African Journal of Pharmacy and Pharmacology Vol. 6(7), pp. 480-486, 22 February, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.767
ISSN 1996-0816 © 2012 Academic Journals

*Full Length Research Paper*

# Effects of *Citrus aurantium* extract on spermatogenic cell density, antioxidant activity and testosterone level in male mice

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Accepted 20 January, 2012

**The aim of this study was to investigate the effects of *Citrus aurantium* extract on sperm characteristics, spermatogenic cell density, antioxidant activity and testosterone level of male mice. Twenty-eight adult male Balb/C mice were divided into four groups; each having seven mice. Two milliliter distilled water, *C. aurantium* extract with low concentration (0.01 mg/kg), medium concentration (0.02 mg/kg) and high concentration (0.04 mg/kg) was given daily for seven weeks by gavage to mice in the experimental groups, respectively. Body and reproductive organ weights, spermatogenic cell density, levels of antioxidants, testosterone and lipid peroxidation antioxidant enzyme activities were investigated. All analyses were done only once at the end of a seven-week study period. Data were compared by analysis of variance (ANOVA) and the degree of significance was set at $P < 0.05$. A significant decrease in malondialdehyde (MDA) level and significant increases in glutathione (GSH), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were observed in mice treated with *C. aurantium*. *C. aurantium* extract provided an increase in sperm concentration, spermatogenic cell density when compared to the control group. Compared to the control group, significant decrease in sperm motility and testosterone level were also observed in experimental groups.**

**Key words:** *Citrus aurantium*, spermatogenic cell, testosterone, antioxidant activity, mice.

## INTRODUCTION

*Citrus aurantium* is also known as Seville orange, sour orange or bitter orange, because it tastes bitter and is orange. This orange tree is big, evergreen and its height is about 5 m with white flowers that first was found in Asia, but nowadays is cultivated in many areas of the world including Mediterranean regions. *C. aurantium* is not popularly eaten, but the ripe fruit is eaten in Iran (Fugh-Berman and Myers, 2004) and Mexico (Facciola, 1998).

*C. aurantium* contains around 0.2 to 0.5% essential oil, which can be distilled from the unripe; green fruit and its peel. Limonoids, flavonoids and monoterpenes are some compounds included in the oils. The peel contains the alkaloid synephrine and carotene pigments. Though, in very small natural amounts, synephrine is also found primarily in the adrenal glands of humans.

Neroli, which is the essential oil from the flowers, is used in perfumes and also as flavoring for sweets (Fugh-Berman and Myers, 2004). The hydrolate of its flowers has been used for the treatment of mild depression and sedation in traditional medicine (Zargari, 1986; Aynehchi, 1991). In Chinese folk medicine, the peel of this immature bitter orange has been used for relief of indigestion, constipation, dysenteric diarrhea and abdominal pain. It is also believed to have similar biological activity and may provide suppress appetite, an energy boost, and increase metabolic rate and caloric expenditure (Hansen et al.,

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2011; Jyotsna, 2011).

Treatment of digestive problems is one of the most common uses of *C. aurantium* (Bisset and Wichtl, 1994).

It has been reported that the smell of *C. aurantium* is useful in lessening of stress and is also especially functional for cases of moderate depression (Mantel, 2002). Reactive oxygen species (ROS) are molecules that are highly disruptive to cellular function, in general, and have free radicals. ROS are produced in the testis as a usual physiological event; the modifications of the synthesis of ROS motivate the oxidation and DNA damage of cells (Sikka, 1996). The sperm plasma membrane has polyunsaturated fatty acids, which are sensitive to peroxidative damage. The lipid peroxidation demolishes the formation of the lipid in the membranes of spermatozoa, hence, reduces the sperm's motility and results in defects to membrane integrity (de Lamirande et al., 1997; Sanocka and Kurpisz, 2004; Henkel, 2005).

Antioxidants are compounds that help to control ROS and lipid peroxidation. Glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide-dismutase (SOD) are some of the famous biological antioxidants (Sikka, 1996; Varnet et al., 2004). Arbo et al. (2009) reported the antiestrogenic potential of *C. aurantium*. Hence, it can inevitably be linked with fertility in female animals. However, no study has so far reported either any positive or negative effects of *C. aurantium* or its extract on male fertility. The present study is carried out in an attempt to surveying the effects of *C. aurantium* extract, a potent antioxidant, on male fertility. Defferental sperm characteristics, spermatogenic cell density, anti-oxidant enzyme activity as well as level of testosterone and lipid peroxidation in mice given *C. aurantium* extract orally for seven weeks were examined.

