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Relationship of Glutathion Concentrations with Cytotoxicity of Cisplatin in Different Cell Lines after Confront Vitamin C and E.

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Abstract: One of the well-known cellular defenses after exposure to cytotoxic agents is the glutathione (GSH) related mechanisms. Resistant to cisplatin (DP) chemotherapy has been strongly correlated to GSH-mediated mechanisms in many articles. In this study, we have evaluated the effects of cisplatin on the cellular total GSH level in different tumor and normal cell lines. Five different cell lines of human hepatic carcinoma (HePG2), human lung adenocarcinoma (A549), human ovarian carcinoma (SKOV3), dog kidney (LLCPK1), Chinese Hamster Ovary (CHO) and Human gingival fibroblast (GHF1) cell lines were exposed to their respected IC_{50} concentrations of cisplatin for two hours. Cisplatin cytotoxicity was measured using clonogenic assay and the total cellular GSH level was analyzed using a photometrical assay. The results showed that cisplatin had different degrees of cytotoxicities on different cell lines as shown by IC_{50} values; 0.87± 0.07 for HepG2, 3.27±0.35 for A549, 0.99±0.08 for SKOV3, 5.50±0.35 for LLCPK1, 5.50±0.21 for CHO and 1.60±0.21 for GHF1 cell lines. GSH level after exposure to cisplatin (GSH-DP) were also different for different cell lines compare to their controls (GSH-C); 85.33±8 for HepG2, 637.00±81 for A549, 2691.00±416 for SKOV3, 1388.30±261 for LLCPK1, 412.60±32 for CHO and 783.24±30 for GHF1 cell. It is shown that compare to the matched controls, the cellular GSH level increased in LLCPK1, A549, SKOV3 and GHF1 cell lines, but decreased in CHO and HepG2 cell lines. The highest significant variation of GSH in cancer cell line was belonging to SKOV3 and in normal cell lines to LLCPK1, after treated with cisplatin. It is concluded that the total GSH variation after exposure to cisplatin is different for different cell lines. We were not able to correlate between the level of resistance to cisplatin (based on the IC_{50} levels) and GSH level or variations in this study. It might indicate that in spite of many publications so far, the GSH is neither the unique, nor the most important mechanism of resistance to cisplatin in these cell lines. Internal and Eternal GSH level in Studied cell lines will be changed in several ways when contaminated with different concentration of vitamins (for examples, Vit C, Vit E and Vit C+E) and observed that variation was more prominent in cancer cell line.

Key words: Cisplatin, glutathione, cell lines, Vit C, Vit E

INTRODUCTION

The discovery and development of platinum compounds has been one of the greatest achievements of cancer chemotherapy in the past three decades. Cisplatin was accidentally discovered by Rosenberg in 1968 as an antibiotic and very rapidly as a very powerful antiproliferative agent against tumor cells. It was rapidly introduced in clinical use, principally for the treatment of germ cell tumors of testis and ovary (Rosenberg, 1985).

Cisplatin (cis-dichlorodiammineplatinum-II) gained a widespread use against various malignant tumors in different experimental animals (Cummings and Schnellmann, 2002; Prasad and Giri, 1994) and in a variety

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of human malignancies (Fokkema *et al*., 2002). Many of the biological properties and effects of cisplatin have been well documented (Rosenberg, 1985; Blasiak *et al*., 1985) with numerous reports indicating that the cellular DNA could be the primary target in its anticancer activity (Zamble and Lippard, 1995). However, the therapeutic efficacy of cisplatin is limited due to the development of drug resistance (Bosscha and Mulder, 1992) and major side effect, nephrotoxicity (Krakoff, 1979). An increased carcinogenic risk with the development of secondary malignancies in animals/patients treated with cisplatin has also been reported (Kempf and Ivankovic, 1986; Greene, 1992). In an attempt to overcome these impediments, the development of many new cisplatin analogues (Christian, 1992) or the use of cisplatin in combination with other agents have been tried with different degrees of success (Treskes and Vijgh, 1993).

Cisplatin is known to generate oxygen free radicals, (Masuda *et al*., 1994). Though the role of free radicals in cisplatin induced nephrotoxicity remains a matter of debate, (Vermeulen and Baldew, 1992), free radical scavengers have been shown to ameliorate the toxicities of cytotoxic agents (Torii *et al*., 1993).

