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Garlic (*Allium sativum*) juice protects from semen oxidative stress in male rats exposed to chromium chloride

J. Ghiasi Ghalehkandi[1]

Department of Veterinary Medicine, Shabestar Branch, Islamic Azad University, Shabestar, Iran.

Abstract

Semen oxidation is one of the major testimonies of infertility. The aim of present study was to examine the effects of fresh garlic juice on semen malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant status (TAS) compared with chromium chloride ($CrCl_3$) in male rats. A hundred and sixty-two male rats were allocated into 9 treatment groups. Group 1 served as control. In group 2, rats gavage 60 mg/kg garlic juice. In group 3, rats were offered 120 mg/kg garlic juice. Group 4 drenched 4 mg/kg $CrCl_3$. In group 5, 8 mg/kg $CrCl_3$ was offered to rats. Group 6 was treated with 60 mg/kg garlic juice + 4 mg/kg $CrCl_3$. Group 7 gavage 60 mg/kg garlic juice + 8 mg/kg $CrCl_3$. Group 8 consumed 120 mg/kg garlic juice + 4 mg/kg $CrCl_3$. In group 9 rats received 8 mg/kg $CrCl_3$ + 120 mg/kg garlic juice. After 4 weeks animals were killed and semen samples used to determine MDA, SOD, GPx and TAS activity. According to the results, garlic juice (120 mg/kg) significantly declined semen MDA activity compared to control group ($P < 0.05$). Also, garlic juice (120 mg/kg) significantly attenuated effects of $CrCl_3$ on TAS compared to control group ($P < 0.05$). These results suggest that garlic juice presumably protects semen oxidation in rat testes.

Keywords: chromium chloride, garlic juice, oxidative enzymes, rat, semen.

Introduction

Every day the number of medical reports increases about infertility rate in the world. Infertility is a multi-parametric phenomenon with a wide range of factors that influence spermatogenesis and sperm quality. In clinical manifestations, insufficient nutrition and toxins are the most prominent (Khaki *et al*., 2009; Vincent *et al*., 2012). Metal induced toxicity is plentifully reported in previous literature (Akunna *et al*., 2012; Babaei and Abshenas, 2013). Chromium is a trace element and extracts from chromite. Chromium appears in both trivalent and hexavalent forms. Trivalent chromium (Cr III) has a biological role while the hexavalent is more toxic. Hence, it is reported that organic forms of chromium are more toxic than mineral forms (Akunna *et al*., 2012; Ghalehkandi *et al*., 2012e).

Allium is the largest and most prominent representative plant genus of the Alliaceae family and broadly cultivated in the northern hemisphere. Since ancient times, garlic (*Allium sativum L.*) has been used to cure diseases. The first citation of medical application of garlic as a remedy is found in the Codex Ebers (1550 B.C.), an Egyptian medical papyrus. Numerous therapeutic effects of garlic are largely attributed to (I) anti-diabetic, anti-atherosclerotic, anti-thrombotic, anti-hypertensive property (II) stimulation of immune function (III) detoxification (IV) hepato protection (V) anti-microbial and (VI) antioxidant effect (Lanzotti, 2006; Bozcuk *et al*., 2011; Khaki *et al*., 2012). Nowadays, there is a worldwide increased interest regarding folk medicine. People desire to consume much more medical plants due to their medicinal properties. Garlic has abundant amount of antioxidants, flavonoids and sulfur-containing compounds (Asadpour *et al*., 2013) which can be used in detoxification systems (Ola-Mudathir *et al*., 2008). Thiosulfinates are volatile sulfur garlic compounds responsible for pungent aroma, taste and biological effects (Lanzotti, 2006). Previously, it was reported that oral administration of garlic acts as insulin secretagogues which decreases blood sugar levels in diabetic patients (El-Demerdash *et al*., 2005).