## MATERIALS AND METHODS

### *C. aurantium* extract and chemicals

The dried peel flower of the plant was obtained from local market in Fars province (Southern region of Iran) in June 2010. The plant was powdered and stored in air-tightened, light protected containers. Powdered plant material (200 g) was mixed with 500 ml ethanol and was refluxed for 2 days followed by filtration through filter paper. The filtrate was evaporated to dryness under reduced pressure and weighed. The other chemicals were purchased from Merck chemical company.

### Animals and experimental design

Twenty-eight healthy adult male Balb/C mice (eight weeks old) were included in the present study. We housed all the mice in standard shoebox cages. The mice had free access to maintenance food and water. Animals were maintained under standard laboratory conditions on a 12 h light/dark cycle in a temperature-controlled room at 21± 3°C.

The mice were divided into 4 groups at random; each group containing seven mice. Only 2 ml distilled water was supplied to mice in the first group as the control group. The second group of mice received 0.01 mg/kg *C. aurantium* extract and were named low dose *C. aurantium*. The third group of mice received 0.02 mg/kg *C. aurantium* extract, and were named middle dose *C. aurantium*. 0.04 mg/kg *C. aurantium* extract was given to the mice in the fourth group, and they were named high dose *C. aurantium*. Distilled water and *C. aurantium* extract were given by gavage daily for the period of seven weeks.

### Sample collection

For the biochemical and hormonal analyses, plasma samples were prepared as described by Turk et al. (2007). Testes and ductus deferens were removed, cleared of stick to connective tissue and weighed. Testis tissues were fixed in Bouin's solution for later histological examinations.

### Evaluation of sperm parameters

The sperm cells were prepared from ductus deferens and counted with the help of a light microscope at 400× magnification as described by Turk et al. (2007) and Sonmez et al. (2007). The percentage of forward progressive sperm motility and the percentage of morphologically abnormal spermatozoa were both evaluated as described by Sonmez et al (2005). Three hundred sperm cells were examined on each slide (2100 cells in each group), and the total abnormality rates of spermatozoa were expressed as a percentage (Turk et al., 2007).

### Biochemical studies

The plasma and sperm lipid peroxidation levels, the reduced glutathione (GSH) components in plasma and sperm samples, the plasma and sperm catalase (CAT) activity, and the plasma testosterone level were all measured as described by Turk et al. (2007).

### Histological examination

To discover the changes in spermatogenic cell density, testis tissues were fixed in Bouin's solution for two days, then were dehydrated through graded concentrations of ethanol, driven in paraffin wax, sectioned at 5 µm thicknesses and stained with Mayer's Hematoxylin and Eosin Staining Protocol (H&E) (Figure 4).

### Data analysis

Data are presented as mean ± standard error of means (SEM). The degree of significance was set at $P < 0.05$. The changes in body weights were analyzed by two-way analysis of variance for repeated measures in the general linear model (GLM) procedure. One-way analysis of variance (ANOVA) and post hoc Tukey-highest significant difference (HSD) test were used to determine the differences among the groups in terms of all the sperm characteristics, biochemical parameters and histological findings. All the analyses were carried out using the SPSS/PC (version 10.0; SPSS, Chicago, IL) software package program.

