Original Article

Bacterial Lipopolysaccharides Pretreatment Protects Against Mutagenic and Immunosuppressor Effects of Cyclophosphamide in Mice

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Abstract

The mutagenic and immunosuppressory effects of cyclophosphamide (CYP) are still the primary limitation to wider application for treating a variety of human malignancies. On the one hand, CYP treatment predisposes transplant recipients and cancer patients to risk of bacterial, fungal, and viral infections, in the other word the, Lipopolysaccharide (LPS), an endotoxin found in cell walls of gram-negative bacteria, has been shown to play a significant role in development a of multiple Immune system responses and can cause a fatal pathological effect. The present investigation is focused on immunization of mice with the endotoxin LPS prior to CYP treatment, in an attempt to reduce mutagenicity and immunosuppressory effects caused by CYP. The in vivo anti-cytotoxicity and anti-mutagenicity of the inflammatory agents of bacterial Lipopolysaccharides (LPS) isolated from Aeromonas hydrophila was evaluated by bone marrow chromosomal aberrations assay,differential white blood cells (WBCs) count and respiratory burst enzymatic assays for phagocytosis in mice exposed to CYP. The data presented in this article indicates that, treatment with low dose of bacterial LPS once a week for four weeks at a dose of ٠,٢ ml of LPS suspension (٥٠ µg/kg mice/week), was non-cytotoxic and un-mutagenic to the animal cells. However, pretreatment with low dose of bacterial LPS significantly increase cellular resistance to the mutagenic and immunosuppressory effects of CYP. In conclusion, this immunization protocol suggests that immunization of mice by LPS prior to CYP treatment may induce a number of adaptive antimutagenic and immune response molecular mechanisms.

Keywords:l ipopolysaccharide,cyclophosphamide ,antimutagenic ,immune response

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Introduction

The Cyclophosphamide (CYP) belongs to class of oxazaphosphorines and it is an alkylating agent extensively used as an anticancer chemotherapeutic agent for childhood[١] and adult malignancies[٢،٣] and other benign diseases [٤]. It also belongs to immunosuppressory drugs that widely used for amelioration of clinical symptoms of autoimmune diseases[٥،٦] and in combination with other compounds, in treatment of leukemias[٧, ٨] The CYP induces profound, transient leucopenia[٩] and neutropenia[١٠] and suppresses humoral[٩،١١] as well as cellular [١٢،١٣] immune response. So that, CYP treatment may lead to an increased susceptibility to microbial infections [١٤،١٥]

The Cyclophosphamide is metabolized in vivo to an alkylating intermediate compound ٤-hydroxycyclophosphamide which then spontaneously interconvert with its tautomer aldophosphamide, through an elimination mechanism, to acrolein and PM [١٦] . Both acrolein and PM are reactive species toward DNA. It has been reported that the covalent DNA adducts formed from PM are intra/inter-strand cross-link DNA adducts and mono adducts at the N٧ position of guanine [١٦] .While DNA adducts formed from acrolein are cyclic adducts between the N١ and exocyclic amino nitrogen's of deoxyguanosine in DNA [١٧] . The DNA adducts from PM and Caroline have been found in rodents after administration of cyclophosphamide[١٨] The In vivo genotoxic activities including chromosome aberrations, sister chromatid exchange, and gene mutation have been reported in both animals and humans after administration of cyclophosphamide [١،٣،١٦] .

The Lipopolysaccharide (LPS) is a potent inflammatory stimulus derived from the outer membrane of gram-negative bacteria. Release of LPS from dying bacteria can initiate a serious systemic inflammatory response to infection[١٩] . Bacterial LPS elicits a multitude of effects on the immune system, including cytokine production, increased of expression of cell adhesion molecules and proinflammatory mediator secretion by monocytes, macrophages and neutrophils, which are recruited into specific host tissues by systemic LPS exposure [٢٠،٢١]

The LPS also contributes to systemic changes seen in septic shock. The response of host to LPS is mediated by the immune modulating molecules such as tumor necrosis factor-a (TNF-a), members of interleukin (IL)family , reactive oxygen species (ROS), and lipids. Overproduction of those mediators induces tissue damage that precedes multiple organ failure [٢٢،٢٣]. Although there is great compositional variation among endotoxin derived from different bacterial serotypes, all endotoxins share a common structural principle. Endotoxins are amphiphilic molecules comprising a hydrophilic polysaccharide component and a covalently bound hydrophobic lipid component, termed lipid-A, a toxic portion of LPS [٢٢] A necessity for activation of cells by the LPS is its interaction with LPS-binding molecules. For example, the LPS binds to LPS-binding protein (LBP) through the lipid-A moiety, and the LPS–LBP complex is then recognized by CD١٤, which accelerates LPS priming of neutrophils. However, there are still various molecules responsible for LPS recognition and signal mediation in various cell types, and physiological relevance of these molecules [٢٠،٥٩] The objective of the present study was to investigate whether immunization of mice with low dose of the inflammatory agents, such as bacterial Lipopolysaccharides (LPS) isolated from Aeromonas hydrophila, may severely diminish resistance to the mutagenic and immunosuppressory effects of cyclophosphamide.

Materials and Methods

Chemicals

The Cyclophosphamide (CYP) and Colchicine were obtained from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France). All other chemicals used in the study were analytical grade.

Animals

Eighty adult male Swiss mice, weighed from ٢٥-٣٠ g were purchased from the Biological Supply Center, Theodore Bilharz Research Institute (TBRI, Cairo, Egypt). The Housing was at ٢٥-٢٨ oC with light from ٨:٠٠ to ٢٠:٠٠ h with free access to water and housed in stainless-steel cages in a pathogen-free University Laboratory Animal Research facility.

Grouping and sampling

Animals were classified into four groups as following: the first group (normal) remained as a control group, and was intra-peritoneal (i.p.) injected with PBS (pH ٧,٤; ٠,٢ ml/mice) at intervals parallel to the treated groups. The second group (CYP) was injected i.p. once with the cyclophosphamide at dose ٥٠ mg/Kg B. wt [٢٤] . The third group (LPS) was injected i.p. with the lipopolysaccharide (LPS) (extraction of LPS is shown below) of Aeromonas hydrophila once per week for four weeks at a dose of ٠,٢ ml of LPS suspension (٥٠ g/kg mice/week). The fourth group (LPS+CYP) was injected with LPS suspension dose once a week for four weeks then injected i.p. with the desired single dose of CYP.

After ٢٤ & ٤٨ hours post-injection with the PBS, CYP or LPS half of the animals (٤٠ mice), five animals for each group, were sacrificed under mild diethyl ether anesthesia. Blood samples were divided into two portions, one portion was added into heparinized tubes for WBCs counting and NBT estimation, while another portion was centrifuged at ٣٠٠٠ r.p.m. for ٢٠ minutes. Also, Liver slices were homogenized in PBS solution (pH ٧,٤) and kept at -٢٠ ٠C until used for the estimation of the respiratory burst enzymes activities. While other half of the animals (٤٠ mice) was used for cytogenetic studies. They were injected with colchicine (٤ mg/kg b.wt.) i.p. after ٢٢ or ٤٦ hours from the last injection in each group prior to sacrifice after the last injection.

