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Ameliorative effect of *Allium atroviolaceum* on sperm quality in cyclophosphamide-treated mice

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Abstract

Background: Cyclophosphamide (CP) is an anti-neoplastic alkylating agent that is extensively used in different chemotherapy regimens. Adverse effects on the reproductive system, especially spermatogenesis, are one of the most important side effects of this drug. It is medically essential to use complementary and alternative drugs. Herbal drugs have long been used as a complementary treatment. Our purpose was to study the effect of hydroalcoholic *Allium atroviolaceum* L. extract on spermatogenesis in CP-treated mice.

Results: CP affected a significant decrease in sperm count, motility, viability, and morphology. Sperm count was significantly higher in the all extract groups than in the group of control ($p < 0.001$) and CP group ($p < 0.001$, $p < 0.01$). Sperm motility was significantly greater in the extract (100 and 200mg/kg) groups than in the group of control ($p < 0.05$ and < 0.001). Sperm immotility and rotational movement were significantly higher in the CP group than in the CP+extract groups ($p < 0.001$). The sperm viability was significantly greater in the CP+extract (200mg/kg) group than in the CP group ($p < 0.001$). The number of headless sperm, sperm with initial tail, with coiled tail, and sperm with curved body, was significantly lower in the CP+extract (200mg/kg) group than in the CP group ($p < 0.001$).

Conclusion: *A. atroviolaceum* extract treatment significantly improved CP-induced reproductive toxicity.

Keywords: *Allium atroviolaceum* L., Infertility, Cyclophosphamide, Spermatogenesis

Background

Infertility refers to development of defect or disorder that prevents reproduction. Scientifically speaking, infertility refers to the inability to conceive after 12 months or more of ordered intercourse without the use of contraceptive methods. Infertility may occur due to various causes [1]. According to the available statistics, approximately 13% of couples are involuntarily childless in reproductive ages, with 35% of the cases due to male factors and 25% of them due to both male and female factors. Therefore, half of infertility cases are due to male factors [2]. The inability to produce sperm and ejaculate,

premature ejaculation, loss of libido, and the inability or unwillingness to have intercourse are certain reasons of infertility of male. However, the most cause of male infertility is the inadequate number of healthy and active sperm [3, 4]. Normal semen samples should have a total sperm count of over 20,000,000 per mL, natural morphology of greater than 20%, and progressive motility of greater than 50% [5]. Certain drugs can also cause secondary infertility [6], including cyclophosphamide (CP). CP is a chemotherapy drug that is used to treat a wide variety of cancers. In addition to therapeutic applications, CP can cause adverse effects such as reproductive toxicity [7, 8]. It has been demonstrated that CP leads to damage by disrupting tissue regenerative and excessively producing oxidative stress [9].

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Complications, failures, and, in certain cases, heavy costs of medical treatments can be reduced by the use of complementary therapies. With the increasing detection of the side effects of chemical drugs in recent years, there has been widespread acceptance of medicinal plants for the treatment of certain diseases, especially infertility [10–13]. Therefore, there is an urgent need for further exploration of this area. The use of medicinal plants can have positive effects and has long been considered to increase fertility and to treat associated conditions such as hormonal imbalance, impotence (sexual weakness), oligospermia, low sperm motility, prostate inflammation, and varicocele. The effects of certain plants from the genus *Allium* to treat diseases such as infertility have been studied [14]. *Allium cepa* and *Allium sativum* are two of the plants whose effects on fertility and the reproductive system have been demonstrated [15]. The genus *Allium* consists of around 750 species. The species of this genus are rich in flavonoids, saponins, saponinins, and volatile sulfur compounds [16, 17]. The antioxidant effects of these species have also been confirmed [18–20]. These plants have potent nutritional and therapeutic effects and have been used as traditional treatments for certain diseases since the past [21]. It has recently been determined that the plants from the genus *Allium* are effective on cardiovascular diseases, tumor growth, and senescence, all of which seem to be associated with the effects of free radicals [22]. Broadleaf wild leek, botanically referred to as *Allium atroviolaceum* L., is a plant from the family Alliaceae and the genus *Allium* [23]. Regarding the optimal effects of other plants from this plant family on fertility, we conducted this research to study the effects of *A. atroviolaceum* on spermatogenesis in mouse.

Methods

Extraction

The aerial parts of *A. atroviolaceum* were collected from Sabzkouh heights between late May 2013 and late June 2013 and identified as the samples of interest by an expert botanist (herbarium no.: 801) at the Herbarium Unit of Medical Plants Research Center. Then, the samples were pulverized in an electric mill. Extraction was conducted by maceration. Briefly, the resulting powder was macerated in 70% ethanol. 72 h later, the resulting combination was filtered using filter paper of Whatman grade 1 and the resulting liquid was concentrated in a rotary evaporator. Then, the obtained extract was incubated at 37°C to dehydrate. The extract was stored in a freezer for future use. We diluted the extract to 50, 100, and 200mg/kg by dissolving it, in appropriate amounts, with normal saline [24].

