The Role of Copper in Development of Drug Resistance in Murine Carcinoma

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Abstract: Multidrug resistance (MDR) is a major obstacle to successful application of cancer chemotherapy and also a basic problem in cancer biology. Studies on the molecular basis of MDR have revealed that a number of proteins over express in multidrug resistant cells viz., multidrug resistant MDR1 gene product P-glycoprotein, the multidrug resistance-associated protein (MRP) and enzymes associated with the glutathione (GSH) metabolism. Decreased expression or altered activity of topoisomerase II has also been implicated in MDR. In the present investigation a number of changes in phase II detoxification parameters have been noticed in drug resistant cells but the novel aspect of the present report is the observation that the metal copper is involved in drug resistance. Although copper plays important roles in many human and other biological systems and even in the treatment of cancer but the relation of Cu and drug resistance has not so far been studied in detailed. The present report describes the novel findings that the level of copper increases with the development of drug resistance in Ehrlich ascites carcinoma and in Lewis lung carcinoma cells and also in serum of mice bearing drug resistant cancer cells compared to mice bearing drug sensitive cells; the work indicates the important aspect of treating drug resistant cancer patients by lowering Cu level in the cancerous cells and serum prior to treatment.

Key Words: Copper, Ehrlich ascites carcinoma (EAC), glutathione (GSH), multidrug resistance (MDR), Lewis lung carcinoma (3LL).

INTRODUCTION

Multidrug resistance (MDR) is one of the most significant problems in cancer chemotherapy and also is a basic problem in cancer biology. Studies on the molecular basis of MDR have revealed that a number of mechanisms operate behind this phenomenon. Drug resistant cells differ from the drug-sensitive cells in many ways e.g., by (i) reduced accumulation of cytotoxic drugs, due to decreased drug influx and or increased drug efflux; (ii) altered expression and or activity of certain cellular proteins and (iii) physiological changes that alter the intracellular milieu [1]. Several proteins have been found to be over expressed in multidrug resistant human cancer cells, including the multidrug resistance MDR1 gene product P-glycoprotein [2], the multidrug resistance-associated protein MRP [3] and enzymes associated with the glutathione (GSH) metabolism [4-7]. Moreover atypical multi drug resistance has been ascribed to decreased expression or altered activity of topoisomerase II [8]. Although each of these proteins has been associated with a unique profile of cellular drug resistance, the drug resistance patterns may be partially overlapping. In some cell systems several mechanisms are involved in drug resistance [9].

In the present report we intend to describe the relation of metal copper and drug resistance in murine tumor models. We have also studied the changes in phase II detoxification parameters with the development of drug resistance. Although a number of changes in phase II detoxification parameters have been noticed the novel aspect of the present work is the observation that the metal copper is explicitly involved in the phenomenon of drug resistance. It has been reported that metal binding transporters of copper homeostasis pathway can mediate resistance to platinum drugs in cancer cells [10], the changes in Cu-level with gradual increase in drug resistance in murine model has been discussed for the first time. As the study of the relation of copper with gradual increase of resistance is not possible in human patients, we have performed the work on mouse model.

Copper plays important roles in human and other biological systems e.g., copper is essential for the proper functioning of copper-dependent enzymes (cytochrome C oxidase, superoxide dismutase, tyrosinase, dopamine hydroxylase, lysyl oxidase, clotting factor V, ceruloplasmin) [11-12]. Copper [Cu (II)] is also involved in the causation and cure of cancer [13-14]. Copper administration suppresses rat hepatoma induced by chemical carcinogen [14]. Copper (II) complexes cause tumor cells to redifferentiate into normal cells. [15]. Angiogenesis is a crucial process of tumor development and copper acts as an essential cofactor in several angiogenic growth factors [16-18].

Although the role of copper in physiological systems is controversial there is no doubt that copper is an essential component of several endogenous antioxidant enzymes [19].

The role of copper in treatment of cancer has been documented [18-21]. Serum copper concentration increases as cancer progresses and correlates with tumor incidence and

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burden [18]. Cancer patients have higher amount of copper in serum than healthy individuals [20]. Copper chelating agent, tetrathiomolybdate (TM) shows anticancer properties and acts by lowering the level of Cu in the serum [10]. The patients who respond to therapy or surgery usually have a return to normal serum copper levels, while non-responders had a persistently elevated serum copper level [12, 19-21]. Does the level of serum copper indicate any relation to drug resistance or drug sensitivity?

Although work has been done on the role of Cu-transporters in the development of Pt-drug resistance [17] the relation of Cu and drug resistance has not been studied in detailed so far.

We have observed that the level of Cu is elevated in serum of cancerous male Swiss albino mice bearing drug resistant Ehrlich ascites carcinoma (EAC/Dox) cells and in drug resistant Lewis lung carcinoma (3LL/CTx) cells compared to drug sensitive mice. We are going to describe in this preliminary report for the first time that the level of copper increases with the development of drug resistance in both the types of cells (EAC and 3LL) and in serum of mice bearing drug resistant cancer cells compared to mice bearing drug sensitive cells. Based on the limited data the present work develops a hypothesis about the relation of Cu and drug resistance. The hypothesis needs to be justified in elaborate experimentation.

MATERIALS AND METHODS

Chemicals:

Doxorubicin hydrochloride, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), 5,5'-dithio bis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Compamy, St. Louis, USA. Ortho dianisidine, Xanthene oxidase were purchased from Acros Organics, Belgium. The other chemicals used were of highest purity available.

Biological Materials

All animals were collected from our animal colony.

Cell Line

Ehrlich ascites carcinoma (EAC) cell line is maintained in male Swiss albino mice, weighing 18-20 g (6-8 weeks old) and water was supplied *ad libitum.* Animals were collected from our own animal colony.