Thiol (sulfhydryl) groups, such as those on glutathione (L-y-glutamyl-L-cysteinyl-glycine; GSH) and metallothionein (MT), defend the cell against cisplatin, (Kraker *et al*., 1985; Masahiko *et al*., 2000; Zhang *et al*., 2001; Tsuruya *et al*., 2003). Since the thiolate anion has a high affinity for Pt, Pt ions entering the cell may preferentially bind to sulfur atoms rather than the bases of DNA, (Dedon and Borch, 1987; Lai *et al*., 1989; Ishikawa *et al*., 1994). Although it is easy to overwhelm this protective mechanism in first-time patients receiving cisplatin, continues exposure to the drug ultimately produces resistance due to increased sulfhydryl levels (e.g., GSH and MT) (Takehiko *et al*., 2001; Godwin *et al*., 1992a). Plant and animal cells eliminate a broad range of hydrophobic toxins from the cytosol to the extra cellular space after their conjugation with GSH, (Ishikawa, 1992). This transport is mediated by a novel class of organic anion transporters belonging to the family of ATP-binding cassette carriers, the GS-X pumps, (Ishikawa *et al*., 1997). GS-X pump activity was found in canalicular and basolateral rat hepatocyte plasma membeanes, heart sarcolemma vesicles, human erythrocytes and tumor ceils (Ishikawa *et al*., 1997; Muller *et al*., 1994). Many publications have claimed that Glutathione is the key component of a ubiquitous antioxidant system that defends the cell against the toxic effects of cisplatin (Ishikawa *et al*., 1997).

α-Tocopherol protects against lipid peroxidation. This pro-tection is achieved via the scavenging of lipid of lipid peroxyl radicals by α-Tocopherol. The α-tocopherol radicals thus formed can be regenerated in the membrance by cytosolic antioxidants such as vitamin C or GSH. Nevertheless, some of the α-tocopherol radicals will not be captured by this recycling process and will be oxidized further to α-tocopherol quinone, which has no antioxidant properties (Kashif and Banu, 2004).

Vitamin C (ascorbic acid), an essential nutrient and active reducing agent, is involved in numerous biological effects. The chemo preventive/therapeutic roles of vitamin C against cancers have been widely reported. However, the definite role of vitamin C in cancer treatment still remains controversial (Giri *et al*., 1998).

In addition to the importance of the glutathione status of cells for resistance to oxyradical generating drugs, the ability of the cell to maintain the natural antioxidant vitamins, α-tocopherol and L-ascorbic acid in their reduced forms is essential. Both vitamins play vital roles in scavenging toxic oxygen free radicals and they are closely integrated with one another and the glutathione redox system. Previous investigations of human tumor cells, *in vitro*, for relative sensitivity to cisplatin have not considered the importance of ascorbic acid in intracellular compartments, because the vitamin was omitted from the culture medium. The half-life of ascorbic acid in tissue culture media is estimated to be 0.9 h and the potential prooxidant action of ascorbic acid in the presence of transition metal ions, especially iron, promotes lipid membrane per oxidation. Therefore, ascorbic acid is often omitted from media for human cells (William *et al*., 1995; Meijer *et al*., 1994).

Whether GSH is the principle parameter in the cisplatin cytotoxicity is the main question of this study. If it is so, the intracellular amount of initial GSH, should be able to present a measurable scale of cellular resistance in different cell lines. On the other hand, it should be possible that variations in cellular GSH content and consumption after the exposure to cisplatin represent the degree of sensitivity and resistant to this drug in different cell lines. In this manuscript, we are presenting the cellular toxicity of cisplatin in different cell lines, as well as the cellular GSH levels before and after the exposure to cisplatin and vitamins (C, E, C+E) to examine the accuracy of above hypothesis.

This study was accomplished Faculty of Pharmacy, Sheheed Beheshtis University of Medical Science, Tehran, Iran, in the year 2005.

MATERIALS AND METHODS

Chemicals: All chemicals that used in this study as following: (cisplatin, media serum DMEM F12 (Gibco BRL), antibiotic (Streptomycin, Penicillin Gibco BRL), FBS

(Fetal Bovine Serum Gibco BRL), OPT (O. Phtaldialdehyde Sigma), EDTA buffer, buffer IV included 750 mL Krebs and 9 mL HEPES, Krebs included: 1000 mL H_2O, 6.95 g NaCl, 0.355 g KCl, 0.16 g KH_2PO_4 0.6 $MgSO_4$, 0.383 g $CaCl_2$, 2.1 g $NaHCO_3$, tris buffer TCA (Tricholoroacetic Acid Merck)% 10, DMSO (Dimethylsolfoxide Sigma), HEPES (Gibco BRL), Trepsin (Gibco BRL) Standard GSH (Sigma GSH Sigma) solutions were prepared freshly for each experiment in 6% (v/v) met phosphoric acid and diluted in phosphate-EDTA buffer (pH 7.4). Vit C and Vit E, Soybean oil, DMSO (Sigma).