## RESULTS

### Body and reproductive organ weights

None of the doses had statistically significant effect on

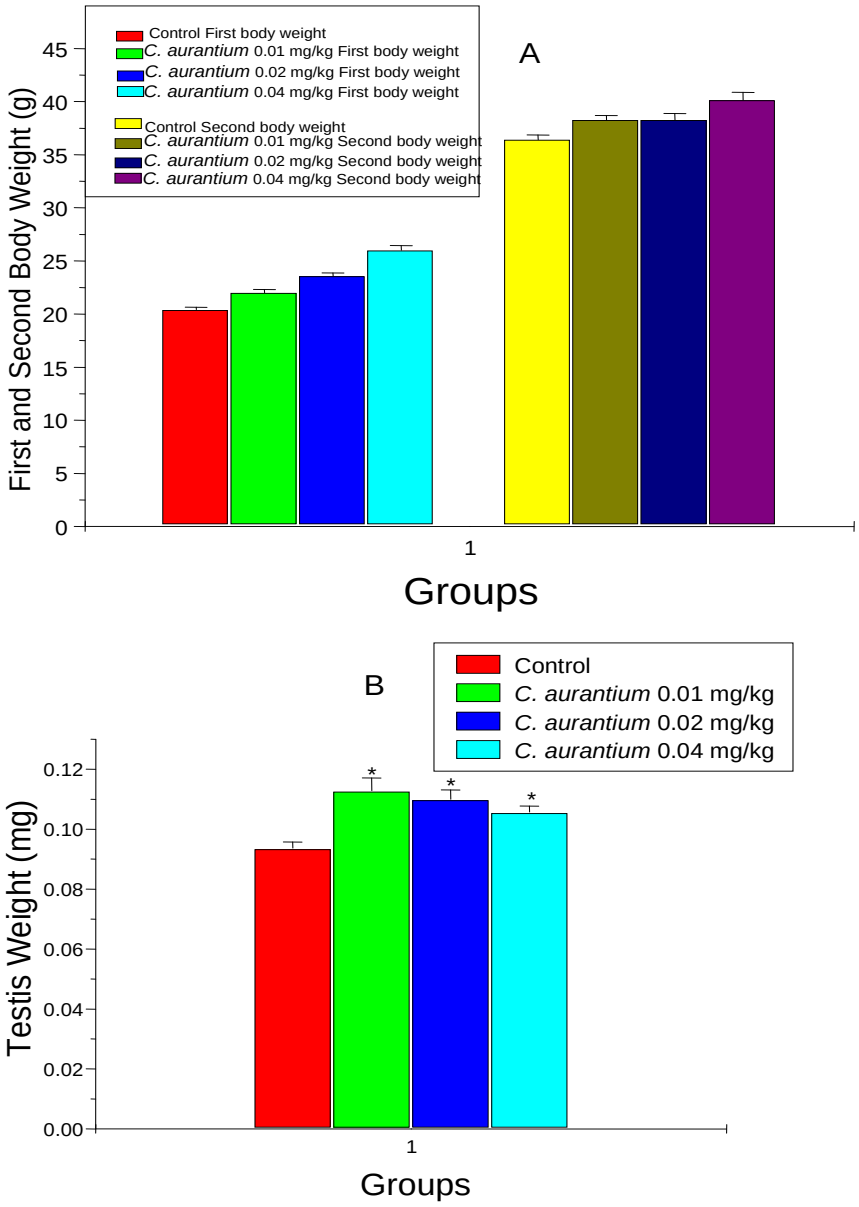


**Figure 1.** Effect of *C. aurantium* on body weight (A) and testis weight (B). All the values are expressed as mean ± SEM.

body weights of the mice during seven weeks when compared to the control group. The weights of testes of mice, treated with different doses of extract were numerically higher than those of the control group and the differences between the groups were statistically significant ($P < 0.05$) (Figure 1).

## Plasma lipid peroxidation level and antioxidant enzyme activities

The MDA and GSH levels, GSH-Px and CAT activities of all the groups are presented in Table 1. All the doses of *C. aurantium* extract caused significant decreases in plasma MDA levels ($P < 0.01$) and all of them increased GSH level significantly in comparison to the control group ($P < 0.01$).

Comparing to the control group, high dose of *C. aurantium* extract caused significant increases in plasma GSH-Px ($P < 0.05$). All doses of *C. aurantium* extract increased plasma CAT activities significantly when compared to the control group ($P < 0.01$). However, increases observed in plasma CAT activities of high dose *C. aurantium* extract group was statistically significant when compared to the control and the other groups ($P < 0.01$) (Table 1).

