ORIGINAL ARTICLE

STUDY OF PLASMA AND CYTOSOLIC GLUTATHIONE IN CADMIUM TOXICITY

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ABSTRACT

Background: Cadmium causes oxidative stress because this metal lacerate antioxidant defense system in intracellular as well as extra cellular compartments resulting in depletion of reduced glutathione or GSH contents in these areas. This study was designed to estimate spectrophotometrically the depletion of GSH in plasma/cytosolic fraction of human blood for which Ellman's method was used.

Material & Methods: This experimental study was conducted in PhD Research Laboratory, Faculty of Pharmacy, Gomal University D.I.Khan during the month of December 2012. Fresh human volunteer's 12000μl venous blood was taken, for which 4 healthy volunteers age 22-27 years with no previous history of illness or long illness (having no history of any disease or abnormality) were selected. Plasma/cytosolic fraction GSH was exposed to various concentrations of cadmium and paired t-Test was applied to test the statistical significance.

Results: It was found that even lowest most concentration of cadmium has statistically significantly ($p<0.001$) depleted GSH up to 3.662 μM (75.15%) in plasma and 3.408 μM (67.73%) in cytosolic fraction with respect to plasma and cytosolic fraction GSH control respectively. Moreover with the passage of time this drop in GSH level was greater showing that with the passage of time cadmium is more toxic toward antioxidant defense system as cadmium is accumulated in human body for a long period of time.

Conclusion: Our study suggests that cadmium has strong chemical binding affinity towards reduced form of glutathione resulting in depletion of this master antioxidant thus rendering human body to have compromised immune system.

KEY WORDS: Glutathione; Oxidative Stress; Cadmium; Carcinogens; Cytosol.

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INTRODUCTION

L-γ-Glutamyl-L-cystenyle-glycine (GSH) is found in free as well as in bound form in cytosol, plasma and in mitochondria.[1] It is widely distributed in almost all living organisms and inside the cells its concentration is very high approximately 0.1 to 10mM.[2,3] Its concentration in mitochondria is 15-20% and is also present in nucleus and endoplasmic reticulum.[4,5]

Carcinogenesis frequently generate oxygen radicals,[6] while the effect of GSH on free radical detoxification[7] is very important, inhibiting carcinogenesis which is produced by a number of mechanisms.[8] The prostate cancer is an example.[9] An intriguing relationship is present between cancer cells GSH and GSH precursors or cysteine pro-drugs and this phenomenon has been proved by in-vitro studies. There is strong possibility of a direct effect of cysteine delivery system on tumor cells because in 1986 Russo et al has observed that GSH levels were 7 fold higher in a human lung adenocarcinoma cell line than a normal human fibro blast line. In tumor cell line OTZ (Oxothiazoline-4-carboxilate) yield cysteine for GSH synthesis was treated in-vitro had no effect but the GSH level of 140 to 170 % of control were achieved in the normal fibroblast line.[10] GSH provide protection to the cells against many toxic compounds, heavy metals, oxidative damage and radiation.[11,12] Reduced glutathione forms complexes with heavy metals including Hg, Cd, Al, As[13] and thus play a protective role against metals toxicity.[14,15]

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From toxicological view point cadmium is ranked at 6th position among the substances for significant human health hazards by U.S poison and disease registry.[16] It is on top priority position in list of studies on food contamination in the World Health Organization.[17] Cadmium also increases the production of oxygen species which leads to oxidative stress.[18] The increase production of ROS is considered to be mediated due to super oxide generation in the mitochondria because of cadmium induced inhibition of electron transfer chain.[18] GSH provides a basic antioxidant system to cellular components and prolong failure to maintain enough intracellular level is detrimental to the cells.[19] There are many enzymes and enzymes systems in human body while glutathione S-transferases (GSTs) is a family of iso-enzymes which conjugate GSH with electrophilic compounds and play a role in detoxification of xenobiotics like drugs, environmental pollutants, pesticides, herbicides, carcinogens like cadmium etc. Due to high toxic nature, accumulation in human being for long time, presence in the environment and food chain, large scale industrial practice, Cd was chosen as test chemical for this study to investigate the effect of cadmium on GSH level of human blood plasma as well as cytosolic fraction.