Cell lines and clonogenic assay: Human hepatoma cell (HepG2) was purchased from the Pasteur institute in Tehran (Iran). Human lung carcinoma (A549), Human ovary carcinoma (SKOV3), Dog renal normal cell (LLCPK1), Chinese Hamster Ovary (CHO) and Human gingival fibroblast (GHF1) were as generous gift from Dr. Rakesh Goel, Ottawa Regional Cancer Center, Ottawa, Canada. All cells were grown in DMEM/F12 media (Gibco BRL, USA) supplied with 10% fetal bovine serum and 1% antibiotic. Cells were maintained in a humidified 37°C incubator with 5% CO_2 for three passages before the start of experiments. Cells were exposed to the different concentrations of cisplatin in a range of 0.5 to 10 μM for 1 h. Clonogenic assay was carried out after the cell exposure to cisplatin based on Von Hoff method, (Von Hoff *et al*., 1985; Conney, 2003).

Sample preparation and analysis of vitamins: For preparation of different concentrations of Vitamin C, 0.031 g Vit C powder sample was weighted and then dissolved in 10 mL DDW. From this solution AC (17.6 mm), another solution prepared with using of sterile PBS.2 mL of solution AC plus 2 mL PBS used for preparation of solution BC (8.8 mM) and then 2 mL of solution BC plus 2 mL PBS used for making of solution CC (4.4 mM) and finally 2 mL of solution CC plus 2 mL PBS mixed for preparation of solution DC (2.2 mM). on the other hand for Preparation of different concentrations of vitamin E (act fast as this is sensitive to light and air), 1 mL of vit E powder sample added to 4 mL of DMSO in a tube under the hood for making the solution called, solution AE (465.11) and then 2 mL of solution AE mixed with 2 mL PBS to making solution BE (232.5 mM) afterward 2 mL of solution BE combined to 2 mL PBS for preparation the solution of CE (116.2 mM) and at last 2 mL of solution CE mixed to 2 mL PBS to make solution DE (85.12 mM).

Final procedure was about Preparation of different concentrations of complex Vitamin C and Vitamin E at first 40 μL each of these solutions added to each three Petri-dishes with specific codes as observe (col), control vit C (SS, SC), different concentration (AC, BC, CC and DC), control vit E (SE1, SE2, SE3) different concentration vit E (AE, BE, CE and DE) and maxi vit E+C (AEC, BEC, CEC and DEC).

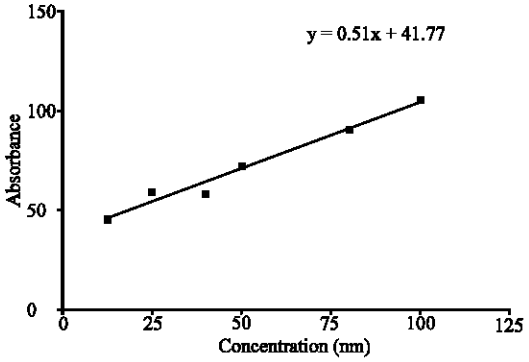

Fig. 1: Standard curve of GSH

GSH assay: GSH analysis was performed spectrophotometrically according to the enzymatic method of Tietze and Floreani, (Neuschwander and Roll, 1989; Blum and Fridovich, 1985). In brief, different cell lines (HepG2, A549, SKOV3, LLCPK1, CHO and GHF1) were grown to 80% confluences as was described above, in 25 mm plates. Experiment and control cells were harvested and collected in 1 mL of PBS, Commented and subsequently stored at -20°C. Determination of GSH was performed by a modification of the method of Cohn and Lyle. To 0.5 mL of the 100,000 g supernatant, 4.5 mL of the phosphate-EDTA buffer, pH 8.0 was added. The final assay mixture (2.0 mL) contained 100 μL of the diluted cell lines supernatant, 1.8 mL of phosphate- EDTA buffer and 100 μL of the OPT solution (Fluorescence material which compound in the GSH).

After thorough mixing and incubation at room temperature for 15 min, the solution was transferred to quartz cuvette. Fluorescence at 420 nm was determined with the excitation at 350 nm (Hissin and Hilf, 1976; Griffith, 1980).

Figure 1 mark the total amount of glutathione in the samples was determined as GSH (μmol) Per million cells using a standard curve obtained by plotting the known amounts of GSH (100, 80, 50, 40, 25 and 12.5 μm), incubated under the same experimental conditions, versus the rate of change of absorbance at 420 nm [r = 0.989].