Antioxidants are a group of compounds that resist against oxidant formation (Erguder *et al*., 2007). Reactive oxygen species (ROS) are a wide group of materials that oxide DNA and cell membrane. They are responsible for inflammation, neurodegenerative disease, infertility and even cancer (Chi *et al*., 2008). It is reported that spermatozoa contain high levels of polyunsaturated fatty acids (PUFAs) which make them vulnerable to ROS (Hsieh *et al*., 2006). Malondialdehyde is the end product of lipid peroxidation and an elevation in testicle MDA level is a sign of lipid peroxidation. So an increase in MDA levels has irreversible effects on sperm fertility and leads to infertility (Hsieh *et al*., 2006). Glutathione peroxidase is an enzyme family with peroxidase activity whose main biological role is to protect organisms from oxidative damage and lipid peroxidation (Lee *et al*., 2012). It is suggested that GPx acts against sperm peroxidation (Hsieh *et al*., 2006). In diabetes, oxidative stress and ROS arise due to a compromise in natural antioxidant mechanisms. Previously, it is reported that treatment with garlic decreased ROS levels in diabetic rats (El-Demerdash *et al*., 2005).

[1]Corresponding author: Ghiasi_jam@yahoo.com
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A correlation exists between excessive ROS generation in semen and infertility (Hsieh *et al*., 2006). Recent researches threw light on protective effects of medical plants on reproduction and fertility. Lines of evidence suggest that aqueous garlic extract (AGE) is rich in antioxidants and able to scavenge ROS by amplification of intercellular antioxidant enzymes e.g. SOD, GPx and CAT (Corzo-Martínez *et al*., 2007). Toxic metal exposure infertility is still one of the concerns in reproduction (Akunna *et al*., 2012). Several lines of evidences reported that abnormal spermatozoa increase in $CrCl_3$-exposed rat and rabbit (Marouani *et al*., 2012). Studies suggest that oral administration of hexavalent chromium declines spermatogenesis and epididymal sperm (Zahid *et al*., 1990; Kumar *et al*., 2005). It is reported that garlic has positive effects in testicular functions and spermatogenesis in rats (Kasuga *et al*., 2001). Therefore, based on previous literature on the adverse effects of $CrCl_3$ on fertility, as well as the antioxidant property of garlic, our hypothesis was to investigate possible involvement of garlic juice on fertility. So the aim of this study was to evaluate the protective effect of garlic fresh juice on semen oxidative status in $CrCl_3$-exposed rats.

Materials and Methods

Animals

One hundred and sixty-two male Wistar albino rats (230-250 g) were purchased from Razi Vaccine and Serum Research Institute, Iran, and randomly allocated into 9 treatment groups (each include 3 groups and 6 replicates). Experimental animals were housed in individual stainless steel wire-bottomed cages under standard laboratory conditions at temperature 23.1-25.8°C, relative humidity 55-60% and 12 h lighting period (started at 8:00 AM) based on European community suggestions for laboratory animals. All animals had *ad libitum* access to chow pellets (Azarbayjan Co. Iran) and fresh water.

Plant material

The *Allium sativum* was obtained from local market-Tabriz, East Azarbayjan province, Iran, in August 2012. Samples of *Allium sativum* were identified at division of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Iran.

Preparation of aqueous garlic extract

Thirty g of garlic added to 100 ml distilled water were crushed and juice was obtained using a fruit juice extracting machine. The resultant homogenized mixture was filtered three times through a cheese cloth. Then, centrifuged at 200 g for 10 min, the clear supernatant was quickly collected and kept in pyrogen-free bottles until used. Based on weight of the starting material (30 g per 100 ml), concentration of prepared garlic was considered to be 500 mg per ml. All extracts were were prepared each day just before the experiments (Thomson *et al*., 1998; Suru, 2008; Khaki *et al*., 2012; Asadpour *et al*., 2013).

Experimental procedure

Garlic juice (60 or 120 mg/kg) was provided to rats once daily as gavage (gastro-oral). Chromium chloride purchased from Merk (© Merck KGaA, Darmstadt, Germany), 4 and 8 mg/kg were dissolved in water and orally administrated to animals. Doses of garlic and chromium chloride were calculated based on pilot and previous studies (Ernst, 1990; Suru, 2008; Ghalehkandi, 2012a, b, c, d, e, f; Asadpour *et al*., 2013). In order to adapt the animals to new experimental condition, rats received a basal diet for 1 week then were grouped as follows:

Groups 1: basal diet + distilled water (control),
Groups 2: basal diet + 60 mg/kg fresh garlic juice,
Groups 3: basal diet + 120 mg/kg fresh garlic juice,
Groups 4: basal diet + 4 mg/kg $CrCl_3$,
Groups 5: basal diet + 8 mg/kg $CrCl_3$,
Groups 6: basal diet + 60 mg/kg fresh garlic juice + 4 mg/kg $CrCl_3$,
Groups 7: basal diet + 60 mg/kg fresh garlic juice + 8 mg/kg $CrCl_3$,
Groups 8: basal diet + 120 mg/kg fresh garlic juice + 4 mg/kg $CrCl_3$,
Groups 9: basal diet + 120 mg/kg fresh garlic juice + 8 mg/kg $CrCl_3$.