**Table 1.** Effect of *C. aurantium* on plasma CAT, GSH-Px, GSH and MDA levels.

| Group | Control | *C. aurantium* (0.01 mg/kg) | *C. aurantium* (0.02 mg/kg) | *C. aurantium* (0.04 mg/kg) |
|---|---|---|---|---|
| Catalase (kUL) | 30.51 ± 0.16 | 36.48 ± 0.16** | 39.47 ± 0.21** | 59.96 ± 0.41** |
| Glutathione peroxidase (IuL) | 8.06 ± 0.04 | 8.27 ± 0.04 | 8.25 ± 0.04 | 8.90 ± 0.06* |
| Glutathione (nmol/ml) | 0.09 ± 0.002 | 0.09 ± 0.001 | 0.1 ± 0.004** | 0.1 ± 0.003** |
| Malondialdehyde (nmol/ml) | 1.50 ± 0.08 | 0.75 ± 0.01** | 0.91 ± 0.01** | 0.87 ± 0.01** |

All the values are expressed as mean ± SEM, (n = 7), *P < 0.05, **P <0.01.

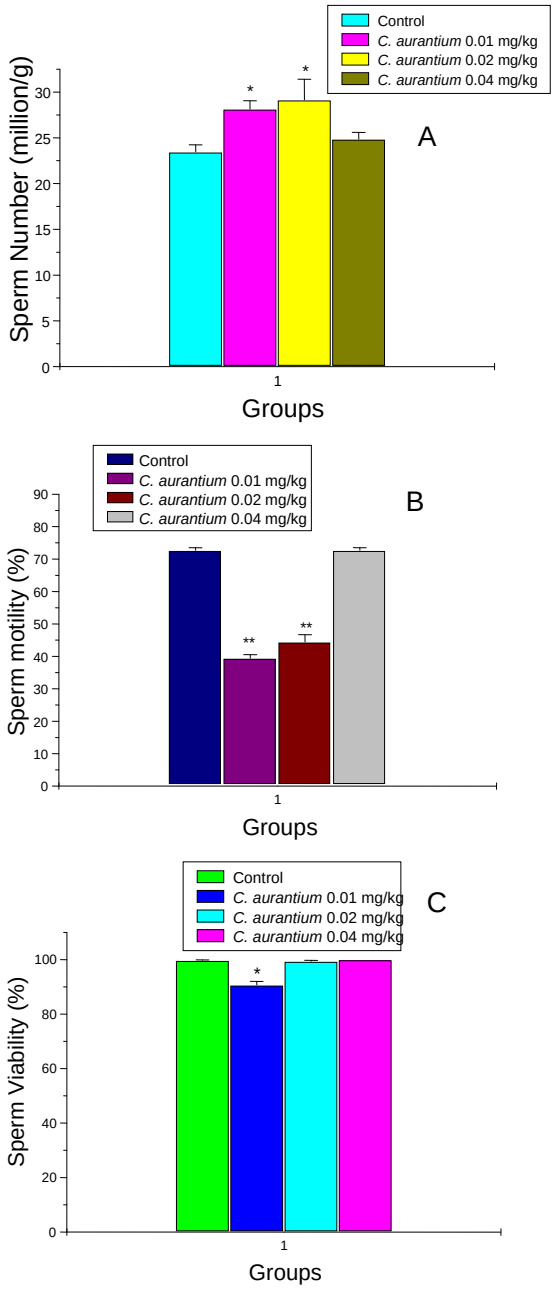


**Figure 2.** Effect of *C. aurantium* on sperm number (A); sperm motility (%) (B) and sperm viability (%) (C). All the values are expressed as mean ± SEM, (n = 7), *P < 0.05, **P < 0.01.

## Differential sperm characteristics

The effects of different doses of *C. aurantium* extract on defferental sperm concentration, sperm motility and sperm viability are presented in Figure 2. While both low and middle dose of *C. aurantium* extract significantly increased the sperm concentration (P < 0.05), when compared to the control group, no significant changes were observed in the high dose *C. aurantium* extract group. Furthermore, sperm concentration of mice in middle dose *C. aurantium* extract group was also significantly higher than those in the high dose *C. aurantium* extract group (P < 0.05).

Both low and middle dose *C. aurantium* extracts provided significant decreases in sperm motility (P < 0.01) and with low dose *C. aurantium* extract, significant decreases were observed in sperm viability when compared to the control group (P < 0.05). However, both the decrease in sperm motility (P < 0.01) and approximately no change in total sperm viability of mice in high and middle dose *C. aurantium* extract groups were not statistically significant in comparison to the control group.

