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Protective effect of *Ferula hermonis* root extract against cycram-induced DNA, biochemical and testicular damage in rats

Shenouda M. Girgis*, Amira Abd ElRaouf and Halima S. Abdou

Department of Cell Biology, National Research Centre, 33 El-Bohouth St. (former El Tahrir St.) -Dokki, Giza, P.O. 12622, Affiliation ID: 60014618, Egypt

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Abstract

Cycram (CYC), or cyclophosphamide is an alkylating drug that has been widely used in the acute treatment of various neoplastic diseases and in the chronic treatment of autoimmune disorders. The major limitation of CYC chemotherapy is the injury of normal tissue, leading to multiple organ toxicity and alter male fertility in mice, rats and human, leading to testicular damage and decreasing in RNA, DNA and protein synthesis. The present study aims to evaluate the protective role of methanol extract of *Ferula hermonis* (FH) against CYC induced changes in testicular RNA, DNA and protein content as well sperm morphology, count and motility, blood levels of testosterone (T) and hemoglobin (Hb) in adult male albino rats. Ninety adult albino male rats were divided into 3 main treated groups (30 animals each) according to treatment periods (15, 30 and 60 days), every one divided to 3 subgroups according to CYC dose (50, 100 and 200 mg/kg bw/day, 10 animals each), and each subgroup divided to 2 sub-sub groups treated with CYC and CYC+ FH 0.025 ml/100 g bw/day by gavage, respectively, (5 animals each), besides the control (10 animals). Animals were sacrificed, sperm morphology with its count and motility, as well RNA, DNA and protein content, and testosterone and hemoglobin levels were determined. The results revealed that CYC treatment induced alterations in RNA, DNA and protein synthesis, as well as affected T and Hb levels and sperm abnormalities of male rats. However, supplementation of FH extract protects these parameters against CYC toxicities. The protective actions of FH extract seem to be closely involved with the suppressing of plasma lipid peroxidation and increasing of antioxidant enzyme activities. Therefore, FH extract might be used in combination with CYC in cancer patients' therapy, transplantation and autoimmune diseases as a protective agent against CYC-induced reproductive toxicity.

Keywords: *Ferula hermonis*, protect, Cycram, DNA, Damage, Testicular, Biochemical, Rats.

1. Introduction

Cycram (CYC) or cyclophosphamide is a cytotoxic alkylating drug that has been widely used in the acute treatment of various neoplastic diseases and in the chronic treatment of autoimmune disorders. The major limitation of CYC chemotherapy is the injury of normal tissue, leading to multiple organ toxicity mainly in the heart, testes and urinary bladder (Fraiser *et al*., 1991; Ghobadi *et a*l., 2017). CYC was reported to alter human fertility and is cytotoxic to rapidly dividing cells, which makes the highly proliferative testes a target for the damaging effects of this drug. Use of CYC for the treatment of cancer in male patients increases the incidence of oligo- and azoospermia and results in male infertility (Howell and Shalet, 1998). Previous studies have demonstrated that chronic administration of CYC to male rats/mice leads to decreased testicular weight, transitory oligospermia, decreased DNA synthesis in spermatogonia and protein synthesis in spermatids (Anderson *et al.*, 1995; Meistrich *et al*., 1995; Kaur *et al*., 1997).

Molecular components of sperm like RNA have the potential to be important indicators of reproductive toxicity. This is especially important for long-term exposures, such as those experienced in chemotherapeutic regimens, in which cytotoxic drugs are often given for months rather than days. Exposures that target germ cells for extended periods of time, as chemotherapy drugs do, may produce changes in the molecular contents of the sperm that can serve as biomarkers of testicular dysfunction (Dere *et al*., 2013). Rats treated with CYC were displayed lowest weight of testicles and epididymis (reproductive organs) and the lowest motility, progression, viability, and sperm count (Shabanian *et al.*, 2017), a decrease in serum hormone levels (testosterone [T], follicle stimulating hormone [FSH], and luteinizing hormone [LH]) (Johari *et al.*, 2011), as well as 20 % reduction in the hemoglobin (Hb) level were found (Cengiz, 2018).