Animals

This study was conducted with 64 male mice weighing 25–30g. The mice were bought from Razi Institute of Iran and then kept in the Animals House at (23±2)°C under 12-h/12-h light–dark cycle, with ad libitum access to food and water. The mice were permitted to acclimate to their environment for 1 week before the beginning of the experiments. The mice were assessed for general health, and all steps of the study were implemented according to the rules of Guide for the Care and Use of Laboratory Animals of National Research Council Committee.

Design of study

In this experimental research, the mice were assigned to eight groups of eight each as follows: Normal saline receiving (0.5 mg); *A. atroviolaceum* extract (50, 100, and 200mg/kg) receiving groups; *A. atroviolaceum* extract (50, 100, and 200mg/kg)+CP (6.1mg/kg) receiving groups; and CP (6.1mg/kg) receiving group. All injections were performed intraperitoneally once a day for 30 days [25]. Eventually, mice were deep anesthetized by co-administration of ketamine (60 mg/kg, intraperitoneally) and xylazine (10 mg/kg, intraperitoneally) [26, 27] and then they euthanized. Finally, testes were dissected out and prepared for histopathological examinations [8].

Measuring sperm parameters

After the testicles and epididymes were removed, they were separated from each other. The epididymes were sliced and incubated in Ham's F10 at 37°C for 5 min to eliminate the sperm. The sperm count was calculated and the sperm motility was determined by hemocytometer using 5λ of the sperm-containing media placed on neobar lam. The samples were analyzed using an optical microscope at ×40 magnification, and 10 fields of lam were studied to calculate the sperm count and determine the sperm motility [15, 28, 29]. To evaluate the sperm count, sperm suspension was diluted in 3% normal saline in a proportion of 1–9, and then a drop of the above solution using a micropipette was gently transferred to the neobar slide. After 5 min using a magnification of 40 spermatozoa with head, middle, and tail pieces were counted [8].

Antioxidant capacity of *A. atroviolaceum*

Fifty microliters of *A. atroviolaceum* extract was blended with 200 μL of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution in methanol. After 15 min at temperature of room, the absorbance was read at 517 nm using a UV–Vis spectrophotometer [30].

Total phenol and flavonoid content measurement

Aluminum chloride colorimetry and Rutin method was applied to measure the total flavonoids. Concentration levels of 25, 50, 100, 250, and 500 ppm of standard solutions of Rutin in methanol were prepared. One milliliter from these solutions was moved into the tubes of test and blended with 1 mL of chloride aluminum 2%. Six milliliters of potassium acetate 5% was added, and after 40 min, the optical density level was recorded at 415 nm wavelength. Then, 0.01–0.02 g of the extracts was dissolved in methanol 60%, reaching 10 mL, and using chloride aluminum colorimetry, the total level of flavonoids was estimated. The total flavonoid level was determined in milligram per 1 g extract, equivalent to Rutin [31].

The amount of total phenolic compounds was measured by colorimetric method using the Folin–Ciocalteu reagent. Five mL of extract or gallic acid (standard phenolic compound) was blended with Folin–Ciocalteu reagent (1:10 diluted with distilled water) and aqueous Na₂CO₃ (4 mL, 1 M). The mixtures were put it aside for 15 min, and the total phenols were measured by colorimetry at 765 nm. A standard curve get ready using 0, 50, 100, 150, 200, and 250 mg/L solutions of gallic acid in methanol: water (50:50, vol/vol). Total phenol values were stated

in terms of gallic acid equivalent (in mg/g). The test was repeated in triplicate [32].

Data analysis

Data analysis was conducted by one-way ANOVA and Tukey’s test in the Prism software version 5. Pearson’s correlation coefficient was used to investigate correlation.