## Spermatogenic cell density

It was observed that all the doses of *C. aurantium* extract provided marked increases in all the spermatogenic cells ranging from spermatogonia, spermatocytes and spermatozoa compared to the control group (P < 0.05) (Figure 3).

## Plasma testosterone level

Testosterone level decreased significantly in mice that received *C. aurantium* extract dose dependently as compared to the control group (P < 0.01) (Figure 5).

## DISCUSSION

To our knowledge, this is the first study demonstrating that daily consumption of *C. aurantium* extract for seven weeks caused increased spermatogenic cell density,

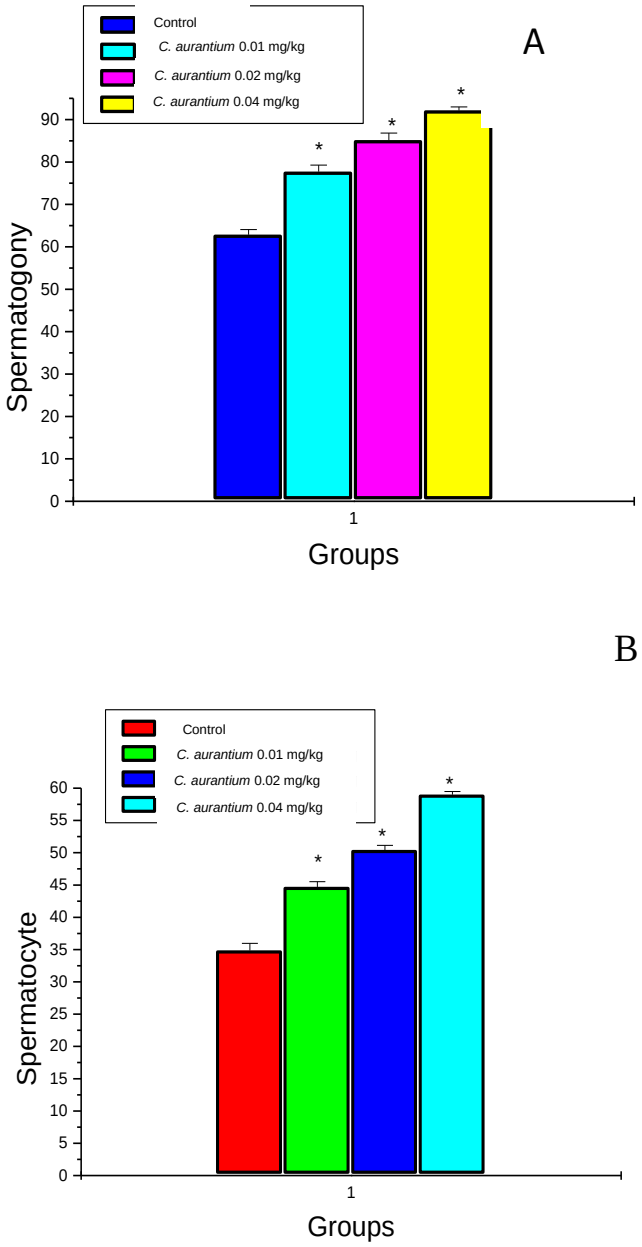


**Figure 3.** Effect of *C. aurantium* on spermatogony (A) and spermatocyte numbers (B). All the values are expressed as mean ± SEM, (n = 7), *P < 0.05.

increased defferental sperm concentration, decreased sperm motility and viability related with decreased lipid peroxidation in male mice.

The most active components in *C. aurantium* fruit are synephrine (also called p-synepherine or oxedrine) and octopamine. The peel also contains flavonoids, including limonene, hesperidin, neohesperidin, naringin and tangaretin. Furanocoumarins are also present (Haung et al., 1995).

*C. aurantium* extract is one of the richest herbal sources of antioxidant vitamins, such as C, E and β- Carotene (Tribble, 1999). A well-known concept is that insufficient vitamins intake can cause deleterious effects on spermatogenesis and production of normal sperm. On the other side, sufficient consumption of vitamins and natural antioxidants can protect sperm DNA from oxidative stress and hence it improves male fertility (Hala and Wahba, 2011). According to the recent studies, flavonoids have essential pharmacologic effects, such as preventing oxidation of lipoproteins with low molecular weight (LDL) (Mahmoodi et al., 2005). *C. aurantium* is rich in vitamin C and flavonoids and is well-known as a fruit with clinical effects (Haung et al., 1995).