Lewis lung carcinoma (3LL) is a gift from Professor Per H. Basse, University of Pittsburg Cancer Institute, Pittsburg, USA. 3LL was maintained in C57BL/6J mice as well as in male Swiss albino mice weighing 18-20 g (6 weeks old).

Development of Drug Resistant EAC Cell Line (EAC/Dox) *In Vivo*

EAC was maintained as an ascitic tumor in male Swiss albino mice weighing 18-20 g (6-8 weeks old), obtained from our own animal colony. A Dox (Meiji Keika Keisha Ltd., Tokyo, Japan) resistant subline was developed following the reported methods [22, 23] by sequential transfer of EAC cells to subsequent generation of host mice with continuous Dox treatment. The treatment regime consisted of 2.0 mg/kg /week Dox intraperitonaelly (i.p.). The daily treatment dose was 0.4 mg/kg for five days. The drug was started 24 hours after inoculation of $1x10^6$ ascites tumor cells i.p. to mice.

The development of EAC/Dox cell line with mean survival time (MST) in each generation is presented graphically in Fig. **1**.

The MST ± standard error of the mean (SEM) of untreated male Swiss mice bearing EAC cells was 19.4 ± 1.5 days (n=20). The MST ± SEM of the host mice bearing this tumor after 4 months treatment with Dox was 32.4 ± 3.1 days (n=20) whereas, MST of the 17th transfer generation of host mice was 20.1 ± 1.7 days (n=20). After this degree of resistance had been developed, the dose of Dox was

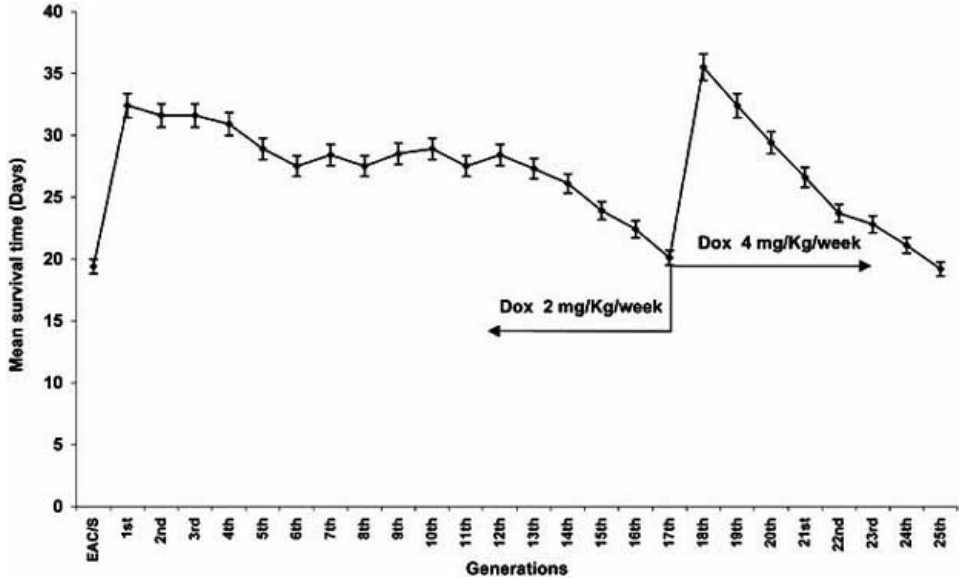

Fig. (1). Mean survival time (MST) of EAC bearing male Swiss albino mice in different generations after Dox treatment.

increased to 4 mg/kg/week (daily treatment dose was 0.8 mg/kg for five days), which resulted in 35.5 ± 1.7 days MST (n=20). When this tumor sub line was retreated with Dox after 24th transfer, MST was 21.1 ± 1.4 days (n=20). After 25th transfer, MST was noted to be 19.2 ± 2.9 days (n=20). The increased survival (35.5 ± 1.7 days) of Dox treated (2 mg/kg/week for four months) mice was statistically significant ($p<0.05$) in comparison to untreated mice group (19.4 ± 1.5 days). The animals with drug resistant cells (up to 25th generation) had the survival (19.2 ± 2.9 days), close to that of animals with drug sensitive cells (19.4 ± 1.5 days).

Development Drug Resistant Lewis Lung Carcinoma (3LL/CTx) Cell Line *In Vivo*

3LL was maintained as solid tumor in male Swiss albino mice weighing 18-20 g (6-8 weeks old) obtained from our animal colony. A cyclophosphamide (CTx) resistant sub line was developed following the method of Teicher *et al.* [24] by sequential transfer of $1x10^6$ 3LL cells subcutaneously (s.c.) in dorsal hind scapula to subsequent generation of host mice with continuous CTx treatment. The treatment regime consisted of 300 mg/kg/week CTx intraperitoneally (i.p.) (single dose). The drug was injected 24 h prior to cell collection. The primary tumor observed (PTO) was within 25±2.4 days (n=18), cell yield $24x10^7$. The PTO and cell yield reduced within 12th transfer generation and remained within a narrow range up to 17th generation.

The development of 3LL/CTx with primary tumor observed (days) and cell yield in each generation is presented graphically in Fig. **2a**.

Measurement of Cellular GSH

GSH was measured according to the method of Sedlack and Lindsay [26]. Briefly, to $2x10^5$ cells in 0.2 ml PBS, 4.8 ml EDTA (0.2 M) was added and kept on ice bath for 10 minutes. Then 4 ml deionised water and 1 ml of 5 % trichloroacetic acid (TCA) were added. The mixture was again kept on ice for 10 to 15 minutes and then centrifuged at 3,000 rpm for 15 minutes. 2 ml of supernatant was taken and 4 ml of 0.4 M tris buffer (pH 8.9) was added. 0.1ml of 5,5'-dithio bis (2-nitrobenzoic acid) (DTNB) solution was added and vortexed thoroughly. Optical density (O.D.) was read (within 2 to 3 minutes after addition of 0.1 ml 0.01M DTNB) at 412 nm against a reagent blank. Appropriate standards were taken and protein was measured according to Lowry [25]. The experiment was repeated for three times.