Experimental animals were treated with diets for 4 weeks.

Surgical procedure

At the end of 4 weeks, rats fasted overnight, and were intraperitoneally (i.p) injected using 40 mg/kg pentobarbital for tranquilizing. Peritoneum was opened by an incision to the width of the abdomen and the testes removed. Semen samples were collected from cauda epididymis and homogenized in 10% (W/V) ice cold buffer (0.1 M phosphate buffer, pH 7.4+150 mM Kcl). The homogenate was centrifuged at 9000 rpm for 20 min to obtain supernatant which was used for SOD, MDA, GPx and TAS estimations (Sharma *et al*., 2012). Then, the animals were euthanized using CO_2 gas in a 2 h period. All experimental procedures were done between 10-12 AM. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (USA) and the current laws of the Iranian government. All experiments were executed in accordance to the Guide for Care and Use of Laboratory Animals to investigate experimental pain in animals (Zimmermann, 1983). All protocols for animal

experimentation were approved by the institutional animal ethical committee.

Semen biochemical assay

Malondialdehyde

Malondialdehyde is used as standard to determine free radical damage in blood. A detection kit was purchased from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). Malondialdehyde is formed as an end product of lipid peroxidation and treated with thiobarbituric acid (TBA) to produce a color product which is measured at 532 nm (Placer *et al*., 1966).

Superoxide dismutase

The role of SOD is to accelerate dismutation of the toxic superoxide radical (O_2), produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. Detecting kit (Cat. no. SD125) was purchased from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye measured at 505 nm (Woolliams *et al*., 1983).

Glutathione peroxidase

The commercial kit was obtained from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom; Cat. no. RS504).. In the presence of glutathione reductase and NADPH, oxide glutathione reduces via change in oxidation of NADPH to $NADP^+$ in absorbance at 340 nm (Paglia and Valentine, 1967).

Total antioxidant status

Total antioxidant status detecting kit was obtained from Randox (Randox Laboratories Ltd., Crumlin, Antrim, United Kingdom; Cat. no. NX2332). Antioxidants added to samples cause suppression in color production measured at 600 nm (Miller *et al*., 1993).

Statistical analysis

Study design was a factorial 3 x 3 experiment (3 levels of fresh garlic juice and 3 levels of chromium chloride). Data is presented as mean values ± SEM following one-way analysis of variance using the general linear models (GLM). All statistical analyses were performed using SAS version 9.1. When significant difference among the means was found, means were separated using Duncan's Multiple Range tests. P = 0.05 was the level set to consider the significance of the differences between groups. The following model was used:

$Yijk = \mu + \alpha i + \beta j + (a\ \beta)\ i\ j + eijk$

Where,

Yijk = All dependent variable

M = Overall mean

αi = The fixed effect of garlic levels (i = 1, 2, 3)

βj = The fixed effect of chromium chloride levels (j = 1, 2, 3)

eijk = The effect of experimental error

Results

The effects of fresh garlic juice administration on semen MDA, SOD, GPx and TAS in $CrCl_3$-exposed male rat are presented in Table 1. As noticed, single administration of garlic juice (120 mg/kg) significantly decreased semen MDA activity compared to the control group after 4 weeks ($P < 0.05$). In addition, 4 weeks of treatment with $CrCl_3$ had no significant effect on MDA content of seminal fluid ($P > 0.05$). Interestingly, simultaneous administration of fresh garlic juice and $CrCl_3$ caused a significant diminishing in MDA levels and the greatest decline was observed in the group which received 120 mg/kg garlic juice + 8 mg/kg of $CrCl_3$.