Animal study: The animals (male mice 10-12 week old, weighing 25-30 g) were divided into nine groups of 8 animals each :

Experimental protocol

Group 1: Normal control, received only the normal saline (5 mg kg^{-1}, i.p.) for 5 days.

Group 2: Positive control, received only single does of cisplatin (8 mg kg^{-1}, i.p.) only at 6th day.
Group 3: Control group with carrier of Vit E, received only soybean oil (10 mg kg^{-1}, i.p.) for 5 days.
Group 4: Control group with Vit C, received only vit C (100 mg kg^{-1}, i.p.) for 5 day.
Group 5: Control group with Vit C, received only vit E (500 mg kg^{-1}, i.p.) for 5 day.
Group 6: Control group with Vit C and Vit E, received does of Vit C (100 mg kg^{-1}, i.p.) and Vit E (500 mg kg^{-1}, i.p.) for 5 days.
Group 7: Treatment group: received Vit C (100 mg kg^{-1}, i.p.) for 5 days and single does of cisplatin (8 mg kg^{-1}, i.p.) only at 6th day.
Group 8: Treatment group: received Vit E (500 mg kg^{-1}, i.p.) for 5 days and single does of cisplatin (8 mg kg^{-1}, i.p.) only at 6th day.
Group 9: Treatment group: received Vt C (100 mg kg^{-1}, i.p.) with Vit E (500 mg kg^{-1}, i.p.) for 5 days and single does of cisplatin (8 mg kg^{-1}, i.p.)At 6th day.

Twenty four and 96 h after 6th day, the animal was killed and taken 2CC blood sample for biochemical analysis and extracted the whole liver and kidney and then fixed in formalin (9%) solution for later pathological studies.

Statistical analysis: The results are expressed as mean±SEM. Differences between means were elaborated by one way analysis of variance or the Turkey-kramer method for multiple comparisons. Differences at $p<0.05$ were considered to be significant. IC_{50} was calculated using Graph pad prism software.

RESULTS

Table 1 represents the IC_{50} amounts of cisplatin in different under-investigation cell lines (HepG2, A549, SKOV3, LLCPK1, CHO and GHF1). The IC_{50}s of cisplatin in these cell lines are HepG2: 0.87±0.07, A549: 3.27±0.35, SKOV3: 0.99±0.08, LLCPK1: 5.5±0.35, CHO: 5.5±0.21 and GHF1: 1.60±0.21.

As is shown in Fig. 2, the rank order of IC_{50}s are A549> SKVO3> HepG2 for cancer cell lines and CHO> LLCPKl>GHF1 in normal cells. There is a statistically significant difference ($p<0.05$) between the IC_{50} of cisplatin among normal and cancer cell lines. Based on the information presented in Fig. 2, among normal cell lines (between LLCPK1 and CHO with GHF1 and then cancer cell lines between HePG2 and SKOV3 with A549, a significant relation was seen ($p<0.05$).

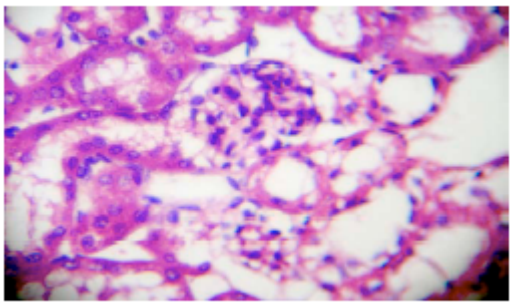

Fig. 2: Pathologic kidney tissue sample after contamination with cisplatin including fatty chanfes of renal tubular

Table 1: Comparison of cisplatin IC_{50} (µmol) on different cell line

	Cell lines		Cis $\bar{\chi}$ ±SEM
IC_{50} (µg mL^{-1})	Cancer cell lines	HEPG2	0.87±0.07
		A549	3.27±0.35
		SKOV3	0.99±0.08
	Normal cell line	LLCPK1	5.5±0.35
		CHO	5.5±0.21
		HGF1	1.6±0.21

Table 1-4 represents the concentrations of GSH in these cell lines in nmol/millinon cells, before and after the 1h exposure to the IC_{50} concentration of cisplatin for each cell line. Based on this figure, the rank order of total GSH concentration for under investigation cell lines is as LLCPKl>GHF1>CHO in normal cell lines and SKOV3> A549>HepG2 in tumor cell lines. The rank order of cellular GSH level among these cell lines remains the same before and after the exposure to cisplatin.

