Protective Effect of some Antioxidants against Ccl_4-Induced Toxicity in Liver Cells from BRL3A Cell Line.

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Abstract: An in vitro experiment was conducted to investigate the protective effect of ascorbic acid, mannitol and aminoguanidine at different concentrations against carbon tetrachloride induced toxicity and oxidative stress in hepatocytes cell line (BRL3A) from buffalo rats. Results were compared with those of vitamin E as standard hepatoprotective agent. Treatment of BRL3A with CcL_4 lead to generation of free radicals detected after two hours incubation using ESR technique and produced cell injury demonstrated by increased leakage of LDH, ALT and AST to the media. Exposure to CcL_4 caused apoptosis to cells but did not induce lipid peroxidation as tested by the TBARS technique. Treatment with vitamin E has significant hepatoprotective effect by lowering the leakage of intracellular enzymes, reducing the oxidation of proteins and decrease incidence of apoptosis. Ascorbic acid, mannitol and aminoguanidine were ineffective against CcL_4 toxicity. [Journal of American Science 2010;6(10):992-1003]. (ISSN: 1545-1003).

Keywords: in vitro; ascorbic acid; mannitol; aminoguanidine

1. Introduction:

The liver occupies a vital role in the main functions of the organism. It is particularly susceptible to chemically induced injury due to its extensive metabolic capacity and cellular heterogeneity. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) formation and scavenging by antioxidants. Excess generation of ROS can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis (Khan and Sultana, 2009).

In vitro liver systems represent a better experimental approach to screen potential hepatotoxic compounds and to investigate the mechanism by which chemicals induce liver lesions *(Kucera et al., 2006).* Liver cell lines are characterized by unlimited subcultivation and cell availability in large number) Guillouzo, *1998* .)The BRL3A cell line is an epithelial cell line from buffalo rat liver which is able to divide in the absence of serum.

Carbon tetrachloride (CcL_4) has long been known as a model toxicant and has been the focus of many *in vitro* and *in vivo* toxicological studies *(Manibusan et al., 2007).* The liver is the major target organ of CcL_4 toxicity owing to its high content of cytochrome P-450 *(Södergren et al., 2001).* Antioxidants are used to antagonize the deleterious action of free radicals and to protect hepatocytes from damage. Vitamin E (α-tocopherol) is considered the most important lipophilic antioxidant in biological tissues *(Weber et al., 2003).* Ascorbic acid is an important dietary antioxidant. It significantly decreases the adverse effect of reactive species such that can cause oxidative damage to macro-molecules such as lipids, DNA, and proteins *(Alpsoy et al., 2009).* Mannitol is a polyol which develops an antioxidant activity by scavenging ROS *(Mendoza et al., 2007).* A number of physiological functions have been ascribed to mannitol, including serving as a reserve carbon source, as an antioxidant, and to store reducing power *(Ruijter et al., 2003).* Aminoguanidine is an irreversible inhibitor of the inducible form of nitric oxide synthase (iNOS), which also inhibits endothelial NOS (eNOS) and neuronal NOS (nNOS) *(Mohamad et al., 2009).*

In the present study, *in vitro* antioxidant activities of ascorbic acid, mannitol and aminoguanidine were assesed in comparison with vitamin E against hepatotoxicity induced by carbon tetrachloride in liver cell line from rats.

2. Material and methods:

a. Material:-

CcL_4 (99.5%), vitamin E "α-tocopherol acetate" and L (+) ascorbic acid "vitamin C" were purchased from Wako Pure Chemicals Co., Japan. D-mannitol and aminoguanidine hemisulfate were obtained from Sigma-Aldrich

Chemical Co., St. Louis, MO, USA. Ham's F-12K media was used for hepatocytes'cell line culturing (Wako Pure Chemicals Co., Japan). LDH cytotoxicity detection kit (Takara Company, Tokyo, Japan), mitochondria extraction kit (Sigma-Aldrich Chemical Co., St. Louis, MO, USA.), Oxyblot protein oxidation detection kit (Chemicon

International Co., USA), Qproteome for detection of Apoptosis and In situ cell death detection kit (Roche Company, Germany).

b. Methods:

1- Cell culture, liver cells injury induction and treatment with different antioxidants:-

Liver cell line BRL3A derived from buffalo rats (*Nissley et al., 1977*) was used. Approximately 1x 10^5 cells/ml (5 ml final volume) were cultured into a 25 ml flask and incubated under standard conditions (37 °C and 5 % CO2) in Ham's F-12K medium with L-Glutamine, phenol red and sodium pyurvate supplemented with 10% Fetal Bovine Serum (FBS) and penicillin and streptomycin as antibiotics. Carbon tetrachloride (7.5 µl) was added in the 25 ml headspace of the flask to a paper attached to the stopper. During incubation, CcL_4 evaporated and equilibrium was reached between the gas phase and the medium (*Azri et al., 1990*). Media samples were collected at 30 min, 1, 3, 6, 9, 12, 14, 24 and 48 hrs. Different antioxidants (vitamin E, ascorbic acid, mannitol and aminoguanidine) were added separately to the media just before the addition of CCL_4. Each agent was individually tested on the cells using a dose range. For vitamin E this was 100, 50 and 25 µM; for ascorbic acid 500, 250 and 125 µM; for either mannitol or aminoguanidine the dose range was 400, 200 and 100 µM. Media samples were collected at 14, 24 and 48 hrs.