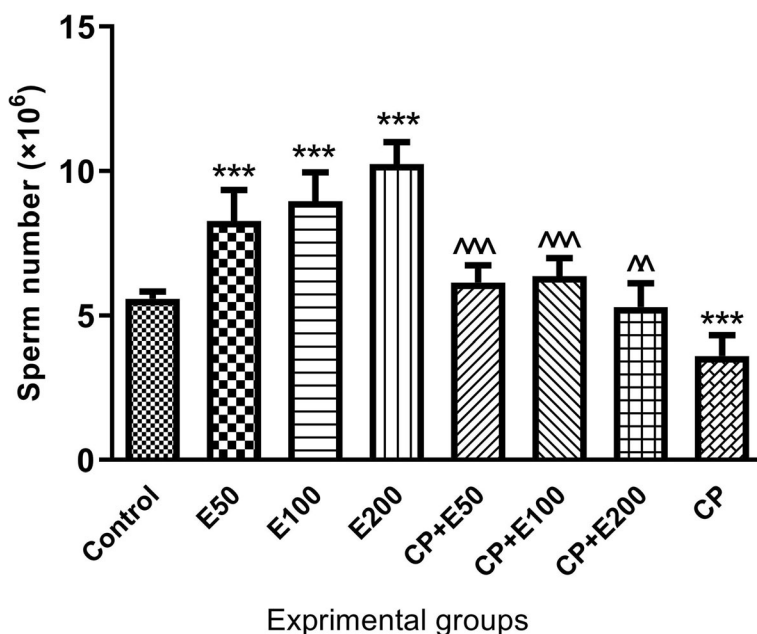
Results

Effect of *A. atrovioleaceum* extract on count of sperm

The results exhibited a significant difference in sperm count between the groups ($p < 0.001$). The sperm count was significantly lower in the CP group than in the group of control ($p < 0.001$). In addition, the sperm count improved significantly in the CP+ extract (50, 100, and 200mg/kg) groups when compared to the CP group ($p < 0.001$, $p < 0.01$). Sperm count was significantly greater in the extract (50, 100, and 200mg/kg) groups than in the group of control ($p < 0.001$) (Fig. 1).

Effect of *A. atrovioleaceum* extract on sperm motility

One-way ANOVA exhibited a significant difference in sperm motility among the groups ($p < 0.001$). The motility of sperm reduced significantly in the CP group when



***P<0.001; compared with control group
 ^^P<0.01, ^^P<0.001; compared with CP group

Fig. 1 The effect of *Allium atrovioleaceum* L. extract (E) on sperm count in cyclophosphamide (CP)-treated mice

evaluated to the group of control ($p < 0.001$). Sperm motility was significantly higher in the extract (100 and 200mg/kg) groups than in the group of control ($p < 0.05$ and < 0.001 , respectively). Sperm motility was also significantly greater in the CP+extract (50, 100, and 200mg/kg) groups than in the CP group ($p < 0.001$) (Fig. 2).

Effect of *A. atrovioleaceum* extract on sperm immotility and rotational movement

The results showed a significant difference in sperm immotility between the groups ($p < 0.001$). The sperm immotility increased significantly in the CP group when compared to the group of control ($p < 0.001$). Besides that, sperm immotility decreased significantly in the CP+extract (200mg/kg) group when evaluated to the group of control ($p < 0.05$). Sperm immotility was significantly higher in the CP group than in the CP+extract (50, 100, and 200mg/kg) groups ($p < 0.001$) (Fig. 3). The results showed a significant difference in sperm rotational movement between the groups ($p < 0.001$). The sperm rotational movement was significantly higher in the CP group than in the control group ($p < 0.001$). Besides that, the sperm rotational movement was significantly higher in the CP group than in the CP+extract (50, 100, and 200mg/kg) groups ($p < 0.001$) (Fig. 4).

Effect of *A. atrovioleaceum* extract on viability of sperm

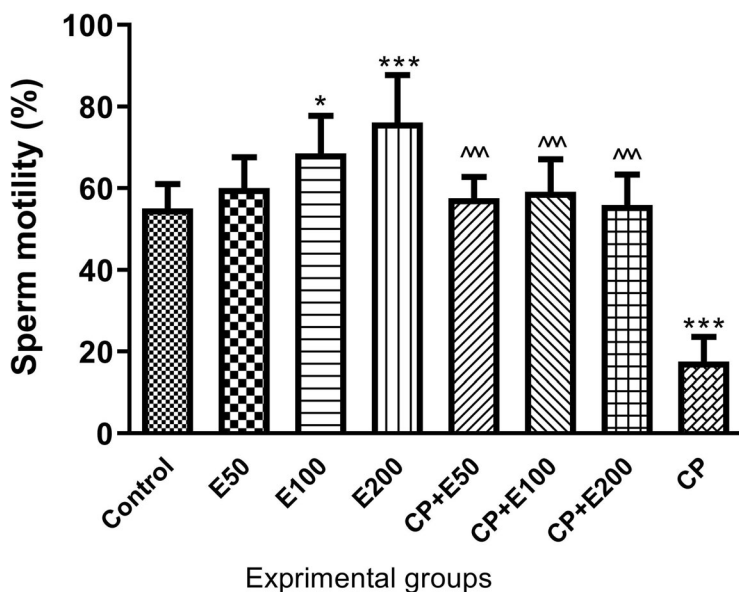
One-way ANOVA exhibited a significant difference in sperm viability among the groups ($p < 0.001$). The viability of sperm decreased significantly in the CP group when compared to the group of control ($p < 0.001$). The sperm viability was significantly higher in the CP+extract (200mg/kg) group than in the CP group ($p < 0.001$). The sperm viability was also significantly higher in the extract (100 and 200mg/kg) groups than in the group of control ($p < 0.01$ and < 0.001 , respectively) (Fig. 5).