Correlation between HEPG2 (cancer cell line) with cisplatin in Internal GSH levels with main blank (col: without any treatment) was seen as SS (with IC_{50} of cisplatin), SC (with IC_{50} of cisplatin plus normal saline control group) and SE2 (with IC_{50} cisplatin + soybean oil) that Correlation between main blank (col) with BC, CC, DC, AE and DE group, was significant ($p<0.0001$) and then Correlation between SS and SC with AC, BE, AEC, BEC and CEC was significant at $p<0.001$ and finally Correlation between SE2 with DE, AEC, BEC, DEC and CEC was significant at $p<0.001$. This results with external GSH level showed that correlation between main blank (col) with BC, CC, DC, AE, DE, AEC, BEC and CEC was significant at $p<0.001$ and relation between SS and SC with AC, BE, CE, AEC, BEC, CEC and DEC was significant ($p<0.001$) and at last correlation between SE2 with DE, AEC, B EC, CEC and DEC was significant at 0.001 (Table 3 and 4).

Above relationship with SKOV3 (cancer cell line) about Internal and external GSH level showed that

Table 2: GSH levels in cancer and normal cell lines before and after the exposure to IC_{50} concentration cisplatin

	Cancer			Normal		
Cell line	HepG2	A549	SKOV3	LLC-PK1	CHO	HGF1
GSH-Cis	177±19	353±53	957±108	1035±81	480±25	635±23
GSH+Cis	85±8.14	637±81.8	2691±416	1288±261	412±32	738±30

Table 3: Comparison mean and mean standard error GSH levels in the cancer cell lines after confront cisplatin and Vitamins concentration

		GSH nmol/10^6cell Cancer cell line								
		HEPG2			A549			SKOV3		
		In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM	In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM	In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM
	COL	51±5	126±10	177±19	70±8	203±19	353±53	157±14	808±68	957±108
Control	SS	24±2	61±4	85±8.14	127±5	510±20	637±81.8	449±51	2242±66	2691±416
	SC	19±1	47±2	66±6	120±6	481±16	601±48	421±86	2107±91	2528±224
Vit C	AC	60±3	151±6	211±8	141±4	564±25	705±33	474±42	2368±104	2842±328
	BC	21±675	52±3	73±7	87±3	453±21	540±27	467±64	2336±115	2804±275
	CC	20±0.86	50±3	70±6	102±6	407±24	509±41	455±27	2275±89	2730±294
	DC	16±0.5	41±2	57±8	94±4	376±31	470±18	452±31	2256±68	2708±186
Control	SE1	26±1	64±2.5	90±6	101±6	405±27	506±26	323±15	1615±91	1938±242
	SE2	42±1.5	104±3	146±13	132±8	526±32	658±42	452±24	2258±86	2710±317
	SE3	33±1.5	84±2.5	117±12	118±3	472±14	590±38	450±30	2248±57	2698±356
Vit E	AE	33±2	83±3.2	116±13	142±6	569±17	711±44	489±28	2442±62	2931±425
	BE	45±2.2	112±3	157±11	137±4	548±28	685±41	489±34	2445±98	2934±335
	CE	43±3	109±2.8	152±10	129±3	517±33	646±52	452±17	2261±83	2713±284
	DE	25±1	63±2	88±6	138±4	511±40	639±34	45±29	2248±57	2698±308
Vit C+E	AEC	146±6	365±6.7	511±12	167±6	666±28	833±47	492±31	2461±128	2954±432
	BEC	78±2	196±7.2	274±11	142±7	567±35	709±31	502±42	2509±201	3011±278
	CEC	66±2.5	167±7.2	233±12	132±3	529±24	661±33	470±19	2351±115	2821±194
	DEC	58±2	144±6	202±14	128±4	514±23	642±29	455±31	2273±93	2728±252

Table 4: Comparison means and means standard error GSH levels in the normal cell lines after confront cisplatin and Vitamins concentration