MATERIAL AND METHODS

Disodium edetate (Reidel Dehan AG sleeze Hannover), sodium chloride, NaCl (Merck), sodium hydroxide (Fluka AG), chloroform (Merck), DTNB of Sigma company, cadmium acetate (Sigma), Reduced form of GSH of Fluka, HCl 35%, (Kolchlight), distilled water double refined, potassium dihydrogen phosphate (Merck), water for injection (Elexor laboratories).

Spectrophotometer (U.V-visible) shimadzu Japan, analytical balance (sortorius), centrifuge model H-200 Kokusan Ensik Japan, pH meter model Nov 210 (Nove scientific co ltd, Korea), Magnetic stirrer, Oven Memmert model-U30 Schwaback of Germany, Hot plate 400, Japan, Micropipettes of various capacities including 100 μl, 200 μl, 500 μl and 1000 μl (Socorex Swiss Finland), Siliconised test tubes, Eppendolf's tubes plastic-101, Disposable rubber gloves otsuka, Japan, Disposalbe pyrogen-free syringes otsuka, Japan.

Fresh human volunteer's 12000μl venous blood was taken, for which 4 healthy volunteers age 22-27 years with no previous history of illness or long illness (having no history of any disease or abnormality) were selected.

A centrifuge tube of capacity 10ml containing 200 μl to 400 μl of 0.5M /liter edetate disodium (anticoagulant) was taken. 10000 μl fresh venous blood of human volunteer was collected by sterile pyrogen free disposable syringe (capacity 10ml) and was immediately pored to above mentioned centrifuge tube. A set of 6 Eppendolf's tubes were prepared each containing 2000 μl of mixture (blood plus different concentrations of $CdAc_2$) in such a way that from this pool of blood, 1000 μl was taken carefully and added to Eppendolf's tubes and to these six Eppodolf's tubes 1000 μl of different concentrations of $CdAc_2$ (.0001-2.0mM) was added (1:1 mixture of whole blood plus different concentrations of $CdAc_2$). These 6 Eppendolf's tubes were incubated for 10 minutes in order to allow the different concentrations of $CdAc_2$ to react with plasma GSH still present in whole blood (yet not separated) and to allow these concentrations of $CdAc_2$ to enter into RBCs to interact there with cytosolic fraction GSH. After 10 minutes these six reaction mixtures were centrifuged at 10000 rpm for 5 minutes. After centrifugation, supernatant fluid from each reaction mixture was separately removed by Pasteur Pipette and was transferred to sample tubes which were kept on ice till further use. Below the supernatant, remaining portion was packed RBCs which were processed for cytosolic fraction.

After the separation of plasma, packed cells (RBCs) were twice washed with isotonic solution and after washing RBCs were lysed at 4°C with 1:1 volume of double refined cold distilled water for 60 minutes, after 60 minutes volume by volume 3:5 cold mixture of chloroform and ethanol was mixed to 2000 μl of lysed cells to precipitate the hemoglobin, following by the addition of 3000 μl distilled water. This mixture was centrifuged and pale yellow colored supernatant fluid was cytosolic fraction which was separated by Pasteur Pipette and the cytosolic fraction was transferred in sample tube and the tube was stored on ice till further use.

For plasma and cytosolic control, equal volume of whole blood and 0.9% NaCl was mixed, centrifuged, supernatant portion (plasma) was separated (without metal concentration) and remaining pack cells were processed for isolation of cytosolic fraction (without metal concentration).

By using Ellman's method (Ellman's, 1959), standard curve of L-glutathione was constructed. The various final concentrations of GSH for preparing standard curve were in order of lowest to highest concentrations i.e 13.33 μM, 26.66 μM, 40.00 μ, 53.33 μM and 66.66 μM. (Figure 1)

RESULTS

A trend of significantly ($p<0.001$) decreasing concentration of plasma level of GSH was found due to the effect of $CdAc_2$ on GSH level of plasma as shown in Fig. 2. Only lowest used concentration of $CdAc_2$ has exhausted plasma GSH level upto 3.662 μM (75.15%) as compare to plasma control while with the increase in concentration of $CdAc_2$ there was

Table 1: Effect of different concentrations of cadmium acetate on the chemical status of plasma-glutathione with time.