The antioxidant and free radical scavenging activity of phenolic compounds derived from C. aurantium and vitamin C have already been reported. In this study, the reproductive organ weights were increased and testosterone level decreased significantly when compared to the control group.

Metabolism of many compounds by cells causes an increase in the levels of the electrophilic radicals that can react with oxygen causing an increase in ROS. Some of the main sources of free radicals are hydrogen peroxide ($H_2O_2$), singlet-oxygen ($^1O_2$), hydroxyl radical (*OH) or peroxynitrite. In many cells and in the spermatozoa, although, ROS are naturally produced in several major metabolic processes, the formation of toxic lipid peroxides can be induced by extreme production of ROS either by spermatozoa themselves (Aitken and Clarkson, 1987; de Lamirande et al., 1997). Accumulation of ROS triggers a defensive mechanism within the cells to use different antioxidant enzymes. CAT and GSH are two major detoxifying systems for peroxides. Activity of CAT destroys $H_2O_2$ that can form a highly reactive OH* in the presence of iron as a catalyst. Excess $H_2O_2$ and lipid peroxides are removed by the participation of GSH together with GSH-Px in the glutathione redox cycle (Sikka, 1996; Calvin et al., 1981; Sanocka and Kurpisz, 2004). Phenolic compounds derived from C. aurantium, vitamins C, E, melatonin (Sonmez et al., 2007), have been used as antioxidant agents. In this study, a significant decrease in MDA level, by-product of lipid peroxidation and marked increases in GSH, GSH-Px and CAT activities of plasma samples of mice that received different doses of *C. aurantium* extract were observed. The findings show that *C. aurantium* extract has a potent anti-oxidative effect.

As mentioned, ROS are highly reactive and can react with many intracellular molecules, chiefly unsaturated fatty acids (phospholipids, glycolipids, glycerides and sterols) and transmembrane proteins with oxidizable amino acids. The oxidation of the mentioned molecules increases the cellular membrane penetrability. The unsaturated bonds of the membrane lipids in an auto-catalytic process can be attacked by ROS resulting in genesis of peroxides, alcohol and lipidic aldehydes as by-products. Therefore, oxidative breakdown of polyunsaturated fatty acids in cells membrane can be induced by the increase of free radicals in cells (de Lamirande et al., 1997;