		GSH nmol/10^6 cell Cancer cell lines								
		LLC-PK1			CHO			HGF1		
		In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM	In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM	In $\bar{\chi}$±SEM	Ex $\bar{\chi}$±SEM	Total $\bar{\chi}$±SEM
	COL	370±24	695±32	1035±81	142±6	338±17	480±25	152±5	403±15	635±23
Control	SS	423±51	86566	1288±261	118±5	294±12	412±32	180±7	558±13	738±30
	SC	397±42	795±91	1192±195	114±8	287±14	401±24	175±5	542±16	717±24
Vit C	AC	433±67	865±104	1298±215	128±6	322±20	450±29	196±6	609±13	805±26
	BC	440±64	881±89	1321±120	126±8	315±21	441±25	193±8	599±12	792±25
	CC	438±27	878±68	1316±132	120±6	301±13	421±23	190±6	590±17	780±30
	DC	406±38	813±94	1219±98	117±7	293±15	410±27	187±4	578±19	765±37
Control	SE1	362±42	724±91	1086±117	109±9	272±17	381±30	154±10	478±20	632±27
	SE2	431±30	863±86	1294±162	144±11	318±13	462±37	194±6	602±24	796±21
	SE3	428±25	857±75	1285±140	113±10	283±12	396±29	184±12	568±19	752±24
Vit E	AE	461±28	924±62	1385±135	137±8	343±11	480±27	202±5	628±24	830±28
	BE	459±34	919±89	1379±162	137±8	344±15	481±31	202±9	626±19	828±19
	CE	449±41	899±39	1348±134	132±6	330±13	462±37	193±8	598±20	791±17
	DE	439±29	879±57	1318±108	128±4	321±16	449±25	189±13	587±17	776±25
Vit C+E	AEC	470±31	941±78	1411±232	150±5	376±20	526±32	212±15	658±16	870±23
	BEC	463±42	927±79	1390±206	161±6	403±15	564±34	208±13	645±14	853±25
	CEC	447±19	895±31	1342±127	140±8	352±22	492±30	205±14	635±14	840±18
	DEC	440±31	881±52	1321±98	135±9	338±14	472±35	199±11	616±9	815±21

correlation between main blank (col) with all studied groups was significant ($p<0.001$) and between SS, SC and SE2 with all studied groups was non significant. A549 (Cancer cell line) with cisplatin about internal GSH level represented that main blank (col) with AC, AE, BE, DE, AEC, BEC, CEC and DEC, groups was significant at $p<0.05$) and control group SS was significant at $p<0.001$ with BC, AEC and at $p<0.01$ with DC and finally control group SC was significant at $p<0.001$ with BC and $p<0.001$ with AEC. Results with external GSH level showed that Correlation of main blank (col) with all studied groups was significant at $p<0.001$ and control group SS with all SS with all studied groups have not significant. Control group SC with all AEC at $p<0.01$ was significant and finally control group SE2 with AEC group was significant at $p<0.05$. Treatment with LLCPK1 (normal cell line) with

cisplation in internal and external GSH level showed no relationship between control groups with different vitamin groups. correlation between CHO (normal cell line) with cisplatin about internal GSH level presented relation between main blank (col) and SE2 with all studied groups was not significant and between SS, SC and SE2 that was significant only with BEC at $p<0.05$. This relation about external GSH level showed as non-significant correlation between main blank with all studied groups and significant correlation between SS and SC only with DEC group at $p<0.01$ and finally correlation between SE2 was significant with BEC at $p<0.001$ and with AEC at $p<0.01$.Correlation between HGF1 (Normal cell line) with cisplatin in internal GSH Level illustrated significant correlation between main blank (col) with AE, BE, BEC, CEC at $p<0.05$ and with ACE at $p<0.01$ and non-significant correlation between SS, SC and SE2 with all studied groups external GSH Level in this study showed significant correlation between main blank (col) with all studied groups was at $p<0.05$ and between SC, SS control only with AEC at $p<0.05$ and finally correlation between SE2 control was not significant with all studied groups.

According to results in Table 3, reduction in total GSH level in HEPG2 cell line remarkably seen in SC and SS Control group versus blank (col), but in cell line contaminated with Vit C and complex Vit C and E, showed increase in GSH level, versus to blank and control groups. All samples that contaminated with different concentration of vit E, showed remarkably decrease in GSH level versus to control and blank. In the other hand, A549 cell line showed increase in GSH level in SS and SC control group versus blank (col), while vit E, vit C and complex vit C and E groups, showed increase level in GSH that was highest concentration of GSH were seen in AC, AE and BEC groups. SKOV3 cell line showed remarkably increase in total GSH level in SS and SC control versus blank (col) and in Vit C, Vit E and complex, increase in total GSH level was seen that highest level was found in AC, AE and BEC.

According to results in Table 4, LLCPK1 cell line has caused increase in total GSH level in SS and SC control group versus blank and this increase seen in Vit C, Vit E and complex, versus blank and control and highest level was seen in BC, AE and AEC. CHO cell line showed remarkably reduction of total GSH level in SC and SS control group versus blank and this condition seen in all Vit C and Vit E Concentration, but in complex increase in total GSH level seen versus control group that BEC concentration was very obvious. In HGF1 cell line, increase in total GSH level was seen in SS and SC control group versus blank and this condition was seen in vit C, vit E and complex versus control and blank AC, AE and finally AEC concentration was very obvious.

