

The Effects of Hydro-alcoholic Extracts of *Allium sativum L.* and *Orchismaculata L.* on Spermatogenesis Index and Testosterone Level in Cyclophosphamide-treated Rats

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Abstract

Background: One of the most common side effects of cyclophosphamide (CP) is oligo-spermatozoa, azoospermia and elimination of spermatogenic cycles. This study was done to find the efficacy of simultaneous consumption of garlic (*Allium sativum L.*) and orchid (*Orchismaculata L.*) hydroalcoholic extracts on spermatogenesis and sex hormones in rats treated with CP.

Methods: Forty-two male rats were divided into the six groups: control, sham, CP (5 mg/kg), garlic (10 mg/kg) + CP, orchid (40 mg/kg) + CP, garlic+ orchid + CP. All of the agents were administered through oral gavage for 28 days.

Results: The number of sperms increased in garlic+ CP and orchid+ CP groups. The percentage of sperm forward motility increased in groups receiving garlic, orchid, and garlic +orchid compared with the group that received only CP.

Antioxidant total capacity and testosterone level showed significant increases in garlic+ orchid, garlic and orchid groups respectively compared with the group that received only CP ($P<0.05$). Also, the tissue and serum malondialdehyde levels reduced in the group received garlic +orchid compared with the group that received only CP.

Conclusion: Garlic and orchid could increase the number and the motility of sperms, index of sertoli cell, antioxidant capacity and serum testosterone level.

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Introduction

In recent years, the prevalence of infertility in men and its impact on the reproduction process have been considered (1).

Among the factors that can disrupt reproduction, some

medicines such as cyclophosphamide (CP) can be mentioned

(2). CP is one of the most common drugs used in chemotherapy, which has destructive effects on human fertility (3). Studies showed the role of CP in the incidence of oligospermia,

azoospermia as well as the elimination of the spermatogenic cycle in adult males treated with this drug (4-6). Long-term use of this drug causes changes in the antioxidant levels of cells and tissues, decrease in the number of leydig cells, increase of lipid peroxidation and decrease of antioxidant enzymes levels (7-9).

With regard to the evidence, prescribing antioxidants during chemotherapy is likely to lead to a reduction in oxidative stress.

The use of herbal medicines, as a natural source of antioxidant agents, for the treatment and prevention of diseases has a long history (10-12). Garlic (*Allium sativum L.*) is one of the most widely used herbs in Iranian medicine (13, 14), and its use goes back to 1550 B.C. in Egypt (15). In traditional Eastern medicine, the beneficial effects of garlic on the functioning of the testicles was well known (16). Garlic contains the active ingredients like allicin, alliinase enzyme, inulin, and B-group vitamins or vitamins A and C. Studies have shown that allicin captures free radicals and inhibits lipid peroxidation (14). Most studies have shown that the use of garlic while reducing oxidative stress (14) can increase the number of sperms (17). Moreover, it can neutralize the toxic effects of free radicals induced by testicular torsion and distortion due to its antioxidant properties (16). Garlic can not only improve the function of the testicles after the injury to them or after hypogonadism (16), but also prevent testicular hypogonadism induced by warm water treatment (18). The administration of aqueous extract of garlic for 3 months to rats has been able to increase the epididymal spermatozooids (19).

Orchid (*Orchismaculata L.*) is well known as a herbal medicine in Iranian medicine for sexual activity and erectile dysfunction (20). In traditional medicine, certain parts of the orchid plant, like the roots or leaves, have been used to increase fertility, sexual desire and semen production. The studies show that in addition to increasing fertility in male rat, the orchid root

can also lead to an increase in the birth rate of the male newborn in rats (21, 22).

Considering the above-mentioned evidence for the effects of garlic and orchid on sexual functions, the present study was carried out to compare the potential effects of consumption of the two mentioned plants individually and together on the sperm parameters, spermatogenesis index, MDA levels, antioxidant capacity, and serum testosterone level changed by CP.

Materials and Methods

Animals and Ethics Considerations

The locally bred male Wistar rats aged 4 months old (250-300 g) were kept under a 12 h light-dark cycle at room temperature and had free access to food and water. Animal studies were conducted according to the guidelines for the care and handling of animals prepared by the Iranian Ministry of Health (ethics board approval number: IR.ARAKMU.REC.14, Arak University of Medical Sciences) and the internationally accepted principles for laboratory animal use and care as found in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC).