2- Detection of Free radicals by Electron Spin Resonance (ESR) method:-

Electron spin resonance signals were measured by preincubation of the hepatocytes in the presence of 40 mM 5, 5-dimethyl-pyrroline-*N*-oxide (DMPO) *(Matsuki, et al., 1999).* After induction of CcL4 toxicity, media samples were collected after 1, 2, 4 and 6 hrs. A control group was incubated with DMPO only. All ESR spectra were recorded at room temperature. AJEOL Model JES – FA 100 ESR spectrometer (JEOL Co., Tokyo Japan) was used. Spectrometer settings were; magnetic field 335.00±5 mT, sweep time 8.0 min, modulation frequency 100 kHz, modulation amplitude 0.2 mT, receiver gain $8x10^3$, time constant 0.03 S, microwave frequency 9.418GHz and microwave power 12 mW.

3- Leakage of intracellular enzymes as (Lactate dehydrogenase (LDH) and aminotransferase (ALT and AST) leakage in cell culture medium: Leakage of LDH enzyme in the cultured media was measured by a kit while the activities of ALT and AST were determined by using automatic multifunction-biochemical analyzer (DRI-CHEM 5500, Japan).

4- Measurement of Lipid Peroxidation: It was carried out by measuring thiobarbituric acid – reactive species (TBARS) according to *Kikugawa et al., (1992).*

5- Protein oxidation detection:

Using Qproteome mitochondria extraction kit, mitochondria was extracted from control and treated cells after 24 hours incubation with CcL4 to assess the formation of protein carbonyl groups as an index of protein oxidation. Protein concentration was determined by the Bradford assay (Bio-Rad) according to Bradford (1976). An OxyBlot protein oxidation detection kit (Chemicon International Co., USA.) was used according to the manufacturer's detailed protocol. Subsequently, 5 µL protein sample was added with 5 µL of 12% SDS and 10 µL of 1× DNPH solution into a tube. Ten microliters of 1× neutralization solution instead of the DNPH solution served as the negative control. Tubes were incubated at room temperature for 15 minutes. Neutralization solution (7.5 µL) was added to each tube. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by Western blotting. Following electrophoresis and transfer to nitrocellulose membranes, the membranes were blocked in Tris buffered saline containing 0.1% Tween 20 and 1% bovine serum albumin for 1 hour at room temperature. Membranes were incubated overnight at room temperature with the primary antibody stock (1:150) and then incubated with secondary antibodies (1:3000) at room temperature for 1 hour. Blots were developed by an enhanced chemiluminescence detection system. Proteins that underwent oxidative modification (i.e., carbonyl group formation) were identified as a band in the derivatized sample, but not in the negative control. Levels of oxidatively modified proteins were quantified and expressed via measurement of optical density using molecular analyst alias program.

6- Analysis of oxidative DNA strand breaks by TUNEL assay:-

The principle of the test is that cleavage of DNA during apoptosis may yield double stranded as well as single strand breaks "nicks" which can be identified by labeling with modified nucleotides in an enzymatic reaction (TUNEL staining, according to Gavrieli et al., 1992. Cells were grown on a small cover glass which was placed inside the flask. After induction of CcL_4 toxicity, cells were washed in PBS and fixed with 4 % paraformaldehyde buffered saline for 1 h at 15-25 °C. Permeablisation of the cells was carried out by incubation with 0.2% Triton X for 2 min. on ice (2-8 °C). Positive control cells were made by treatment with DNAse I for 10 minutes to break up the DNA.

TUNEL reaction mixture (50 µl) was added on sample, for the negative control, 50 µl of label solution was added for 60 min. at 37 °C in a humidified atmosphere in the dark. The samples were analyzed under a fluorescence microscope using an excitation wavelength in the range of 450-500 nm. Converter AP (50 µl) was then added on sample, and incubated in a humidified chamber for 30 min. at 37 °C. Substrate solution (100 µl) was added to the slides which were incubated for 10 min. at 15-25 °C in the dark. Cells were washed in PBS between each of these steps. The slides were counterstained with haematoxyline and eosin, examined under light microscope, and the apoptotic cells were counted in 10 random fields.

c. Statistical analyses:-

Data are expressed as mean values ± SE. Student's t-test was used for observations. One-way analysis of variance (ANOVA) was used to assess significant differences among treated groups followed by Tukey's test. Statistical analyses were performed using GraphPad prism software (GraphPad, Inc., California USA). Differences were considered statistically significant when $p < 0.05$.

3. Results

I- Toxic effect of CcL4 on BRL3A cell line:-

a- Generation of free radicals

The ESR signals were observed after 2 hours incubation of BRL3A cell line with CcL_4 and DMPO (Fig.1, A) reached its peak at 4 hr and began to decline at 6 hr (Fig.1, D&E).

b. Leakage of intracellular enzymes:-

Activities of LDH, ALT and AST enzymes were increased significantly ($P<0.05$) at 14 and 48 hrs. (Table, 1).

c- Lipids peroxidation:-

Lipids peroxidation revealed no significant change between control and treated cells with CcL_4 (Table, 1).

d -Oxidation of proteins:-

The amount of protein carbonyls was markedly increased in cells treated with CcL_4 compared with that of normal control group (Fig. 2, B).

e -Oxidative DNA strand breaks-:

Examination of control cells by the fluorescent microscope revealed no signals, while CcL4 treated cells showed fluorescent signals. The percent of apoptotic hepatocytes stained with the TUNEL method was significantly greater in the CcL4-treated cells (4.2%) than that in the control group (0.66%) (Fig. 3) (Photo, 1)

II- Effect of different antioxidants against CcL4-induced hepatotoxicity in BRL3A cell line:-