According to animal study protocol, BUN and creatinin were measured in time of 24 and 96 h after contamination with cisplatin and vitamins (C, E, Complex E + C) and no significant different was seen between nine test groups.

Pathological assessment from mice liver and kidney Tissue samples by fluorescent microscopy showed that Considered alteration in tissue samples remarkably seen in group that contaminated directly with cisplatin (Group 1).

Main alteration in kidney tissue sample after contamination with cisplatin including fatly changes of renal tubular epithelium, Tubular epithelium is necrotic in areas and preserved epithelial lining of the tubules (Fig. 2 and 3).

Basic alteration in hepatic tissue sample after contamination with cisplatin including Apoptotic cell, Necrotic hepatic parenchyma cells, central vein dilatation and kuppfer cell hyperplasic (Fig. 4 and 5).

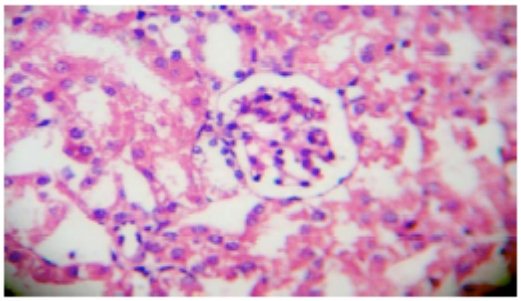

Fig. 3: Pathologic kidney tissue sample after contamination with cisplatin including tubular epithelium is necrotic in areas

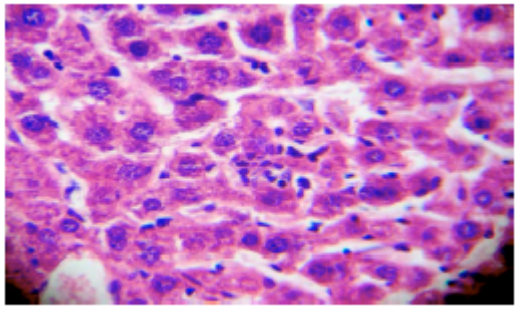

Fig. 4: Pathologic hepatic tissue sample after contamination with cisplatin including focal cellular necrosis, kuppfer cell hyperplasic and apoptotic cell

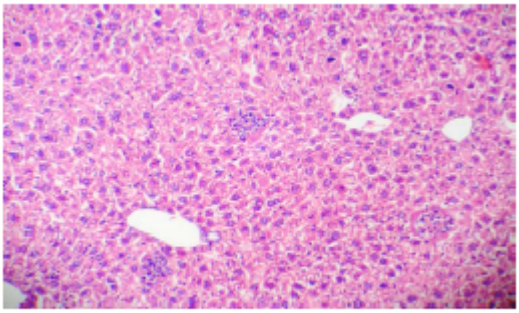

Fig. 5: Pathologic hepatic tissue sample after contamination with cisplatin including apoptotic cell, necvotic hepatic pavenchyma cell, central vein dilatation

DISCUSSION

Cell lines from many different tumors may develop resistance to cisplatin. In most cases, the level of resistance is less than 50-fold, although there are reports of up to 1000-fold resistance (Neuschwander and Roll, 1989). Nevertheless, even a small increase in resistance of a tumor to cisplatin can be clinically important.

Biochemical studies have not succeeded in identifying conclusively the basis of resistance in any type of cell selected with cDDP, but they have defined several mechanisms that can contribute to resistance. The effectiveness of cell killing is a function of how much drug gets into the cell, how much of this actually reacts with DNA, how tolerant the cell is of lesions in its DNA and how effectively it removes these adducts from DNA (Rooseboom and Schaaf, 2002).

Thiol (sulfhydryl) groups, such as those on glutathione (GSH) and metallothionein (MT), defend the cell against cisplatin (Kraker *et al*., 1985; Masahiko *et al*., 2000; Zhang *et al*., 2001; Tsuruya *et al*., 2003). Since the thiolate anion has a high affinity for platinum atom, Pt ions entering the cell may preferentially bind to sulfur atoms rather than the bases of DNA (Dedon and Borch, 1987; Lai *et al*., 1989; Ishikawa *et al*., 1994).