Experimental protocol

Forty two male rats were divided into the six groups: control group which received only water and food, sham group received 2 ml normal saline, cyclophosphamide (CP) group, received CP (Baxter, Germany) 5 mg in 2 ml normal saline /kg (for each mice, 0.175mg of CP was solved in 2ml normal saline) (23, 24), garlic group received 10 mg/kg of hydro-alcoholic garlic extract (for each mice, 0.35mg of garlic extract was solved in 2ml normal saline) along with CP (14, 25), orchid group received 40 mg/kg hydro-alcoholic orchid extract (for each mice, 1.4mg of orchid extract was solved in 2ml normal saline) (26) along with CP, and orchid and garlic group treated

with hydro-alcoholic orchid and garlic extracts, along with CP. All of the agents were administered through oral gavage for 28 days.

Preparation of the hydro-alcoholic extract

Garlic seeds were prepared from Tafresh (Markazi province, Iran). They were peeled off, washed, then cut into small pieces and finally dried under shade. One kilogram of powder was soaked in a solvent with the same ratio of 96% ethanol alcohol and water for 72 hours, and after straightening, was put into an oven at 40° C to evaporate water and alcohol. In the next step, the different amounts of the extract were dissolved in distilled water to obtain the desired concentrations. The orchid used in this research, was collected from the highlands of Khomein (Markazi province, Iran). After preparation of herbarium specimens, they were identified by the botanist of Kashan Barij Essence Company and registered with voucher number 195-1.

To provide the hydro-alcoholic extract, the roots were rinsed, dried out, then grinded to become a fine powder. Afterwards, the extraction process was carried out by the same method as above (20).

Sampling

Animals were euthanized by ether exposure in a specific device 24 hours after the last oral gavage. Testes and epididymides were quickly dissected out, and then cleared of adhered connective tissue. The right testicles were separated for Hematoxylin and eosin (H&E) and histological studies. To measure the oxidative stress factors and MDA and ferric reducing ability of plasma (FRAP), the left testicles were put into liquid nitrogen, and kept in the freezer at -80° C. The distal part of the epididymis was dissected to measure the sperm parameters. Blood samples were taken from the heart and

serum levels of testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), MDA, and FRAP were measured after serum separation.

Sperm motility

The distal segments of the epididymis were placed in 10 ml of Ham's F10 medium. To remove sperm, the epididymis was split using a sterile scalpel, then was incubated for 5 minutes at 37° C. The evaluation of sperm motility was done when 10 µl of culture medium of sperm suspension was put on the Neobar slide. The forward motility, the vibration and motionless (stationary) of sperms were viewed under a light microscope (Labophot-2, Nikon, Japan) at 200× magnification. Finally, the percentages of those were calculated (27).

Sperm count

The sperm counts were carried out according to the World Health Organization (WHO) guidelines. Sperm suspensions were diluted with 2% formalin (1:9) in different groups. Sperm counts were performed on the Neobar slide under a light microscope (Labophot-2, Nikon, Japan) at 40× magnification (28, 29).

After counting the sperm in squares, their number per 1ml volume of the sample was calculated using the following formula:

$$N = a \times 5 \times b \times 10000$$

Which "N" is the number of sperms per 1 ml volume of the sample, "a" is the number of sperms per 5 squares, "5" is the constant number, "b" is the dilution factor, and "1000" is obtained by dividing 1 ml (1000 µl) to 0.1 µl of the volume of the square number 1.

To calculate the total sperm numbers of the total sample size, the number of sperms counted per 1ml was multiplied by

the total volume of the sample. The count was done twice for each sample and the average was expressed (30).

Abnormal sperm morphology

To investigate the sperm morphology, culture media of sperm were stained with eosin-nigrosin. A thin layer of the sample was spread over the slide. After spreading the thin layer of the sample onto the slide, 100 sperms were evaluated under a light microscope at $100\times$ magnification in each sample. The abnormalities were expressed as percentages (29, 30).

Stereological analysis

One hundred cross-sectional of the seminiferous tubules (STs) were randomly examined per section. All spermatogenic and Sertoli cells were counted. Tubular differentiation index (TDI), spermiogenesis index (SPI), Sertoli cell index (SCI), and meiotic index (MI) were evaluated as spermatogenesis indices.

TDI is defined as the percent number of STs that were showing more than three layers of differentiated germinal cells from spermatogonia type A. To calculate the SPI, the ratio of the number of STs with sperms to the empty STs was calculated. SCI is the proportion of the total number of germ cells to the number of Sertoli cells. To evaluate MI, the ratio of the number of round spermatids to primary spermatocytes was calculated (27).

Testicular MDA measurement

The testicle tissue was homogenized in KCL, and then added to the stock solution (2 ml, 12% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and 0.25 mol/l hydrochloric acid). Afterwards, the MDA of the tissue was investigated by measurement of thiobarbituric acid-reactive substances (TBARS) (27).