Preparation of crude Aeromonas hydrophila endotoxin

The strain used in this study was identified as Aeromonas hydrophila (A-٤٧) as a subculture slant from that purchased from American Type Cell Culture, a Globule Biosource center, the USA (ATCC; Cat. # ٧٩٦٦) then kindly provided to the author by Microbiology Dept., Faculty of Veterinary, Beni-Sueif University, Beni-Sueif, Egypt.

The crude endotoxin of Aeromonas hydrophila was prepared according to Schill et al. [٢٥] and modified by Austin & Austin[٢٦] . In brief, Aeromonas hydrophila bacteria were subcultured onto nutrient agar medium at ٢٥ ° C for ٧٢ hrs. The subcultured

strain was inoculated into ١٥٠ ml of a liquid medium containing ٢,٥ % peptone, ١,٠٪ yeast extract, ٠,٥٪ NaCl; ٠,٢٥٪ KHPO٤ and ٠,٠٠٠٢٪ CaCl٢ ٢H٢O, ٠,٠٠٠٢٪ MnSO٤ ٦H٢O and ٠,٠٠٠٢٪ CaCl٢ (pH ٧,٠) in an Elemner flask (٥٠٠ ml) and was incubated at ٢٥ ° C for ٢٤ hrs with reciprocal shaking. The culture fluid was transferred into ٥٠٠ ml of the same medium in a shaken flask (٢ Liters) then incubated at ٢٥ ° C for ٢٧ hrs with reciprocal shaking. Bacteria were harvested by centrifuging at ٦٠٠٠-xg for ١٠ min and washed twice with PBS. The result bacterial pellet was resuspended in PBS and frozen at -٢٠ °C.

Extraction and purification of Lipopolysaccharide (LPS)

Extraction of LPS from the frozen bacterial pellet was performed using the phenol-water method of Westphal and Jann [٢٧]. Briefly, one gram of dry bacterial cell mass (A. hydrophila) was mixed with ١٢,٥ ml of phenol and ١٢,٥ ml of water. The mixture was incubated in water bath at ٦٠ ° C with continuous mixing for ١٥ min., then incubated at room temperature for another ١٥ min with continuous mixing, and finally kept for ١ hr at ٥°C. After the previous incubation periods, the obtained mixture was centrifuged at ٢٥٠٠ rpm for ١٥ min. The upper layer (water phase) was carefully removed and kept at ٤ ° C. The same volume of water was added to phenol phase and mixed again and all previous steps were repeated. After last centrifugation, the water phase was removed and combined with the first one and dialyzed overnight against distilled water with several changes and then concentrated by ultra-filtration. The extract was incubated at ٣٧ ° C for ٤ hrs with a ٠,٨٥ ml of ٠,١M Potassium phosphate buffer pH ٧, ٠,٠٢ ml of ١M MgCl٢, ٠,٣M CaCl٢ and containing ٠,٨ mg of DNase and ٠,٤ mg RNase. The LPS was purified from nucleic acids by ultracentrifugation at a speed of ١٠٠٠٠٠ xg for ٦ hrs at ١٠ C. Supernatant including nucleic acids was removed, while the precipitated LPS was collected and lyophilised until use. The Lipopolysaccharides (LPS) was reconstituted in sterile saline (٠,٩٪ NaCl), then divided into aliquot and immediately stored at -٧٠ °C until use. Groups of ١٠ mice (LPS and LPS+CYP groups) were injected intraperitoneally with the LPS at total dose of ٥٠ μg/kg body weight

Total and Differential White blood cells (WBCs) Count

The total white blood cells (WBCs) count was carried out by Neubaur chamber, using Turk's solution for WBCs count as well as differential leukocytic count was calculated according to Dacie and Lewis [٢٨] .

Nitroblue tetrazolium (NBT) index and activity (phagocytosis)

Phagocytic ability of neutrophils (mg format NBT reduction per one ml blood and NBT index) was performed according to Siwicki[٢٩] . Briefly, ٠,١ ml of each heparinized blood sample was mixed with ٠,١ ml of ٠,٢ % NBT solution in sterile plastic test tubes for ٣٠ minutes at room temperature. Extinction was determined in ١ cm cuvettes at wave length ٥٤٥ nm (spectrophotometer Specoll ١١ Carl Zeiss, Jena). Values of extinction were transposed according to a standard curve into mg NBT format per ١ ml of blood. Simultaneously, the total number of the leukocytes was examined in order to calculate the absolute number of blood neutrophils. The NBT index was determined from the following equation:

$$\text{NBT index} = \frac{\text{mg of NBT formate/one ml blood}}{\text{Neutrophils count in thousands}}$$

Respiratory Burst Enzymatic Assays for Phagocytosis

A part of liver (٠,٥ g) was ice-cooled, homogenized in ٥ ml phosphate buffer pH ٧,٤ (٢٠٪ w/v). The homogenate was centrifuged at ٣٠٠٠٠-xg for ١٥ min at ٤ °C. The supernatant was collected and preserved at -٢٠ °C until used. Catalase activity was analyzed according to the method described by Cohen et al [٣٠]. and modified by Sinha[٣١] by monitoring the enzyme-catalyzed decomposition of hydrogen peroxide using potassium permanganate. Briefly, ٠,٠٥ ml of each sample (or tissue homogenate) was mixed with ٠,٢ ml of ٢M H٢O٢ in a test tube and incubated on ice for ٣ min. ١N H٢SO٤ was used to stop the reaction. Finally, KMnO٤ solution was added and absorbance was recorded at ٤٨٠ nm. In this assay, ١ unit of enzyme activity equals k/(٠,٠٠٦٩٣), where k = log (S٠/S٢) x (٢,٣/t), whereas S٠ = absorbance of standard – absorbance of blank, S٢ = absorbance of standard – absorbance of sample, and t = time interval.

On the other hand, total peroxidase activity in liver tissue homogenate was estimated according to the method described by Kar & Mishra[٣٢] . Heme peroxidases (myeloperoxidase and eosinophil peroxidase) activity in the liver of normal and treated mice was measured using a pyrogallol as a

substrate in presence of H٢O٢ according to the method of Joseph et al. [٣٣]. Briefly, ٢ ml phosphate buffer solution (pH ٦,٨), ٠,١ ml ١,٤٪ pyrogallol and ٠,١ ml ١,١ mmole H٢O٢ were added to ٠,٠٢٥ ml of hepatic supernatant. After exactly ٥ minutes, color density of formed purpurogallin was measured against the blank by taking absorbance at ٤٢٠ nm. The enzyme activity (in units) for each sample was obtained from the standard curve performed by using different dilutions of heme peroxidase (EC.١,١١,١,٧) (Sigma-Aldrich Company, USA).