Measurement of Cellular GST

GST activity was determined spectrophotometrically according to the method of Habig *et al.* [27] with the use of 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate. Glutathione conjugates formed in the presence of the enzyme were quantified spectrophotometrically and the specific activity was expressed as nmol/min/mg of protein.

Measurement of Glutathione Peroxidase (GPx)

GPx activity was measured from the tissue homogenate. Tissue homogenate was prepared following the method of Hafemann *et al.* [28]. In brief, the animals were sacrificed; the liver was dissected, dried and weighed. The homogenate was prepared with 0.15 M KCl solution and centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was analyzed according to the reported method [28, 29].

Measurement of Catalase

Catalase was measured by the reported method [28, 30]. In brief, tissue homogenate was prepared using 0.1 M phosphate buffer solution, pH 7.0 and centrifuged at 1,00,000 g for 1 h at 4°C. Tissue homogenate was transferred in 0.1 M phosphate buffer solution (PBS), pH 7 containing 0.45 M H_2O_2. Aliquots of the mixture (0.5 ml) were removed at 20 second intervals and added to 2.0 ml of a solutions

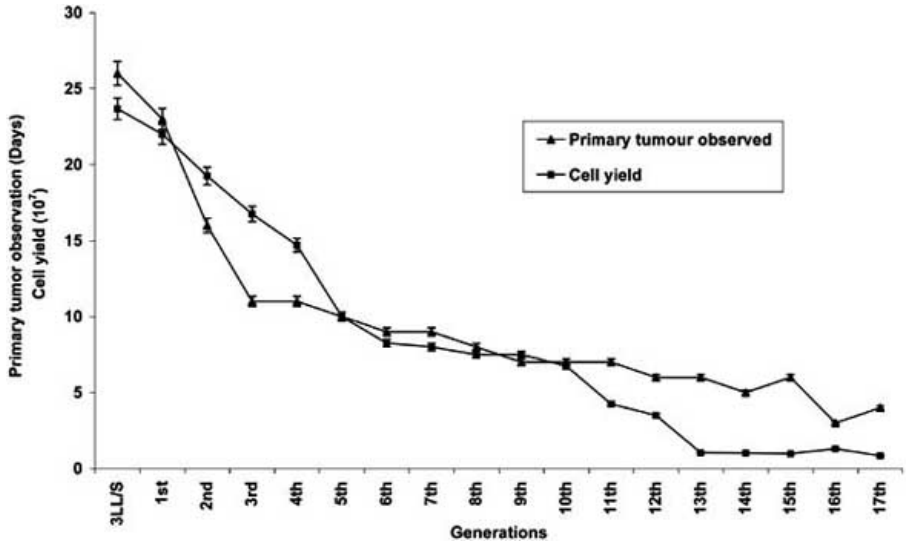

Fig. (2a). Progression of 3LL primary tumor with the development of MDR in male Swiss albino mice.

containing 0.2 mg/ml O-dianisidine, 0.015 mg/ml peroxidase and 0.81mg/ml sodium azide. After a 10-mins incubation at room temperature, 50% H_2SO_4 solution was added to stop the reaction. The absorbance of the reaction mixture was measured at 530 nm. One unit of enzyme activity (k) was calculated as follows:

k= 2.303/t log a/(a-x) [a, starting conc. of H_2O_2; a-x, H_2O_2 conc. after time t].

Measurement of Superoxide Dismutase (SOD)

SOD was measured by the reported method [28, 31]. In brief, tissue homogenate was prepared using 0.1 M PBS, pH 7 and centrifuged at 1,00,000 g for 1 h. at 4°C. The supernatant was dialyzed overnight against 0.1 M PBS, pH 7.0 and transferred to a reaction mixture containing 0.043 M Na_2CO_3 buffer (pH 10.2), 0.1mM xanthine, 0.1 mM EDTA, 0.05 mg/ml bovine serum albumin, 0.025 mM nitro blue tetrazolium (NBT) and the sample. After 10 min pre incubation at 25°C, the reaction was started with 0.1 ml xanthine oxidase and incubation was performed for 20 min at 25°C. After addition of 0.2 mM $CuCl_2$, the absorbance of the solution at 560 nm was measured. The activity of SOD required to inhibit the ratio of NBT reduction by 50% was defined as 1 unit of activity.

Measurement of Copper in Serum

Blood was collected directly from the heart and kept for clotting. Serum was collected with the help of micropipette. 100 µl serum was mixed with 2.5% of 3.9 ml nitric acid solution and vortexed for 2 minutes. To avoid co-precipitation each sample was centrifuged at 2,000 rpm at 37°C. The supernatant was kept for atomic absorption spectroscopy (AAS) study [Varian Spectra 200FS, hollow cathode lamp, Flame type: Air acetylene; replicate 3; wave length 324.8 nm].

Measurement of Copper in Liver Cells

Liver cells were collected after sacrificing the mice. The liver was removed, cut to pieces and washed repeatedly in 0.9% normal saline (NS). The liver was then homogenized slowly in hand tissue homogenizer in NS to make single cell suspension. $1x10^7$ liver cells per ml were lysed in 0.15 M KCl solution, the concentration was adjusted to $1x10^6$ cells/ml and centrifuged at 10,000 rpm for 20 mins. at 4°C. 100 µl of the supernatant of the liver soup was mixed with 2.5% of 3.9 ml nitric acid solution and vortexed for 2 minutes. To avoid co-precipitation each sample was centrifuged at 2000 rpm at 37°C. The supernatant was kept for atomic absorption spectroscopy (AAS) study.