Effect of *A. atrovioleaceum* extract on headless sperm count

The results showed a significant difference in headless sperm count between the groups ($p < 0.001$). The headless sperm count was significantly higher in the CP group than in the of control ($p < 0.001$). Besides that, the headless sperm count was significantly lower in the CP+extract (50, 100, and 200mg/kg) groups than in the CP group ($p < 0.05$ and < 0.001) (Fig. 6).

Effect of *A. atrovioleaceum* extract on a number of sperm with initial tail

The results exhibited a significant difference in the number of the sperm with initial tail among the groups ($p < 0.001$). The number of the sperm with initial tail was significantly higher in the CP group than in the group of



*P<0.05, ***P<0.001;compaired with control group
^^^P<0.001; compaired with CP group

Fig. 2 The effect of *Allium atrovioleaceum* L. extract (E) on sperm motility in cyclophosphamide (CP)-treated mice

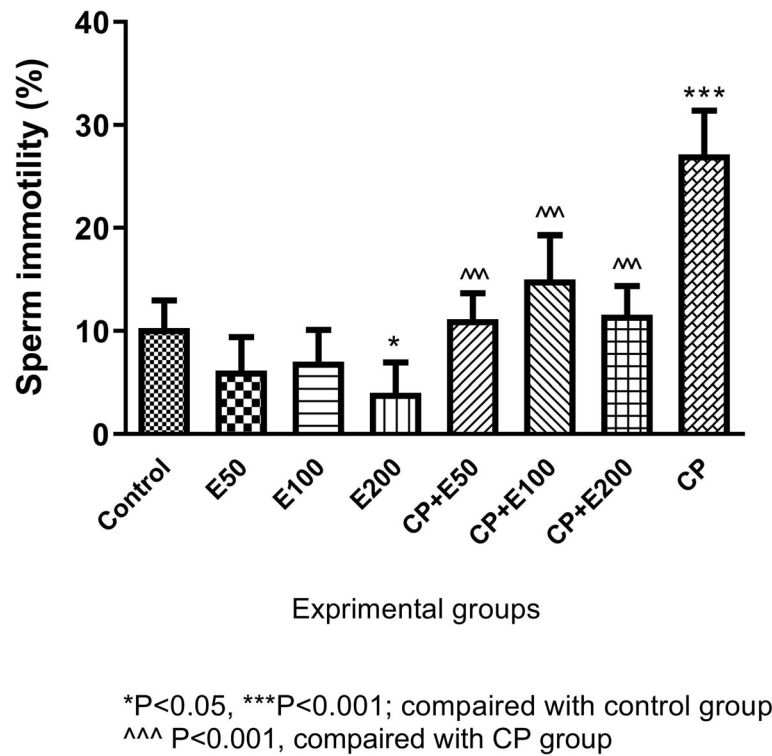


Fig. 3 The effect of *Allium atrovioleaceum* L. extract (E) on sperm immotility in cyclophosphamide (CP)-treated mice