Numerous studies have shown that CYC exposure can disrupt the redox balance of tissues to suggest that biochemical and physiological disturbances may result from oxidative stress (Selvakumar *et al.*, 2004). It is, therefore, important to continue the search for an effective natural compound that will protect against CYC-induced reproductive toxicity associated with chemotherapy. These studies suggest that natural compounds with antioxidative

* Corresponding author e-mail: shenoudag2009@hotmail.com.

** **Abbreviations :** FH: Ferula hermonis; CYC: Cycram; ANOVA: Analysis of variance; T: testosterone; Hb: hemoglobin; FSH: follicle stimulating hormone; LH: luteinizing hormone.

properties may have the potential to ameliorate CYC-mediated testicular injury. In the past decade, the bioactivities of flavonoids on human health have given rise to much attention, especially the antioxidant activity (Zhang, 2005). The protective role of flavonoids involves several mechanisms of action: direct antioxidant effect, inhibition of enzymes of oxygen-reduction pathways and sequestration of transient metal cations (Rice-Evans, 2001).

To prevent these toxic side effects, there is a need for novel agent or natural product such as *Ferula hermonis* (FH) that may protect against CYC-induced reproductive toxicities in male rat. FH is a medicinal plant and a known natural antioxidant that belongs to the apiaceae family and grows abundantly in the Mediterranean region and Middle East (Naguib, 2003; Auzi *et al*., 2008), and used as a tonic, stimulant and aphrodisiac and as an anti-impotency medication for both sexes, as well as increasing the "stamina" of animals. Other traditional uses for FH are cauterizing wounds, curing animal infections and increasing the milk production of cows (Canogullari *et al*., 2009). Ferula hermonis (FH) is reported to have diverse therapeutic effects (Abutaha *et al.*, 2019). Pharmacological reports have revealed that FH has several bioactivities which are useful to treat reproductive dysfunction, menopausal disturbances, neurological disorders (Zanoli *et al*., 2005) and diabetes (Raafat and El-Lakany, 2015). The plant is also reported to have antimicrobial, anti-inflammatory (Saab *et al.*, 2012; Al-Ja'fari *et al.*, 2013), anticancer activities (Saab *et al.*, 2012; Galal *et al.*, 2001) and antioxidant effect due to its flavonoid composition (Abarikwu *et al.*, 2012).

There are a little studies about effects of FH on the reproductive system and sexual appetite. The ethanolic extract of seeds and roots of FH called "masculine" was examined on male fertility and sexual functioning in rats and humans and was exhibits a high level of safety in rats, humans and cultured human fibroblasts and increases erection in rats. In addition; consumption of one tablet of masculine daily for 3 months could increase sperm number and sperm motility in men (Kassisa *et al.*, 2009). In addition, Rajeh and Al-Shehri (2019), found that FH ameliorates sperm count and T serum level due to its antioxidant activity against testicular toxicity in male rats. Another study reported that a single oral dose of FH extract can increase serum T concentration in adult male rat up to three-folds (Gunes and Fetil, 2000).

Therefore, the present study was designed to evaluate the protective effects of FH extract against CYC-induced reproductive toxicities which include sperm morphology, count and motility; RNA, DNA and protein content in rat testes as well measurement of testosterone and hemoglobin levels has been attempted.

2. Materials and Methods

2.1. Animals and treatment

Adult male albino rats (weighing 200–220 g), were obtained from animal house of National Research Centre, Dokki, Cairo, Egypt. They were housed in a standard conditions of temperature (22 ± 2°C), relative humidity (55 ± 10%), and 12/12 h light/dark cycle, and fed with a standard rat feed and water *ad libitum.* The experimental designs and protocols for study conform the requirement of Ethics Committee of our Institute in accordance with the standard guide for the care and use of laboratory animals. Animals were divided into 3 main treated groups (30 animals each) according to treatment periods (15, 30 and 60 days every one divided to 3 subgroups according to CYC dose (50, 100 and 200 mg/kg bw/day, dissolved in distilled water following pretreatment with olive oil by gavage, 10 animals each), and each subgroup divided to 2 sub-sub groups treated with CYC and CYC+ FH (0.025 ml/100 g bw/day dissolved in olive oil by gavage), nd5 animals each, besides the control group (10 animals).

