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Assessment of the protective role of hesperidin against genotoxic and biochemical effects of cytoxan in mice

A.Abd El Raouf [1], S.M.Girgis[1]*, H.S.Abdou[1], Eman M.Saleh[2]

[1]Cell Biology Department, National Research Center, 12622-Dokki, Cairo, (EGYPT)

[2]Biochemistry Dept., Fac. of Sci., Ain Shams Univ., Cairo, (EGYPT)

E-mail : shenoudagirgis10@yahoo.com

ABSTRACT

Hesperidin (HDN) is one of naturally occurring flavonoid widely found in citrus fruits. Flavonoids were found to inhibit DNA and chromosomal damage induced by cyclophosphamide. Chemoprevention is one of the most promising and realistic approaches in the prevention of side effects produced by cytoxan treatment as an anticancer drug. Therefore, the present study was designed to evaluate the protective effect of HDN against genotoxic and biochemical effects of cytoxan (anti-tumor drug) in Swiss albino male mice. Mice were divided into 19 groups (5 animals each). The 1st gp served as control. The 2nd, 3rd, 4th, gps had divided into 9 subgroups and received orally 50, 100 and 200mg/kg bw of cytoxan for one, two and three weeks, respectively. The 5th, 6th, 7th gps had divided into 9 subgroups and received 50, 100 and 200 mg/kg bw of cytoxan in combination with 50 mg of HDN for 1, 2 and 3 weeks, respectively. At the end of the experimental periods, animals were subjected to genetic and biochemical analysis. The results revealed that MN frequency significantly increased in cytoxan treated mice. However, HDN treatment showed a significant decreament in MN frequency in mice bone marrow cells compared to control. As well, a significant inhibition in DNA content using comet assay in cytoxan treated groups with 3 fold lower than control, whereas the treatment with HDN in improved that content. Also, there was a significant reduction in DNA, RNA and protein content in brain and kidney tissues and functions (creatinine and urea level elevation). However, administration of HDN in combination with cytoxan significantly improved all parameters studied in brain and kidney tissues. A significant decrease in the level of tissue antioxidants like superoxide dismutase (SOD), cholinesterase (CHE) and increase in malondialdhyde (MDA), cholesterol, glucose and triglycerollevels was found in cytoxan treated groups. In conclusion, HDN supplementation significantly ameliorate these parameters due to antioxidant activity, thereby showing potent antigenotoxic and chemopreventive effects against toxicity induced by cytoxan in mice.

KEYWORDS

Hesperidin;
Antioxidant;
Cytoxan;
Genotoxicity;
Biochemical;
Mice.

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INTRODUCTION

Hesperidin (HDN) is one of naturally occurring flavonoid widely found in citrus fruits.Hesperidin as a flavonoid was found to inhibit DNA & chromosomal damage induced by cyclophosphamide (CPA). Flavonoid compounds have many biological properties, including hepatoprotective, antibacterial, antimutagenic and anticancer activities[1]. HDN is a flavonone glycoside, belonging to the flavonoid family. This natural product is found in citrus species. Citrus extract had a significant protective effect on genotoxicity induced by cyclophosphamide[2]. HDN was reported to have many biological effects including anti-inflammatory, antimicrobial, anticarcinogenic, antioxidant effects, and decreasing capillary fragility[3].

Cytoxan (Cyclophosphamide, CPA) is an alkylating anti-tumor drugused for the treatment of various cancer and noncancer disorders. It is a member of oxazophorine group and its chemical formula is C7H15Cl2N2O2P[4]. Cytoxan (CPA) shows antitumor activities against a broad range of cancers including malignant lymphomas, myeloma, leukemia, neuroblastoma, adeno-carcinoma, retino-blastoma, and breast-carcinoma[5-7]. CPA given at low doses act as either an anti-angiogenic or an immuno-stimulatory agent in combination with other immunotherapies in the treatment of cancer[8,9]. CPA is also used for the mobilization of hematopoietic progenitor cells from the bone marrow into peripheral blood[10,11]. It is also a well known immunosuppressive agent used for graft rejection in case of renal, hepatic, and cardiac transplantation[12]. Its therapeutic use as anticancerous drug is limited due to its side effects.