Glutathione (GSH) is a tripeptide thiol, y-glutamylcysteinylglycine. Concentrations of 0.5-10 mM make it the most abundant thiol in the cell. Glutathione is synthesized in a two-step pathway involving the ATP-dependent enzymes glutamylcysteine synthetase and glutathione synthetase (Meister, 1991). The first step is rate-limiting and inhibited by glutathione itself and by buthionine sulfoximine (BSO). As a potent nucleophile, GSH reacts with alkylating agents as well as cisplatin. The reaction of GSH and cisplatin in a 2:1 molar ratio forms a GSH-platinum complex that is then eliminated from the cell by an ATP-dependent glutathione S-conjugate export pump (Meister, 1991; Rooseboom and Schaaf, 2002). GSH may protect cells by intercepting reactive platinum complexes before they can react with DNA. GSH also protects cells by supporting DNA repair, possibly by stabilizing repair enzymes such as DNA polymerase (Rooseboom and Schaaf, 2002). Increased glutathione levels have been found in some cisplatin resistant cells but not in all cell lines. On the other hand, continued exposure to the drug in patients receiving cisplatin, ultimately produces resistance due to increased sulfhydryl levels (e.g., GSH and MT) (Meister, 1991; Godwin *et al*., 1992b).

It has been suggested that the capacity of the cell to synthesize GSH in response to stress may be more important than the steady state GSH level (Schilder *et al*., 2002). We have therefore examined the hypothesizes that different cell lines have variable potency in adapting a higher level of GSH level as a protective mechanism in long-term or short-term exposure to platinum compounds, which might reflect in their level of sensitivity to cisplatin.

To investigate on this postulation, we have examined the effects of cisplatin on the cellular total GSH level of three tumor (HePG2, A549, SKOV3) and normal (LLCPK1, CHO, GHF1) cell lines. Present results showed that different cells have different levels of GSH. A comparison of normal GSH level with the IC_{50} of cisplatin for each cell line did not reveal any kind of correlation, to conclude that intracellular amount of GSH might be the critical index for the cell line resistance to this drug. As an example, although LLCPK1 cell line has the highest IC_{50} value and also GSH level among these cell lines (1035.7±81 nM/10^6 cells), but the CHO cell line with the same concentration of IC_{50} contain a median level of GSH (480.1±25 nM/10^6 cells) within this group of cells. Our results were therefore failed to show any correlations between the level of intracellular GSH and resistance to cisplatin.

To examine the importance of GSH increase in response to stress, cells were exposed to cisplatin. IC_{50} concentrations in the same exposure time were selected for the cisplatin to be exposed for each cell line to produce the same level of potency for the drug and stress on the cell line. As is shown in this study, compare to the matched controls, the cellular GSH level following exposure to cisplatin were increased in LLCPK1, A549, SKOV3 and GHF1 cell lines, but decreased in CHO and HepG2 cell lines. The cellular total level of GSH in various cell lines after exposure to the corresponding IC_{50} concentrations of cisplatin for each cell line was hence different and not as a good indicator of sensitivity to this drug (Cummings and Schnellmann, 2002; Tsuruya *et al*., 2003).

On the other hand, intracellular GSH level was evaluated in some but decreased in other cell lines after exposure to cisplatin. We have therefore tried to find any correlation between the decrease and/or increase in GSH and cellular resistant to cisplatin, but was not successful to find any pattern. As examples, SKOV3 (IC_{50} = 0.99±0.08) and HepG2 (IC_{50}=0.87±0.07) have shown almost the same level of sensitivity, however, after the exposure to cisplatin the GSH level was increased dramatically (about 2.5 times), but decreased significantly (about 2 times) in these cell lines, respectively. LLCPK1 and CHO cell lines with the same level of IC_{50} showed the same pattern, as GSH level increased in LLCPK1, but decreased in CHO cell line after exposure to the same toxic level of cisplatin for the same duration of time (Paolicchi *et al*., 2003; Hanigan *et al*., 2001).

There was also not significant difference between normal and tumor cells in their pattern of GSH level or variation for cisplatin. The highest significant GSH variation in cancer cell line was belong to SKOV3 and between normal cell lines to LLCPK1 after treated with cisplatin (Schilder *et al*., 2002; Hanigan *et al*., 2001).

Answer to questions of cellular recognition of stress after exposure to cisplatin, level and speed of cellular reaction to express GSH related genes and the two-step process of GSH production in different cells and specially possible differences in the cellular uptake and hence the actual exposure of cellular GSH molecule exposure to cisplatin, are remained to be answered in further more detail studies. However, in spite of many publications on the GSH critical role, but the fact that our study was not able to show any kind of correlation between GSH level and/or variations with cisplatin sensitivity, suggests a more important role for other cellular thiol pools (e.g., MT and thiol groups on the chromatin) and resistance mechanisms (e.g., cellular entrance and DNA) repair in cisplatin clinical response (Masahiko *et al*., 2000; Tsuruya *et al*., 2003; Schilder *et al*., 2002; Chu, 1994).

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