S. No.	Conc: of CdAc2 Used (mM)	Final Conc: of CdAc2 (μM)	0 mint	20 mint	40 Mint	60 mint	90 Mint	120 Mint
			Conc	Conc	Conc	Conc	Conc	Conc
1	0.0001 mM	0.003 μ M	3.662	3.554	3.529	3.452	3.376	3.261
2	0.001 mM	0.03 μ M	3.420	3.306	3.236	3.134	3.299	3.108
3	0.01 mM	0.33 μ M	3.210	3.083	3.006	2.917	2.873	2.580
4	0.1 mM	3.33 μ M	2.701	2.592	2.535	2.439	2.344	2.121
5	1.0 mM	33.33 μ M	2.338	2.172	2.083	1.955	1.854	1.732
6	2.0 mM	66.66 μ M	2.019	1.962	1.917	1.860	1.783	1.701
Plasma GSH Control			4.873	4.873	4.873	4.873	4.873	4.873

Table 2: Effect of different concentration of cadmium acetate on the chemical status of cytosolic fraction-glutathione with time.

S.No.	Conc. of CdAc2 Used (mM)	Final Conc: of CdAc2 (μM)	0 mint	20 mint	40 mint	60 mint	90 Mint	120 mint
			Conc	Conc	Conc	Conc	Conc	Conc
1	0.0001 mM	0.003 μ M	3.408	3.331	3.210	3.140	2.924	2.847
2	0.001 mM	0.03 μ M	3.338	2.981	2.911	2.815	2.688	2.580
3	0.01 mM	0.33 μ M	2.860	2.771	2.694	2.599	2.529	2.414
4	0.1 mM	3.33 μ M	2.471	2.401	2.293	2.134	2.064	1.936
5	1.0 mM	33.33 μ M	2.248	2.121	2.006	1.783	1.707	1.580
6	2.0 mM	66.66 μ M	1.898	1.828	1.752	1.586	1.484	1.452
Cytosolic Fraction GSH Control			5.032	5.032	5.032	5.032	5.032	5.032

Table 3: T-test, Paried two sample for means in case of effect of cadmium acetate on plasma GHS.

t-Test: Paired Two Sample for Means		
	CdAc2+plasma	GSH Blank
Mean	2.891666667	4.873
Variance	0.415666667	0
Observations	6	6
Pearson Correlation	0	
Hypothesized mean Difference	0	
Df	5	
T-stat	-7.527669995	
P(T<==t) one-tail	0.00032747	
T Critical one-tail	2.015048372	
P (< =t) two-tail	0.000654941	
T Critical two-tail	2.570581835	

Table 4: T-test, Paired two sample for means in case of effect of cadmium acetate on cytosolic fraction GSH.

t-Test: Paired Two Sample for Means		
	CdAc2+CF	GSH Blank
Mean	2.7038333	5.032
Variance	0.366753767	0
Observations	6	6
Pearson Correlation	0	
Hypothesized mean Difference	0	
Df	5	
T-stat	-9.4167795	
P(T<==t) one-tail	0.0001139	
T Critical one-tail	2.0150484	
P (< =t) two-tail	0.0002279	
T Critical two-tail	2.5705818	

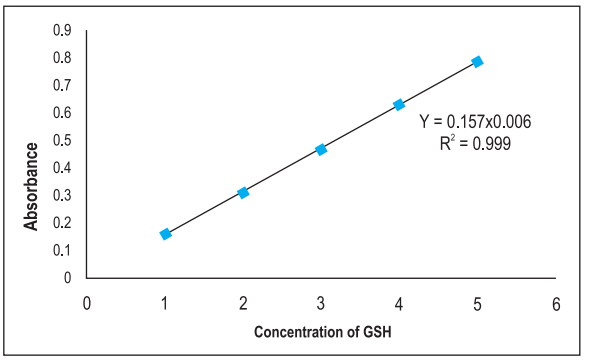

Figure 1: Standard curve of GSH.

