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RESEARCH ARTICLE

CORRELATION BETWEEN HEPCIDIN HORMONE AND SOME IMMUNOLOGICAL AND HEMATOLOGICAL PARAMETERS ALTERED BY SUBACUTE TOXICITY OF DIAZINON WITH THE PROTECTIVE EFFECT OF CURCUMIN IN MALE RATS

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ABSTRACT

Hepcidin, a small peptide hormone produced mainly by the liver, plays a central role in iron status regulation in humans and other mammals. Thirty six male albino rats were classified randomly into equally six groups (G). The obtained results revealed that low (17.5) and high (35) mg/kg b.w. DZN dose (G2 and G3, respectively) administration for 28 days significantly increased the pro-inflammatory cytokines (IL-6, TNF-α) and hepcidin, decreased erythropoietin and significantly decreased hemoglobin concentration, red blood cells counts, serum iron and its related parameters in comparison to untreated healthy control G1. Moreover, the groups co-administrated with curcumin (CUR) mixed with diet for 28 days (G5 and G6) revealed improvement of these parameters in comparison to those of corresponding groups. Thus it was concluded that subacute toxicity of DZN induces alteration in some immunological and hematological parameters which in turn cause alteration in hepcidin and CUR administration had a protective effect against these adverse effects of DZN.

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INTRODUCTION

Organophosphorus (OP) compounds are widely used in agriculture and industry. OP pesticides, in addition to their intended effects like the control of insects or other pests, are sometimes found to affect non-target organisms including humans (Chaudhuri *et al*., 1999). Organophosphate compounds are useful as pesticides due to their ability to inhibit acetylcholinesterase, an enzyme responsible for the inactivation of the neurotransmitter acetylcholine (Pesando *et al*., 2003). However, both short-term and long-term regulatory studies often reveal no anticholinesterase effects, such as delayed polyneuropathy, immunotoxic endocrine effects, hepatic injury, genotoxicity, and developmental of neurochemical and neurobehavioral impairments (Aboul-Soud *et al*., 2011). Diazinon (DZN) is a contact organophosphate pesticide that is extensively used, both in agriculture and households to control insects. Agricultural spray contains 85–90% DZN. After its application on crops and plants, DZN is easily washed by surface water and thus enters the ground water. Eventually, it enters the aquatic environment in large quantities (Ferrari *et al*., 1997). DZN degrades rapidly, but under conditions of low temperature, low moisture, high alkalinity, and lack of suitable microbiological degraders, it may remain biologically active in soils for six months or longer (Burkepile *et al*., 2000). There is significant experimental evidence that acute OP intoxication elicits a robust inflammatory response, and emerging evidence suggests that chronic repeated low-level OP exposure also upregulates inflammatory mediators (Banks and Lein, 2012). It has been shown that OP pesticides stimulate the release of cytokines, such as interleukin (IL)-1beta (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α) (Chadban *et al*., 1998). Hepcidin is a small peptide hormone secreted mainly by the liver. It was discovered in 2000, and appears to be the principal regulator of iron homeostasis in humans and other mammals (Ganz, 2003). Hepcidin function is to regulate iron transport across the gut mucosa, thereby preventing excess iron absorption and maintaining normal iron levels within the body. Also, hepcidin inhibits transport of iron out of macrophages (site of iron storage and transport). Thus, in states of high hepcidin levels

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(including inflammatory states), serum iron levels can drop because iron is trapped inside macrophages. This may lead to anemia (De Domenico *et al*., 2010). Inflammation and infection increase hepcidin synthesis. Macrophages are stimulated during the inflammatory process and this stimulation depends on the severity of inflammation. Activated macrophages release a network of cytokines. Among them is IL-6 which is one of the primary inducers of hepcidin expression then an increase in hepcidin levels that finally results in hypoferremia (Peyssonnaux *et al*., 2006). The sole known molecular target of hepcidin is ferroportin, which functions as a transmembrane conduit for the transfer of cellular iron to plasma. Most cells contain very little ferroportin and do not export iron, using it only for their own metabolic needs. The professional iron exporters, including macrophages, duodenal enterocytes, hepatocytes, and placental syncytiotrophoblasts, express ferroportin and provide iron for the entire organism (Nemeth *et al*., 2004). Ferritin scavenges free iron and enables its sequestration in macrophage reticuloendothelial stores. Transferrin, the principal iron carrier, regulates the total iron-binding capacity (TIBC) of blood. Also, ferritin expression is dependent on IL-6 signalling (Isaacs *et al*., 2013).