In somatic cells, CPA has been shown to produce gene mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells. It has also produced chromosome damage and micronuclei in rats, mice and Chinese hamsters, and gene mutations in the mouse spot test and in the transgenic lacZ construct of Mutant Mouse. It acts as both a mutagen and a carcinogen[13-16].

For micronucleus (Mn) assay, the studies of[17,18] showed that oral administration of CPA in mice and rats resulted in a significant increase in the frequency of Mn and that depends on the dose and sampling times. In addition, when CPA was administered to rat a significant increase in chromosomal aberration and Mn formation was found in a dose-dependent manner in BM cells of rat[19].

Administration of citrus extract before CPA treatment significantly reduced the frequency of Mn in mice compared with the group treated with CPA alone. Citrus extract, particularly flavonoids constituents with antioxidant activity, reduced Mn 2.8 fold against genotoxicity induced by CPA[2]. The same findings were found by[20,21]. A significant increase in comet tail length and % Mn in both acute (single dose) and subacute (multiple doses repeated every 24 h for 7 times) studies of CPA treated mice[22,23]. The same finding was reported by[24]. DNA is the primary target in terms of the teratogenic, mutagenic, and antineoplastic effects of CPA. Effects of CPA on DNA have been reported widely in mammalian cells, both of somatic and germ cell origin[12,25]. CPA is supposed to exert its cytotoxicity via the cross-linking of cellular DNA, and some studies demonstrated that following drug exposure there is occurance of interstrand and DNA-protein cross-links, but no single strand breaks[26]. CPA inhibits embryonic DNA synthesis and does so prior to its effect on RNA or protein synthesis[27].

Although CPA is known to produce DNA cross-links, other DNA lesions are produced as well. CPA mediates G0/G1 and S phase arrest. Accumulation ofcells in G0/G1 in comparison to the control, whereas higher concentrations causes dose-dependent G0/G1, S phase and G2/M phase inhibition[28]. However, orange juice was found to reduced the extent of DNA damage caused by CPA in mice due to its antigenotoxic effect[29,30]. The protective effect against that damage was associated with modulation of lipid peroxidation as well as an increase in GSH and the GSH-dependent enzyme glutathione S-transferase[30]. These findings indicate that intake of HDN can lead to protection against *in-vivo* genotoxicity and oxidative stress.

Effect of CPA on biochemical parameters studied by[31,32] in rats showed the decreased level of serum total protein and increased levels of urea and creatinine than the control animals.

An increase in MDA level and decrease in SOD level was found in CPA treated Wistar male rats, that

also happened in mice due to reproductive toxicity[33,34]. However, HDN has shown to increase the level of SOD in the mice and rats[35,36].

An increase in cholesterol, glucose, triglycerol, MDA and Mn frequency levels in CPA treated mice, whereas a decrease in total protein level where found by[37]. To our knowledge this is the first report to study the effect of HDN as a chemopreventive agent in combination with cytoxan on cholesterol, glucose and triglycerol levels in mice.

Therefore, the present study was designed to evaluate the protective effect of hesperidin (HDN) against genotoxic and biochemical effects of cytoxan (anti-tumor drug) treated Swiss albino male mice.

MATERIALS AND METHODS

Animals

Adult Swiss albino male mice weighting 25-30 gm were used in the present study. Animals were housed in cages of the Animal house laboratory of the National Research Center, Dokki, Cairo, Egypt and had free access to water and pellet diet for one week for adaptation.

Drugs

I – Cytoxan were purchased from Sigma Aldrich Chemical Private Ltd., India is used in the treatment of mice under study.

II- Hesperidin was supplied by Sigma Aldrich Chemical private Ltd., India and suspended in distilled water and administered orally and used as a protective.

Treatment

Mice were divided into 19 groups (5 animals each). The 1stgp served as control. The 2rd, 3th, 4th, gps had divided into 9 subgroups and received orally 50, 100 and 200mg/kg bw of cytoxan for one, two and three weeks, respectively. The 5^{h}, 6th, 7th gps had divided into 9 subgroups and received 50, 100 and 200 mg/kg bw of cytoxan in combination with 50 mg of HDN for 1, 2 and 3 weeks, respectively. At the end of the experimental periods animals were anaesthetized and sacrificed by cervical dislocation and subjected to genetical and biochemical analysis.