Next, we determined the effect of garlic juice and $CrCl_3$ on SOD levels in seminal fluid. According to the results, sole administration of fresh garlic juice or $CrCl_3$ for 4 weeks did not attenuate semen SOD levels in rat ($P > 0.05$). Furthermore, co-administration of garlic juice plus $CrCl_3$ had no significant effect on semen SOD concentration ($P > 0.05$). We then determined whether there was a change on GPx levels in rats. As it appears, no significant change was observed by sole garlic juice or $CrCl_3$ administration after 4 weeks in rats ($P > 0.05$). Also, semen GPx level was not affected in rats receiving garlic juice + $CrCl_3$ ($P > 0.05$).

In this study there was no significant effect on semen TAS in animals that received garlic juice or $CrCl_3$ daily for 4 weeks ($P > 0.05$). Noticeably, single administration of $CrCl_3$ for 4 weeks significantly lessened TAS in rat ($P < 0.05$). Also, in garlic juice-treated rats, the level of 120 mg/kg significantly attenuated $CrCl_3$ effects on semen TAS compared to the control group ($P < 0.05$).

Table 1. Effects of fresh garlic (*Allium sativum*) juice on semen malondialdehyde, superoxide dismutase, glutathione peroxidase and total antioxidant status in chromium chloride exposed male rat.

Treatment groups		MDA (nmol/ml)	SOD (IU)	GPx (IU)	TAS (mmol/ml)
Garlic (mg /kg)					
0 (control)		232.50[a]	190.00	7191.67	13.66
60		207.50[a]	190.00	7158.33	12.33
120		170.00[b]	177.14	7200.00	11.50
P-value		0.01	0.34	0.58	0.36
SEM		10.96	7.53	31.06	1.03
$CrCl_3$ (mg /kg)					
0 (control)		233.33	177.50	7208.33	12.75
4		231.67	190.83	7150.00	11.75
8		247.26	187.14	7192.86	12.78
P-value		0.48	0.44	0.41	0.65
SEM		10.96	7.53	31.06	1.03
Combination administration					
Garlic (mg /kg)	$CrCl_3$ (mg /kg)				
0	0	260.00[a]	185.00	7225.00	18.25[a]
	4	217.50[abcd]	185.00	7200.00	9.50[b]
	8	222.50[abc]	162.50	7200.00	10.50[b]
60	0	255.00[ab]	200.00	7125.00	10.00[b]
	4	182.50[cd]	185.00	7225.00	12.75[ab]
	8	247.50[ab]	187.50	7100.00	12.25[ab]
120	0	192.50[bcd]	185.00	7225.00	13.00[ab]
	4	157.50[de]	197.50	7175.00	15.25[ab]
	8	120.00[e]	181.67	7183.33	11.16[b]
P-value		0.61	0.83	0.62	0.03
SEM		18.99	13.04	53.81	1.79

MDA: malondialdehyde, SOD: superoxide dismutase, GPx: glutathione peroxidase, TAS: total antioxidant status, $CrCl_3$: chromium chloride. SEM: standard error mean. [a,b,c,d,e]Different letters indicate significant differences between treatments ($P < 0.05$). Data are presented in mean ± SEM.

Discussion

Spermatogenesis is a complex process where clinical and pathological manifestations affect fertility (Akhondi *et al*., 2013; Barkhordari *et al*., 2013). This study was designed to determine effects of fresh garlic juice on semen MDA, SOD, GPx and TAS in $CrCl_3$-exposed male rats. To our knowledge, the effect of $CrCl_3$ and garlic juice on sperm oxidation is not discussed previously. In the present study, to identify possible modifications, cauda epididymis sperm samples were used (Hammami *et al*., 2008). As seen in the results, daily gavage of fresh garlic juice decreased adverse effects $CrCl_3$ on semen oxidative enzymes in rat. It is reported that hexavalent chromium (as sodium chromate) at levels of 2 and 4 mg induces seminiferous tubules atrophy and decrease epididymal sperm numbers (Ernst, 1990). Chromium is a mineral which is required in a trace amount by the body. Previously, Marouani *et al*. (2012) reported that epididymal spermatozoa and sperm motility decrease in chromium-exposed rat. Several factors are suggested for adverse effects of chromium. It is described that $CrCl_3$ may impress its effects by histological lesions on Sertoli cells and/or lumen of seminiferous tubule (Marouani *et al*., 2012). Phytochemical substances present in garlic act as transition chelator for several metals such as cadmium, cupper and chromium which result in improving spermatozoa survival (Nijveldt *et al*., 2001). Recently, it has been reported that $CrCl_3$-induced testicular toxicity is mediated through oxidative stress (Akunna *et al*., 2012). In our previous study, we found that garlic juice (120 mg/kg) improves sperm number and decreases its mortality rate (Ghalehkandi *et al*., 2012c).