Curcumin (CUR) which is a polyphenolic compound present in the rhizomes of the turmeric (*Curcuma* (*C.*) *longa* Linn.) family (*Zingiberaceae*) (Sweetman, 2009). CUR has been shown in last two decades to be a potent immunomodulatory agent that can modulate the activation of T cells, B cells, macrophages, neutrophils, natural killer cells, and dendritic cells (Jagetia and Aggarwal, 2007). This compound exhibits numerous biological activities including anti-inflammatory, antiprotozoal, antibacterial, anti-HIV and anti-cancer activities against several malignancies. Also, hepato, neuroprotective, hypoglycemic, and antirheumatic effects of CUR were reported. The safety of CUR at very high doses has been proved in various animal and human studies (Anand *et al*., 2007). Moreover, it has been shown to inhibit the production of the inflammatory cytokines TNF-α, IL -1, -2, -6, -8, and -12 (Abe *et al*., 1999, Goel*et al*., 2008). So this work aimed to provide new insight into correlation between hepcidin (iron-regulatory hormone), some immunological factors (IL-6 and TNF-α), erythropoietin and some haematological parameters altered by subacute toxicity of DZN. Also, the possible protective effect of CUR against this adverse effect was studied in male rats.

MATERIALS AND METHODS

Experimental Animals

Thirty-six mature Wistar male rats weighing (180-200 g) were purchased from Theodore Bilharz Research Institute, Giza, Egypt. The animals were housed in plastic cages and kept under laboratory conditions with a 12-hour (hr) light/dark cycle and a room temperature of 21±3ºC. The animals were provided with food and water *ad libitum* and were acclimatized for two weeks before starting the experiment. The study was approved by the Institutional Animal Care and Use Committee (IACUC).

Chemicals

DZN was obtained from ADWIA 60% EC (emulsifiable concentrate), Cairo, Egypt. The median lethal dose (LD_{50}) of DZN was determined according to Sine (1990) and its value was 350 mg/kg b.w. DZN was emulsified in distilled water before use and orally administrated by the stomach tube to rats at 17.5 and 35 mg/kg b.w which represent 1/20and 1/10 $LD_{50,}$ respectively (Rady, 2009). CUR, obtained from Sigma, St. Louis, Mo, USA, was mixed with diet at a dose of 200 mg/kg of diet (Messarah *et al*., 2013).

Experimental design

Animals were classified at random into six groups (G) each of six as follows

G1: rats received tap water, fed on basal diet and were considered as the control group.

G2: rats received 17.5 mg/kg b.wDZN(1/20 LD_{50}) as a low dose over a period of 28 days (5 days/week) (El-Kashoury and Tag El –Din, 2010).

G3: rats received 35 mg/kg b.w DZN (1/10 LD_{50}) as a high dose over a period of 28 days (5 days /week).

G4: rats fed on basal diet mixed with CUR at a dose of 200 mg/kg diet for 28 days.

G5: rats fed on basal diet mixed with CUR four days before and along with the administration with low dose of DZN orally (5 days/week) for 28 days.

G6: rats fed on basal diet mixed with CUR four days before and along with the administration with high dose of DZN orally (5 days/week) for 28 days.

Collection of samples

Rats were subjected to diethyl ether anesthesia on day 29. Blood samples were collected from the orbital sinus of each animal by using heparinized capillary tubes. The blood samples were divided into two parts: The first part was put into dry clean tube and left to clot for sera preparation and kept at -20°C for biochemical and immunological analysis. The second part of blood samples was transferred to test tubes containing EDTA for hematological parameters

Determination of Serum Cytokines

Quantitative determination of serum IL-6 and TNF-α concentration was done using ELISA Kit (KOMA BIOTECH INC, www.komabiotech.com) with Catalog No: K0331229 and K0331196, respectively, and standard range of 125-8000 pg/ml and 47-3000 pg/ml, respectively

Hormonal assay

Quantitative determination of serum rat hepcidin and erythropoietin concentrations were done using ELISA Kit (Glory Science Co., Ltd, USA), Catalog No: 30981 and 94783, respectively. The detection range of the kits is 5-100 µg/L and 1 -20 IU/L, respectively.

Determination of iron-related parameters

Estimation of serum iron and total iron binding capacity (TIBC) was carried out by Coral Clinical Systems, Goa, India (Catalog No: IRT010) according to ferrozine/magnesium carbonate method of Siedel *et al*. (1984). Unsaturated Iron Binding Capacity (UIBC) according to the equation:

UBIC in µg/dl = TIBC in µg/dl - Iron in µg/dl

Percentage iron saturations were calculated as: iron level/TIBC × 100 (Jiao *et al*., 2009).