Genetic analysis

Micronucleus assay

Mice were sacrificed and both femurs of mice were removed and aspirated with fetal calf serum[38]. The bone marrow smears were made, fixed and stained with Giemsa[39]. 2000 polychromatic erythrocytes were scored per animal.

Comet assay

The comet assay were performed according to Comet assay reagent kit for single cell gel electrophoresis assay (Catalog ≠ 4250-050-k). DNA migration, image length, nuclear size and DNA damage were calculated in mice brain cells.

Determination of nucleic acids

Nucleic acids (DNA and RNA) were determined using a simplified method for determination of specific DNA or RNA[40].

Biochemical analysis

Blood samples were collected in heparinized tubes and centrifuged at 5000 rpm for 10 min for quantitative measurement of lipid peroxidase malondialdhyde (MDA) according to[41]. Superoxide dismutase (SOD) activity was assayed by the method of[42]. Cholinesterase (CHE) was assayed by the method of[43]. Biochemical estimation of cholesterol was developed according to[44]. Triglyceride was measured according to[45] and urea, creatinine and glucose were determined by a differential pH technique according to[46]. Total protein content was measured colorimetrically according to[47].

Statistical analysis

Data are presented as Means ± SE. One way analysis of variance (ANOVA) and Tukey's HSD test were used for multiple comparisons of data.

RESULTS AND DISCUSSION

In the present study, HDN was investigated for its potential antigenotoxic and chemopreventive effects in CP treated Swiss albino mice by genetic (Mn and comet assays) and biochemical analysis.

The results of the present study revealed that the

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TABLE 1 : Effect of HDN on cytoxan induced micronuclei in male mice

Treatment period	Dose mg/kg	Number of animals	Number of examined cells	Cytoxan		Cytoxan + HDN	
				% of cells with micronuclei	M ± S.E.	% of cells with micronuclei	M ± S.E.
1 Week	50.0	5	2000	2.80	11.2±0.88*	2.15	8.6±0.60
	100.0	5	2000	3.10	12.4±1.04**	2.20	8.8±0.55
	200.0	5	2000	3.40	13.8±0.65***	2.25	9.0±0.61
2Weeks	50.0	5	2000	3.00	12.0±0.79**	2.30	9.2±0.42
	100.0	5	2000	3.20	13.0±0.80***	2.35	9.4±0.57
	200.0	5	2000	3.70	15.0±1.00***	2.40	9.6±0.57
3Weeks	50.0	5	2000	3.60	14.4±1.20***	2.50	10.0±0.80
	100.0	5	2000	3.90	15.6±1.03***	2.60	10.4±0.84
	200.0	5	2000	4.10	16.4±0.91***	2.70	10.8±1.08
Control	0.0	5	2000	2.05		8.4±0.57	

Results are shown as mean ± SE; The last row indicate the control group data; *=P<0.05, **=P<0.01 and ***=P<0.001.

TABLE 2 : Mean comet tail length (μm) of mice brain exposed to cytoxan and /or HDN

Treatment period	Dose mg/kg	Mean comet tail length (Mean ± S. E)	Mean comet tail length (Mean ± S. E)
		Cytoxan	Cytoxan + HDN
1 Week	200.0	18.15±1.839*	13.94±1.034
2 Weeks	200.0	22.29±1.72***	17.842±2.078**
3 Weeks	200.0	29.34±2.872***	20.828±1.301***
Control	0.00	13.17±1.072	

Results are shown as mean ± SE; The last row indicate the control group data; *= P<0.05, **= P<0.01 and ***= P<0.001

treatment with cytoxan drug resulted in significant increase in the frequency of MN with 3 fold compared to control (TABLE 1). However, hesperidin treatment showed a significant decrease in MN frequency in mice bone marrow cells compared to cytoxan treated groups.

The comet assay (mean comet tail length) results showed a significant increase in comet tail length in cytoxan treated groups with 3 fold higher than control, whereas the treatment with HDN improved that results (TABLE 2).