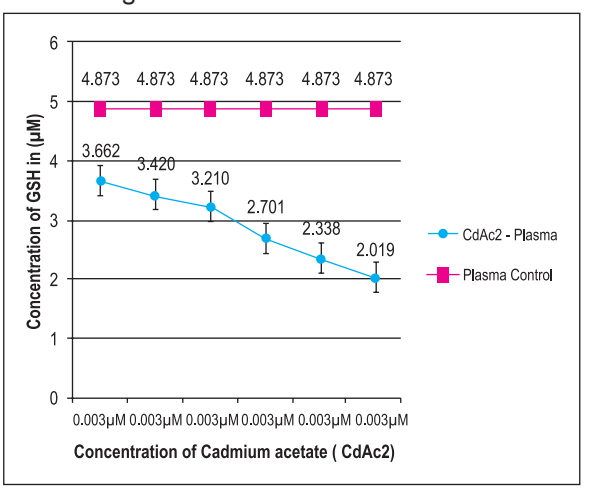

Figure 2.● Effect of various concentrations of cadmium acetate (CdAc2) on the chemical status of plasma-GSH level (concentration effect). ■ Plasma-GSH control (1:1, 1000 µl blood: 1000µl 0.9% normal saline solution). Results are the mean ±SE of 3 experiments of plasma fraction.

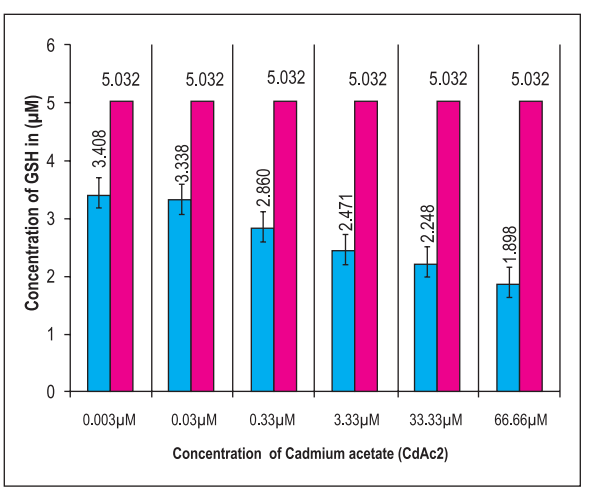

Figure 3.■ Effect of different concentrations of cadmium acetate (CdAc2) on the chemical status of cytosolic fraction-GSH level (concentration effect). ■ Cytosolic fraction-GSH control (1:1, 1000 µl blood: 1000µl 0.9% normal saline solution). Results are the mean ±SE of 3 experiments of cytosolic fraction.

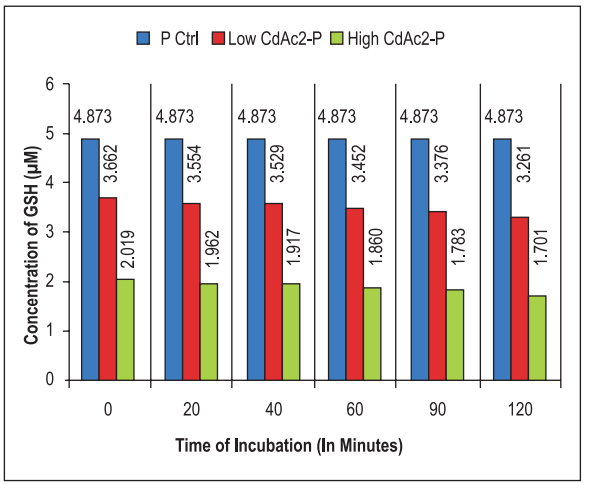

Figure 4 Effect of cadmium acetate (CdAc2) on Plasma GSH with time of incubation from 0 min: to 120 min:
■ Control (1000µl of blood: 1000 µl of 0.9% normal saline 1:1)
■ Effect of lowest used CdAc2 concentration (0.0001 mM).
■ Effect of highest used CdAc2 (66.66 mM). Results are the mean ± SE of 3 experiments of Plasma GSH.