Quantitative determination of ferritin concentration (ng/ml) in rat sera was done using ELISA Kit Immunospec Corporation Catalog No: E29-013, Esdoornlaan, The Netherlands, according to Valberg (1980) the minimal sensitivity of the test is 5.0 ng/ml.

Hematological Parameters

Blood samples, including RBCs count, hemoglobin (Hb), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBCs), lymphocyte percent and platelet (PLT) count, were examined by using the hematology analyzer HEMAVET multispecies hematology system-HV950FS (Drew Scientific, Dallas, TX) per the manufacturer's instructions.

Statistical analysis

Data obtained were executed by the aid of the statistical package for social science (SPSS) version 22 software (SPSS-22, 2013). Duncan's test of homogeneity was used to estimate the similarities for each group separately, at all the experimental periods. All the results were expressed as a mean (M) of six rats± standard error of mean (SE).

RESULTS

Pro-infammatory cytokines

The mean level of serum IL-6 and TNF-α (Table 1) was significantly ($P < 0.05$) elevated in DZN and CUR treated groups when compared with normal control. Whereas, the level of IL-6 in G5 and G6 was significantly declined when compared with G2 and G3, respectively. No significant change was recorded in G4 in comparison with G1. As regards, the level of TNF-α was significantly ($P < 0.05$) increased in G2 and G3, while in G4 and G6 were significantly declined than G2 and G3. The level of TNF-α in G5 was not significantly decline than G2 and G3.

Level of hepcidin and erythropoietin

As depicted in Table (2), the mean level of hepcidin in serum was significantly ($P < 0.05$) elevated in both DZN G2 and G3 than G1 and G4. The levels of hepcidin in G5 and G6 were declined than G2 and G3, while the decrease in G5 was significant. Also, the results indicated an insignificant change in G4 as compared with G1 and G5. As regard erythropoietin, the mean level of erythropoietin showed significant decline in G3 than G1, G4 and G5, however insignificant change was recorded in G5 and G6 in comparison with G2 and G3.

Hematological parameters

The present data in Table (3) revealed that RBCs count was significantly ($P < 0.05$) declined in G3 in comparison with all different experimental groups. Also, a significant decrease of RBCs count was recorded in G2 and G3 as compared to G1. While, CUR treated groups were significantly increased RBCs counts in comparison with G3. Hb level was significantly decreased in G2 and G3 in comparison with all different experimental groups. However, a significant increase was detected in G5 and G6 in comparison with G2 and G3. The level of MCV demonstrated insignificant variation in the different experimental groups. Meanwhile, MCH in G2 was significantly decreased in comparing with the other experimental groups. Also, there was a significant decrease in MCHC level in G2 when compared with control group, however, no significant variation was recorded between G5 and G6 in comparison with G2 and G3, respectively. HCT was significantly declined in G3 in comparing with the other experimental groups. Also, WBCs count, LYM % and PLT count showed insignificant change in all the experimental groups.

Iron profile

Data presented in Table (4) showed that the level of serum iron according to Duncan test recorded significant ($P < 0.05$) elevation in G5 and G6 than G2 and G3 but still lower than G1 and G4. The level of TIBC and consequently UIBC was significantly declined in G3 when compared with all different studied groups. Serum iron levels and then TIBC, UIBC and percent of iron saturation recorded significant decrease in G2 and G3 than G1 and the decline in G3 followed by G2 compared with the other experimental groups, also, a significant increase was recorded in G5 and G6 when compared with G2 and G3. However, serum ferritin level showed that there was a significant increase in G2 and G3 as compared with G1, and insignificant decrease in G5 and G6 in comparing with G2 and G3.

Correlation between hepcidin and TNF-α, IL-6, erythropoietin and iron components

Concerning the correlation between hepcidin and different parameters in G2 was recorded in Table (5). It was found that hepcidin exhibited positive correlation with IL-6, TIBC and UIBC but it was negatively correlated with % of iron saturation. The correlation in G3, Table (6) showed that hepcidin was positively correlated with TNF-α, IL-6, ferritin and % of iron saturation. But hepcidin showed strong negative correlation with erythropoietin, TIBC and UIBC. In G4, Table (7) hepcidin showed positive correlation with iron, UIBC and strong positive correlation with TIBC, but hepcidin with IL-6 and erythropoietin showed weak positive correlation. While, it was negatively correlated with ferritin and % of iron saturation and only strong negative correlation was detected with TNF-α. As shown in Table (8), G5 hepcidin showed very strong negative correlation with IL-6, TIBC and UIBC. Moreover, strong negative correlation with erythropoietin, and average negative correlation with TNF-α, While very strong positive correlation with % of iron saturation" changed into "it showed strong negative correlation with erythropoietin, and average negative correlation with TNF-α, while very strong positive correlation with % of iron saturation was also detected in G5. In G6, hepcidin showed average positive correlation with TNF-α, IL-6 and strong positive correlation with iron, ferritin and % of iron saturation. Meanwhile, a negative correlation was detected with UIBC (Table 9).