Measurement of Copper in EAC and 3LL Cells

$1x10^7$ EAC and 3LL cells each per ml were lysed in 0.15 M KCl solution; the concentration was adjusted to $1x10^6$ cells/ml and centrifuged at 10,000 rpm for 20 minutes. At 4°C 100 µl of the supernatant of the EAC/Dox cell soup was mixed with 2.5% of 3.9 ml nitric acid solution and vortexed for 2 minutes. To avoid co-precipitation each sample was centrifuged at 2,000 rpm at 37°C. The supernatant was kept for atomic absorption spectroscopy (AAS) study.

RESULTS

Mean survival time (MST) of EAC bearing male Swiss albino mice with the development of drug resistance in different generations after doxorubicin (Dox) treatment is presented in Fig. **1**. The details have been described in 'Materials and methods'.

With administration of Dox in EAC bearing Swiss albino mice MST increased; the value of MST remain higher in comparison to Dox-untreated mice up to 16^{th} generation. At 17^{th} generation the value of MST was the least (20.1± 1.7) and the dose of Dox increased to double (4 mg/kg). The MST increased to 35 days at 18^{th} generation and gradually fell down in subsequent generations. The MST of animals bearing drug resistant cells fell down to 19.2 and remained almost in that level at 23^{rd}, 24^{th}, 25^{th} generations when cells become resistant.

Progression of 3LL solid tumor with the development of MDR in male Swiss mice is given in Fig. **2a**. Swiss mouse bearing 48 days of growth of 3LL/S tumor is given in Fig. **2b**.

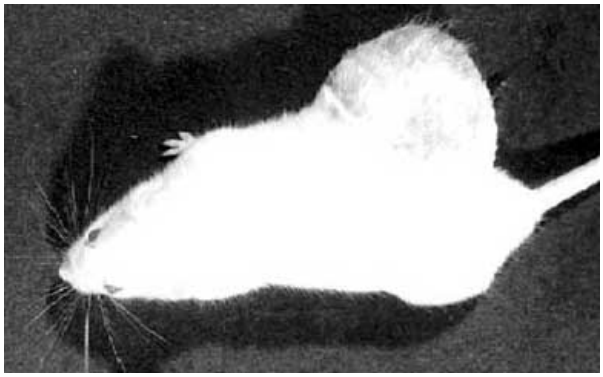

Fig. (2b). Drug resistant 3LL primary tumor (48 days growth) in male Swiss albino mice.

Concentration of GSH, GST, GPx in EAC/Dox and EAC/S cells are presented in Table **1**.

The level of GSH increased to 146% in EAC/Dox cells compared to EAC/S cells. GST and GPx decreased in EAC/Dox compared to drug sensitive cells. The level of GSH and GST found lower in liver, heart and higher in kidney in EAC/Dox bearing animals compared to EAC/S bearing animals. The change in the level of GPx in heart, kidney and liver were not significant except lung tissue.

Concentration of GSH, GST, GPx in 3LL/CTx and 3LL/S cells are presented in Table **2**.

The level of GSH increased to 158% in 3LL/CTx cells compared to 3LL/S cells. GPx increased significantly to 346% in drug resistant 3LL cells compared to drug sensitive cells. GST decreased in drug resistant cells compared to drug sensitive cells. The level of GSH and GST was found to be higher in significant amount in all the organs like heart, kidney, liver and lung in 3LL/CTx bearing animals compared to 3LL/S bearing animals.

Table 1. Level of GSH, GST and GPx in Male Swiss Albino Mice Bearing EAC/S and EAC/Dox

	Normal	Sensitive	Resistant	Change %
GSH (μg/mg of Protein)				
EAC	---	5.36 ± 0.49	13.2 ± 1.1	+ 146
Heart	49.66 ± 3.9	13.50 ± 1.1	4.03 ± 0.04	- 70
Kidney	30.91 ± 2.89	9.71 ± 0.9	13.29 ± 1.29	+ 37
Liver	23.64 ± 2.1	9.62 ± 0.82	3.32 ± 0.29	- 66
Lung	11.75 ± 1.1	17.22 ± 1.4	7.39 ± 0.69	- 57
GST (Unit/mg of protein/min.)				
EAC	---	1.76 ± 0.3	1.64 ± 0.11	- 7
Heart	24.70 ± 1.9	4.34 ± 0.4	1.44 ± 0.12	- 67
Kidney	34.39 ± 2.3	5.47± 0.35	8.64 ± 0.71	+ 58
Liver	19.32 ± 1.3	3.76 ± 0.31	2.29 ± 0.18	- 39
Lung	32.57 ± 2.8	5.17 ± 0.48	6.99 ± 0.54	+ 35
GPx (Unit/mg of protein/min.)				
EAC	---	30.67 ± 2.8	21.29 ± 1.7	- 31
Heart	49.55 ± 4.3	55.20 ± 4.1	46.82 ± 3.8	- 15
Kidney	79.40 ± 5.3	78.26 ± 5.7	80.54 ± 4.8	+ 3
Liver	148.03 ± 11.6	143.68 ± 11.3	157.61 ± 12.3	+ 10
Lung	82.15 ± 6.8	67.49 ± 5.3	86.25 ± 6.7	+ 28

The values are mean ± SD of four independent experiments.

The increase of GSH is significant ($p<0.001$) *in vivo* in male Swiss albino mice in drug resistant cells (EAC/Dox) in comparison drug sensitive cells (EAC/S).