Measurement of serum MDA

0.5 ml of serum was mixed with 1 ml of MDA reagent solution and the samples were placed in boiling water bath for 15 minutes. Then, samples were centrifuged for 10 minutes. The absorbance was read against the blank at 532 nm. The concentration of MDA was calculated by extinction coefficient, which is equal to $1.56\times 10^5 M^{-1} Cm^{-1}$ (nmol/ml) (27).

Evaluation of total antioxidant capacity (TAC) by FRAP

Plasma (100 μ l) or 0.1g of homogenized tissue was poured into the cuvettes, then 3 ml of the FRAP reagent was added. Absorbance at 593 nm was read after 4 minutes. The FRAP value of blood was expressed as nmol/ml (31,32).

Biochemical evaluations

Blood testosterone level (ng/ml) was measured in accordance with the ELISA (enzyme-linked immunosorbent assay) kit EIA-37K5J5 instruction (Monobind Co, USA). A mixture of testosterone, anti-testosterone solutions, 10 μ l of sample and 50 μ l of conjugation solution were incubated at room temperature for 60 minutes. After washing with deionized water, 100 μ l of substrate was added to the mixture and then was incubated for a minimum of 15 minutes at room temperature and in a dark environment. Finally, the absorption was read at 450 nm. Blood LH and FSH levels were measured in accordance with the Rat, LH ELISA Kit ZB-10179-R9648 and FSH ELISA Kit ZB-10182-R9648 instructions (ZellBio GmbH, Germany). For this, 50 μ l of standard, specimens, and controls were dispensed into wells. Then, 100 μ l of Enzyme Conjugate Reagent were dispensed into each well and were Incubate at room temperature (18-25°C) for 45 minutes. The incubation mixture was removed and the microtiter wells were rinsed and flicked 5 times with deionized water. Then, 100 μ l TMB Reagent was dispensed into each

well and the reaction was stopped by adding 100 μ l of Stop Solution to each well. Finally, the optical density was read at 450 nm.

Statistical Analysis

The analysis of data was done through SPSS software (Statistical Package for the Social Sciences, version 16.0, SPSS Inc, Chicago, IL, USA) and using one-way ANOVA analyzing method followed by Tukey test. Data were reported as mean \pm SD and the significance level was set at $P \leq 0.05$.

Results

Spermatogenesis indices and sperm count

The mean of *SCI* ($P < 0.05$) and sperm count ($P < 0.001$) were significantly lower in the CP group compared to the control group. However, in the garlic+ CP ($P < 0.025$), orchid+ CP ($P < 0.034$) and garlic+ orchid+ CP groups ($P < 0.05$) means of sperm count increased significantly compared to that in the CP group (Table 1).

Table 1. The Mean of *SCI* and sperm count in each group

Groups	<i>SCI</i> (mean \pm SD)	Sperm count (mean \pm SD)
Control	14.15 \pm 1.6 [†]	30 \pm 3.4**
Sham	13.74 \pm 1.9	28.4 \pm 3.4
CP	11.2 \pm 0.6	17.4 \pm 1.5
garlic+ CP	11.64 \pm 2.73*	22.2 \pm 1.9*
orchid+ CP	11.39 \pm 2.65*	22 \pm 2.2*
garlic+ orchid+ CP	11.78 \pm 2.65*	20.1 \pm 1.9*

** : significant difference with cyclophosphamide (CP) group ($P < 0.001$)

* : significant difference with the CP group ($P < 0.05$)

† : significant difference with the CP group ($P < 0.05$)

Sperm motility and morphology As it is seen in table 2, CP treatment led to a significant decrease in the percentage of the sperm forward motility; meanwhile, it increased the vibration (non-progressive motility) and percentage of motionless sperms compared with the control group ($P < 0.001$). However, when CP was administrated along with garlic, orchid or both of them, a significant increase of the forward motility in the same time with decrease of the vibration and motionless conditions of sperms were observed compared with the group that received only CP ($P < 0.05$).