Table 1. The level of IL-6 and TNF-α in the different experimental groups

	(G1)	(G2)	(G3)	(G4)	(G5)	(G6)
IL-6	150.00	249.17	373.50	145.00	194.17	225.83
(Pg/ml)	±2.89 [a]	±5.83 [d]	±2.43 [e]	±4.08 [a]	±10.12 [b]	±9.17 [c]
TNF-α (Pg/ml)	155.17	209.67	394.6	145.00	160.42	175.00
	±1.20 [b]	±4.45 [d]	±2.79 [e]	± 2.22 [a]	±1.83 [b]	±2.19 [c]

Data are represented as mean value ± SE. n=6.
Values in the same raw with the different superscript letter are significantly different (P <0.05).
DZN: Diazinon, **CUR:** Curcumin.
G1: Control, **G2:** DZN (1/20 LD_{50})**, G3:** DZN (1/10 LD_{50}), **G4:** CUR (200 mg/kg diet), **G5:** CUR+DZN (1/20 LD_{50}),
G6: CUR + DZN (1/10 LD_{50}).

Table 2. Serum levels of hepcidin and erythropoietin in the different experimental groups

	(G1)	(G2)	(G3)	(G4)	(G5)	(G6)
Hepcidin	35.83	63.75	77.50	29.17	44.17	54.17
(µg/L)	±2.39 [a]	±8.75 [c]	±4.96 [c]	±4.36 [a]	± 3.91 [ab]	±5.50 [bc]
Erythropoietin (IU/L)	9.32	6.92	4.50	8.42	8.33	5.83
	±1.08 [c]	±0.490 [abc]	±0.775 [a]	±0.455 [bc]	±1.36 [bc]	±0.691 [ab]

Data are represented as mean value ± SE. n=6.
Values in the same raw with the different superscript letter are significantly different (P <0.05).
DZN: Diazinon, **CUR:** Curcumin,
G1: Control, **G2:** DZN (1/20 LD_{50}), **G3:** DZN (1/10 LD_{50}), **G4:** CUR (200 mg/kg diet), **G5:** CUR+DZN (1/20 LD_{50}), **G6:** CUR + DZN (1/10 LD_{50}).

Table 3. Hematlogical parameters and blood indices value of different experimental groups.

	(G1)	(G2)	(G3)	(G4)	(G5)	(G6)
RBCs	4.63	4.09	3.58	4.56	4.42	4.30
(10^{12} /L)	±0.173 [c]	±0.084 [b]	0.196 [a]	±0.159 [bc]	±0.123 [bc]	±0.127 [bc]
Hb	13.80	11.38 ±0.217	10.51	13.52	12.88	12.67
(g/dL)	±0.403 [b]	[a]	±0.530 [a]	±0.468 [b]	±0.331 [b]	±0.433 [b]
MCV	50.98	51.85	49.55	53.12	50.82	52.72
(**fl**)	±0.757 [a]	±1.18 [a]	±1.26 [a]	±1.29 [a]	±1.37 [a]	±1.22 [a]
MCH	29.83	27.80	29.47	29.73	29.23	29.47
(pg)	±0.407 [b]	±0.137 [a]	±0.547 [b]	±0.448 [b]	±0.470 [b]	±0.357 [b]
MCHC	58.60	53.78	59.70	56.03	57.63	56.05
(g/L)	±1.54 [b]	±1.23 [a]	±1.90 [b]	±1.07 [ab]	±1.17 [ab]	±1.26 [ab]
HCT	23.65	21.25	17.80	24.23	22.43	22.65
(%)	±1.03 [b]	±0.873 [b]	±1.25 [a]	±1.17 [b]	±0.716 [b]	±0.995 [b]
WBCs	7.80	8.20	7.47	8.05	6.58	7.70
(10 9 /L)	±0.830 [a]	±0.863 [a]	±0.492 [a]	±0.836 [a]	±0.863 [a]	±0.460 [a]
LYM %	54.45	61.80	62.65	60.53	56.72	62.57
	±4.60 [a]	±2.38 [a]	±1.54 [a]	±1.89 [a]	±6.63 [a]	±1.60 [a]
PLT	458.50	481.33	590.33	497.17	510.50	489.33
(10^{9}/L)	±43.33 [a]	±36.86 [a]	±84.33 [a]	±63.36 [a]	±60.55 [a]	±43.02 [a]