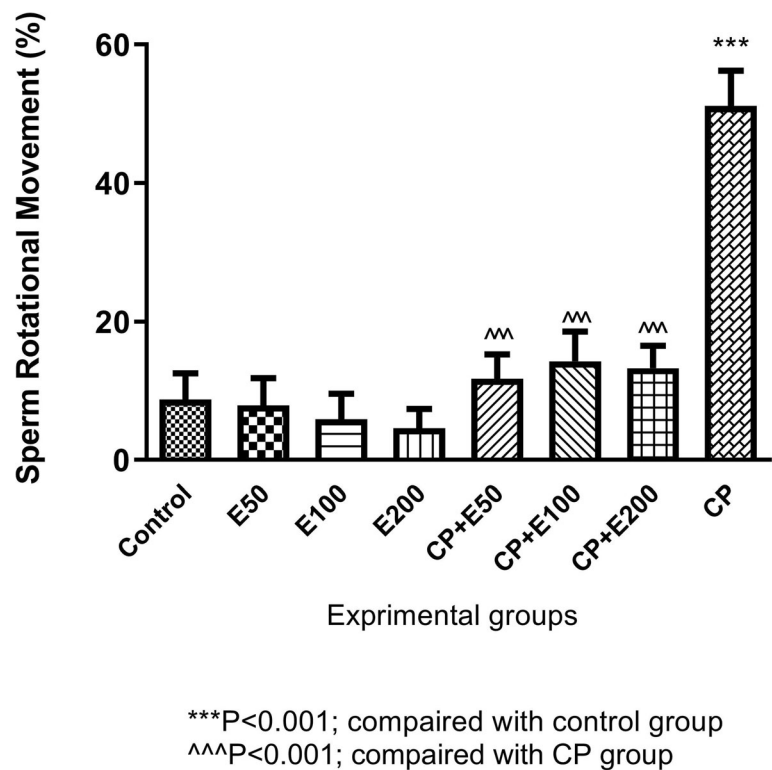
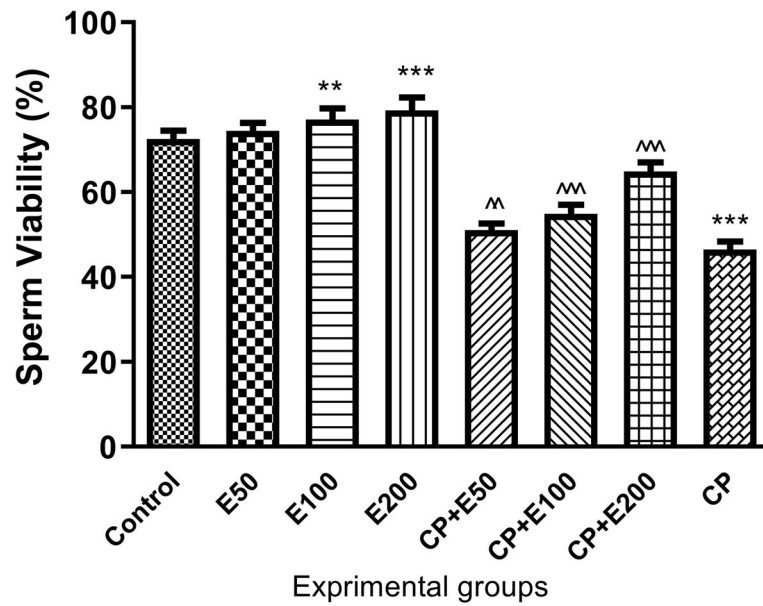
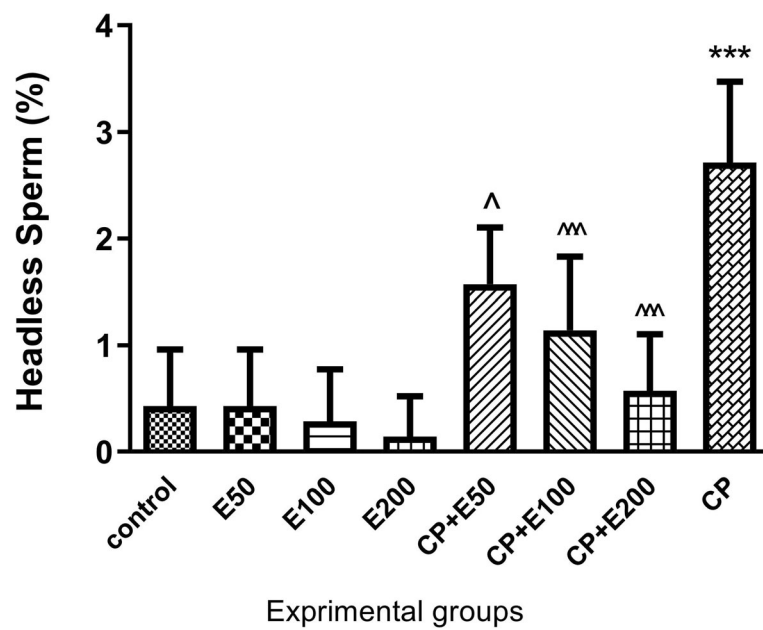


Fig. 4 The effect of *Allium atrovioleaceum* L. extract (E) on sperm rotational movement in cyclophosphamide (CP)-treated mice



P<0.01, *P<0.001; compared with control group
 ^^P<0.01, ^^^P<0.001; compared with CP group

Fig. 5 The effect of *Allium atroviolaceum* L. extract (E) on sperm viability in cyclophosphamide (CP)-treated mice



***P<0.001; compared with control group
 ^P<0.05, ^^P<0.001; compared with CP group

Fig. 6 The effect of *Allium atroviolaceum* L. extract (E) on headless sperm count in cyclophosphamide (CP)-treated mice

control ($p < 0.001$). Besides that, the number of such sperm was significantly lower in the CP+extract (100 and 200mg/kg) groups than in the CP group ($p < 0.005$ and < 0.001 , respectively) (Fig. 7).

Effect of *A. atrovioleaceum* extract on number of sperm with coiled tail

One-way ANOVA exhibited a significant difference in the number of the sperm with dorsal tail among the groups ($p < 0.001$). The number of the sperm with coiled tail improved significantly in the CP group when evaluated to the group of control ($p < 0.001$). The number of the sperm with coiled tail was significantly lower in the CP+extract (100 and 200mg/kg) groups than in the CP group ($p < 0.001$) (Fig. 8).