The antigenotoxic activity of HDN was evaluated by measuring their inhibitory effect on CP (cytoxan) induced genotoxicity in mice. It is indicated that CP induced chromosomal damage in mouse bone marrow cells. These fragmented chromosomes were condensed to form Mn[48]. HDN decreased the CPA induced formation of Mn, which may due to the inhibition of CPA induced chromosomal and DNA damage.

The antitumor agent CPA induced genotoxicity in mice and rats based on evaluation of Mn frequencies[49]. A dose response for CPA –induced DNA damage was detectable in mice and rats by the comet assay. Evaluation of the nature of the CPA– induced Mn in mice and rats revealed that the Mn were primarily due to breakage events and contained chromosomal fragments[49,23]. While[17,18] showed that oral administration of CPA in mice and rats resulted in a significant increase in the frequency of Mn and that depends on the dose and sampling times. As well with[22-24], who reported a significant increase in comet tail length and Mn frequency in cytoxan treated mice.

Consumption of orange juice containing HDN can protect DNA from damage induced by CPA. Such protective effects of orange juice may be mediated by, (1) modulation of phase I and II enzymes; (2) substrate competition for the nucleophilic action of CPA or quenching of CPA metabolities and side-products (reactive species); and (3) enhancement of DNA repair[29].

Oral administration of cytoxan (50, 100 and 200mg/kg bw) to male mice for 1, 2 and 3 weeks caused a significant reduction in DNA, RNA and protein content in brain (TABLE 3) and kidney (TABLE 4) tissues.

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TABLE 3 : Effect of cytoxan and/or HDN on DNA, RNA and protein of mice brain

Treatment period	Dose mg/kg	Cytoxan			Cytoxan + HDN		
		DNA	RNA	Protein	DNA	RNA	Protein
1 Week	50.0	0.39***±0.02	0.272*±0.013	9.046*±0.105	0.491±0.012	0.312±0.012	9.606±0.131
	100.0	0.39***±0.12	0.250**±0.14	8.687**±0.237	0.481±0.240	0.304±0.014	9.453±0.251
	200.0	0.37±0.01	0.235***±0.006	8.473***±0.166	0.469±0.012	0.296±0.012	9.392±0.137
2 Weeks	50.0	0.38***±0.01	0.264*±0.014	8.685**±0.276	0.488±0.025	0.310±0.013	9.501±0.195
	100.0	0.36±0.01	0.244**±0.008	8.356**±0.253	0.450*±0.016	0.294±0.012	9.410±0.187
	200.0	0.35***±0.02	0.218±0.010	8.344***±0.110	0.448*±0.012	0.283±0.0017	9.201±0.223
3Weeks	50.0	0.36***±0.02	0.257**±0.017	8.276***±0.207	0.458*±0.011	0.291±0.008	9.283±0.204
	100.0	0.34***±0.02	0.235***±0.006	8.150***±0.193	0.440*±0.240	0.280±0.014	9.101±0.149
	200.0	0.31±0.02	0.198±0.006	8.080±0.170	0.436*±0.019	0.267±0.001	9.060±0.220
Control	0.0	0.491±0.01	0.323±0.014	9.835±0.283			

Results are shown as mean ± SE; The last row indicate the control group data; *= P<0.05, **= P<0.01 and ***=P<0.001.

TABLE 4 : Effect of HDN on kidney (DNA, RNA, Protein) and kidney function (Creatinine, Urea) of mice treated with cytoxan