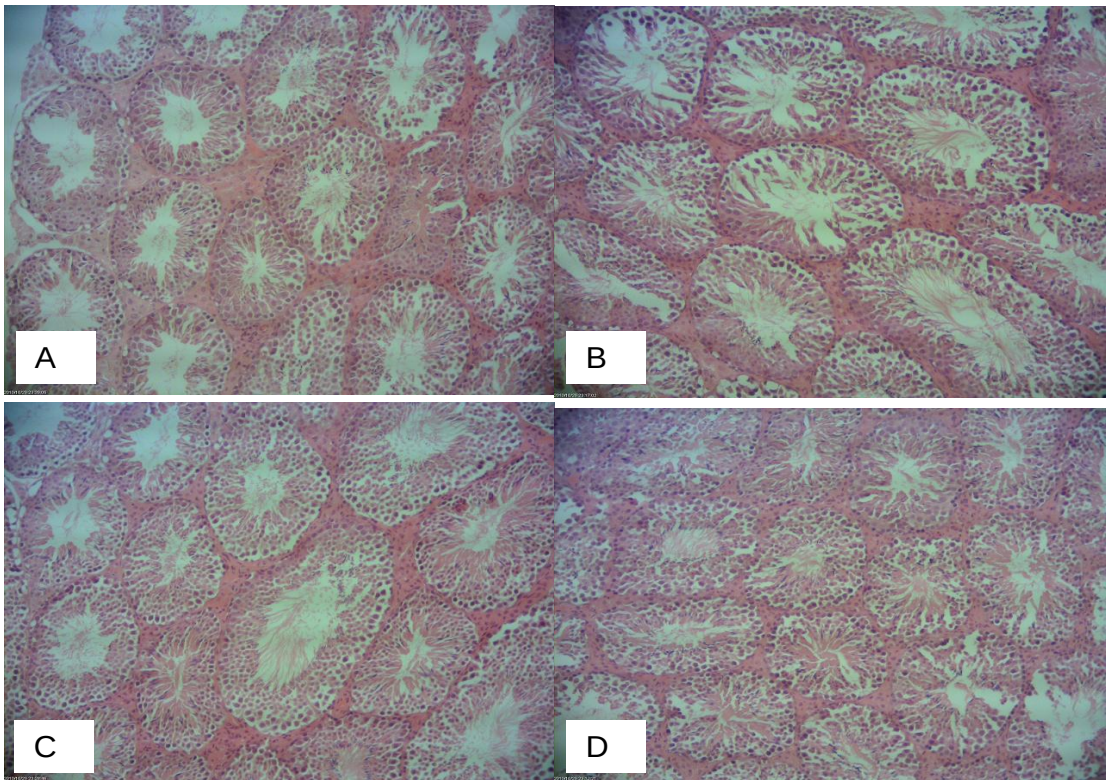


**Figure 4.** Photomicrographs of testis tissue from mice that consumed only distilled water (A), low (B), middle (C) and high doses of *C. aurantium* extract (D). Hematoxylin - eosin (H & E); bar 150 µm. Although, a low dose of *C. aurantium* caused a slight increase in spermatogenic cell density, both middle and high doses of *C. aurantium* provided marked increases in spermatogonia and spermatocytes.

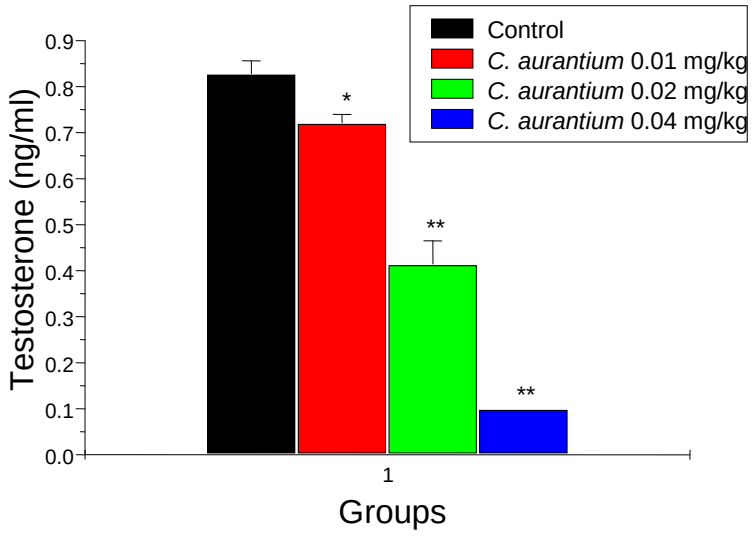


**Figure 5.** Effect of *C. aurantium* on testosterone level. All the values are expressed as mean ± SEM, (n = 7), *P < 0.05, **P <0.01.

Henkel, 2005). Spermatozoa are particularly vulnerable to peroxidative damage as a result of the high concentration of polyunsaturated fatty acids that are involved in regulation of sperm maturation, spermatogenesis, capacitation, acrosome reaction and eventually in membrane fusion, and low antioxidant capacity.

Obviously, the structure of the lipid matrix in the membranes of spermatozoa is destroyed by peroxidation of sperm lipids, and is connected with the fast loss of intracellular ATP. This in turn results in axonemal damage, decreased sperm viability and increased mid-piece morphological defects, and even can completely inhibits spermatogenesis in severe cases (Sikka, 1996; Sanocka and Kurpisz, 2004; Varnet et al., 2004). In the present study, it was observed that defferental sperm concentration of mice that received different doses of *C. aurantium* extract was significantly higher and the sperm motility was significantly lower when compared with the control group. It seems that *C. aurantium* extract has directly affected the spermatogenic cells. The study has also shown that the number of spermatogony cells and primary spermatocytes increased significantly in the experimental groups in comparison to the control group.

## Conclusion

The increase in spermatogenic cell density was due to increased antioxidant capacity that protected spermatozoa against peroxidative damage in healthy mice, but the sperm quality and the level of testosterone decreased significantly. Hence, it can be said that the consumption of *C. aurantium* is not recommended for a long period of time and it seems that there is a negative relation between *C. aurantium* consumption and sperm parameters.