A-Leakage of intracellular enzymes:-

Comparing with the CcL_4 treated group, All the used doses of vitamin E significantly ($p<0.05$) reduced leakage of LDH enzyme to the media. Ascorbic acid at a dose of 500 µM was able to reduce LDH enzyme leakage to the media after 24 and 48 hrs. Mannitol failed to show reduction in leakage of LDH except at a dose of 200 µM at 48 hr, while aminoguanidine failed to show reduction in leakage of LDH. As regards to leakage of ALT and AST enzymes to the media, vitamin E treatment (100, 50 and 25 µM) significantly ($p<0.05$) reduced enzymes' leakage. Ascorbic acid, mannitol and aminoguanidine at all doses were unable to reduce the leakage of ALT and AST enzymes to the culture media compared with CcL_4 treated group throughout the experiment (Tables, 2,3 and 4).

b- Oxidation of proteins:-

Treatment with vitamin E showed marked reduction in the amount of protein carbonyls with all doses. Similar results were observed in Ascorbic acid treated groups. No changes in protein carbonyls were observed in groups treated with different doses of mannitol and aminoguanidine, (Figs. 4A & 4B),

c- Oxidative DNA strand breaks:-

Percentage of apoptotic cells stained with the TUNEL method was markedly decreased in vitamin E (VE) 100 µM treated group. The percentage of positive TUNEL cells was 0.85% in control group, 3.8 % in CcL_4 group, 1.79 % in VE 100 µM group, 2.1 % in VE 50 µM and 2.3 % in VE 25 µM treated group (Fig. 3B). Ascorbic acid (AA) did not reduce the incidence of apoptosis in cells treated with CcL_4. Percentage of positive apoptotic cells were 2.8% in AA 500 µM, 2.5% in AA 250 µM, 2.8% in AA 125 µM and 3.2% in AA 62.5 µM treated group. Treatment with different doses of mannitol (M) did not show protective effect against CcL_4 induced apoptosis in BRL3A cells. The percentages of positive TUNEL cells were 2.8% in M 400µM, 2.6% in M 200 µM and 2.5% in 100 µM (Fig. 3C). Aminoguanidine failed to reduce the incidence of apoptosis occurred in BRL3A cells due to CcL_4 toxicity. The percentage of apoptotic cells were 2.66% in AG 400 µM, 2.42% in AG 200 µM and 2.44% in AG 100 µM group (Fig. 5).

Table (1):- LDH, ALT and AST enzyme activities & TBARS values of BRL3A cell line treated with CcL_4 at different incubation times.

Incubation time		LDH (U/ml)	ALT (U/L)	AST (U/L)	TBARS (µmol/L)
30 min	**Control**	0.026±0.001	1.667±0.333	9.000±1.723	0.006±0.001
	CcL4	0.032±0.002	2.000±0.577	8.000±1.000	0.012±0.002
1 h	**Control**	0.030±0.003	1.333±0.333	10.000±1.000	0.005±0.003
	CcL4	0.025±0.001	1.678±0.333	10.000±1.000	0.014±0.006
3 hs	**Control**	0.041±0.001	1.333±0.333	10.330±1.528	0.005±0.001
	CcL4	0.042±0.002	1.770±0.667	13.670±1.528	0.005±0.003
6 hs	**Control**	0.044±0.000	3.333±0.882	6.670±0.333	0.007±0.002
	CcL4	0.043±0.000	4.330±0.881	8.660±1.667	0.004±0.001
9 hs	**Control**	0.040 ±0.001	4.000±0.577	6.790±0.333	0.016±0.001
	CcL4	0.041±0.001	4.333±0.882	8.560±0.577	0.026±0.007
12 hs	**Control**	0.039±0.001	6.000±0.577	3.667±0.333	0.017±0.001
	CcL4	0.042±0.002	7.333±0.882	4.667±0.067	0.025±0.004
14 hs	**Control**	0.006±0.002	3.000±0.577	5.333±0.333	0.018±0.002
	CcL4	0.023±0.001**	7.335±0.333**	7.667±0.666*	0.024±0.006
24 hs	**Control**	0.013±0.003	4.333±0.333	6.300±0.330	0.022±0.003
	CcL4	0.028±0.001*	9.320±0.881**	11.67±0.333**	0.022±0.002
48 hs	**Control**	0.126±0.002	6.000±0.577	11.33±0.577	0.022±0.000
	CcL4	0.378±0.008**	12.67±1.453*	23.67±2.082**	0.031±0.004

(*) Significantly different from the control group at $P < 0.05$.
(**) Significantly different from the control group at $P < 0.001$.

Table (2):- Activities of LDH, ALT and AST enzymes of BRL3A cell line treated with different doses of vitamin E, ascorbic acid, mannitol and aminoguanidine for 14 hr against toxicity of CcL_4.