2.2. Drug and chemicals

CYC was purchased from Baxter Oncology GmbH, Frankfurt, Germany. All other chemicals used were of the highest purity and analytical grade.

2.3. The herbal remedy

Roots of FH were obtained from the Experimental Station of Medicinal Plants, Ministry of Agriculture, Cairo, Egypt. Sample extract was obtained by stirring 3 g of dry organ powder with 30 ml of methanol for 24 h at 120 rpm using a magnetic stirrer plate. Extract obtained was kept at 4 °C, filtered through a Whatman filter paper (No. 4) and freed of solvent under reduced pressure at 45 °C using a rotary evaporator. The dried crude concentrated extract was stored at −20 °C until further use for analyses (Rahali *et al*., 2018). Phenolic compounds of plant sample were extracted according to the method outlined by (Duke *et al.*, 2003). The identification of the individual phenolic compounds was performed on high liquid chromatography (JASCO HPLC) (Table 1), using hypersil C18 reversed place column (250x4.6mm) with 5 µ particle size.

The herb was given to the treated animals orally (using oral tube), twice weekly as prescribed by herbalist (0.025 ml/ 100 g bw from plant water extract 50%) prepared in the form of water extract (by soaking in boiled water over night) for 8 successive weeks.

Table 1. HPLC of polyphenols in methanol extract of FH.

Ingredients	mg/100 g
Phenol	30
Resorcinol	670
Protocatchuic acid	340
Catchines	8380
Parahydroxy benzoic	3260
Caffeic acid	420
Daidzin	26
Ferulic acid	880
Coumarine	440
Paracoumaric acid unhydrate	290
Rutin	380
Myricetin	1100
Eugenol	1700
3,5 Dihydroxyisoflavon	1500
Quercedin	220
Caumpherol	3840
Pinostrobin	1810

HPLC= High pressure liquid chromatography. FH= Ferula hormones.

2.4. Sampling

At the end of the specified treatment (2, 4 and 8 weeks), the animals were euthanized by ethyl ether exposure and were killed by decapitation. Blood samples were collected in vials containing heparin. The plasma was separated for analysis of testosterone and hemoglobin using specific kits purchased from Rocky Mountain Diagnostic, Inc. 2139 Chuckwagon Road Suite 20. Colorado Springs, CO 80919, USA. Testis were obtained for RNA, DNA and protein analysis.

2.5. Sperm characteristics

Epididymal sperms were collected for sperm count and motility according to (WHO, 2010). Briefly, sperm count and motility within semen should be assessed as soon as possible after scarification, preferably at 30 minutes, but in any case within 1 hour, to limit the deleterious effects of dehydration, pH or changes in temperature on motility. Mix the semen sample well. Remove an aliquot of semen immediately after mixing, allowing no time for the spermatozoa to settle out of suspension. Remix the semen sample before removing a replicate aliquot. For each replicate, prepare a wet preparation approximately 20 m deep. Wait for the sample to stop drifting (within 60 seconds). Examine the slide with phase-contrast optics at ×200 or × 400 magnifications. Assess approximately 200 spermatozoa per replicate for the percentage of different motile categories.

For the analysis of morphological abnormalities such as head (hook less and banana shape), or abnormal tails (coiled and divided), the standard protocol for sperm morphology assay (Wyrobek *et al.,* 1983) was used. Briefly, after the treatment, the animals were sacrificed by cervical dislocation. Both of the cauda epididymises were dissected out, cut into pieces in 5 mL of saline, filtered, and smears were made. The smears were fixed in methanol and stained with 10 % Giemsa in Sörensen buffer for 10 min. A total of 1000 sperms per animal were scored under a microscope (Olympus CX21®, Japan) with 100x10 magnifications. Sperm head and tail abnormalities were determined as having either normal or abnormal morphology.

