GSH content showed gradual decrease in GSH content as shown in table 2 and fig. 4. Intracellular Cytosolic GSH content was also measured at time intervals (0-90-minutes) after the addition of Pd $(NO_3)_2$ to venous blood showing the time dependent decrease in GSH content as shown table 2 and fig. 5.

STATISTICAL ANALYSIS

Statistical analysis applied to the data shows that Palladium Nitrate caused reduction in the GSH concentration of plasma and cytosolic fraction of blood significantly (p value is > 0.05) as shown in the table 3 and 4.

DISCUSSION

Glutathione (GSH) has been suggested to minimize the toxic effect of palladium in various biological systems i.e. it prevents enzyme inhibition induced by Pd (II) complex cation by forming complexes with palladium metal compounds (Katarina Krinulovic *et al.*, 2006). Therefore we have studied the effect of Palladium nitrate on GSH level in plasma and Cytosolic fraction of human venous blood spectrophotometrically. The effect of Palladium nitrate on the chemical and metabolic status of GSH was studied in terms of determination of concentration of GSH at λmax 412nm. This λmax (412nm) is being used for the determination of GSH concentration in samples according to a well known Elman's method (Elman's, 1959).

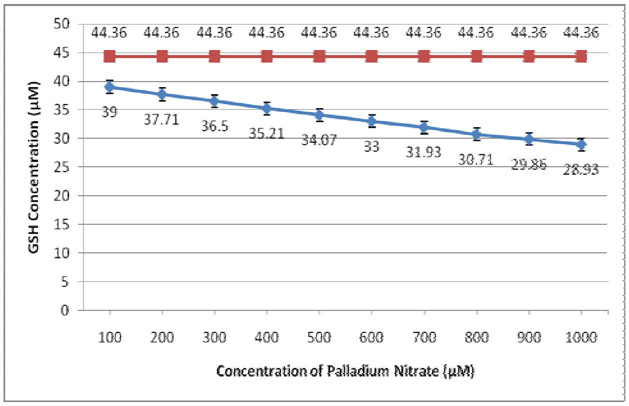

Fig. 4: Effect of (Pd $(NO_3)_2$) Concentration on intracellular CF GSH Content

■Control (1ml 0.9% NaCl/1ml of blood), ♦ (Pd $(NO_3)_2$) (100-1000 µM)
Results are the mean ± SE of 3 experiments of CF GSH

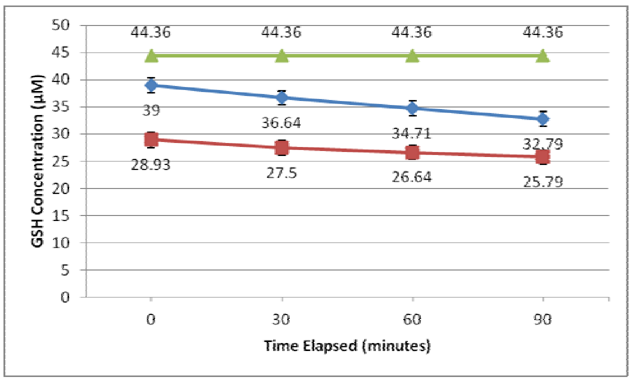

Fig. 5: Effect of (Pd $(NO_3)_2$) Concentration on the intracellular CF GSH content with time incubation period 0-90 minutes.

♦Control (1ml 0.9% NaCl/1ml of blood)
■ (Pd $(NO_3)_2$) (100µM), ▲ (Pd $(NO_3)_2$) (1000µM)
Results are the mean ± SE of 3 experiments of CF GSH

Table 3: t-Test: Paired two sample for means in case of effect of palladium nitrate on gsh in plasma

	GSH + Pd	Control GSH
Mean	35.6	49.36
Variance	21.29402	5.61E-29
Observations	10	10
Pearson Correlation	3.25E-16	
Hypothesized Mean Difference	0	
df	9	
t Stat	-9.42952	
P(T<=t) one-tail	2.91E-06	
t Critical one-tail	1.833113	
P(T<=t) two-tail	5.82E-06	
t Critical two-tail	2.262157	

Thus the interaction of heavy metal with glutathione *in vitro* as a model of *in vivo* reaction will establish further scientific data and will strengthen our knowledge about the toxicological profile of Palladium and the role of GSH in the protection of our body from its harmful effects. According to our findings the Palladium induced the depletion of GSH in a dose dependent manner. These results also show that depletion of glutathione is positively correlated to the increasing concentration of Palladium Nitrate. From our finding the concentration of Glutathione decreased to be 35.57µM when treated with the lowest(100µM) concentration of Palladium nitrate and 24.57µM when treated with the highest(1000µM) concentration of Palladium nitrate compared to glutathione concentration (49.36µM) of control sample in plasma while the concentration of Glutathione dropped to 32.79 µM when treated with the lowest(100µM) concentration of Palladium nitrate and 25.79µM when treated with the highest(1000µM) concentration of Palladium nitrate compared to glutathione concentration (44.36µM) of control sample in Cytosolic fraction of human whole blood. During this study, time dependent effect of the above given concentrations of palladium Nitrate on the metabolic status of glutathione was also investigated and further gradual depletion of reduced Glutathione was observed for a time interval from 0 to 90 minutes. From our findings it appears that the oxidative stress or toxic effect exerted by Pd^{+2} is slightly more severe in extracellular than intracellular level. This evidence further suggests that extracellular compartment is more oxidized than intracellular compartment by the addition of Palladium nitrate solution.

Table 4: t-Test: Paired two sample for means in case of effect of palladium nitrate on GSH in cytosolic fraction

	GSH + Pd	Control GSH
Mean	33.692	44.36
Variance	11.60977	5.61E-29
Observations	10	10
Pearson Correlation	0	
Hypothesized Mean Difference	0	
df	9	
t Stat	-9.90082	
P(T<=t) one-tail	1.94E-06	
t Critical one-tail	1.833113	
P(T<=t) two-tail	3.89E-06	
t Critical two-tail	2.262157	

The exact mechanism of the reaction between Palladium nitrate and depletion of reduced glutathione still need to be fully investigated, but the formation of Pd $(GS)_2$ complex may be proposed. This hypothetical mechanism of action and formation of Pd-SG complex is in agreement with our research work, where antibacterial activity of Palladium Nitrate, GSH and Palladium Nitrate and GSH mixture were examined (not shown).

The results indicated that antibacterial activity of Palladium Nitrate was very low (almost Zero) and Palladium Nitrate and GSH mixture was lower than GSH and higher than Palladium Nitrate.

These results indicating that antibacterial activity of GSH was maximum and GSH and Palladium Nitrate mixture was very low than GSH and Palladium Nitrate had almost Zero, leads to the conclusion that most probably Palladium Nitrate, converts GSH to Pd-SG complex.

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