The SOD [EC ١,١٥,١,١] activity was measured in hepatic supernatant according to the method described by Zou et al[٣٤] , based on the inhibition of pyrogallol auto- oxidatiion. Briefly, an aliquot of cell extract was mixed with Tris-cacodylic buffer (٥٠mM Tris-HCl, ٥٠ mM cacodylic acid, ١mM diethylenetriamine pentaacetic acid, pH ٨,٢), and ٢mM pyrogallol. The auto-oxidation of pyrogallol and inhibition of this reaction were monitored with spectrophotometer. Under the conditions of this assay, ١ unit of superoxide dismutase activity is equivalent to the amount of enzyme that produces a ٥٠٪ inhibition of pyrogallol auto- oxidation. The rate of pyrogallol's autoxidation was manipulated to be ٠,٠٧٠ O.D./min, and ٥٠% depression of autoxidation rate of pyrogallol by SOD was known as ١ U.

Preparation of mice bone marrow cells

Bone marrow cell preparations for the analysis of chromosomal aberrations and mitotic index were produced by colchicine–hypotonic technique. Potassium chloride (٠,٠٧٥ M) was used in this technique.

Table ١: Changes in total and differential leukocytic counts in male Swiss mice injected with Lipopolysaccharide and cyclophosphamide.

Parameters	Groups	Sampling Time	
		٢٤ hours	٤٨ hours
Total WBCs (Cells X ١٠٣)	Normal	٥,٢٦٠ ± ٠,٣٤٤ a	٥,٣٤٠ ± ٠,٣٨٤ a
	CYP	٢,٩٦٠ ± ٠,٣٦١ c	٢,٢٠٠ ± ٠,١٥٤ b
	LPS	٤,١٤٠ ± ٠,٤٤٥ b	٥,٦٨٠ ± ٢,٤٣٥ a
	LPS + CYP	٥,٧٠٠ ± ٠,٤١٩ a	٤,٠٤٠ ± ٠,٧٤٠ a
	P > F	٠,٠٠٠١	٠,٠٠٢٤
	LSD at ٥٪	٠,٥٩٢	١,٧٢٩
	LSD at ١٪	٠,٨١٥	٢,٣٨٢
Neutrophils Cells %	Normal	٤,٦٠٠ ± ٢,٤٠٨ c	٤,٤٠ ± ٢,١٩٠ b
	CYP	٥,٦٠٠ ± ٢,١٩٠ bc	٤,٦٠ ± ١,٥١٦ c
	LPS	١٢,٨٠ ± ١,٩٢٣ a	١٦,٠٠ ± ٤,٠٠٠ a
	LPS + CYP	٨,٨٠٠ ± ٣,٣٤٦ b	٩,٤٠ ± ٣,٥٠٧ c
	P > F	٠,٠٠٠٤	٠,٠٠٠١
	LSD at ٥٪	٣,٣٨٥	٣,٩٨٨
	LSD at ١٪	٤,٦٦٤	٥,٤٩٥
Eosinophils Cells %	Normal	١,٠٠ ± ٠,٧٠٧ a	٠,٦٠ ± ٠,٥٤٨
	CYP	٠,٨٠ ± ٠,٨٣٦ ab	٠,٦٠ ± ٠,٨٩٤
	LPS	١,٦٠ ± ٠,٨٩٤ a	٠,٦٠ ± ٠,٥٤٨
	LPS + CYP	٠,٠٠ ± ٠,٠٠٠ b	٠,٨٠ ± ٠,٨٣٨
	P > F	٠,٠١٩٩	٠,٨٥٧
	LSD at ٥٪	٠,٩٤٨	------
	LSD at ١٪	١,٣٠٦	------
Lymphocytes Cells %	Normal	٨٤,٢٠ ± ١,٠٩٥ a	٨٤,٦٠ ± ١,٥١٦ a
	CYP	٨٣,٨٠ ± ٣,٧٠١ a	٨٦,٨٠ ± ٣,٨٩٨ a
	LPS	٧٧,٠٠ ± ٣,٣٩١ c	٧٥,٤٠ ± ٧,٠٢١ b
	LPS + CYP	٨٠,٨٠ ± ٤,٣٨١ ab	٨١,٨٠ ± ٦,٤١٨ ab
	P > F	٠,٠١٤٠	٠,٠١٨
	LSD at ٥٪	٤,٥٢٧	٦,٩٦٧
	LSD at ١٪	٦,٢٣٧	٩,٥٩٩
Monocytes Cells %	Normal	١٠,٢٠ ± ١,٤٨٣	١٠,٤٠ ± ٢,١٩١
	CYP	٩,٨٠ ± ٢,٠٤٩	٨,٠٠ ± ٣,١٦٢
	LPS	٨,٦٠ ± ٢,٤٠٨	٨,٠٠ ± ٣,١٦٢
	LPS + CYP	١٠,٤٠ ± ٢,١٩٠	٨,[illegible] ± ٦,١٦٤
	P > F	٠,٥٣٠٣	٠,٧١٥
	LSD at ٥٪	------	------
	LSD at ١٪	------	------

After completion of the treatment period, five animals from each group were sacrificed at sampling time of ٢٤ h and ٤٨ h, by cervical dislocation, the Colchicine was given at the dose of ٤ mg/Kg b.wt. intraperitoneally at ٢٢ and ٤٦ hrs respectively prior to sacrificing the animals. The bone marrow smears of animals in each group were prepared according to Preston et al[٣٥] protocol. Slides were stained with Giemsa and ٥٠ well spread metaphase plates/animal were analyzed for chromosomal aberrations and incidence of aberrant cells (in percentages) for each group. The mitotic index was obtained by counting the number of mitotic cells in ١٠٠٠ cells/animal.

Also percentage of suppressed aberrant cells was calculated according to Shukla and Taneja,[٣٦]as follows:

Statistical Analysis

$$١٠٠ - \left\{ \frac{\text{\% of aberrant cells in group IV (LPS+CYP)}}{\text{\% of aberrant cells in group II (CYP)}} \times ١٠٠ \right\}$$

Statistical analysis for difference in the mean number of chromosomal aberrations and mean mitotic index between groups were obtained by using student-t-test (P<٠,٠٥ was considered

Table ٢: Changes in Phagocytic index and some respiratory burst enzymes (antioxidant enzymes) in male Swiss mice injected with Lipopolysaccharide and cyclophosphamide.