Effect of *A. atrovioleaceum* extract on number of sperm with curved body

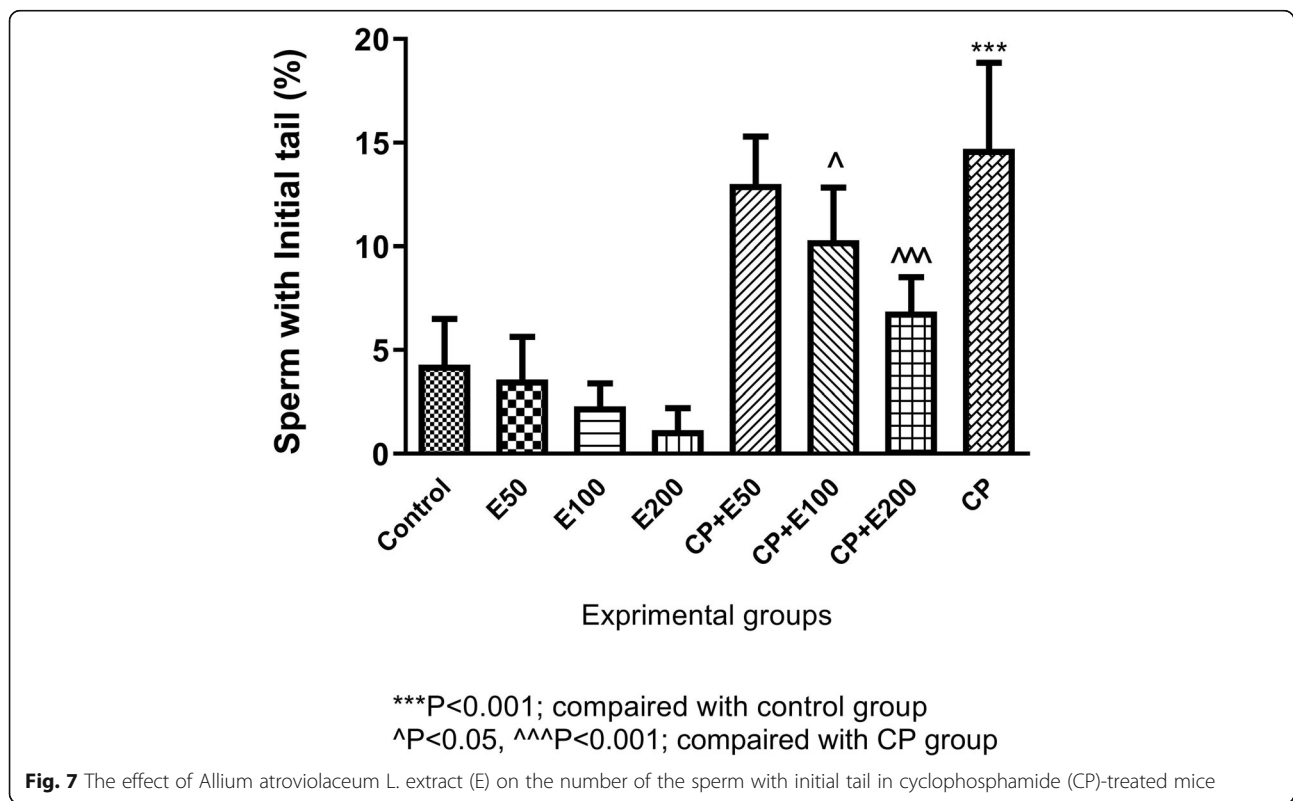
A significant difference in the number of the sperm with curved body was observed among the groups ($p < 0.001$). The number of the sperm with curved body improved significantly in the CP group when evaluated to the group of control ($p < 0.001$). Besides that, the number of such sperm was significantly lower in the CP+extract (200mg/kg) group than in the CP group ($p < 0.001$) (Fig. 9).

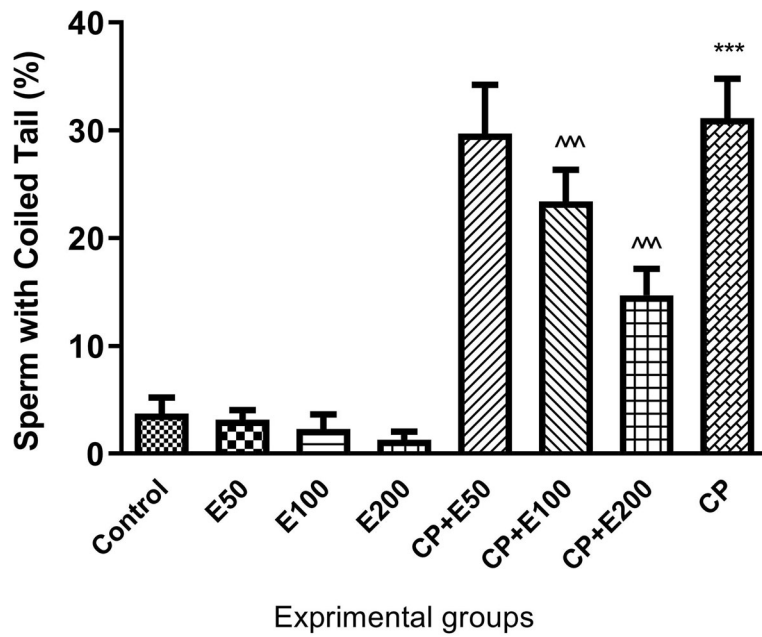
Antioxidant capacity and total phenol and flavonoids content of *A. atrovioleaceum*