Table 2. Level of GSH, GST and GPx in Male Swiss Albino Mice Bearing 3LL/S and 3LL/CTx

	Normal	Sensitive	Resistant	Change %
GSH (μg/mg of protein)				
3LL	---	19.95 ± 1.3	51.55 ± 5.3	+ 158
Heart	49.66 ± 3.9	1.22 ± 0.1	14.66 ± 1.1	+ 1102
Kidney	30.91 ± 2.89	2.09 ± 0.18	10.91 ± 1.4	+ 422
Liver	23.64 ± 2.1	1.90 ± 0.13	15.52 ± 1.33	+ 717
Lung	11.75 ± 1.1	1.10 ± 0.08	8.83 ± 0.67	+ 703
GST (Unit/mg of protein/min.)				
3LL	---	12.92 ± 1.12	4.41 ± 0.38	- 66
Heart	24.70 ± 1.9	4.02 ± 0.32	7.01 ± 0.66	+ 67
Kidney	34.39 ± 2.3	3.82 ± 0.29	6.06 ± 0. 53	+ 59
Liver	19.32 ± 1.3	1.44 ± 0.11	10.44 ± 1.0	+ 625
Lung	32.57 ± 2.8	3.86 ± 0.24	4.75 ± 0.38	+ 23

(Table 2. Contd….)

	Normal	Sensitive	Resistant	Change %
GPx (Unit/mg of protein/min.)				
3LL	---	8.56 ± 0.61	38.17 ± 4.8	+ 346
Heart	49.55 ± 4.3	65.82 ± 4.8	70.83 ± 6.1	+ 8
Kidney	79.40 ± 5.3	104.05 ± 8.3	95.94 ± 7.7	- 8
Liver	148.03 ± 11.6	169.56 ± 13.3	175.44 ± 15.3	+ 4
Lung	82.15 ± 6.8	95.56 ± 6.7	125.41 ± 9.3	+ 31

The values are mean ± SD of four independent experiments.

The increase of GSH is significant ($p<0.001$) *in vivo* in male Swiss albino mice bearing drug resistant cells (3LL/CTx) in comparison to drug sensitive cells (3LL/S).

GSH also increased significantly ($p<0.001$) in heart, kidney, liver and lung in male Swiss albino mice bearing drug resistant cells (3LL/CTx) in comparison to male Swiss albino mice bearing drug sensitive cells (3LL/S).

The changes in the level of GPx in all the organs in animals bearing 3LL/CTx cells were not significant when compared to animals bearing 3LL/S cells except lung tissue.

Concentration of SOD and catalase in drug resistant (EAC/Dox and 3LL/CTx) and drug sensitive (EAC/S and 3LL/S) cells are presented in Table **3**.

The level of SOD and catalase was found to be lower in EAC/Dox cells compared to EAC/S cells. No significant difference in the level of SOD and catalase in liver and kidney of animals bearing EAC/Dox cells were found when compared to animals bearing EAC/S cells.

The level of SOD and catalase was found to be higher in drug resistant 3LL/CTx cells compared to drug sensitive 3LL/S cells. Both SOD and catalase were increased in 3LL/CTx cell bearing mice compared to 3LL/S cell bearing mice.

Table 3. Superoxide Dismutase (SOD) and Catalase in Cancer Cell (EAC & 3LL) with the Development of MDR in Male Swiss Albino Mice

	Normal	Sensitive	Resistant	Change %
SOD (Unit/mg of protein) in EAC bearing Swiss mice				
EAC cell	---	74.67 ± 6.8	14.17 ± 1.41	- 81
Kidney	1.09 ± 0.1	1.30 ± 0.11	0.67 ± 0.05	- 49
Liver	1.66 ± 0.13	1.27 ± 0.11	1.34 ± 0.12	+ 6
Catalase (k'/mg of protein) in EAC bearing Swiss mice				
EAC cell	---	29.16 ± 2.3	7.30 ± 0.67	- 75
Kidney	0.048 ± 0.003	0.101 ± 0.01	0.11 ± 0.01	+ 9
Liver	0.031 ± 0.012	0.030 ± 0.003	0.032 ± 0.003	+ 7
SOD (Unit/mg of protein) in 3LL bearing Swiss mice				
3LL cell	---	4.55 ± 0.38	12.26 ± 1.2	+ 170
Kidney	1.09 ± 0.1	0.65 ± 0.06	5.21 ± 0.5	+ 702
Liver	1.66 ± 0.13	4.15 ± 0.06	4.65 ± 0.43	+ 12
Catalase (k'/mg of protein) in 3LL bearing Swiss mice				
3LL cell	---	0.18 ± 0.013	0.79 ± 0.06	+ 339
Kidney	0.048 ± 0.003	0.65 ± 0.06	0.89 ± 0.07	+ 37
Liver	0.031 ± 0.012	0.42 ± 0.03	1.65 ± 0.13	+ 293

The values are mean ± SD of four independent experiments.

SOD and catalase decreased significantly ($p<0.001$) in drug resistant EAC cells compared to drug sensitive cells *in vivo* in male Swiss albino mice.

SOD and catalase increased significantly ($p<0.001$) in drug resistant 3LL cells compared to drug sensitive cells *in vivo* in male Swiss albino mice.

Concentration of copper in drug resistant (EAC/Dox and 3LL/CTx) and drug sensitive (EAC/Sand 3LL/S) cells are presented in Table **4**.

The level of Cu increased to 281% in drug resistant cells (EAC/Dox) compared to drug sensitive (EAC/S) cells. The level of Cu increased in serum (32%) and decreased in liver (91%) of EAC/Dox cell bearing animals compared to EAC/S cell bearing animals.

The level of Cu increased to 612% in drug resistant Lewis lung carcinoma cells (3LL/CTx) compared to drug sensitive (3LL/S) cells. The level of Cu increased in serum (75%) and decreased in liver (36%) of drug resistant cell (3LL/CTx) bearing animals compared to drug sensitive cell (3LL/S) bearing animals.

Changes in GSH, GST and GPx level of EAC cell with the development of MDR is presented in Fig. **3**.