Parameters	Groups	Sampling Time	
		٢٤ hours	٤٨ hours
Phagocytic activity NBT format (mg/ml blood)	**Normal**	٨,٠٤ ± ١,٣٤٤ bc	٩,٠٨ ± ١,٠٤٢ b
	CYP	٦,٧٦ ± ٠,٤٠٢ c	٧,٥٢ ± ٠,٨٠٧ b
	LPS	١١,٤٤ ± ٢,١٦٥ b	١٤,٢٦ ± ٢,٩٩٦ a
	LPS + CYP	٨,٨٦ ± ١,٠١٨ a	١٣,٧٢ ± ٣,١٢٩ a
	P > F	٠,٠٠٢	٠,٠٠٠٤
	LSD at ٥٪	٢,٠٦٦	٣,٠٣٦
	LSD at ١٪	٢,٨٤٧	٤,١٨٣
NBT index (%) X ١٠ $^{-٣}$	**Normal**	٣٠,٣٥٣ ± ١٢,٤٦٦	٣٤,٢٤٢ ± ١١,٨٠٧ bc
	CYP	١٧,١٦٣ ± ٣,٥٩٨	١٦,٥٤١ ± ٤,٠٨٩ c
	LPS	٣٥,٨٣٧ ± ١١,٨٥٨	٨٠,٦٨٤ ± ٢٥,١٦٥ a
	LPS + CYP	٢٧,٣٥٧ ± ١٦,٠٣٠	٤١,٩٩٦ ± ١٥,٦٧٦ b
	P > F	٠,١٣١٢	٠,٠٠٠١
	LSD at ٥٪	------	٢١,٥٦٩
	LSD at ١٪	------	٢٩,٧١٩
Catalase (U/g)	**Normal**	٤,٤٣١ ± ٠,٥٧٣ a	٤,٧٨٤ ± ٠,٦٢٠ a
	CYP	٢,٤٩٥ ± ٠,٥٦٤ b	٢,٦٠٦ ± ٠,٦٠٢ c
	LPS	٤,٤٣٢ ± ٠,٨١٤ a	٥,٠١٦ ± ٠,٨١٣ a
	LPS + CYP	٣,٦٦٧ ± ٠,٥٨٩ a	٣,٨٨٧ ± ٠,١٧١ b
	P > F	٠,٠٠٠٢	٠,٠٠٠١
	LSD at ٥٪	٠,٧٦٨٥	٠,٨٠٤
	LSD at ١٪	١,٠٥٨٩	١,١٠٧
Peroxidase (U/ml)	**Normal**	١١,٠٩٢ ± ١,٢٨٠ a	١١,٠٩٢ ± ١,٢٨١ a
	CYP	٨,٥٩٣ ± ٠,٧٩٥ bc	٤,٠٩٩ ± ٠,٢٦٦ c
	LPS	١٠,٥٨٦ ± ٢,٠١٤ ab	٩,١٧٩ ± ٠,٤٩١ b
	LPS + CYP	٧,٨٣٩ ± ٢,٣١٨ c	٩,٠٠٦ ± ٢,١٩٥ b
	P > F	٠,٠٢٣٥	٠,٠٠٠١
	LSD at ٥٪	٢,٢٩٤٠	١,٧٤٤٤
	LSD at ١٪	٣,١٦٠٢	٢,٤٠٣٥
Superoxide dismutase (U/g)	**Normal**	٢٤,٨١٣ ± ٧,٤٣٧ a	٢٧,٠٩٨ ± ٤,٩٥١ b
	CYP	١٣,٤٧٥ ± ٤,١٦٣ b	٢١,١٣٥ ± ٤,١٣٨ b
	LPS	٢٦,٢٥٣ ± ٣,٤٤٨ a	٣٢,٠٨٠ ± ٧,٦٠٦ a
	LPS + CYP	١٦,٠٢٣ ± ٢,٥٠٨ b	٢٩,٣١٤ ± ٣,٧٩٢ a
	P > F	٠,٠٠١	٠,٠٣٥
	LSD at ٥٪	٦,٣٨٩٠	٧,٢٨٧
	LSD at ١٪	٨,٨٠٣	١٠,٠٤٠

significant).

While in case of differential white blood cells (WBCs) count and respiratory burst enzymatic assays for phagocytosis the data was analyzed using analysis of variance (ANOVA) [٣٧]followed by LSD analysis to evaluate variations between groups and for multiple comparisons between different groups. The Results are expressed as mean + standard deviation (S.D.). Values of P> ٠,٠٥ were considered as statistically non-significant, while values of P< ٠,٠٥ were considered as statistically significant.

Results

Table -١ recorded the changes in total white blood cells (WBCs) count and showed a decrease in cyclophosphamide (CYP) injected groups with a significant decrease at ٢٤ and ٤٨ hours with P< ٠,٠٥ as estimated by ANOVA test, while a significant decrease and non-significant increase in endotoxin infected groups (LPS) at ٢٤ and ٤٨ hours respectively were recorded. Also, a non-significant increase in LPS+CYP groups as compared with their corresponding control animals was reported. Moreover, studies on stained blood films of male Swiss mice infected with the LPS showed a significant increase in neutrophilis and eosinophilis at ٢٤ and ٤٨ hours except at ٤٨ hours for eosinophils with a non-significant degree (P< ٠,٠٥). Also, a significant decrease in lymphocytes and monocytes in LPS infected animals at ٢٤ and ٤٨ hours were observed. However, in CYP injected groups non-significant decreases in eosinophilic, lymphocytic and monocytic cells were recorded. Also, non-significant increases in neutrophils at ٢٤ and ٤٨ hours were observed.

Regarding the phagocytic ability of neutrophils as represented by NBT format (mg/ml blood) and NBT index reported at Table (٢), it was showed that NBT format was decreased in CYP group and increased in LPS group with highly significant changes (P< ٠,٠٠١) at ٢٤ and ٤٨ hours, while the administration of LPS+CYP elevated the activity in comparison with the control and CYP groups but still less than that in the LPS group. On the other hand, the NBT index (%) was parallel to behaviour of NBT activity and recorded a very highly significant increase in the NBT index within the LPS and LPS+CYP groups (P< ٠,٠٠١) all over the experiment intervals.

Concerning catalase, peroxidase and SOD synthesis by the hepatic tissue, Table (٢) illustrated that the CYP induced a very highly significant decrease (P< ٠,٠٠١) in synthesis of these enzymes all over the experiment intervals. On the other hand, LPS-infected mice exhibited a significant decrease in peroxidase and a non-significant increase in Catalase and SOD activities as compared to control group all over the experimental periods. While the concomitant administration of CYP to the LPS-infected mice induced significant increase in synthesis of catalase, peroxidase and SOD in comparison with the CYP group but still less than that in the control and LPS groups.

The cytogenetic results illustrated in Tables (٣&٤) revealed that, the CYP when is given at a single dose of ٥٠ mg/kg. b.w., cause a high incidence of chromosomal aberrations in Swiss albino mice. The mitotic index was decreased by ٤٦,٤٧٢٪ at ٢٤ hours over controls (P < ٠,٠٥), indicating bone marrow cytotoxicity. However, the LPS alone induced a very low number of chromosomal aberrations as compared to the CYP all over the experimental period significantly confirming its non-mutagenicity (Table ٣). The LPS was also not found to be cytotoxic, as there was no significant changes in the mitotic index over negative control group (٧٤,٥٧٩٪ at ٢٤). Moreover, when pre-treatment of LPS was given prior to CYP treatment, decreased rates of clastogenic changes were observed (Tables ٣ & ٤). All types of chromosomal aberrations induced by the CYP including Chromatid breakages, centromeric attenuations, end to end associations, centric fusion, fragments and other multiple damages were found to be reduced by LPS pre-treatment. The status of mitotic index was found to be increased significantly, ٧٠,٣١٠٪ (P < ٠,٠٥) at ٢٤ hours, indicative of its anti-cytotoxicity toward the CYP. The percentage of aberrant cells which were found to be ٥٥,٢٠ + ٢,٩٩٣ at ٢٤ hours, in CYP treated animals, was reduced significantly to ٣٨,٨٠ + ٢,٧١٣ by LPS pre-treatment (Table ٤).