Data are represented as mean value ± SE. n=6.
Values in the same raw with the different superscript letter are significantly different (P <0.05).
DZN: Diazinon, **CUR:** Curcumin.
G1: Control, **G2:** DZN (1/20 LD_{50})**, G3:** DZN (1/10 LD_{50}), **G4:** CUR (200 mg/kg diet), **G5:** CUR+DZN (1/20 LD_{50}),
G6: CUR + DZN (1/10 LD_{50}).
RBC: Red blood cells; **Hb**: Hemoglobin; **MCV**: Mean corpuscular volume;
MCH: Mean corpuscular hemoglobin; **MCHC:** Mean corpuscular hemoglobin concentration;
HCT: Hematocrit; **WBC:** White blood cells; **LYM%:** Lymphocyte, % **PLT**: Platelet.

Table 4. Levels of serum iron and its related parameters in all the experimental groups

	(G1)	(G2)	(G3)	(G4)	(G5)	(G6)
Iron	52.25	37.48	33.30	50.43	46.75	46.03
(µg/dL	±1.68 [d]	±1.26 [b]	±1.47 [a]	±0.626 [d]	±0.982 [c]	±1.35 [c]
TIBC	140.00	119.83	111.32	135.63	137.53	130.57
(µg/dL)	±3.28 [d]	±1.47 [b]	±1.89 [a]	±1.60 [cd]	±4.37 [cd]	±2.38 [c]
UIBC (µg/dL)	87.75	82.35	79.22	85.20	90.78	84.54
	±2.72 [bc]	±1.08 [ab]	±1.64 [a]	±1.67 [abc]	±3.84 [c]	±2.70 [abc]
%iron saturations	37.37	30.82	29.58	37.20	34.10	35.31
	±1.05 [c]	±0.836 [a]	±1.15 [a]	±0.618 [c]	±0.878 [b]	±1.23 [bc]
Ferritin (ng/ml)	32.58	41.05	50.03	31.18	39.73	43.32
	±2.02 [ab]	±1.51 [c]	±1.03 [d]	±1.78 [a]	±3.40 [bc]	±4.08 [cd]

Data are represented as mean value ± SE. n=6.
Values in the same raw with the different superscript letter are significantly different(P <0.05).
DZN: Diazinon, **CUR:** Curcumin.
G1: Control, **G2:** DZN (1/20 LD_{50}), **G3:** DZN (1/10 LD_{50}), **G4:** CUR (200 mg/kg diet), **G5:** CUR+DZN (1/20 LD_{50}),
G6: CUR + DZN (1/10 LD_{50}).
TIBC: Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

Table 5. Correlation between hepcidin and TNF-α, IL-6, erythropoietin and iron components in G2 (DZN, 1/20 LD_{50})

	Hepcidin	TNF-α	IL-6	Erythropoietin	Iron	Ferritin	TIBC	UIBC	% of Iron Saturation
Hepcidin	1								
TNF-α	-0.02	1							
IL-6	0.42	-0.73	1						
Erythropoietin	-0.03	-0.55	0.46	1					
Iron	0.14	0.50	-0.26	-0.07	1				
Ferritin	-0.24	-0.24	-0.39	-0.27	-0.49	1			
TIBC	0.61	0.12	-0.05	0.07	0.70	-0.10	1		
UIBC	0.68	-0.41	0.24	0.17	-0.21	0.44	0.55	1	
% of Iron Saturation	-0.35	-0.89	0.38	0.50	-0.51	0.49	-0.24	0.27	1

DZN: Diazinon, **TIBC:** Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

Table 6. Correlation between hepcidin and TNF-α , IL-6, erythropoietin and iron components in G3 (DZN, 1/10 LD_{50})

	Hepcidin	TNF-α	IL-6	Erythropoietin	Iron	Ferritin	TIBC	UIBC	% of Iron Saturation
Hepcidin	1								
TNF-α	0.43	1							
IL-6	0.48	0.38	1						
Erythropoietin	-0.67	-0.83	-0.13	1					
Iron	-0.09	0.58	-0.27	-0.52	1				
Ferritin	0.30	0.64	-0.37	-0.73	0.63	1			
TIBC	-0.55	-0.21	-0.59	0.04	0.52	-0.02	1		
UIBC	-0.52	-0.75	-0.41	0.51	-0.32	-0.59	0.64	1	
% of Iron Saturation	0.12	0.76	-0.05	-0.61	0.92	0.73	0.15	-0.66	1