Antioxidant capacity of *A. atrovioleaceum L.* extract was measured as $IC_{50} = 192.3 \pm 0.25 \mu\text{g/ml}$. Also total phenol and flavonoids content of this extract is 39.57 mg equivalent rutin/gr dry weight of extract and 54.40 mg equivalent galic acid per gr dry weight of extract, respectively.

Discussion

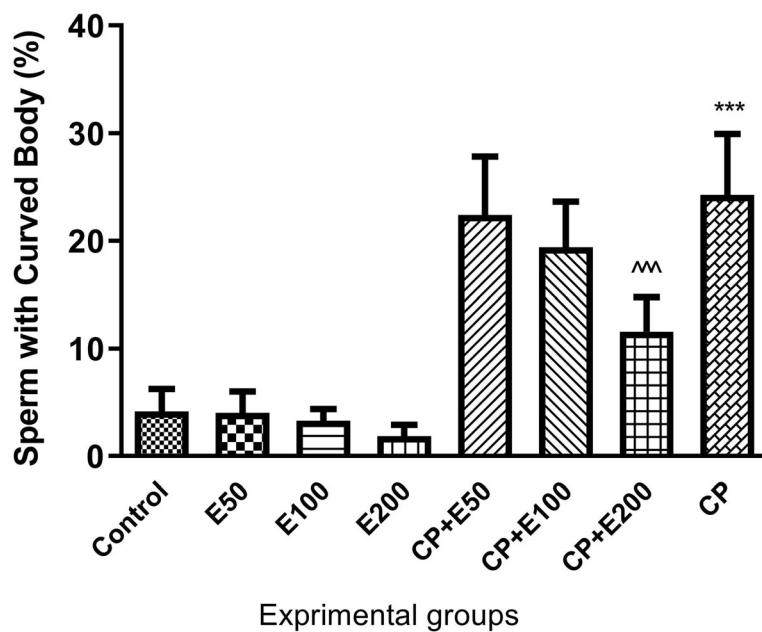
Impaired spermatogenesis is one of the most common causes of infertility in male. CP is one of the chemotherapy agents that are widely used to treat different types of cancers. Although CP has many clinical applications, it leads to adverse side effects such as reproductive toxicity in humans and animals [7]. Although the action mechanism of CP-induced testicular disorders has not yet been fully understood, studies have shown that CP can disrupt tissue regenerative. The resulting biochemical and physiological disorders are due to the excessive oxidative stress, and it is therefore necessary to use antioxidants throughout chemotherapy to reduce CP-induced reduction of oxidative stress and to detoxify the tissues [33]. The current study was an investigation into the effects of hydroalcoholic *A. atrovioleaceum* extract on spermatogenesis in CP-treated mice. Our results showed that the total count of sperm, count of motile sperm,





***P<0.001; compared with control group
 ^^P<0.001; compared with CP group

Fig. 8 The effect of *Allium atroviolaceum* L. extract (E) on the number of the sperm with coiled tail count in cyclophosphamide (CP)-treated mice



***P<0.001; compared with control group
 ^^P<0.001; compared with CP group

Fig. 9 The effect of *Allium atroviolaceum* L. extract (E) on the number of the sperm with curved body in cyclophosphamide (CP)-treated mice

and viability of sperm decreased significantly in the CP-treated mice when compared to the controls. In addition, the count of immotile sperm, the number of the sperm with rotational movement, the count of headless sperm, the quantity of the sperm with abnormal and dorsal tails, and the sperm with curved body was significantly greater in the CP-treated group than in the group of control, reflecting the adverse effects of CP on spermatogenesis.