2.6. Determination of Nucleic Acids and protein

Nucleic acids (DNA and RNA) were determined using a simplified method for determination of specific DNA and RNA using quantitative PCR and an automatic DNA sequencer (Porcher *et al.*, 1992). Total protein is estimated using commercial kits (Peter, 1968).

2.7. Biochemical investigations

At the end of each experimental period and 24 h after the last dose of protective treatment, rats were sacrificed by cervical decapitation after an overnight fast. Blood was collected after 2, 4 and 8 weeks of treatment, and the separated serum was used for assaying the testosterone (T) level. Serum level of T was measured using an enzyme-linked immunosorbent assay (ELISA) purchased from Rocky Mountain Diagnostic, Inc. 2139 Chuckwagon Road Suite 20. Colorado Springs, CO 80919, USA. Hemoglobin level was determined using specific kits purchased from Crystal Chem, 955 Busse Rd, Elk Grove Village, IL 60007 USA .

2.8. Statistical analysis

The values are expressed as mean ± standard deviation (S.D). Differences between groups were assessed by one-way analysis of variance (ANOVA). Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were carried out by least significant difference (LSD) test. Significance at P-values <0.001, <0.01 and <0.05 have been considered in the tables.

3. Results

3.1. Effect of CYC and FH extract on RNA, DNA and protein content

The results of the present study revealed that CYC treatment significantly (P <0.01) decreased RNA, DNA and protein content in male rat testes compared to control (Table 2). RNA and DNA levels decreased specially with the high dose (200 mg/kg bw of CYC) for 15 days of treatment up to the largest dose (200 mg /kg bw) for 60 days of treatment, while protein level decreased gradually in a time and dose dependent manner. However, FH administration in combination with CYC ameliorate these levels compared to CYC only treated groups.

Table 2. Effect of CYC and FH extract on RNA, DNA and protein content in rat testes.

Treatment period	Dose	RNA mg/g tissue		DNA mean ± S.D		g/dl protein	
		CYC	CYC+FH	CYC	CYC+ FH	CYC	CYC+ FH
15 days	50	0.336± 0.030	0.403± 0.029	0.478± 0.019	0.491± 0.020	11.289**± 0.692	12.393± 0.690
	100	0.289± 0.015	0.387± 0.015	0.465± 0.013	$0.482^{N.S}$± 0.018	9.341**± 0.672	11.323*± 0.875
	200	0.268± 0.016	0.369± 0.027	0.430**± 0.039	0.474± 0.016	8.014***± 0.216	10.221**± 0.730
30 days	50	0.320± 0.030	0.392± 0.015	0.464*± 0.019	0.488± 0.030	8.745***± 0.565	10.940**± 0.644
	100	0.278± 0.018	0.376± 0.022	0.446**± 0.030	0.470± 0.025	8.035***± 0.190	9.996**± 1.005
	200	0.252± 0.021	0.330± 0.027	0.398± 0.021	0.398± 0.021	7.47***± 0.420	8.168***± 0.488
60 days	50	0.298± 0.007	0.377± 0.024	0.441**± 0.30	0.463± 0.019	8.243***± 0.547	9.168**± 0.488
	100	0.247± 0.014	0.340± 0.030	0.429± 0.034	0.456± 0.038	7.199***± 0.544	8.273***± 0.538
	200	0.230± 0.012	0.292± 0.050	0.379± 0.018	0.436**± 0.037	6.771***± 0.3199	7.917***± 0.323
Control		0.415± 0.617		0.511± 0.018		13.323± 0.757	

Data are presented as means ± SD. * Significant at P≤ 0.05; ** Significant at P ≤ 0.01;

*** Significant at P ≤ 0.001. CYC= Cycram; FH= Ferula hormones.

3.2. Serum testosterone and hemoglobin levels

Table (3) showed that CYC administration caused significant reductions ($P < 0.001$), in serum testosterone (T) level (9.308±0.399) and hemoglobin (9.393±0.505) ($P < 0.001$), when compared with the control group, especially with the high dose of CYC (200 mg/kg bw) and long period treatment (60 days) in a dose and time dependent manner (9.308±0.399 ng/l and 9.393±0.505 g/dl vs. 15.052±0.841and15.813±0.622 for T and Hb levels in treated and control groups, respectively). However, supplementation of FH improved these parameters compared to CYC only treated groups. FH was able to produce marked effects in CYC-treated rats, as demonstrated by the restoration of the levels of serum T and hemoglobin, to almost normal levels when compared with the control group.