DZN: Diazinon, **TIBC:** Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

Table 7. Correlation between hepcidin and TNF-α , IL-6, erythropoietin and iron components in G4 (CUR group)

	Hepcidin	TNF-α	IL-6	Erythropoietin	Iron	Ferritin	TIBC	UIBC	% of Iron Saturation
Hepcidin	1								
TNF-α	-0.77	1							
IL-6	0.19	-0.37	1						
Erythropoietin	0.08	-0.61	0.45	1					
Iron	0.26	-0.06	0.07	0.05	1				
Ferritin	-0.16	0.15	0.54	0.38	0.19	1			
TIBC	0.52	-0.53	-0.49	-0.04	0.08	-0.84	1		
UIBC	0.40	-0.48	-0.49	-0.06	-0.30	-0.88	0.93	1	
% of Iron Saturation	-0.17	0.34	0.40	0.06	0.70	0.75	-0.66	-0.89	1

CUR: Curcumin, **TIBC:** Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

Table 8. Correlation between hepcidin and TNF-α, IL-6, erythropoietin and iron components in G5 (CUR+ DZN, 1/20 LD_{50})

	Hepcidin	TNF-α	IL-6	Erythropoietin	Iron	Ferritin	TIBC	UIBC	% of Iron Saturation
Hepcidin	1								
TNF-α	-0.36	1							
IL-6	-0.89	-0.06	1						
Erythropoietin	-0.57	0.14	0.68	1					
Iron	-0.06	0.62	-0.31	-0.14	1				
Ferritin	-0.44	0.29	0.39	0.44	0.22	1			
TIBC	-0.78	0.50	0.55	0.49	0.62	0.55	1		
UIBC	-0.87	0.41	0.71	0.60	0.45	0.57	0.98	1	
% of Iron Saturation	0.95	-0.15	-0.96	-0.73	0.05	-0.48	-0.75	-0.87	1

CUR: Curcumin, **DZN:** Diazinon, **TIBC:** Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

Table 9. Correlation between hepcidin and TNF-α , IL-6, erythropoietin and iron components in G6 (CUR+ DZN, 1/10 LD_{50})

	Hepcidin	TNF-α	IL-6	Erythropoietin	Iron	Ferritin	TIBC	UIBC	% of Iron Saturation
Hepcidin	1								
TNF-α	0.22	1							
IL-6	0.42	0.45	1						
Erythropoietin	-0.07	-0.81	-0.35	1					
Iron	0.74	0.41	0.44	-0.45	1				
Ferritin	0.61	-0.04	0.58	0.27	0.59	1			
TIBC	-0.06	0.20	0.29	0.26	0.03	0.57	1		
UIBC	-0.42	-0.02	0.04	0.45	-0.47	0.21	0.87	1	
% of Iron Saturation	0.66	0.26	0.22	-0.54	0.85	0.21	-0.49	-0.86	1

CUR: Curcumin, **DZN:** Diazinon, **TIBC:** Total iron binding capacity, **UIBC:** Unsaturated iron binding capacity.

DISCUSSION

The inflammatory response is initiated via activation of macrophages in the periphery and microglia and/or astrocytes in the central nervous system (CNS), which leads to the release of pro-inflammatory mediators, such as cytokines. These compounds induce the dilation of blood vessels to promote migration of leukocytes, typically neutrophils, to the area of injury (Duffield, 2003). In the current study, there was a significant increase in both IL-6 and TNF-α in DZN treated rats in comparison with the control. Whereas, CUR co-administration with DZN could significantly decrease IL-6 and TNF-α serum levels to approach the normal levels. The increase in TNF-α level coincide with those reported by Hariri *et al*. (2010) who found that serum level of TNF-α was increased significantly by DZN and significantly decreased in group treated with crocin. Also, Ahmed *et al*. (2013) recorded that the oral administration of DZN (25 mg/kg b.w.) significantly increased brain TNF-α as compared to control group. Co-administration of melatonin and DZN significantly reduced TNF-α level as compared to DZN group. Also, other OP compound reported similar results as in Henderson *et al*. (2002) who showed that rats repeatedly exposed to low doses of sarin vapor have elevated levels of IL-1β, TNF-α, and IL-6 in the brain. Moreover the pesticide chlorpyrifos has been reported to modulate IL-6 and TNF-α/NF-κB signaling pathways by downregulating genes that encode signaling molecules in these pathways (Stapleton and Chan, 2009). Also, Pena-Philippides *et al*. (2007) found that in rats exposed to single or repeated subclinical doses of sarin, mRNA expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα is upregulated in the lungs. In addition Mense *et al*. (2006) demonstrated that chlorpyrifos upregulated key inflammatory mediators, including IFNγ and IL-6, as well as glial fibrillary acidic protein (GFAP) a marker of inflammatory astrocytes. These results of IL-6 and TNF-α level in rat exposed to DZN seem to be in agreement with a preliminary report that chlorpyrifos induces expression of the pro-inflammatory cytokines TNFα and IL-6 in addition to the chemokine MCP-1 in the mouse brain in a time- and dose-dependent manner (Hirani *et al*., 2007).