Treatment period	Dose mg/kg	Cytoxan					Cytoxan + HDN				
		DNA	RNA	Protein	Creatinine	Urea	DNA	RNA	Protein	Creatinine	Urea
1 Week	50.0	0.331*** ±0.015	0.264* ±0.018	6.563* ±0.449	0.660* ±0.026	34.900** ±0.488	0.417 ±0.011	0.316 ±0.014	7.859 ±0.490	0.588 ±0.012	30.880 ±0.285
	100.0	0.314*** ±0.019	0.250* ±0.012	6.149* ±0.236	0.699** ±0.030	34.780*** ±0.339	0.394 ±0.009	0.290 ±0.018	7.062 ±0.541	0.616 ±0.012	31.160 ±0.348
	200.0	0.308 ±0.013	0.218** ±0.006	5.761** ±0.382	0.743*** ±0.018	35.110*** ±0.408	0.381 ±0.019	0.278 ±0.009	6.647 ±0.550	0.625 ±0.018	32.050 ±0.618
2 Weeks	50.0	0.328 ±0.013	0.259** ±0.023	6.443* ±0.549	0.670* ±0.028	34.970*** ±0.580	0.407 ±0.110	0.308 ±0.015	7.486 ±0.478	0.600 ±0.010	31.850 ±0.567
	100.0	0.311*** ±0.008	0.203*** ±0.006	5.920** ±0.200	0.701** ±0.021	35.870*** ±0.392	0.391 ±0.008	0.284 ±0.012	6.961 ±0.464	0.626 ±0.010	32.350 ±0.690
	200.0	0.304 ±0.014	0.192*** ±0.010	5.713** ±0.253	0.814*** ±0.013	37.300*** ±0.606	0.377 ±0.008	0.274 ±0.008	6.628* ±0.440	0.639 ±0.020	33.160 ±0.844
3 Weeks	50.0	0.310 ±0.008	0.237** ±0.008	6.401* ±0.516	0.715*** ±0.210	36.920*** ±0.456	0.406 ±0.005	0.282 ±0.009	7.123 ±0.555	0.607 ±0.012	32.060 ±0.340
	100.0	0.306*** ±0.014	0.200*** ±0.007	5.701** ±0.288	0.883*** ±0.025	37.670** ±0.444	0.365* ±0.017	0.270 ±0.017	6.815 ±0.586	0.630 ±0.022	33.820 ±0.847
	200.0	0.295*** ±0.015	0.188*** ±0.014	5.438** ±0.376	1.001*** ±0.009	40.160*** ±0.494	0.343* ±0.014	0.267 ±0.010	6.824* ±0.326	0.649* ±0.016	34.720* ±0.950
Control	0.0	0.429 ±0.02	0.319 ±0.019	8.166 ±0.431	0.571 ±0.023	0.0			30.670 ±0.727		

Results are shown as mean ± SE; The last row indicate the control group data; *=P<0.05, **=P<0.01 and ***=P<0.001

These findings are in accordance with[27], who found that CPA inhibits DNA synthesis and does so prior to its effect on RNA or protein synthesis. As well, a significant damage in kidney function (creatinine and urea) in 3 fold with 3 doses used in the study. However, administration of HDN in combination with cytoxan significantly improved all parameters studied in brain and kidney tissues and that coincide with[32] who said that increased of urea could be due to increased protein level or due to impairment of renal function in CPA intoxication. As well a significant decrease in total protein level was found in CPA treated rats. However, no differences were found in creatinine and urea levels between CPA and morin (a naturally occurring flavonoid) treated groups[32]. CPA mediates G0/G1 and S phase arrest. Accumulation of cells in G0/G1 in comparison to the control, whereas higher concentrations causes dose-dependent G0/G1, S phase and G2/M phase inhibition[28].

Serum cholinesterase (CHE) with antioxidant SOD

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TABLE 5 : Effect of HDN on blood MDA, SOD and CHE of cytoxan intoxicated male mice

Treatment period	Cytoxan Dose (mg/kg)	Number of Animals	Cytoxan			Cytoxan + HDN (50 mg/kg bw)		
			MDA	SOD	CHE	MDA	SOD	CHE
1 Week	50.0	5	9.65±0.69*	6.27±0.33*	6.009±0.40**	7.87±0.55	7.51±0.44	7.604±0.29
	100.0	5	10.19±0.87*	6.11±0.39*	5.773±0.32**	8.36±0.54	7.13±0.35	7.385±0.24
	200.0	5	10.51±0.83	5.78±0.36**	5.193±0.26***	8.96±1.06	6.74±0.42	7.164±0.24
2 Weeks	50.0	5	10.20±0.88*	6.21±0.48*	5.863±0.35**	8.46±0.74	7.38±0.50	7.495±0.23
	100.0	5	11.04±0.93	6.06±0.45*	5.693±0.29**	8.66±0.57	6.83±0.43	7.268±0.28
	200.0	5	11.89±0.58	5.49±0.35***	5.042±0.34***	9.04±0.82	6.64±0.38	7.156±0.22
3 Weeks	50.0	5	11.18±1.13	5.53±0.42**	4.249±0.26***	9.23±0.80	7.06±0.46	6.841±0.25*
	100.0	5	12.39±0.92	5.33±0.34***	4.222±0.24***	9.61±0.88	6.74±0.42	6.542±0.35*
	200.0	5	13.20±0.59	4.75±0.40	3.758±0.24***	9.66±0.65*	6.37±0.63	6.505±0.26*
Control	0.0	5	7.67±0.28	7.90±0.42	8.292±0.52			