Table 3. Effect of CYC and FH extract on serum testosterone and hemoglobin levels in male rats.

Treatments period	Dose	Testosterone hormone (ng/l)		Hemoglobin g/dl	
		CYC	CYC + FH	CYC	CYC + FH
15 days	50	11.776±0.671***	14.372±0.701	12.566± 0.783***	14.872± 0.584
	100	11.462±0.510***	13.668±0.559*	11.593± 0.525***	13.958± 0.867*
	200	11.03±0.424***	13.24±0.567*	10.114***± 0.280	13.017± 0.719**
30 days	50	11.289±0.760***	13.862±0.298*	12.304± 0.651***	14.375± 0.542*
	100	10.346±0.521***	13.526±0.417*	11.359± 0.525***	13.913± 0.655**
	200	10.037±0.292***	12.46±0.515**	9.758± 0.516***	12.755± 0.623**
60 days	50	10.39±0.502***	13.19±0.372**	11.958± 0.893***	13.997± 0.740**
	100	9.734±0.529***	12.728±0.505**	10.662± 0.526***	13.396± 0.543**
	200	9.308±0.399***	12.270±0.830**	9.393± 0.505***	12.708± 0.483**
Control		15.052±0.841		15.813±0.622	

Data are presented as means ± SD. * Significant at P≤ 0.05; ** Significant at P ≤ 0.01;

*** Significant at P ≤ 0.001. CYC= Cycram; FH= Ferula hormones.

3.3. Sperm abnormality in CYC and FH extract treated rats

Sperm abnormality rates in response to various treatments for 8 weeks of treatment are presented in table (4). CYC administration caused statistically significant ($P < 0.01$) increases in tail, head and total abnormality of sperm in comparison with the control group. A significant ($P < 0.01$) decrease in these abnormalities was observed in CYC+ FH groups as compared with alone CYC group. The values of head abnormality were brought near values to control by FH administrations to CYC-treated rats, especially with the low dose of CYC; these administrations could significantly improve this parameter when compared with the alone CYC group.

A significant decrease ($P < 0.01$) in total count and motility was found in CYC treated groups. However, the treatment with FH in combination to CYC ameliorates these parameters compared to alone CYC treated groups and brought these values near to control especially with the low dose of CYC.

Table 4. Sperm abnormality in CYC and FH extract treated rats.

Treatment	Dose	Tail abnormality		Head abnormality		Total Abnormality	Total Count	Motility %
		Divided	Coiled	Without hock	Banana shape			
CYC	50	3.0± 0.581**	2.2± 0.924**	2.0± 0.581**	1.8± 0.924**	9.0± 0.581**	26.522x10^6± 1.476	41.35% ±2.53
	100	3.4± 0.302**	3.0± 0.225**	3.6± 0.789**	2.4± 0.342**	12.4± 0.302**	24.478x10^6± 3.288	39.44% ±3.06
	200	5.0± 0.581**	4.6± 0.140**	4.0± 0.581**	3.2± 0.304**	16.4± 0.140**	22.980x10^6± 1.110	33.59% ±2.75
CYC+ FH	50	1.4± 0.401	1.2± 0.837	1.0± 0.710	0.6± 0.894	4.2± 0.837*	31.124x10^6± 1.755	68.75% ±4.57
	100	2.2± 0.837*	1.6± 0.581*	1.4± 0.140	1.0± 0.707*	6.2± 0.280**	28.100x10^6± 0.945	59.35% ±5.78
	200	3.0± 0.581**	1.2± 0.836	1.6± 0.40*	2.0± 0.837**	7.8± 0.28**	27.85x10^6± 1.069	54.69% ±4.41
Control		0.4± 0.548	0.6± 0.548	0.8± 0.095	0.4± 0.394	2.2± 0.924	32.5 x10^6± 1.714	82.42% ±4.36