During second phase of cell cycle (٤٨ hrs sampling time) the incidence of aberrant cells in the positive control group (CYP treated group), was found to be relatively low (٤٢,٨٠ %, P < ٠,٠٥) but significantly higher than the control group (Table ٤). The cytotoxic potential of CYP was still evident in CYP treated animals, as there was significant decrease in mitotic index (٥٦,٩٤٩٪, P < ٠,٠٥). In LPS treated animals, a very low significant increase in aberrant cells and non significant decrease in the mitotic index were observed when compared to negative control group, further indicating a non-mutagenic and non-cytotoxic response of LPS (Table ٤). Different types of chromosomal damage caused by the CYP were decreased significantly in LPS+CYP treated animals (Table ٣). The incidence of aberrant cells was found to be ٤٢,٨٠ + ٣,٩١٩ in the CYP treated group, but

declined to ۳۰,۸۰ + ۲,۰۳۹ in the LPS+CYP treated group.

Table ٣: protective effect of LPS pre-treatment on Cyclophosphamide induced different types of chromosomal aberrations in Swiss male mice bone-marrow cells.

	Groups	Number of examined cells	Structural Chromosomal Aberrations						Numerical Chromosomal Aberrations		
			Chromatid Breakage	**Centric Attenuation**	**Centric Fusion**	**End to end Association**	**Total of Structural Aberrations**	**Number of cells with fragmented chromosomes**	**Polyploidy**	**Endomitosis**	**Total of Numerical Aberrations**
٢٤ Hours	**N**	٢٥٠	١٩ (٠,٠٧٦)	-----	١ (٠,٠٠٤)	٣ (٠,٠١٢)	٢٣ (٠,٠٩٢)	-----	١ (٠,٠٠٤)	٧ (٠,٠٢٨)	٨ (٠,٠٣٢)
	CYP	٢٥٠	١٠٨ (٠,٤٣٢)	١ (٠,٠٠٤)	١٦ (٠,٠٦٤)	٢٠ (٠,٠٨٠)	١٤٥ (٠,٥٨٠)	٣٠ (٠,١٢٠)	١٥ (٠,٠٦٠)	٦ (٠,٠٢٤)	٢١ (٠,٠٨٤)
	LPS	٢٥٠	٢٨ (٠,١١٢)	٣ (٠,٠١٢)	٣ (٠,٠١٢)	٦ (٠,٠٢٤)	٤٠ (٠,١٦٠)	-----	٧ (٠,٠٢٨)	١٢ (٠,٠٤٨)	١٩ (٠,٠٧٦)
	LPS+CYP	٢٥٠	٥٥ (٠,٢٢٠)	٢ (٠,٠٠٨)	٣ (٠,٠١٢)	١٧ (٠,٠٦٨)	٧٧ (٠,٣٠٨)	٣٦ (٠,١١٤)	٧ (٠,٠٢٨)	٦ (٠,٠٢٤)	١٣ (٠,٠٥٢)
٤٨ H٠urs	**N**	٢٥٠	٢١ (٠,٠٨٤)	----	----	١ (٠,٠٠٤)	٢٢ (٠,٠٨٨)	----	٢ (٠,٠٠٨)	٨ (٠,٠٣٢)	١٠ (٠,٠٤٠)
	CYP	٢٥٠	١٠٣ (٠,٤١٢)	٣ (٠,٠١٢)	٥ (٠,٠٢٠)	٢٢ (٠,٠٨٨)	١٣٣ (٠,٥٣٢)	٢٥ (٠,١٠٠)	١١ (٠,٠٤٤)	٦ (٠,٠٢٤)	١٧ (٠,٠٦٨)
	LPS	٢٥٠	٣١ (٠,١٢٤)	٢ (٠,٠٠٨)	----	٤ (٠,٠١٦)	٣٧ (٠,١٤٨)	----	٨ (٠,٠٣٢)	١٠ (٠,٠٤٠)	١٨ (٠,٠٧٢)
	LPS+CYP	٢٥٠	٥١ (٠,٢٠٤)	١ (٠,٠٠٤)	١ (٠,٠٠٤)	١٣ (٠,٠٥٢)	٦٦ (٠,٢٦٤)	١٧ (٠,٠٦٨)	١٠ (٠,٠٤٠)	٨ (٠,٠٣٢)	١٨ (٠,٠٧٢)

Number of metaphase cells analyzed per animal group = ٢٥٠ cells.

Table ٤:P rotective effect of LPS pretreatment on Cyclophosphamide induced changes in number of chromosomal aberrations /cell,incidence of aberrant cells and mitotic index in Swiss mice bone-marrow cells.

	Groups	Mitotic Index [a]	Incidence of aberrant cells [a] (%)	Number of aberrations/Cell [a]	Suppressive effect (%)
٢٤ Hours	N	٨٢,٨٣٩ ± ٥,٩٧٧	١٢,٤٨٠ ± ١,٤٩٦	٠,٠٢٥٠ ± ٠,٠٠٢٩	
	CYP	٤٦,٤٧٢ ± ١٢,٢٨٥ b	٥٥,٢٠٠ ± ٢,٩٩٣ b	٠,١٦١٠ ± ٠,٠١٨٠ b	
	LPS	٧٤,٥٧٩ ± ٩,٨٧٧ c	٢١,٦٠٠ ± ٣,٤٤١ bc	٠,٠٤٨٠ ± ٠,٠٠٨٠ bc	
	LPS+CYP	٧٠,٣١٠ ± ٥,٣٧٩ c	٣٨,٨٠٠ ± ٢,٧١٣ c	٠,١٠١٢ ± ٠,٠٠٩٠ c	٢٩,٧١٠
٤٨ Hours	N	٨٣,٧٥٢ ± ٤,٨٥٩	١٢,٨٠٠ ± ٢.٩٩٣	٠,٠٢٦٠ ± ٠,٠٠٥٩	
	CYP	٥٦,٩٤٩ ± ٤,٣١٥ b	٤٢,٨٠٠ ± ٣,٩١٩ b	٠,١٣٣٠ ± ٠,٠١٩٠ b	
	LPS	٦٩,٩٧٠ ± ٦,٥٩٠ c	١٩,٦٠٠ ± ٢,٦٥٣ bc	٠,٠٤٥٠ ± ٠,٠٠٦٤ bc	
	LPS+CYP	٧٠,٤٩٣ ± ٣,٦٥٩ c	٣٠,٨٠٠ ± ٢,٠٣٩ c	٠,٠٨٢٠ ± ٠,٠٠٤١ c	٢٨,٠٣٧

Number of metaphase cells analyzed per animal group = ٢٥٠ cells
[a] Values represent mean ± S.E. of five animals.
[b] Significantly different from untreated control (N) *P*< ٠,٠٥.
[c] Significantly different from positive control (CYP) *P*< ٠,٠٥.