The level of GSH is significantly higher in drug resistant cells compared to drug sensitive cells. With the development of drug resistance GSH increased up to 7^{th} generation and gradually decreased and reached to lowest level at 17^{th} generation. At 18^{th} generation the dose of Dox doubled and GSH increased sharply and then reached the steady level at 22^{nd} to 25^{th} generation.

The level of GST and GPx in drug resistant and drug sensitive cells were almost the same.

Copper concentration in serum, liver and cell (EAC) in male Swiss albino mice in different generations with the development of drug resistance is presented in Fig. **4**

Table 4. Copper Concentration in Cancer Cell (EAC & 3LL) with the Development of MDR in Male Swiss Albino Mice

	Normal	Sensitive	Resistant	Change %
Copper concentration (μg/ml) of EAC bearing Swiss mice				
EAC cell	---	0.79 ± 0.06	3.01 ± 0.3	+ 281
Serum	2.65 ± 0.021	2.99 ± 0.02	3.95 ± 0.28	+ 32
Liver	1.69 ± 0.015	1.48 ± 0.012	0.13 ± 0.012	- 91
Copper concentration (μg/ml) of 3LL bearing Swiss mice				
3LL cell	---	0.34 ± 0.031	2.42 ± 0.021	+ 612
Serum	2.65 ± 0.021	2.34 ± 0.022	4.10 ± 0.36	+ 75
Liver	1.69 ± 0.015	0.44 ± 0.041	0.28 ± 0.023	- 36

The values are mean ± SD of four independent experiments.

The level of copper increased significantly ($p<0.001$) in drug resistant EAC cells compared to drug sensitive cells and in serum in drug resistant cell bearing male Swiss albino mice compared to mice bearing drug sensitive cells.

The level of copper also increased significantly ($p<0.001$) in drug resistant 3LL cells compared to drug sensitive cells and in serum in drug resistant cell bearing male Swiss albino mice compared to mice bearing drug sensitive cells.

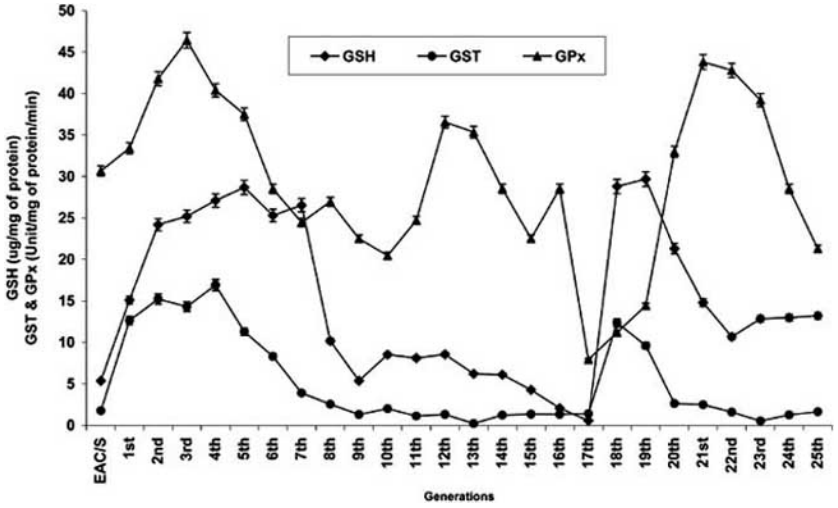

Fig. (3). GSH, GST and GPx level in each generation with the development of drug resistance in EAC cell.

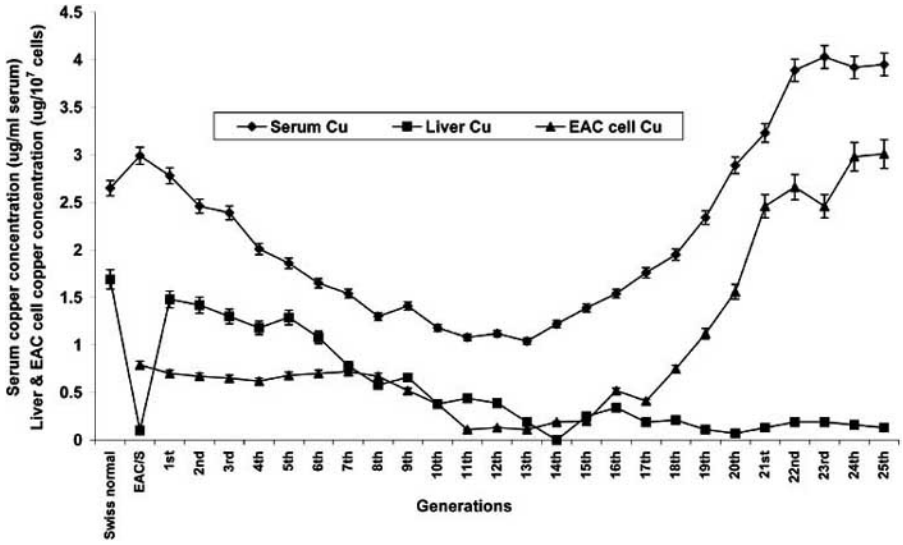

Fig. (4). Copper concentration in serum, liver and EAC cell of EAC cell bearing male Swiss albino mice with the development of MDR.

The level of copper in the serum gradually lowers from first generation and reaches at the lowest level at 13th generation. At 18th to 20th generation the level is slightly higher and reaches steady state from 21st to 25th generation when drug resistance is developed.

However, the concentration of copper is higher in the serum of Swiss albino male mice bearing drug resistant cells (EAC/Dox) compared to male mice bearing drug sensitive (EAC/S) cells.

The level of copper in liver of EAC bearing mice gradually lowers from first generation and reaches at the lowest level at 14th generation. At 15th to 20th generation the level is slightly higher and reaches steady state from 21th to 25th generation when drug resistance is developed. However, the concentration of copper is less in liver of drug resistant cell (EAC/Dox) bearing animals compared to Swiss albino male mice bearing drug sensitive (EAC/S) cells.