Antioxidant	Groups	LDH ml)/(U	ALT L)/(U	AST L)/(U
Vitamin E (VE)	**Control (0.5%ethanol)**	002.005±0.0	333.4.33±0	333.670±0.3
	CcL_4	003 [a].077±0.0	333 [a].9.670±0	202 [a].330±1.11
	VE 100 µM+ CcL_4	001 [b].066±0.0	333 [b].5.330±0	453 [b].330±1.4
	VE 50 µM + CcL_4	000 [b].068±0.0	333 [b].6.330±0	202 [b].667±1.4
	VE 25 µM+ CcL_4	000 [b].062±0.0	7.000±0.577 [b]	155 [b].000±1.3
Ascorbic acid (AA)	**Control**	000.028±0. 0	577.00±0. 4	3.67±0.333
	CcL4	001 [a].036±0.0	577 [a].00±0.9	8.00±0.577 [a]
	AA 500 µM+ CcL_4	001.033±0.0	333.67±0.7	6.00±0.577
	AA 250 µM+ CcL_4	000.036±0.0	333.33±0.7	6.33±0.881
	AA 125 µM+ CcL_4	001.034±0.0	577.00±0.7	7.00±0.577
Mannitol (M)	**Control**	000.009±0.0	667.33±0.3	4.33±0.333
	CcL4	002 [a].030±0. 0	33 [a].33±0.7	9.00±0.577 [a]
	M 400 µM+ CcL_4	002.049±0.0	667.67±0.6	9.67±0.333
	M 200 µM+ CcL_4	003.041±0.0	577.00±0.6	9.33±0.333
	M 100 µM+ CcL_4	000.039±0.0	33.33±0.5	8.33±0.333
Aminoguanidine (AG)	**Control**	000.009±0.0	667.33±0.3	3.67±0.333
	CcL4	000 [a].022±0. 0	33 [a].33±0.7	10.67±0.333 [a]
	AG 400 µM+ CcL_4	001.039±0.0	882.34±0.7	10.33±0.333
	AG 200 µM+ CcL_4	002.028±0.0	66.33±0.6	9.67±0.333
	AG 100 µM+ CcL_4	002.027±0.0	67.67±0.5	10.00±0.577

- ([a]) Significantly different from the control group at $P<0.05$.
- ([b]) Significantly different from the CcL4 treated group at $P<0.05$
- Other values proved no significance.

Table (3):- Activities of LDH, ALT and AST enzymes of BRL3A cell line treated with different doses of vitamin E, ascorbic acid, mannitol and aminoguanidine for 24 hr against toxicity of CcL_4.

Antioxidant \ groups		LDH (U/ml)	ALT (U/L)	AST (U/L)
Vitamin E (VE)	Control (0.5% ethanol)	0.004±0.000	8.000±0.577	7.670±0.333
	CcL_4	0.045±0.002 [a]	15.330±0.882 [a]	14.660±0.333 [a]
	VE 100 µM+CcL4	0.007±0.000 [b]	6.330±0.881 [b]	8.660±0.333 [b]
	VE 50 µM+ CcL4	0.017±0.001 [b]	7.000±1.13 [b]	9.000±0.577 [b]
	VE 25 µM+ CcL4	0.020±0.000 [b]	10.000±0.577 [b]	9.330±0.333 [b]
Ascorbic acid (AA)	Control	0.029±0.000	5.00±0.577	4.67±0.333
	CcL4	0.041±0.001 [a]	10.33±0.333 [a]	10.00±0.577 [a]
	AA 500 µM+ CcL4	0.032±0.001 [b]	10.00±0.577	9.00±0.577
	AA 250 µM +CcL4	0.042±0.000	12.00±0.577	9.00±0.577
	AA 125 µM+ CcL4	0.042±0.001	9.00±0.577	9.33±0.333
Mannitol (M)	Control	0.027±0.001	4.00±0.577	5.67±0.333
	CcL4	0.040±0.000 [a]	9.00±0.577 [a]	12.33±0.333 [a]
	M 400 µM +CcL4	0.073±0.000	7.33±0.33	11.30±0.667
	M 200 µM +CcL4	0.069±0.004	7.33±0.33	11.33±0.333
	M 100 µM +CcL4	0.047±0.001	9.33±0.33	10.67±0.333
Aminoguanidine (AG)	Control	0.027±0.001	4.00±0.577	5.00±0.577
	CcL4	0.04±0.002 [a]	9.00±0.577 [a]	12.33±0.333 [a]
	AG 400 µM +CcL4	0.052±0.001	8.33±0.577	10.67±0.333
	AG 200 µM +CcL4	0.045±0.000	8.00±1.15	11.33±0.333
	AG 100 µM +CcL4	0.038±0.001	8.00±0.000	11.67±0.333

- ([a]) Significantly different from the control group at P<0.05. - ([b]) Significantly different from the Ccl4 treated group at P<0.05
- Other values proved no significance.

Table (4):- Activities of LDH, ALT and AST enzymes of BRL3A cell line treated withdifferent doses of vitamin E, ascorbic acid, mannitol and aminoguanidine for 48 hr against toxicity of CCL_4.

Antioxidant	Groups	LDH ml)/(U	ALT L)/(U	AST L)/(U
Vitamin E (VE)	Control (0.5%ethanol)	000.004±0.0	577.000±0.6	18.000±0.577
	CcL_4	001 [a].031±0.0	577 [a].000±0.11	577 [a].000±0.25
	VE 100 µM+ CcL4	000 [b].004±0.0	577 [b].000±0.7	577 [b].000±0.12
	VE 50 µM+ CcL4	000 [b].012±0.0	577 [b].000±0.5	577 [b].000±0.14
	VE 25 µM+ CcL4	000 [b].014±0.0	577 [b].000±0.7	577 [b].000±0.13
Ascorbic acid (AA)	Control (0.5%ethanol)	001.047±0.0	577.00±0.4	10.33±0.333
	CcL_4	002 [a].068±0.0	577 [a].00±0.8	22.67±0.333 [a]
	VE 100 µM+ CcL4	002 [b].058±0.0	577.00±0.8	20.00±0.577
	VE 50 µM+ CcL4	001.070±0.0	577.00±0.7	21.33±0.333
	VE 25 µM+ CcL4	002.068±0.0	577.00±0.6	21.33±0.882
Mannitol (M)	Control (0.5%ethanol)	003.018±0.0	577.00±0.5	12.00±0.577
	CcL_4	004 [a].031±0.0	577 [a].00±0.8	23.00±0.577 [a]
	VE 100 µM+ CcL4	001.041±0.0	33.33±0.7	21.00±0.577
	VE 50 µM+ CcL4	001.045±0.0	577 [b].00±0.5	21.00±0.577
	VE 25 µM+ CcL4	001.048±0.0	577.00±0.6	20.67±0.333
Aminoguanidine (AG)		003.017±0.0	577.00±0.5	9.00±0.577
		004 [a].030±0.0	577 [a].00±0.8	22.00±0.577 [a]
		002.046±0.0	882.6.67±0	20.33±0.333
		001.034±0.0	577.00±0.6	20.30±0.333
		000.027±0.0	33.5.33±0	19.67±0.333