The chromosomal and chromatid type aberrations per cell in LPS+CYP treated animals were also inhibited significantly (٠,٠٨٢٠ + ٠,٠٠٤١, P < ٠,٠٥). the Mitotic index in the LPS+CYP treated group at ٤٨ hrs sampling time, when compared with the CYP treated group was found to be increased by ٧٠,٤٩٣% P < ٠,٠٥).

However, as a result of decease in the number of aberrations per cell, both chromosome and chromatic type, were observed in LPS pre-treated and CYP post-treated groups, the inhibition capacity (calculated suppressive effect) of ٢٩,٧١٠% and ٢٨,٠٣٧% (at ٢٤ and ٤٨ hrs respectively) of LPS pre-treatment against CYP induced cytogenetic damage was recorded (Table ٣).

Discussion

According to aforementioned results, the major finding of the present study was as follows: Pre-treatment with a low dose of Aeromonas hydrophila LPS induces a number of adaptive antimutagenic and immune response molecular mechanisms, which increases the cellular resistance to the mutagenic and the immunosuppressory effects of Cyclophosphamide (CYP). Previous studies indicated that, such mechanisms include stimulation of Kupffer cells and macrophages[٣٨،٣٩] release of cytokines [٤٠،٤١،٤٢] activation of the transcriptional factors such as nuclear factor kappa B and AP-١ [٤٣], induction of the acute phase protein such as metallothionein [٤٤], induction of the antioxidant enzymes such as manganous superoxide dismutase [٤٥], and stimulation of tissue repair machinery[٤٦] .

The data presented in this article indicate that, treatment with a low dose of LPS induces a significant increase in phagocytic and killing activities by macrophages and neutrophils, a significant increase in respiratory burst enzymes activities and a very low significant increase in the frequency of aberrant cells. Therefore it seems reasonable to assume that treatment with the LPS at low doses was non-cytotoxic and un-mutagenic over the animal cells. These investigations agree with some of earlier studies conducted with the microbial LPS. Contrary to potentiation of hepatotoxicity when the LPS was administered simultaneously with hepatotoxicants [٤٧،٤٨], a sub-lethal dose of LPS pretreatment (٢٤h) is hepatoprotective. Indeed, a low dose of LPS, IL-١, or TNF-α induces a number of adaptive mechanisms, which in affects the magnitude and progression of subsequent toxic insults in turn[٤٠،٤١،٤٤،٤٩] . Also, Chorvatovicova et al.[٥٠]) reported that, cell wall polysaccharides isolated from yeasts and fungi possess ability to enhance immune system of higher organisms. Pharmacologically they are classified as biological response modifiers (BRM) which revealed high inhibitory activity against murine sarcoma tumors.

Also, the present study indicated that animals treated with single dose of CYP showed several-fold increase in the frequency of aberrant cells, decrease in the mitotic index, decrease in phagocytic index and decrease in some respiratory burst enzymes activity. This agrees with the previous investigations which reported the ability of CYP to react with electron rich areas of the susceptible molecules such as nucleic acid and proteins [٥١]. Therefore, CYP targets rapidly dividing cells, disrupting cell growth, mitotic activity, differentiation, and functions via alkylation of DNA at the $N^{٧}$ position of guanine [١٦]. The cytotoxicity mediated by formation of DNA-DNA cross-links, DNA-protein cross-links, and single-strand

breaks is greater than what in other drugs; however, many damaged cells survive exposure to cyclophosphamide [١٦،٥١]. The nuclear damage is responsible for mutagenicity while the effect on proteins will further aggravate the malfunction of host cell[٥٢]. Also it has been used to evaluate the mutagenic as well as anti-mutagenic agents and was reported to induce chromosomal damage and micronucleus formation in rats, mice, Chinese hamsters and even in transgenic mouse [٥١]

However, the results of the present investigation exposed that, administration of LPS at low dose (٥٠ mg/kg mice/week) for four weeks before treatment with a single dose of CYP (٥٠ mg/Kg B. wt) revealed the immunomodulatory activity and the antimutagenic potential of LPS against the mutagenic and immunosuppressor effects of CYP. I did not succeed in finding any previously published data on the antimutagenic effects of LPS in the literature. So that, the results of the present investigation confirmed the antimutagenic effect of LPS isolated from Aeromonas hydrophila against CYP in mice. Administration of LPS prior to CYP injection decreased significantly the frequencies of chromosomal aberrations and increased the mitotic index in bone marrow compared to the CYP-treated group.

The most serious and frequent complication of CYP chemotherapy is suppression of the immune system, immunological dysregulation, and increase about intracellular amount of reactive oxygen species and glutathione depletion, such compounds can exert clastogenic effects, especially by acting as spindle inhibitors, thereby causing c-anaphasis (abnormal mitosis) and consequently aneuploidy and/or polyploidy [٥٣-٥٧]. Based on above, it seems reasonable to assume that pre-transplantation and cancer chemotherapy immunization, aim to down regulate the inflammatory effect of endotoxins and the mutagenic effect of such anticancer alkylating agents. Likewise, stimulation of the immune system by the LPS could induce changes in macrophages, antigen-presenting cells, regulatory T cells, natural killer (NK) cells and NKT cells.

In summary, this study shows that LPS pretreatment protects mice, from clastogenic and immunosuppressory effects produced by the CYP. However, there could be other simple potential explanations for the anti-mutagenic and immunomodulatory effects observed following administration of low dose of LPS prior to CYP treatment: (١) LPS-mediated exhaustion and stimulation of the innate immune system response. These nonlethal toxic effects of LPS resulting in desensitization and reduced xenobiotic bioactivation could potentially increase the immune system response of CYP treated animals to the effects of LPS and other endotoxins in gram-negative bacteria. (٢) the LPS increases induction of the antioxidant enzymes like catalase, peroxidase and Superoxide dismutase. The activities of these enzymes may decrease intracellular amount of reactive oxygen species (ROS), which lead to decrease in the frequencies of chromosomal aberrations and increase in the mitotic index of bone marrow cells. These explanations are in a good agreement with the literatures [٢٠, ٤٤،٤٢،٥٨]

Finally, the present investigation concluded that severity of anticancer chemotherapeutic and immunosuppressive agents may be reduced by immunizing the cancer-treated patients and the recipients or donors prior to organ transplantation, with a sub-lethal dose of Gram-negative bacteria lipopolysaccharide (LPS).

References

١. *Thomson AB, Critchley HO, kelnar C, et al. Late reproductive sequelae following treatment of childhood cancer and options for fertility preservation. Best Pract. Res Clin Endocrinol Metab.* ٢٠٠٢; ١٦: ٣١١-٣٤.