The adverse effects of CP on sperm morphology have been reported. The study of Buchanan et al. showed that spermatogenesis decreased significantly in the patients under CP treatment all of whom developed azoospermia after 6 months of CP treatment [34]. The study of Rezvanfar et al. also showed that CP caused impairment of spermatogenesis and fertility. Rezvanfar et al. observed that the sperm quality significantly declined in the CP-treated mice, which was associated with DNA damage and decline in chromatin quality [35]. Selvakumar et al. observed that the sperm count and motility decreased significantly and died and abnormal sperm count increased significantly in the CP-treated mice. Besides that, lipid peroxidation and protein carbonyl groups increased significantly in the epididymal sperm of the CP-treated mice [36]. The study of Çeribaşı et al. indicated that CP treatment was related with a significant improve in sperm tail and midpiece abnormalities in the mice [37], which is in agreement with the current study. We observed that treatment with *A. atrovioleaceum* extract at different concentrations significantly increased the count of sperm, motility, and viability and significantly decreased the sperm immotility and rotational movement, the sperm with abnormal and dorsal tails as well as the sperm with curved midpiece. The doses of 50, 100, and 200mg/kg of extract significantly improved the sperm motility, viability, and morphology when compared to the control group.

ROS play significant roles in sperm physiological processes such as capacitation, acrosome reactions, and sperm-oocyte binding, while the excessive production of ROS is associated, under certain conditions, with sperm DNA damage and decreased fertility [38]. In this condition, sperm during maturation lose a large proportion of their cytoplasm as residues and create cytoplasmic droplet in their bodies. The sperm containing these droplets have not fully matured and are still dysfunctional. The cytoplasmic residue contains large amounts of inner cytoplasmic and antioxidant enzymes. The lack of a large proportion of cytoplasm leads to decline in the antioxidant defense system. On the other hand, sperm is very disposed to oxidative stress because of large

concentrations of unsaturated fatty acids in its plasma membrane and low concentrations of antioxidants. CP causes reproductive toxicity by increasing ROS and decreasing glutathione content and glutathione peroxidase (GPx) activity [33]. As an antioxidant agent, GPx contributes significantly to protecting the sperm in the testicular and epididymal tissues, and therefore, its reduction in the body leads to infertility. GPx protects sperm against free radicals and contributes to complete sperm maturation and evolution by staying on the sperm plasma membrane and nucleus and epididymal region and fluid [39]. The ability of CP to generate free radicals and to cause lipid peroxidation and oxidative stress in rats has been reported [35]. Cao et al. reported that CP caused decrease in the main enzymatic and non-enzymatic antioxidants in Leydig cells and thus decrease in testosterone synthesis and secretion through increasing oxidative stress, and acted as an effective factor for impairment of spermiogenesis and thus significant decrease in the epididymal sperm count [40]. The investigation of Aziz et al. confirmed that there was a significant association between the production of ROS in sperm and the proportion of the number of the sperm with abnormal morphology [41]. Given that *A. atrovioleaceum* extract has exhibited activity of antioxidant in both in vitro and in vivo studies [42], it can be argued that this extract prevents sex cell damage by decreasing the levels of free radicals and ROS.

As far as we searched, no study has yet been conducted on the protective effects of *A. atrovioleaceum* on the reproductive system but other species of the genus *Allium* have been investigated for such property. The study of Khaki et al. on *Allium cepa* effect on spermatogenesis in mice revealed that this plant caused increase in the sperm viability, motility, and count [4]. It has also been argued that the organic sulfur compounds present in the species from the genus *Allium* significantly decrease free radical-induced toxic effects on sperm DNA via increasing glutathione levels and GPx activity. Organic sulfur compounds increase the activities of GPx and superoxide dismutase in the liver, kidney, breast, and testicular cells and protect the cells by influencing peroxide forms and oxidative reduction [43].

Conclusion

Our results confirmed the protective effect of *A. atrovioleaceum* extract against spermatogenesis in the CP-treated mice. With regard to the nutritional value of *A. atrovioleaceum*, this plant can be used in the diets of the people who use CP to prevent or decrease its side effects. The properties of *A. atrovioleaceum* extract, however, should be further studied with human subjects.

Abbreviations

CP: Cyclophosphamide; *A. atrovioleaceum*: *Allium atrovioleaceum*

Acknowledgements

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Plant authentication

The aerial parts of *A. atrovioleaceum* were collected from Sabzkouh heights between late May 2013 and late June 2013. After identification by expert botanist (Shirmardi Hamzeh Ali, PhD., Research Center of Agriculture and Natural Resources, P.O. Box 415, Shahrekord, Iran), a specimen was deposited in herbarium at the Medical Plants Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran (Herbarium no.801).

Authors' contributions

ZLG and MSH conceptualized the project and gave technical inputs in conducting the study and preparing manuscript. AH, SHA, and NA performed the study and prepared manuscript. MA and AY gave technical inputs in conducting the study. ZLG and SHA analyzed the data. The authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration

Ethics approval and consent to participate

All experimental procedures and protocols used in the study were reviewed and approved by the Shahrekord University of Medical Sciences ethical committee dated October 29, 2016. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All stages of experimentation were carried out in accordance with the regulations of the University and the Guide for the Care and Use of Laboratory Animals of National Institutes of Health (Ethics code: IR.SKUMS.REC.1395.193).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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