- ([a]) Significantly different from the control group at P<0.05. - ([b]) Significantly different from the CcL4 treated group at P<0.05
- Other values proved no significance.

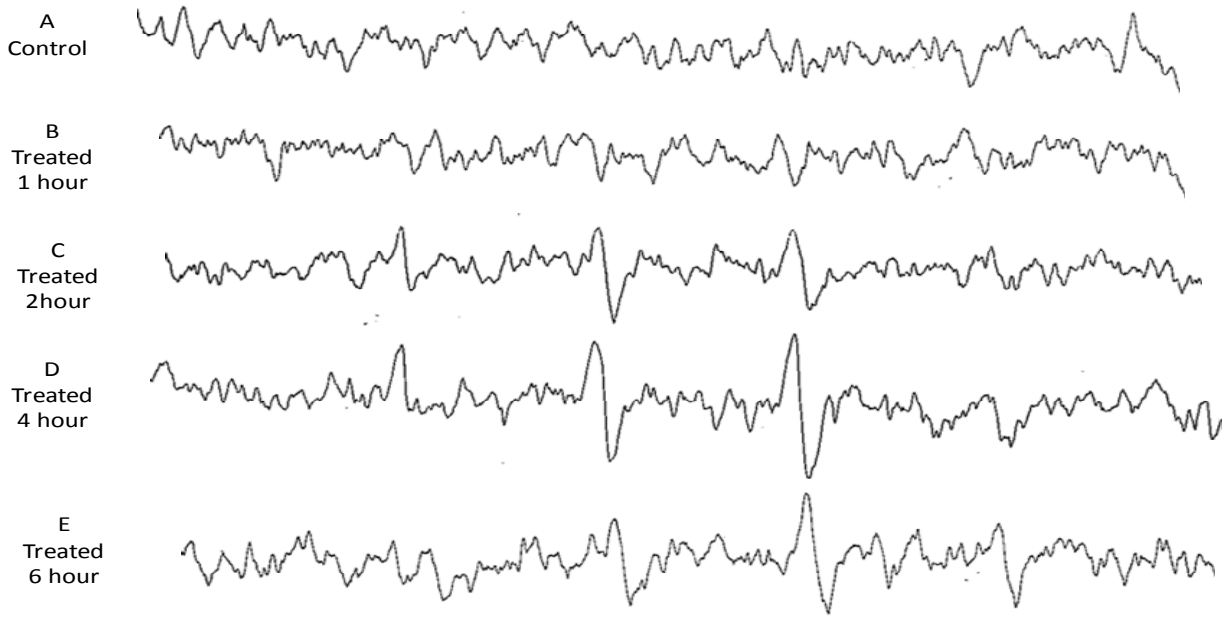
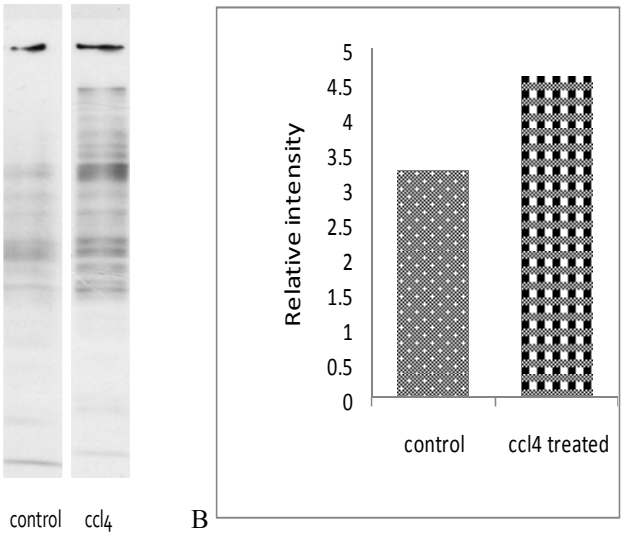


Fig. (1):- ESR spectra of supernatants of BRL3A cell line treated with CCL4 at different periods of incubation.

(Spectrum **A**) ESR spectra of the 40 mM DMPO adduct detected in BRL3A cell line. (Spectrum **B**) Same as in spectrum A, but cells were incubated with Ccl4 for one hour. The spectrum shown in **C** is from cells incubated 2 hours with CcL4, while the spectrum in **D** after 4 hours incubation with CcL4. Spectrum shown in **E** after 6 hour incubation period with CcL4. Instrumental settings of a JEOL Model JES-FA 100 ESR spectrometer: magnetic field 335.000±5 mT, sweep time 8.0 min, modulation frequency 100 kHz, modulation amplitude 0.2 mT, receiver gain 8x103, time constant 0.03 S, microwave frequency 9.418GHz and microwave power 12 mW.