٢. *COLVIN OM. An overview of cyclophosphamide development and clinical applications. Curr Pharm.* ١٩٩٩; ٥: ٥٥٥-٦٠.

٣. *Triphaty DN, JenaGB . Astaxanthin inhibits cytotoxic and genotoxic effects of cyclophosphamide in mice germ cells. Toxicol.* ٢٠٠٨; ٢٤٨(٢-٣): ٩٦-١٠٣.

٤. *Jorge I, Gonzalez B, Amadeu C,et al. The Mutagenic Potential of the Furylethylene Derivative ٢-Furyl-١-nitroethene in the Mouse Bone Marrow Micronucleus Test. Toxicol Scien.* ٢٠٠٣; ٧٢: ٣٥٩-٦٢.

٥. *Mok CC, Ho CT, Chan KW, et al. Outcome and prognostic indicators of diffuse proliferative lupus glomerulonephritis treated with sequential oral cyclophosphamide and azathioprine. Arthritis Rheum.* ٢٠٠٢; ٤٦: ١٠٠٣ ١٣.

٦. *Weiner HL, Cohen, J.A. Treatment of multiple sclerosis with cyclophosphamide: critical review of clinical and immunologic effects.Mult Scler .* ٢٠٠٢; ٨: ١٤٢-٥٤.

٧. *CAI Y, Wu MH, Ludeman SM, et al. Role of O٦-Alkylguanine-DNA Alkyltransferase in Protecting against Cyclophosphamide-induced Toxicity and Mutagenicity. Cancer Research.* ١٩٩٩; ٥٩: ٣٠٥٩-٦٣.

٨. *Asou N, Suzushima H, Nishimura S, et al. Long-term remission in an elderly patient with mantle cell leukemia treated with low-dose cyclophosphamide. Am J Hematol.* ٢٠٠٠; ٦٣: ٣٥-٧.

٩. *Santosousso M, Divangahi M, Zganiacs A. Reduced tissue macrophage population in the lung by anticancer agent cyclophosphamide: restoration by local granulocyte macrophage-colony-stimulating factor gene transfer. Blood.* ٢٠٠٢; ٩٩: ١٢٤٦-٥٢.

١٠. *Vogels MT, Hermsen CC, Huys HL, et al. Roles of tumor necrosis factor alpha, granulocyte–macrophage colony-stimulating factor, platelet-activating factor, and arachidonic acid metabolites in interleukin-١-induced resistance to infection in neutropenic mice. Infect Immun.* ١٩٩٤; ٦٢: ٢٠٦٥ ٧٠.

١١. *Morato EF, Gerbase-Delima M, Gorczynski RM. Conditioned immunosuppression in orally immunized mice. Brain Behav Immun* ١٩٩٦;١٠: ٤٤–٥٤.

١٢. *Levesque JP, Hendy J, Takamatsu Y, et al. Mobilization by either cyclophosphamide or granulocyte colony-stimulating factor transforms the bone marrow into a highly proteolytic environment. Exp Hematol.* ٢٠٠٢;٣٠: ٤٤٠-٩.

١٣. *Levesque JP, Hendy J,Takamatsu Y, et al. Disruption of the CXCR٤/CXCL١٢ chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide.J Clin Invest .* ٢٠٠٣; ١١١: ١٨٧-٩٦

١٤. *Mu HH, Penny R, SEWELL WA. Interleukin-٥ is necessary for eosinophilia induced by cyclophosphamide in immunized mice.Immunol .* ١٩٩٣; ٧٩: ٤٥٢–٨.

١٥. *Buisman AM, Van Zwet TL, Langermans JA, et al. Different effect of granulocyte colony-stimulating factor or bacterial infection on bone-marrow cells of cyclophosphamide-treated or irradiated mice. Immunol.* ١٩٩٩; ٩٧: ٦٠١-١٠.

١٦. *Anderson D, Bishop JB, Garner RC, et al. Cyclophosphamide. Review of its mutagenicity for an assessment of potential germ cell risks.Mut Res .* ١٩٩٥; ٣٣٠: ١١٥-٨١.

١٧. *Chung F, Young R, Hecht SS. Formation of cyclic* ١, *N* ٢*-propanodeoxyguanosine adducts in DNA upon reaction with acrolein or crotonaldehyde. Cancer Res.* ١٩٨٤; ٤٤: ٩٩٠-٥.

١٨. *Jacobson-kram D, Albertini RJ, Branda R. Measurement of chromosomal aberration sister chromatid exchange, hprt mutation and DNA adducts in peripheral lymphocytes of human populations at increased risk for cancer.Environ Health Perspect .* ١٩٩٣; ١٠١: ١٢١-٥.

١٩. *Zaks-Zilberman M, Salkowski CA, Elsasser T, et al. Induction of Adrenomedullin mRNA and Protein by Lipopolysaccharide and Paclitaxel (Taxol) in Murine Macrophages.Infection Immunity .* ١٩٩٨;٦٦(١٠): ٤٦٦٩ ٧٥.

٢٠. *Yong JN, Sang BH, Jong SK, et al. Lactoferrin works as a new LPS-binding protein in inflammatory activation of macrophages.Int Immunopharmacol.* ٢٠٠٤; ٤: ١١٨٧ ٩٩.

٢١. *Robert SM, Alan WV. Shield as signal: lipopolysaccharides and the evolution of immunity to gram-negative bacteria. PLoS Pathog.* ٢٠٠٦; ٢(٦): ٦٧.

٢٢. *Heine H, Rietschel ET, Ulmer AJ. The biology of endotoxin.Mol Biotechnol .* ١٩٩٩;١٩(٣): ٢٧٩–٩٦.

٢٣. *Van-Deusen JB, Shah MH, Becknell B, et al. STAT-١-mediated repression of monocyte interleukin-١٠ gene expression in vivo. Eur J Immunol.* ٢٠٠٦;٣٦ (٣): ٦٢٣-٣٠.

٢٤. *Pandurangarao VL, Blazina S, BHERJE R. Cadmium chloride strongly enhances cyclophosphamide-induced chromosome aberrations in mouse bone marrow cells. Eniron Molecul Mutagenesis.* ١٩٩٧;٢٩: ١٩ ٢٣.

٢٥. *SCHILL WB, PHELPS SR, PYLE SW. Multilocus electrophoretic assessment of Yersinia ruckeri. App. Environ Microbiol.* ١٩٨٤; ٤٩: ٩٧٥-٩.

٢٦. *AUSTIN B, AUSTIN DA. Methods for the microbiological examination of fish and shellfish. Ellis Harwood Limited (publisher Chichester).* ١٩٨٩.

٢٧. *Westphal O, Jann K. Bacterial lipopolysaccharides: extraction with phenole-water and further applications of the procedure.Methods Carbohydr .Chem .* ١٩٦٥;٥: ٨٣-٩١.

٢٨. ٢٨. *Dacie SJ, LEWIS SM. Practical haematology.* ٧*th Edition.Churchill Livingstone .* ١٩٩١.