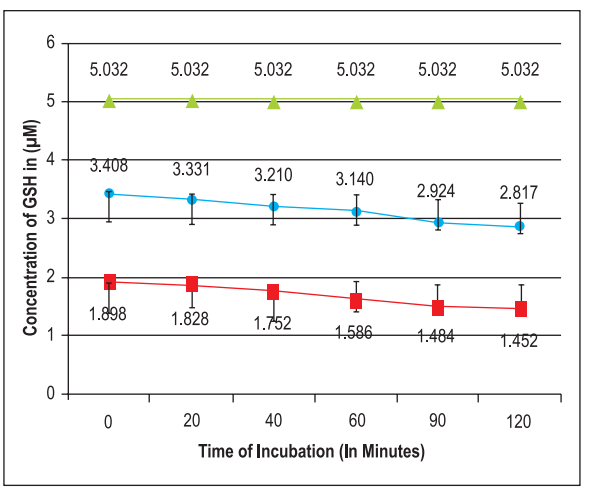

Figure 5 Effect of cadmium acetate (CdAc2) on cytosolic fraction GSH with time of incubation from 0 min: to 120 min:
▲ Control (1000µl of blood: 1000 µl of 0.9% normal saline 1:1)
● Effect of lowest used CdAc2 concentration (0.0001 mM).
■ Effect of highest used CdAc2 (66.66 mM). Results are the mean ± SE of 3 experiments of cytosolic fraction GSH.

further depletion in plasma GSH .The drop in plasma GSH by other used concentrations of $CdAc_2$ was 3.420 µM (70.18%), 3.210 µM (65.87%), 2.701 µM (55.43%), 2.338 µM (47.98%) and 2.019 µM (41.43%) respectively fig.2. Time-dependent effect of $CdAc_2$ on plasma-GSH level was also investigated which

was noted that as the time of incubation increases, there is further decrease in plasma GSH level fig. 4, the time of incubation were 0, 20 ,40,60,90 and 120 minutes. The lowest used concentration of $CdAc_2$ has caused a total decrease up to 66.92 % in 120 minutes, the effect of lowest used as well as highest used concentration from 0 to 120 minutes is shown in Fig. 4 while the details of decrease in plasma GSH levels by all the used concentrations of $CdAc_2$ in different time of incubation is shown in Table 1.

Cytosolic fraction was exposed to several concentrations of $CdAc_2$ to investigate the effect of cadmium acetate on cytosolic fraction GSH level. (Fig. 3) It was found that there is considerable effect of $CdAc_2$ on GSH level of cytosolic fraction in such way that $CdAc_2$ has significantly depleted cytosolic fraction GSH. It was noted that minimum concentration of $CdAc_2$ that was used against cytosolic fraction GSH has decreased the GSH contents up to 3.408μM (67.73%) as compare to the cytosolic fraction GSH control. The remaining concentrations of cytosolic fraction GSH after its exposure to various concentrations (0.001 mM, 0.01 mM, 0.1 mM, 1.0 mM, 2.0 mM) of $CdAc_2$ were 3.338 μM (66.33%), 2.860 μM (56.84%), 2.471 μM (49.10%), 2.248 μM (44.68%) and 1.898 μM (37.72%) respectively.

Scientifically it was valuable to evaluate the effect of $CdAc_2$ on reduced form of glutathione level in this fraction of blood at different times of intervals. (Fig. 5) The times of intervals used to check the interaction of $CdAc_2$ with cytosolic fraction GSH level were 0, 20, 40, 60, 90 and 120 minutes. It was found that with the increase in time of interaction between $CdAc_2$ and cytosolic fraction GSH there was further significant decrease in the GSH level of cytosolic fraction GSH. The decrease in cytosolic fraction GSH was 56.58% from 0 to 120 minutes by lowest used concentration of $CdAc_2$ table 2 while the second concentration of $CdAc_2$ against which cytosolic fraction was exposed has depleted cytosolic fraction GSH from 0 minute to 120 minutes upto 51.27% and rest of the $CdAc_2$ concentrations have depleted cytosolic fraction GSH from 0 to 120 minutes upto 47.97%, 38.47%, 31.40% and 28.86% by 3rd, 4th, 5th and 6th used concentrations of $CdAc_2$ table 2.

Statistical analysis applied to the data shows that cadmium acetate has caused depletion in plasma-GSH as well as cytosolic fraction GSH concentration significantly ($p<0.001$). (Table 3 & 4)

DISCUSSION

Along with a number of established roles of GSH including cell proliferation, antioxidant properties[20] and xenobiotic metabolism,[21] it has a strong chemical affinity for heavy metals especially for cadmium, mercury, arsenic and form complexes with various heavy metals and hence might function in protection of cells against metal toxicity.