### REFERENCES


Aitken RJ, Clarkson JS (1987). Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. Reprod Fertil J., 81: 459-469.

Arbo MD, Franco MT, Larentis ER, Garcia SC, Sebben VC, Leal MB, Dallegrave E, Limberger RP (2009). Screening for *in vivo* (anti)estrogenic activity of ephedrine and synephrine and their natural sources *Ephedra sinica* Stapf. (Ephedraceae) and *Citrus aurantium* L. Rutaceae in rats. Arch. Toxicol., 83(9): 95-99.

Aynehchi Y (1991). Pharmacognosy and Medicinal Plant of Iran. Tehran University Pub., pp. 272-295.

Bisset NG, Wichtl M (1994). Herbal Drugs and Phytopharmaceuticals. Stuttgart, Germany: Medpharm GmbH Scientific Publishers, and Boca Raton. FL: CRC Press. pp. 91-95.

Calvin HI, Cooper GW, Wallace EW (1981). Evidence that selenium in rat sperm is associated with a cysteine-rich structural proteins of the mitochondrial capsule. Gamete Res., 4: 139-145.

De Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C (1997). Reactive oxygen species and sperm physiology. Rev. Reprod., 2: 48-54.

Facciola S (1998). Cornucopia II: A source book of edible plants. Vista, CA: Kampong Publications, p. 506.

Fugh-Berman A, Myers A (2004). *Citrus aurantium*, an ingredient of dietary supplements marketed for weight loss: Current status of clinical and basic research. Biol. Med., pp. 698-703.

Hala MA, Wahba (2011). Protective Effect of *Nigella Sativa*, Linseed and Celery Oil against Testicular Toxicity Induced by Sodium Valproate in Male Rats. J. Am. Sci., 7(5).

Hansen DK, Juliar BE, White GE, Pellicore LS (2011). Developmental toxity of *Citrus aurantium* in Rats. Birth Defects Res. B., 80: 451-472.

Huang YT, Wang GF, Chen CF, Chen CC, Hong CY, Yang MCM (1995). *Fructus Aurantii* Reduced Portal Pressure in Portal hypertensive rats. Life Sci., 57: 201-220.

Henkel R (2005). The impact of oxidants on sperm functions. Andrologia, 37: 205-6.

Jyotsna A (2011). An overview of *Citrus Aurantium* used in treatment of various diseases. Afr. J. Plant Sci., 5(7): 390-395.

Mahmoodi M, Shamsi Meimandi M, Foroumadi AR, Raftari Sh, Asadi SM (2005). Antidepressant effect of sour orange flowers extract on lipopolysaccharide-i. J. Kerman Uni. Med. Sci., 4(12): 244-251.

Mantel F (2002). The role of alternative medicine in treating postnatal depression. Complementary Therapies in Nursing Midwifery, 8: 197-203.

Sanocka D, Kurpisz M (2004). Reactive oxygen species and sperm cells. Reprod. Biol. Endocrinol., 2(12): 1-7.

Sikka SC (1996). Oxidative stress and role of antioxidants in normal and abnormal sperm function. Front Biosci., 1: 78-86.

Sonmez M, Turk G, Yuce A (2005). The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. Theriogenology, 63: 2063-2072.

Sonmez M, Yuce A, Turk G (2007). The protective effects of melatonin and vitamin E on antioxidant enzyme activities and epididymal sperm characteristics of homocysteine treated male rats. Reprod. Toxicol., 23: 226–231.

Tribble DL (1999). Antioxidant Consumption and risk of Coronary Heart Disease: Emphasis on Vitamin C, Vitamin E, and-β Carotene: A Statement for Healthcare Professionals Form American Heart Association. Circulation, 99: 591-595.

Turk G, Atessahin A, Sonmez M, Yuce A, Ceribasi AO (2007). Lycopene protects against cyclosporine A-induced testicular toxicity in rats. Theriogenology, 67: 778-785.

Varnet P, Aitken RJ. Drevet JR (2004). Antioxidant strategies in the epidydimis. Mol. Cell Endocrinol., 216: 31-39.

Zargari A (1986). Medicinal Plants. Vol. 1, 4th ed. Tehran University Press. pp. 478-485.