A

Fig. (2,A):- Oxyblot of mitochondria of BRL3A in both control and CcL_4 treated groups using oxyblot technique.

Fig. (2,B):-Densitometeric measurement of the oxyblot lanes of fig. (2,A)

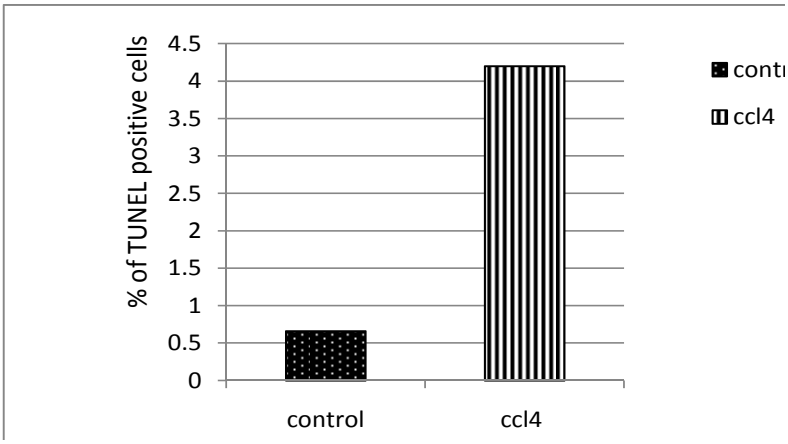


Fig. (3):- Quantitative evaluation of TUNEL positive cells in BRL3A cells treated with CcL_4.

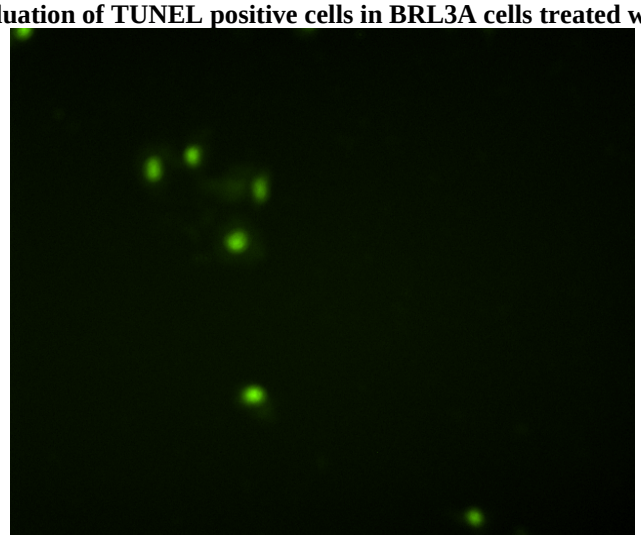


Photo (1):- Cell apoptosis of BRL3A cell line after exposure to Ccl4 as detected with TUNEL assay under fluorescent microscope.

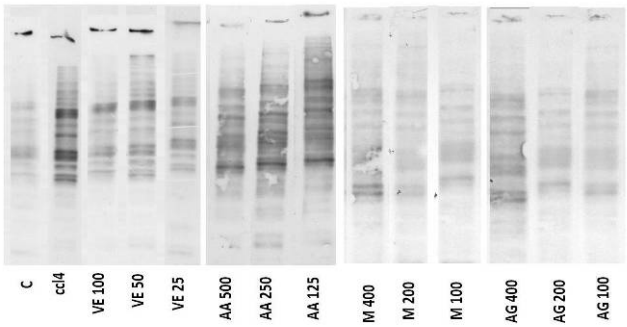


Fig. (4,A):- Oxyblot of mitochondria of BRL3A incubated with Ccl4 and different dose of vitamin E (VE), ascorbic acid (AA), Mannitol (M) an aminoguanidine (AG).

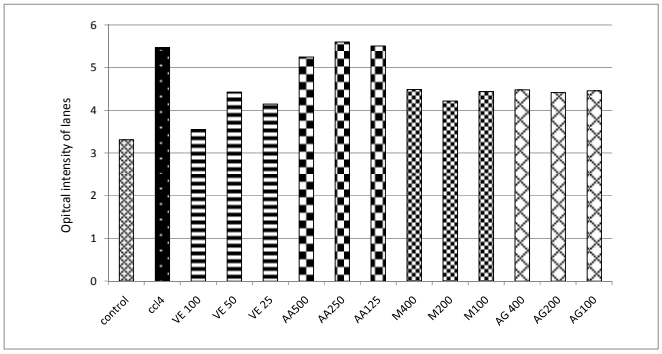


Fig. (4,B):- Densitometeric measurement of the oxyblot lanes of Fig. (3,A)