In this study we sought to evaluate the role of glutathione in the detoxification of cadmium by using Ellman's method of thiol quantification.[22] Exposure of plasma and cytosolic fraction of human blood to various concentrations of cadmium acetate results in marked depression in GSH level of plasma and cytosolic fraction. (Table1,2) It is clear that even the minimum concentration of CdAc2 to which plasma and cytosolic fraction were exposed has depleted GSH level in these compartments to 3.662 μM (75.15%) and 3.408 μM (67.73%) with respect to plasma and cytosolic fraction GSH control. This is consistent with previous studies which show that Cd^{2+} forms complexes with glutathione. The finding that with the passage of time there is further decrease in GSH level in these compartments offer evidence that Cd^{2+} accumulates in the body and form complex with GSH.

All these findings are consistent with the role of glutathione in protection against cadmium toxicity. A comparison between the effect of cadmium acetate on plasma GSH and cytosolic fraction GSH is very clear in figure 4 and figure 5 respectively which shows that cytosolic fraction GSH has depleted more than depletion in plasma GSH by cadmium acetate indicating the penetrating of Cd^{2+} in RBC(s), as various concentrations of Cd^{2+} were incubated with whole blood and later on both the fractions (blood plasma and cytosolic fraction) were separated. It means before separation of plasma and cytosolic fraction, large amount of Cd^{2+} has entered to the RBC(s) and when RBC(s) were lysed, the cytosolic fraction GSH was proved to be depleted more than the plasma GSH depletion as the results of thiol quantification by Ellman's method shows in figure2,3. Table 1 and 2 also shows that decrease in reduced form of glutathione is directly proportional to the increase in incubation time but yet this decrease is more profound in cytosolic fraction portion of blood as compare to plasma GSH which is consistent to concentration dependent effect of Cd^{2+} on plasma GSH and cytosolic fraction GSH. GSH and heavy metal like Cd^{II} interaction in-vitro conditions as a model of in vivo reaction will increase our scientific knowledge regarding toxicological profile of Cd^{2+} and protective role of GSH against Cd^{2+} toxicity. The decrease in plasma GSH level by lowest used concentration of cadmium acetate was 3.662 μM with respect to plasma GSH control while the decrease in cytosolic fraction GSH level by this Cd^{2+} concentration was 3.408 μM indicating more profound effect of Cd^{2+} on cytosolic fraction GSH showing greater penetrating power of Cd^{2+} into RBC(s) and enrich GSH environment inside RBC(s) as compare to plasma compartment. This fact also proves that oxidative stress due to Cd^{2+} is a little bit more severe in RBC(s). The highest used concentration of Cd^{2+} has depleted

plasma GSH to 2.019 μM while this concentration has depleted cytosolic fraction GSH level to 1.898 μM as compare to plasma and cytosolic fraction GSH control respectively. Several evidences are present that Cd^{2+} is associated with oxidative stress because this metal can alter the antioxidant defense system in various tissues of animals, causing a depletion in level of GSH and alteration in activity of antioxidant enzymes and a change in the structure of cellular membrane through a process of lipid peroxidation.[23] Hence it is reasonable to assume that glutathione form complex with Cd^{2+}. The possible proposed reactions may be.

$$Cd^{2+} + 2GSH \longrightarrow Cd(GS)_2 + 2H^+$$

$$GS^{-1} + GS^{-1} \longrightarrow GSSG$$

Reduced form of glutathione can easily lose its one electron and thus it attains the form of a radical having an unpaired electron and is called as thiyl identity. In this way two thiyl combine with a single divalent cadmium ion (Cd^{2+}) as shown in equation first above or two thiyl radical combine to form one molecule of oxidized form of glutathione as shown above in equation second. Cadmium has long half life in human beings and according to a vast range of literature it remains inside human body for up to 30 years resulting in more harmful results in the form of various complications which occur due to the depletion of reduced form of glutathione.

CONCLUSION

Our study suggests that cadmium has strong chemical binding affinity towards reduced glutathione so the industries using cadmium extensively in different products should have a look and check and balance on human hazards of this toxic metal and its compound and should think over the substitute of this metal in their products.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
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None declared.