Data are presented as means ± SD. * Significant at P≤ 0.05; ** Significant at P ≤ 0.01;

*** Significant at P ≤ 0.001. CYC= Cycram; FH= Ferula hormones.

4. Discussion

The present work was performed to evaluate the protective role of FH against CYC induced changes in testicular RNA, DNA and protein content, sperm morphology, count and motility, as well as blood levels of T and Hb in adult male albino rats. The main findings of this study indicated that CYC induced deleterious effect on these parameters. That is in accordance with those of (Anderson *et al.*, 1995; O'Flaherty *et al.*, 2010; Bujan *et al.*, 2013) who found that administration of CYC leads to decreased RNA, DNA and protein synthesis in male rats/mice.

Oxidative DNA damage is caused by hydroperoxide derivative of CYC through generation of H_2O_2 (Murata *et al.*, 2004). Also, acrolein has been found to interfere with

the tissue antioxidant defense system (Arumugam *et al*., 1997) and produces highly reactive oxygen free-radicals (Mythili *et al.*, 2004) that are mutagenic to mammalian cells (Kawanishi *et al*., 1998). From these studies, it is clear that the administration of a potent and safe antioxidant such as FH extract might reduce CYC-induced reproductive toxicity.

The reduction in T and Hb levels in rats exposed to CYC, especially with the high dose coincides with those of (Motawi *et al*., 2010; Mohammadi *et al*., 2014). The decline in serum T level after CYC treatment in consistent with previous reports, and this decline could be due to CYC-induced membrane lipid peroxidation (Das *et al*., 2002). The decrease in serum T level in CYC-treated rats demonstrates defect in the testis. The decreased level of Hb observed in the CYC-treated group in our study may be due to the interaction of CYC with sulfhydryl-containing proteins and/or testicular Ca^{+2} overload (Selvakumar *et al*., 2006).

The level of abnormalities in sperm morphology, total count and motility in CYC treated rats was high; however, FH administration improved these parameters. That is in line with the results of (Abarikwu *et al*., 2012; Watcho et al., 2019) who found that CYC-treated rats had higher abnormal sperm morphology than the control group. However, the CYC plus Rutin (one of the FH component) treated rats had lower abnormal sperm morphology rates than the CYC-treated rats only ($P < 0.05$).

Our results support those of Ilbey *et al.* (2009), who reported that CYC treatment induced irregular and diminished seminiferous tubules containing a few germ cells counted. As well with Mohammadi *et al*. (2014), who found that spermatogonia, spermatocyte, spermatid and sperm count in seminiferous tubules were decreased significantly in CYC treated rats. These results suggest that CYC inhibits spermatogenesis. The sperm abnormalities observed in CYC-treated rats are a serious problem of reproductive function, since abnormal sperm cannot reach the oviduct after intravaginal ejaculation (Izawa *et al*., 2008)**.**

The positive effects of antioxidants on human health have attracted more attention in recent times. Most of the FH component biological actions seem to be associated with its potency as an antioxidant. Administration of antioxidant FH along with CYC has been shown to prevent the testicular injury and reproductive dysfunction as revealed by restoring the normal level of sperm counts, morphology and motility, DNA, RNA and protein as well T and Hb levels. The protective role of FH involves several mechanisms of action: direct antioxidant effect, inhibition of enzymes of oxygen-reduction pathways and sequestration of transient metal cations (Rice-Evans, 2001).

5. Conclusion

In conclusion, this study suggests that CYC treatment induced a significant alteration in RNA, DNA and protein synthesis and affected T and Hb levels and sperm (morphology and motility) abnormalities of male rats. However, supplementation of FH protects morphological structure of sperms and RNA, DNA, protein content as well blood Hb and T levels against CYC toxicities. These protective actions of FH seem to be closely involved with the suppressing of plasma lipid peroxidation and increasing of antioxidant enzyme activities. Therefore, FH might be considered as an alternative drug in combination with CYC in cancer patients, transplantation and autoimmune diseases to prevent CYC-induced reproductive toxicity in patients receiving CYC chemotherapy.

Competing interests

The authors declare that they have no competing interests.

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