The level of copper in the EAC cells gradually decreases from first generation and reaches at the lowest level at 14th generation. From 15th generation onward the level of copper gradually goes up and reaches steady state from 20st to 25th generation when drug resistance is developed. However, the concentration of copper is significantly higher in the drug resistant cells (EAC/Dox) compared to drug sensitive cells (EAC/S) (Table **4**).

The level of copper in liver gradually lowers from first generation and reaches at the lowest level at 13th generation. At 14th to 17th generation the level is slightly higher and reaches steady state from 18th to 25th generation when drug resistance is developed. However, the concentration of copper is less in liver of drug resistant cells compared to drug sensitive cells in Swiss albino male mice bearing 3LL cells.

The level of copper in the serum decreases from first to third generation and then gradually reaches at the highest level at 9th generation and then slightly decreases. The level reaches steady state from 12th to 17th generation when drug resistance is developed (Fig. **5**). However, the concentration of copper is significantly higher in the serum of drug resistant cell (3LL/CTx) bearing animals compared to drug sensitive cell (3LL/S) bearing Swiss albino male mice.

The level of copper in 3LL/CTx cells gradually increases from first generation and reaches at the highest level at 11th generation and then reaches steady state from 12th to 17th generation when drug resistance is developed (Fig. **5**). However, the concentration of copper is significantly higher in the drug resistant cells (3LL/CTx) compared to drug sensitive cells (3LL/S) (Table **4**).

Changes in biochemical parameters with the development of drug resistance in 3LL cells are presented in Fig. **6**.

The level of GSH and GPx gradually decreases up to 10th generation and then gradually increases in the cells (3LL/CTx) with the development of drug resistance. The level of GSH and GPx reaches steady state at 15th to 17th generation. However, the level of GSH and GPx is significantly higher in drug resistant cells (3LL/CTx) compared to drug sensitive cells (3LL/S) (Table **2**).

The level of GST remains almost unchanged with the development drug resistance in 3LL cells.

DISCUSSION

The development of drug resistant EAC cells has been reported previously; drug resistance follows non P-gp pathway with change in phase II detoxification parameter [23]. In the present investigation also P-gp has not been detected in drug resistant cells up to 25th generation; GSH has been found to be significantly higher in drug resistant (EAC/Dox) cells than drug sensitive (EAC/S) cells. With the development of drug resistance the changes in phase II detoxification parameters like GSH, GST, GPx have been studied in the cells and various organs like heart, kidney, liver and lung in Swiss albino male mice.

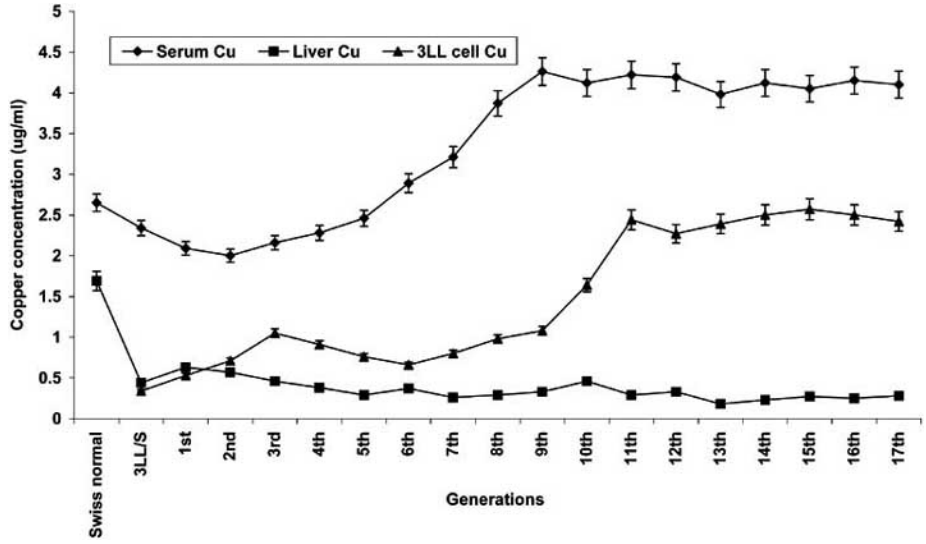

Fig. (5). Copper concentration in serum, liver and 3LL cell of 3LL cell bearing male Swiss albino mice with the development of MDR.

In lung no significant change in GSH, GST and GPx has been observed; GSH and GST have been decreased in liver and heart and increased in kidney with the development of drug resistance (Table **1**).

In the present report drug resistant 3LL cells i.e., 3LL/CTx has been developed in male Swiss albino mice following the reported method [24]. GSH has been found to be significantly higher in drug resistant (3LL/CTx) cells than drug sensitive (3LL/S) cells. With the development of drug resistance the change in phase II detoxification parameters has been noticed in the cells and in various organs like heart, kidney, liver and lung of Swiss albino male mice; in kidney and liver GSH and GST increased significantly but the level of GPx remains unaltered. Moreover, the level of GSH in the heart of animals bearing 3LL/CTx cells is tremendously higher compared to animals bearing 3LL/S cells, the reason remains unclear (Table **2**).

Although there are differences in the biochemical properties of drug resistant EAC cells and drug resistant 3LL cells, but in both the drug resistant cells (3LL/CTx and EAC/Dox) higher level of GSH has been noticed when compared with drug sensitive cells.