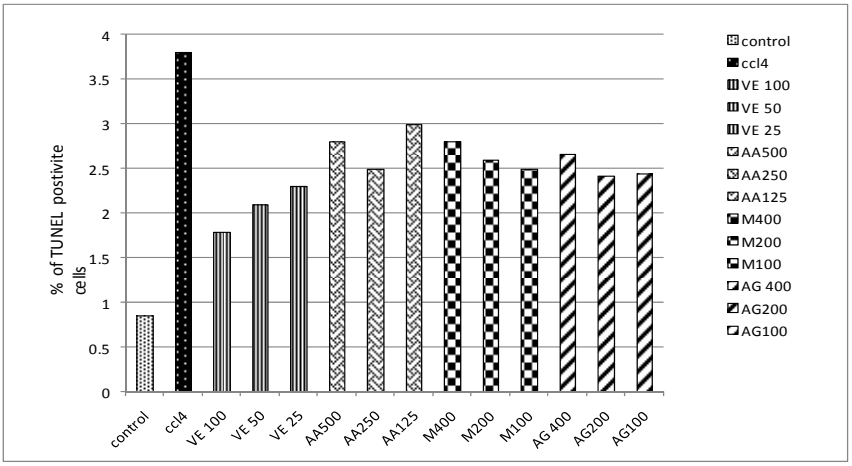


Fig (5):- Quantitative evaluation of TUNEL positive cells in BRL3A cells treated with ccl4 and different doses of vitamin E (VE), ascorbic acid (AA), Mannitol (M) and aminoguanidine (AG).

4. Discussion

Various pharmacological or chemical substances are known to cause hepatic injury. It is believed that the occurrence of drug/chemicals-induced liver injury is especially associated with oxidative stress and a cellular imbalance between the production and elimination of free radicals (*Castro and Freeman, 2001*). Overproduction of free radicals could directly injure hepatocellular membrane by lipid peroxidation, or other means, followed by a series of cascades of cellular events such as massive release of inflammatory mediators or cytokines, which eventually lead to liver injury (*Higuchi and Gores, 2003*). Therefore, it is valuable to identify

natural drugs or compounds that can antagonize the deleterious action of free radicals and act as an antioxidant to protect hepatocytes from damage.

In the present experiments, hepatocytes cultured cells were utilized. In an *in vitro* system, compounds affect the cells directly and continuously until the removal of compound -containing medium. In addition, when cells are cultured using single type of cells, there are no interactions from other interstitial cells *(Kikkawa et al., 2006).*

Cytotoxicity of CcL_4 has been selected as a model for induction of oxidative damage. Carbon tetrachloride is one of the most extensively studied hepatotoxicants (*Bhattacharjee and Sil, 2007*). It is now generally believed that CcL_4 hepatotoxicity depends on its reductive dehalogenation catalyzed by cytochrome P-450 enzyme in the endoplasmic reticulum of hepatic cells leading to the generation of an unstable complex trichloromethyl radical. The superoxide anion, H_2O_2, and the hydroxyl radical (OH) are reactive oxygen species (ROS) mainly produced in mitochondria (*Wang et al., 2007*).

The possible protective effect of some antioxidants (ascorbic acid, mannitol and aminoguanidine) has been attempted on hepatocytes

cultured cells treated with CcL_4. Trials were compared to vitamin E known to be important lipophilic antioxidant in biological tissues. The activity of an antioxidant may depend on its reactivity towards particular radicals, its ability to concentrate near the critical target in the cell or its inhibitory action on radical formation (*Anderson and Phillips, 1999*).

Electron spin resonance (ESR) was employed to measure directly reactive oxygen species (ROS) production in the liver of CcL_4-treated hepatocytes cell line. Results indicated that ROS production was highly elevated in the cultured media of the cell line at 4 h after CcL_4 treatment, thus further confirming that free radicals and oxidative damage certainly play a vital role in the pathogenesis of acute liver injury (*Wu et al., 2007*).

Cell viability depends directly on the structure of the membrane, and damage to the cellular membrane can be detected by enzyme leakage. It has been generally accepted that leakage of the cytosolic enzyme LDH correlates well with cellular viability, thus being a useful indicator of plasmatic membrane damage (*Grajeda-Cota et al., 2004*). In the present study, observations revealed that CcL_4 induced significant increase in the leakage of LDH, ALT and AST enzymes into the medium after 14 hr. The obtained results are in harmony with those reported by *Chandan et al. (2007).* It is interesting to note that there was difference in the degree of leakage of ALT and AST enzymes, where AST enzyme leakage was higher than ALT enzyme leakage. *McQueen and Williams (1982)* reported differences in the extent of leakage of enzymes from the cytosolic fraction of hepatocytes. These differences could be due to differences in the tightness of binding of cytosolic enzymes to cellular organelles and in the size of the enzyme molecule (*Nakamura et al., 1985*). Moreover, AST is present in two isozymes, one located in the cytoplasm and the other in the mitochondria (Latner, 1975). The presence of enzymes outside the cell represents damage to the hepatic cell.

Involvement of lipid peroxidation in the mechanism of carbon tetrachloride-induced hepatotoxicity has been a point of controversy. The present results denoted that CcL_4 did not induce lipid peroxidation as there was no significant difference in the level of thiobarbituric acid-reactive substances (TBARS) between CcL_4 treated cells and control cells. These data are comparable to that of *Ikeda et al. (1998)* who reported that TBARS, which are widely used index of lipid peroxidation, don't increased significantly in the liver of rats treated with CcL_4. Previous investigators reported absence of lipid peroxidative degradation products in mice after exposure to carbon tetrachloride and have used this evidence against the hypothesis that lipid peroxidation is an integral part of the events that cause tissue damage *(Lee et al., 1982).* On the contrary, *Krithika et al. (2009)* recently proved the involvement of lipid peroxidation on exposure to CcL_4.