Table 2. Comparison of sperm motility and morphology in different groups

Groups	Forward motility (%)	Vibration (%)	Non-motile (%)
Control	78.2 \pm 3.8**	6.8 \pm 2.2**	15 \pm 3.5**
Sham	80.4 \pm 5.7	7.8 \pm 2.7	13.4 \pm 4
CP	39.2 \pm 1.3	25.2 \pm 1.3	35.6 \pm 1.1
garlic+ CP	66.2 \pm 6.5*	5.2 \pm 2.3*	26.6 \pm 3.6*
orchid+ CP	68.2 \pm 5.6*	12.4 \pm 2.2*	19.8 \pm 6.2*
garlic+ orchid+ CP	67.4 \pm 3.9*	8 \pm 1.6*	26 \pm 3.7*

** : significant difference with the CP group ($P < 0.001$), * : significant difference with the CP group ($P < 0.05$)

MDA and TAC level

The results showed a significant increase of the serum and tissue MDA level in rats treated with CP compared with the control group ($P < 0.01$). However, the administration of garlic and orchid along with CP could decrease the MDA significantly compared with that in the CP group ($P < 0.05$). Moreover, the TAC level of serum and tissue in rats treated

with CP decreased significantly compared to that in the control group ($P < 0.05$). However, when CP was administrated along with garlic, orchid or both of them, a significant increase of the TAC level of serum and tissue was observed in comparison to the group that received only CP ($P < 0.001$), so that the TAC reached to the level of the control group (Table 3).

Table 3. The mean of serum and tissue MDA and TAC levels

Groups	tissue level of MDA (nmol/mg)	serum level of MDA (nmol/ml)	serum level of TAC (nmol/ml)	tissue level of TAC (nmol/ml)
Control	0.08±0.06	0.45±0.15	0.49±0.04	0.56±0.08
Sham	0.09±0.01	0.57±0.017	0.48±0.02	0.57±0.05
CP	0.13±0.02**	0.62±0.07**	0.36±0.03*	0.50±0.05*
garlic+ CP	0.1±0.01	0.5±0.01	0.50±0.03†	0.55±0.03†
orchid+ CP	0.08±0.01	0.54±0.29	0.48±0.02†	0.55±0.09†
garlic+ orchid+ CP	0.07±0.02+	0.44±0.04+	0.48±0.03†	0.56±0.01†

** : significant difference compared to the control group ($P < 0.001$)

* : significant difference compared to the control group ($P < 0.05$)

+ : significant difference compared to the CP group ($P < 0.05$)

† : significant difference compared to the CP group ($P < 0.001$)

Serum testosterone level

As it is seen in table 4, CP treatment led to a significant decrease in the serum testosterone level compared with the

control group ($P = 0.03$). However, when CP was administrated along with garlic ($P = 0.03$) or orchid ($P < 0.001$), a significant increase in the serum testosterone level was observed compared with the group that received only CP ($P < 0.05$).

Table 4. The mean ± SD of serum testosterone in different groups

Groups	Mean concentration of serum testosterone (ng/ml)
Control	1.47±0.1
Sham	1.66±0.04
CP	0.51±0.36*
garlic+ CP	1.48±1.04†
orchid+ CP	2.34±0.22**
garlic+ orchid+ CP	0.74±0.04

* : significant difference with the control group ($P = 0.03$)

** : significant difference with the CP group ($P < 0.001$)

† : significant difference with the CP group ($P = 0.03$)

Discussion

In the present study, use of CP reduced the count of MI, SCI and sperm count. While, the administration of hydro-alcoholic extracts of garlic and orchid increased the number of sperm compared to that in the CP group. Our results are consistent with other studies that have showed the reduction of stereological parameters and spermatogenesis activities (24), sperm count (24, 33), and apoptosis of sex cells in CP-treated subjects (23). CP increases the pre-apoptosis gene expression and decreases the transcription of anti-apoptosis genes. Furthermore, CP decreases the transcription of c-Kit significantly in spermatogonia cells. The c-Kit is a member of a transmembrane tyrosine kinase family that acts as a receptor for stem cell factor (SCF) and as a ligand which plays a key role in the survival of stem cells and differentiation during spermatogenesis (34). CP reduces the level of Nrf2. The Nrf2 is a vital gene for the regulating of proteins that cause cell survival under oxidative stress. CP also increases the expression of heat shock protein (Hsp70) in spermatid. Hsp70 plays an important role in the regulation of apoptosis and its down regulation is important in the pathogenesis of male infertility (35, 36).

In some studies, the orchid extract has increased the testosterone level and the number of sperms, which is consistent with our results (37, 38). Polyphenols and flavonoids like quercetin, as the most important antioxidant of orchid, have a protective role against toxins and free radicals. Also, by increasing the capacity of antioxidant enzymes such as glutathione reductase, glutathione peroxidase and catalase, they protect cells against glutathione (GSH) evacuation (39). GSH is one of the main antioxidants in the body and neutralizes the toxicity of various toxic substances such as xenobiotics,

peroxide compounds, and other free radical molecules, and thus plays an effective role in cellular protection (31, 39). The other compound found in orchid, like glucomannan, provides the required energy for sperm production in the seminiferous tubules (40).