The report of increase of GSH in drug resistant cells compared to drug sensitive cells has been documented [23, 32, 33]. GSH, an important intracellular antioxidant is the most abundant non-protein thiole present in the cells. GSH reduces anticancer drug-derived free radical species and

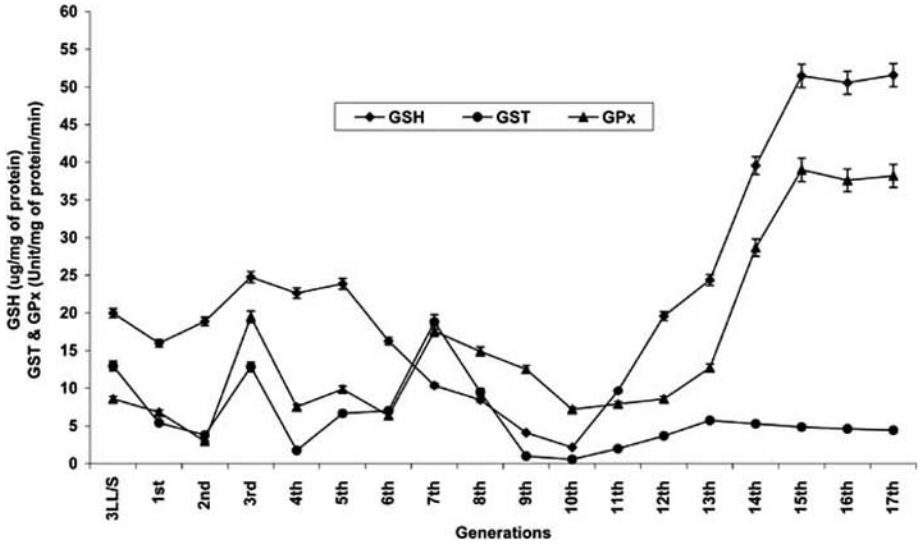

Fig. (6). GSH, GST and GPx level in each generation with the development of drug resistance in 3LL cell.

hence decreases cytotoxicity of the drug [34]. It has been reported that increasing level of GSH is parallel with the development of drug resistance in tumor cells [35].

Many reports have been published on the role of GSH in drug resistance [32-36]. The most important feature of the present investigation lies in reporting the changes in level of copper with gradual increase of drug resistance from one generation of mice to other. We have observed that Cu-level increases in two types of drug resistant cells (EAC/Dox and 3LL/CTx) compared to their drug sensitive counterparts (EAC/S and 3LL/S). Moreover, the level of copper in the serum of animals bearing drug resistant cells (EAC/Dox and 3LL/CTx) is significantly higher when compared with animals bearing drug sensitive cells; the level of copper in the liver of animals bearing drug resistant cells (EAC/Dox and 3LL/CTx) is significantly lower when compared with animals bearing drug sensitive cells. To summarize, the Cu-level increases in the cancer cells from drug sensitive cases to drug resistant cases. The opposite feature is reflected in the liver where Cu-level decreases from drug resistant cases to drug sensitive cases.

Some recent reports disclose the relation of copper and cis-platin resistance; Safaei *et al.* [10] have reported that cells selected for resistance to copper or Pt-drugs display bi-directional cross-resistance, parallel defects in the transport of Cu or Pt-drugs. The altered expression of Cu-transporters is consistent with the concept that Cu-homeostasis proteins regulate sensitivity to the Pt-drugs. Cu-homeostasis proteins may be markers and contribute to Pt-resistance [10]. Many features of the relation of Cu and drug resistance need to be deciphered. Cancer patients have higher amount of Cu in the serum than healthy individual. Moreover, patients responding to therapy or surgery usually have a return to normal serum copper levels, while non-responders, i.e., resistant cases had a persistently elevated serum copper level [12, 19, 20].

In the present work we have measured the level of copper in cells and various organs of animals bearing sensitive and drug resistant cells to decipher the role of copper in drug resistance or drug sensitivity.

We have found that in both types of cells when drug resistance is developed the level of copper is increased compared to drug sensitive cells, 281% in EAC/Dox and 612% in 3LL/Ctx (Table **4**). Also, the level of copper in serum increases in animals bearing drug resistant cells compared to animals bearing drug sensitive cells, 32% in EAC/Dox and 75% in 3LL/Ctx (Table **4**). Why the level of Cu is elevated in drug resistant cells and in serum of animals bearing drug resistant cells is not completely understood.

Cu is an essential trace element and is integrated into many enzymatic reactions in prokaryotic and eukaryotic systems. Cu is metabolized in human liver and excess level of copper is transported to the extracellular environment by an energy dependent system. Defect in copper transport causes a number of diseases like Wilson's disease (WND), Menkes disease characterized by chronic liver and kidney damage [33]. WND gene encodes a Cu-transporting P-type ATPase (ATP7B) whereas Menkes disease is caused due to mutation in ATP7A gene. ATP7B is a member of a class of heavy metal transporting P-type ATPases that pump out Cu, Cd, Zn, Ag and Pb [38]. Over expression of ATP7B renders cells resistant to carboplatin as well as copper and cis-platin; this feature is associated with parallel reductions in cellular accumulation of both copper and Pt-drugs [39]. Whether the increase in Cu level causes a change in ATP7B in two different drug resistant cell lines viz., EAC/Dox and 3LL/CTx warrants further study.

The mechanism of elevation of Cu in EAC/Dox and 3LL/CTx cells compared to their drug sensitive counterpart remains unknown at the present stage. The increase in Cu-level may be secondary to the development of resistance, based on other prior molecular changes, e.g., change Cu-transporters, enzyme systems [10]. One possible reason of Cu elevation is perhaps due to some defect in copper-transport in the drug resistant cells as metal transporter ATP7B also confers resistant to cis-platin [40].

High level of increase of copper in the cells and serum of both types of drug resistant cells (EAC/Dox and 3LL/CTx) deserves attention and further study.

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ABBREVIATIONS

CTx = Cyclophosphomide

Dox = Doxorubicin hydrochloride

EAC = Ehrlich ascites carcinoma

GSH = Glutathione

LLC = Lewis lung carcinoma

MDR = Multidrug resistance

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