Results are shown as mean ± SE; The last row indicate the control group data; *=P<0.05, **=P<0.01 and ***=P<0.001.

TABLE 6: Effect of HDN on blood cholesterol, glucose and triglycerol of cytoxan intoxicated male mice

Treatment period	Dose mg/kg	Cytoxan			Cytoxan + HDN (50 mg/kg bw)		
		Cholesterol mg/dL	Glucose mg/dL	Triglycerol mg/dL	Cholesterol mg/dL	Glucose mg/dL	Triglycerol mg/dL
1 Week	50.0	159.55±0.79*	72.86±1.02	97.29±1.84*	155.57±1.50	70.57±0.56	92.37±0.69
	100.0	161.54±0.79**	73.54±0.81*	99.73±1.66***	156.60±0.93	70.59±0.33	93.40±1.04
	200.0	163.54±0.53***	74.99±0.92**	100.82±1.47***	157.46±0.79	71.16±0.46	93.89±3.09
2 Weeks	50.0	160.48±0.80*	73.84±1.1*	97.91±1.66**	156.40±1.12	70.81±0.34	92.50±0.82
	100.0	162.25±0.76**	74.99±0.92**	100.92±1.99***	157.51±0.96	71.35±0.58	93.74±0.94
	200.0	165.95±0.55***	75.53±0.92***	101.01±2.27***	158.46±0.71	71.85±0.90	94.11±1.08
3Weeks	50.0	161.59±0.85***	74.15±0.98***	100.39±1.16***	157.57±0.96	71.81±0.35	93.66±1.01
	100.0	164.20±0.99***	76.2±0.48***	101.69±2.32***	158.98±0.88	72.26±1.06	95.90±4.01
	200.0	167.07±0.96***	78.43±0.80***	102.73±1.93***	159.48±1.07	72.86±1.02	97.15±2.98
Control	0.0	15.42±1.5	70.54±0.50	91.02±1.1			

Results are shown as mean ± SE; The last row indicate the control group data; *=P<0.05, **=P<0.01 and ***=P<0.001.

and MDA values were significantly decreased ($p \leq 0.01$) with cytoxan treatment compared to control. That happened in mice due to reproductive toxicity[34]. However, the treatment with HDN improved these values in all treated groups (TABLE 5).

Determination of serum lipids and glucose showed a significant increase ($p \leq 0.01$) in cholesterol, triglyceride and glucose values in the cytoxan treated groups compared to control group (TABLE 6). However, administration of hesperidin in combination with cytoxan improved these values.

The treatment with HDN in combination with cytoxan significantly ($p < 0.05$) lowered the degradation of membrane lipids than the cytoxan alone. Cytoxan induction provokes lipid bilayer repercussion by breaking down membrane phospholipids, therefore MDA, product of lipid peroxidation acts as a marker for lipid bilayer damage[50]. As the membrane damage progress, it results in the buildup of free radicals in normal animals which leads to greater membrane damage and inactivation or alteration of membrane bound enzymes[51]. On treatment with rats, HDN decreased MDA level signifying attenuation in lipid peroxidation thereby proving its stabilizing power on membranes[52]. Enough evidence has been garnered for HDN proving to be effective antioxidant in CPA mediated oxidative stress[21].

In conclusion, these findings substantiate the chemoprotective and antigenotoxic potential of HDN against toxicity induced by cytoxan in mice. It is clear that HDN may has antioxidative activity which reduced

the oxidative stress, genotoxicity, and DNA damage induced by cytoxan in mice.

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