Garlic also plays roles in accelerating testosterone secretion and the proliferation of spermatogenesis cells (41). It can stimulate the conversion of round spermatids into elongated spermatids (15). The role of garlic in the secretion of testosterone, the appearance of healthy spermatogenic cells, Leydig cells and natural lumens has been clearly indicated (41). In addition, a dose-dependent relationship has been reported between the garlic intakes and changes in the number of seminiferous tubules (15). The consumption of garlic for more than one month can increase apoptosis in spermatid cells by activation of caspase-3 and the pre-apoptotic protein like Smac (42). Furthermore, treatment with different doses of raw garlic for one month resulted in ultrastructural defects in Sertoli and interstitial cells in adult male rats. The tissue and ultrastructural changes in testicular cells have happened in adult rats following treatment with 20% raw garlic (43). This difference can be related to the dose, duration of treatment, and the method of garlic extract preparation. The garlic extract at a dose of 4 ml/kg for 14 days significantly increases sperm count (18).

On the other hand, CP decreased the sperm forward motility and increased the vibration of sperms. Consumption of garlic alone or at the same time with orchid can increase the number of progressive sperms and reduce the number of vibrating sperms. CP exposure markedly enhances the expression of Hsp70 gene. Hsp70 is present in the eukaryotic flagella and is probably involved in the assemblage of axonemes. This represents the mechanism through which CP

affects the formation of flagella and sperm motility. In accordance with the present study, in a study was shown significant decrease of the percentage of sperm motility in rats treated with CP (33).

According to a study, orchid accelerates sperm motility through oxidative phosphorylation (22). There is no study on the effect of low dose of garlic on sperm motility, but garlic might increase sperm motility due to its antioxidant properties.

In the present study, increase of serum and testicular tissue MDA levels were seen in CP treated rats, while garlic and orchid reduced this parameter. Furthermore, CP reduced the mean TAC of the serum, while simultaneous treatment with garlic and orchid increased it. CP can induce oxidative stress, free radical production, lipid peroxidation and thereby, adenosine triphosphate (ATP) exertion. ATP deficiency reduces sperm motility (3, 33). Treatment of adult male mice with CP can increase the MDA level of testicular tissue and it has been suggested that this is due to the decreased activity of peroxidase and catalytic enzymes (44). CP decreases the level of antioxidant enzymes and vitamins E and C and consequently increases the susceptibility of cells to oxidative damage, which increases the peroxidation of endogenous lipid and MDA (45). Furthermore, another report showed that, the rats treated at CP doses of 250, 500 and 1000 mg /kg had a reduced level of MDA (46). The administration of garlic also reduced MDA levels, which can confirm the beneficial role of this plant as an antioxidant (47). Therefore, reduction of MDA level in the garlic group in the present study is probably related to the antioxidant activity of the compounds in garlic. GSH is the most important antioxidant present in the body cells. Garlic has the potential to undermine harmful changes in GSH levels, which may be the result of garlic ability to eliminate free

radicals and / or reduce homeostasis in antioxidant systems in rats by reducing the ability to produce testicular GSH. Antioxidant properties of this extract can also be due to the presence of flavonoids and tannins, which has high antioxidant activity and inhibits the formation of tumor and reduces inflammation (15).

The mean serum testosterone concentration in the CP-treated group decreased in comparison to that in the control group and increased in the garlic+ CP and orchid+ CP groups. Reduced plasma testosterone levels have been reported after 28 days of CP administration (24, 44). It can also reduce the activity of testicular 17 β -Hydroxysteroid dehydrogenases (17 β -HSD) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and spermatogenesis (44). In accordance with the present research, orchid extract could increase testosterone level (37, 38). Through an effect on pituitary axis, orchid extract increases the level of androgenic hormones as a result of increased testosterone secretion (22). Also, garlic intake in rats increases serum testosterone levels (48). The main components of garlic include zinc and selenium, which play an important role in testicular activity, especially the production of steroidal enzymes like testosterone. This issue has also been mentioned by prominent scholars of Persian medicine (41, 49).

Conclusion

The present study revealed that CP causes severe tissue and cell damages in testicular tissue and reduces sperm motility and spermatogenesis. On the other hand, hydro-alcoholic extract of garlic and orchid can prevent the stress-oxidative damages caused by CP. It is therefore recommended that hydro-alcoholic extract of garlic and orchid be used as a supplement to prevent undesirable effects of CP.

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Conflict of interest

The authors declare no conflict of interest.

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