A significant decrease in IL-6 and TNF-α in rats fed on a diet containing CUR was due to its inhibitory effect on inflammatory mediators (Gulcubuk *et al*., 2006). The reduction of pro-inflammatory cytokines level observed in rats that received CUR and DZN in this study, is in harmony with previously reported *in vitro* and *in vivo* abilities of antioxidants to reduce TNF-α (Boghdady, 2013) The inflammation caused by DZN lead to elevation of hepcidin hormone level in G2 and G3 compared to G1 and G4. Indeed, inflammation is a potent inducer of hepcidin expression (Nemeth *et al*., 2003). Inflammatory regulation of hepcidin gene expression involves transcriptional control through a signal transducer and activator of transcription (STAT) site on the hepcidin promoter was evaluated by VergaFalzacappa *et al*. (2007). It was found also that IL-6 is a major hepatic regulator of the acute phase response to inflammatory stimuli. Ligand binding to the IL-6 receptor in turn leads to activation of Janus kinases (Jak) that phosphorylate STAT3. Translocation of STAT3 to the nucleus results in upregulation of hepcidin gene expression (Wrighting and Andrews 2006). Accordingly, the current study indicated that hepcidin was positively correlated with TNF-α and IL-6 in G3.

In this study, co-administration of CUR to DZN-treated rats was decreased hepcidin in G5 and G6 than G2 and G3. In agreement to our result Bharti *et al*. (2003) reported that CUR was found to be a more rapid and more potent inhibitor of STAT3 phosphorylation than AG490, where CUR suppresses the action of IL-6 through the downregulation of STAT3 activation. The decrease of erythropoietin level significantly was observed on administration of high dose of DZN in G3 in comparison with G1, G4 and G5. Many studies showed that erythroid colony formation in response to erythropoietin is impaired in the presence of pro-inflammatory cytokines (Kurzrock, 2001). In addition, Morceau *et al*. (2009) reported that impaired erythropoiesis is most likely due to apoptosis induction, cell growth inhibition and erythropoietin receptor downregulation as a result of a local increased production of the cytokines, but also iron metabolism damage. Also, Macciò *et al*. (2005) found that pro-inflammatory cytokines affect erythropoietin either by inducing inhibition of its production by kidney or by preventing its physiological functions at the cellular level. The defect of erythropoiesis was due to four pro-inflammatory cytokines; TNFα, TNF-related apoptosis-inducing ligand (TRAIL), IFNγ and IL-6. The results were confirmed by the negative correlation between hepcidin and erythropoietin indicated in G3. Administration of CUR showed insignificant increase in erythropiotein in G5and G6 in comparison with G2 and G3. These findings may be due to the inhibitory effect of CUR on proinflammtory cytokines which in turn improve expression of erythropiotein. There was a significant decrease in Hb concentration along with decrease in RBCs count in DZN treated rats as compared to untreated control one and HCT% significantly decreased in G3 than all experimental studied groups. The current study is in agreement with El Shenaway *et al*. (2009) who reported a significant decrease in Hb, RBCs and HCT in high dose of DZN compared to control which was dose dependent and leading to anemia after 14 days of treatment. Similar result by Yassa *et al*. (2011) who found decrease in Hb and RBCs count in DZN administrated groups. Patil *et al*. (2003) reported that pesticide residues play a role in the development of anemia due to interference of Hb biosynthesis and shorting of the life span of the circulating erythrocyte. The decrease in Hb concentration along with the decrease in RBCs count might be due to the effect of pesticide on erythropoietic tissue (Kalender *et al*., 2006). One of the most important factors to be considered in reduction of RBCs count is production of hormone erythropoietin (Morowati, 1998). Cytokines can affect different erythropoiesis stages. Immune activation involves accessory cells of the hematopoietic microenvironment. T cells produce TNF-α and IFN-γ, and monocytes produce TNF-α and IL-6. These proinflammatory cytokines inhibit proliferation of erythrocyte progenitor cells and antagonize the antiapoptotic actions of erythropoietin. Moreover, this direct negative effect on erythrocyte progenitor cells may be primarily due to changes in sensitivity to the action of erythropoietin (Icardi *et al*., 2013). Improved RBCs count and Hb concentration in groups fed on diet mixed with CUR and co-treated with low and high dose of DZN were