As regards to oxidation of proteins, the present study was able to detect that there was a significant increase in the formation of carbonyl proteins in the CcL_4 treated cells comparing to control cells. Free radical-mediated oxidation of proteins results in the formation of carbonyl groups in quantities that reflect the intensity of the oxidative stress (*Robinson et al.; 1999*). Protein carbonyl content is widely used as both a marker for oxidative stress and a measure of oxidative damage (*Luo and Wehr, 2009*). Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains when they are oxidized. These moieties are chemically stable, which is useful for both their detection and storage (*Dalle-Donnea et al., 2003*). Protein oxidation may play a role in the pathogenesis of CcL_4 induced liver injury and that the accumulation of oxidized proteins may be an early indication of CcL_4 induced liver damage*(Sundari et al. 1997)*. The usage of protein (CO) groups as a marker may have some advantages in comparison with lipid peroxidation products because the formation of protein bound (CO) groups seems to be a common phenomenon of protein oxidation and because of the relatively early formation and stability of oxidized proteins *(Dalle-Donnea et al., 2003)*.

Apoptosis is characterized by morphological changes such as membrane "blebbing", nuclear condensation and fragmentation (Ramage, et al., 2006). These morphological changes are a result of a cascade of biochemical changes occurring within the cell, resulting in activation of caspases and DNA fragmentation (Hengartner, 2000). The present data indicate that along with oxidative damage, apoptosis plays a crucial role in CcL_4 induced hepatotoxicity which was indicated by marked increase in DNA fragmentation (increase in the percent of TUNEL positive cells) in the CcL_4 treated cells compared to control one. Apoptosis represents one of the mechanisms of cell death after CcL_4 induced liver injury (Shi et al, 1998).

Effects of antioxidants on oxidative damage in vitro systems are variable. In most cases, endogenous antioxidant enzymes, particularly catalase, are very effective in preventing damage. Exogenous antioxidant chemicals, on the other hand, can have a pro-oxidant effect at high doses. The chemical composition of culture media and the doses of antioxidants that can be applied in culture systems

are major factors in confounding interpretation of in vitro results (*Anderson and Phillips, 1999*).

Vitamin E antioxidant functions mainly in and around the membrane/lipid bilayers acting as the chain breaking antioxidants (*Janero and Burghardt, 1989*). Results of the present experiments showed that incubation of hepatocytes with vitamin E at concentrations of 100, 50 and 25 µM induced hepatoprotective effect against CcL_4 induced toxicity. This was manifested by an increase in the stability of the cell membrane and decreased leakage of cytoplasmic enzymes (LDH, ALT and AST). Although we cannot rule out a direct scavenging action of vitamin E on CcL_3 radical, inhibition of lipid peroxidation is not a likely explanation for vitamin E effects in the present study. Although the ability of CcL_4 to initiate lipid peroxidation in various biological systems has been equivocal, an important observation of the present study was that there was no evidence of CcL_4 -initiated lipid peroxidation.

Ascorbic acid, in the present study, was ineffective against the elevation of enzymes leakage (LDH, ALT and AST). Also it was not able at any concentration to reduce the oxidation of protein. Ascorbic acid did not show any protective effect against DNA damage caused by CcL_4. Similar results were obtained by *Winter et al. (2005)* who reported that ascorbic acid was ineffective at reducing oxidative DNA damage produced by camphorquinone which generates reactive oxygen species and causes oxidative DNA damage in vitro. On the contrary *Qin et al. (2006)* found that ascorbic acid reduced myocyte apoptosis and ameliorated myocardial damage after acute myocardial infarction. Park and Lee (2008) reported that high ascorbic acid concentrations might act as a pro-oxidant due to its auto-oxidizing properties.

Mannitol is generally accepted as being a hydroxyl radical scavenger (Van Zandwijk, 1995). Previous studies have used mannitol to determine whether hydroxyl radicals are the predominant species responsible for Copper mediated DNA damage (Li et al., 1995). However, these studies have found that mannitol is generally ineffective at preventing Cu- mediated oxidative damage in the presence of an ROS-generating system and at mannitol concentrations less than 100mM. The present results showed that mannitol did not effectively reduce CCL_4 induced hepatotoxicity. It showed no reduction in the leakage of cytoplasmic enzymes, protein oxidation or DNA damage. *Winter et al. (2005)* reported that mannitol treatments of 10.0, 5.0, 2.5, 1.0, and 0.5 mM did not influence the extent of DNA damage generated by camphorquinone. In explanation, it is possible that some ROS generated from CcL_4 are not effectively scavenged by mannitol. Additionally, mannitol may not be a very potent hydroxyl radical scavenger or mannitol may not have adequate access to the damaging ROS (*Winter et al., 2005*). *Shinar et al. (1983)* demonstrated that mannitol effectively scavenged hydroxyl radicals at concentrations greater than 1M.

In the present experiments, aminoguanidine (AG) treatment against CCL_4 induced hepatoxicity was ineffective in reducing the leakage of enzymes, the oxidation of protein, and the DNA damage. A notable finding was that AG at a dose of 400 µM significantly increased leakage of LDH into the media comparing with CcL_4 group. Our results confirmed reports of other investigators (*Ou and Wolff, 1993 and Skamarauskas et al., 1996*) that AG has pro-oxidant activity where it generates H_2O_2 at a low rate in vitro.

In conclusion, the present study indicates that CcL_4 has a potential cytotoxic effect in BRL3A cell culture, and apoptosis represents one of the mechanisms of cell death after CcL_4- induced liver injury. There was no evidence of CcL_4 -initiated lipid peroxidation. Vitamin E seems effective and provides complete protection against CcL_4 induced hepatotoxicity in culture cells. Ascorbic acid, mannitol and aminoguanidine were ineffective against CcL_4 toxicity. Aminoguanidine, in addition, generates H_2O_2 in vitro, so it has pro-oxidant activity.

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