٢٩. *SIWICKI AK. Immunostimulating influence of levamisole on nonspecific immunity in carp (Cyprinus carpio). Dev Comp Immunol.* ١٩٨٩; ١٣(١): ٨٧-٩١.

٣٠. ٣٠. *COHEN G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Anal Biochem.* ١٩٧٠; ٣٤: ٣٠-٣٨.

٣١. *SINHA AK. Colorimeteric assay of catalase. Anal. Biochem* ١٩٧٢; ٤٧: ٣٨٩-٩٥.

٣٢. *Kar M, Mishra D. Catalase, Peroxidase and polyphenoloxidase activities during rice leaf senescence. Plant Physiology.* ١٩٧٦;٥٧: ٣١٥-١٩.

٣٣. *Joseph P, murthy KR Nelson JL, et al. Peroxidase: a novel pathway for chemical oxidation in human term placenta. Placenta.* ١٩٩٢; ١٣(٦): ٥٤٥-٥٤.

٣٤. *Zou GL Gui XF, Zhong XL, et al. Improvements in pyrogallol auto-oxidation method for the determination of SOD activity. Progress in Biochemistry and Biophysics.* ١٩٨٦; ٤: ٧١-٣ .

٣٥. *Preston RJ, Dean BJ, Galloway S, et al.Mammalian in vivo cytogenetic assays-analysis of chromosomal aberrations in bone marrow cells.Mut Res .* ١٩٨٧; ١٨٩: ١٥٧-٦٥.

٣٦. *Shukla Y, taneja P. Antimutagenic effects of garlic extract on chromosomal aberrations. Cancer Lett.* ٢٠٠٢;١٧٦: ٣١-٦ .

٣٧. *PC-STAT One-way Analysis of Variance Procedure. Georgia Univ.* ١٩٩٥.

٣٨. *Hafenrichter DG, Roland CR, Mangino MJ, et al. The Kupffer cell in endotoxin tolerance: mechanism of protection against lethal endotoxemia.Shock .* ١٩٩٤،٢: ٢٥١-٦.

٣٩. *Freudenberg MA,Galanos C. Induction of tolerance to lipopolysaccharide (LPS)-D-galactosamine lethality by pretreatment with LPS is mediated by macrophages. Infect Immun.* ١٩٩٨;٥٦: ١٣٥٢-٧.

٤٠. *Alexander HR, Doherty GM, BLOCK MI, et al. Single-dose tumor necrosis factor protection against endotoxin-induced shock and tissue injury in rats. Infect. Immun.* ١٩٩١; ٥٩: ٣٨٨٩-٩٤.

٤١. *LeonP , Redmond HP, ShouJ , et al. Interleukin-*١ *and its relationship to endotoxin tolerance. Arch Surg.* ١٩٩٢; ١٢٧: ١٤٦-٥١.

٤٢. *Abdul-hai A, Weiss L, Slavin S, et al. Improved survival following induction of GVHD following lipopolysaccharide immunization. Ex Hematol.* ٢٠٠٦;٣٤: ٥٤٩–٥٣.

٤٣. *YOZA B, LA-RUE K, McCALL C. Molecular mechanisms responsible for endotoxin tolerance. Prog Clin Biol Res.* ١٩٩٨;٣٩٧: ٢٠٩-١٥.

٤٤. LIU J, LOUIS ES, ANDREW P, et al. Endotoxin pretreatment protects against the hepatotoxicity of acetaminophen and carbon tetrachloride: role of cytochrome P ٤٥٠ suppression.Toxicology . ٢٠٠٠; ١٤٧: ١٦٧-٧٦.

٤٥. GUIDOT DM. Endotoxin pretreatment in vivo increases the mitochondrial respiratory capacity in rat hepatocytes. Arch Biochem Biophys. ١٩٩٨; ٣٥٤: ٩-١٧.

٤٦. Calabrese EJ, Mehendale HM. A review of the role of tissue repair as an adaptive strategy: why low doses are often non-toxic and why high doses can be fatal.Food Chem Toxicol. ١٩٩٦; ٣٤: ٣٠١-١١.

٤٧. Laskin DL. Sinusoidal lining cells and hepatotoxicity. Toxicol Pathol. ١٩٩٦; ٢٤: ١١٢-٨.

٤٨. Roth RA, Harkema JR, Pestka JP, et al. Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents?. Toxicol Appl Pharmacol., ١٩٩٧; ١٤٧: ٣٠٠-٣١١.

٤٩. Ishikawa M, Tanno K, Sasaki M, et al. Antidotal effect of lipopolysaccharide against acetaminophen-induced mortality in mice.Pharmacol Toxicol . ١٩٩٠; ٦٧: ٣٨٧-٩١.

٥٠. Chorvatovicova D, Machova E, Sandula J, et al. Protective effect of the yeast glucomannan against cyclophosphamide-induced mutagenicity. Mut Res. ١٩٩٩; ٤٤٤: ١١٧-٢٢.

٥١. Barton TS, Wyrobek AJ, Hill FS, et al. Numerical Chromosomal Abnormalities in Rat Epididymal Spermatozoa Following Chronic Cyclophosphamide Exposure. Biol Reprod. ٢٠٠٣; ٦٩: ١١٥٠-٧ .

٥٢. Zhang QH, Wu CF, DuanL , et al. Protective effects of ginsenoside Rg(٣) against cyclophosphamide-induced DNA damage and cell apoptosis in mice. Arch Toxicol. ٢٠٠٨; ٨٢(٢): ١١٧-٢٣.

٥٣. De-Flora S, Bennicelli C, Bagnasco M. Genotoxicity of mercury compounds.Mut Res . ١٩٩٤; ٣١٧(١): ٥٧-٧٩.

٥٤. Heddle JA, Moody JA, Thompson LU, et al. New approaches to antimutagenesis. J Env Pathol Toxicol Oncol. ١٩٩٩; ١٨: ٩٥-١٠١.

٥٥. Schurz F , Sabater-Vilar M, Fink-Gremmels J. Mutagenicity of mercury chloride and mechanisms of cellular defence: the role of metal-binding proteins. Mutagenesis. ٢٠٠٠; ١٥(٦): ٥٢٥-٣٠

٥٦. Rao MV, Chinoy NJ Suthar MB, et al. Role of ascorbic acid on mercuric chloride-induced genotoxicity in human blood cultures. Toxicol. ٢٠٠١; ١٥(٦): ٦٤٩-٥٤.

٥٧. Oh MS, Chang MS, Park W, et al. Yukmijihwang-tang protects against cyclophosphamide-induced reproductive toxicity. Reprod Toxicol. ٢٠٠٧; ٢٤(٣-٤): ٣٦٥-٧٠.

٥٨. Warren GW, Poloyac SM, Gary DS, et al. Hepatic cytochrome P ٤٥٠ expression in TNF-a (p٥٥:p٧٥) knock out mice following endotoxin administration. J Pharmacol Exp Ther. ١٩٩٩; ٢٨٨: ٩٤٥-٥٠.

٥٩. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. ٢٠٠١; ٢(٨): ٦٧٥-٨٠.