observed in our study. Abu Aita *et al*. (2012) reported that rats administered profenofos (OP) in combination with propolis showed a significant improvement of RBCs count, PCV% and Hb concentration in comparable to profenofos administered group. Propolis induced extensive proliferation of hematopoietic cells in the spleen and bone marrow (Orsolić and Basić, 2005). High content of flavonoids in propolis improves the expression level of erythropoietin and accelerates the generation of erythrocyte and Hb (Li-Wei *et al*., 2005).

In this study, there was no significant change with MCV, MCH, MCHC, and PLT count in different studied groups, Meanwhile, PLT count was insignificantly increased in G3 in comparison with the different studied groups. This may be due to the different doses of DZN could not induce thrombopoiesis. Similarly El-Shenaway *et al*. (2009) showed no significant changes in MCV, MCH, MCHC, but increasing thrombocyte due to DZN toxicity.

As regards WBCs there was not any significant change in WBCs and percentage of lymphocytes. While, El-Shenaway *et al*. (2009) and Yassa *et al*. (2011) observed increase in WBC count and percentage of lymphocytes, and Abu Aita *et al*. (2012) who showed that Leukogram revealed significant leukopenia with neutropenia and lymphopenia in profenofos intoxicated rats compared to control group. On the other hand, the present study agree with Baconi *et al*. (2013) who studied the immunotoxic effect of DZN which induced no significant changes of lymphocytes counts. Almost two thirds of the body's iron is found in Hb in circulating erythrocytes (Dewey *et al*., 2002). In the present work, decline levels of serum iron, TIBC, UIBC and percent of iron saturation in DZN-treated groups were detected. Similar finding reported by Shamoushaki *et al*. (2012) who found long-term exposure to DZN in *Rutilus frisiikutum* causes a decrease in iron. It was found that IL-6 is one of the primary inducers of hepcidin expression; an increase in hepcidin levels finally results in hypoferremia (Peyssonnaux *et al*., 2006). TIBC is a medical laboratory test that measured the blood capacity to bind iron with transferrin. It is performed by drawing blood and measuring the maximum amount of iron that it can carry, which indirectly measures transferring since transferrin is the most dynamic carrier (Yamanishi *et al*., 2003).TIBC is less expensive than a direct measurement of transferrin (Gambling *et al*., 2009**)**. The low level of serum iron, leading to decreased release of iron to the bone marrow and thus favoring anemia (Silverberg *et al*., 2011), even hepcidin regulates serum iron level during inflammation and infection (Ganz, 2003). So, hepcidin antagonist could be useful without iron supplementation (Vyoral and Petrák, 2005). Iron treatment can be harmful because it can trigger the growth of pathogens and/or contribute to neoplastic transformation because of its ability to unbalance immune functions (Weiss, 2002**)**. However, ameliorating effect of CUR was significantly increased in the iron parameters than G2 and G3. This may be because CUR has the ability to suppress STAT3 phosphorylation, inhibit IL-6 signaling, down-regulate the expression of IL-6 the primer producer of hepcidin(Wrighting and Andrews, 2006). Serum ferritin levels were significantly increased in G2 and G3 as compared with G1, while, insignificant change was recorded in G5 and G6 in comparing with G2 and G3. Serum ferritin is also an indirect marker of the total amount of iron stored in the body; hence serum ferritin is used as a diagnostic test for iron deficiency anaemia (Wang *et al*., 2010).Weiss and Goodnough (2005) and Silverberg *et al*. (2011) recorded that pro-inflammatory cytokines induced ferritin expression and stimulate iron storage and retention within macrophages which lead to a reduction in the levels of iron in the circulation and thus their availability for erythroid cells. In addition, they inhibited erythropoietin production in the kidneys, resulting in inappropriately low levels in the blood and hence worsening of anemia. This was confirmed by negative correlation between ferritin, erythropoietinand iron in G2, and the positive correlation between ferritin and hepcidin, TNF-α in G3.

So we concluded that subacute toxicity of DZN induces alteration in some immunological parameters which in turn cause alteration in hepcidin hormone secretion from hepatic cells leading to anemia. And CUR administration has a protective effect against this adverse effect of DZN .

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