Reactive oxygen species play a physiological role on sperm maturation and capacitation while excessive generation of ROS is associated with DNA damage and sperm infertility (Hsieh *et al*., 2006). Excessive ROS production and subsequent oxidative damage is the main mechanism of cadmium and chromium induced toxicity on spermatozoa (Ola-Mudathir *et al*., 2008; Marouani *et al*., 2012). Seminal plasma is endowed with frequent enzymatic antioxidants that include SOD, GPx, MDA (Fingerova *et al*., 2007). ROS react with these enzymes via cell membrane lipid oxidative damage in sperm (Chi *et al*.,

2008). Spermatozoa are uniquely rich in polyunsaturated fatty acids (PUFAs) which are susceptible to be attacked by ROS. In this study, daily fresh garlic juice gavage decreased semen MDA activity in rat. In this regard, Verma and Kanwar (1999) revealed that promoted MDA level is an index for pathologic lipid peroxidation of sperm. In this study, co-administration of garlic juice and $CrCl_3$ decreased seminal MDA levels in rat. In contrast, in our previous study, we found that serum MDA levels diminished after 4 weeks in $CrCl_3$-exposed rats treated with garlic aqueous extract (Ghalehkandi *et al*., 2013). To our knowledge, direct mechanism for protective effects of fresh garlic juice against free radical damage on sperm is not fully identified but it seems that garlic may act via antioxidant defense mechanisms such as regulation of cytochrome P450 enzymes. However, the accuracy of this mechanism is still controversial and needs to be investigated further (Chitra *et al*., 2003; Hsieh *et al*., 2006). Also, it seems that polyphenols in the garlic bind to lipid peroxides acting as an antioxidant ligand (Hsieh *et al*., 2006; Mansouri and Abdennour, 2011).

In the current study, co-administration of garlic juice + $CrCl_3$ had no significant effect on semen SOD concentration. Testis is resistant to oxidative damage by its antioxidants (Mansouri and Abdennour, 2011). Superoxide dismutase is a fundamental part of cellular antioxidant defense system. It is the first defense line against oxidative stress with dismutation of superoxide anion radicals to H_2O_2 (Suru, 2008; Asadpour *et al*., 2013). Previous studies demonstrated that administration of garlic extract enhanced SOD level in testis (Mansour**i** and Abdennour, 2011). In our former study, serum SOD levels significantly increased in $CrCl_3$-exposed rat treated with aqueous garlic extract (Ghalehkandi *et al*., 2013). Based on the results, we think levels of garlic juice used in the current research were not sufficient to amplify SOD levels in rat semen. The method used to obtain garlic extract, as well as juice preparation affects its biological activity (Thomson *et al*., 1998). In the current study, garlic aqueous extract was provided according to previous techniques described by Thomson *et al*. (1998) and Suru (2008). Garlic contains water-soluble organosulfur compounds such as S-allylmercaptocysteine (SAMC), SAC, lipid-soluble allyl sulphides (DAS) and diallyl polisulphides (DADS) which have antioxidant properties (Kim *et al*., 2013).

Recently, antioxidants attract widespread attention in medicine research (Khaki *et al*., 2012). It is well documented that toxic metal cause oxidation (Akunna *et al*., 2012). In the present study there was no significant fluctuation on semen GPx level after garlic juice administration in $CrCl_3$-exposed rat. Glutathione peroxidase is a lipid peroxidative antioxidative enzyme and its elevated activity is a biomarker for tissue damage. GPx is related to GSSG (reduced form of glutathione) and GSH (oxidized form of glutathione) which reduced glutathione and can neutralize hydroxyl radicals and detoxify peroxides (Hsieh *et al*., 2006). Dandekar *et al*. (2002) reported that a positive correlation exists between GPx concentration and asthenozoospermia in which GPx improves sperm motility by catalyze ROS. In this regard, Asadpour *et al*. (2013) reported that oral administration of 400 mg/kg aqueous garlic extract had no significant effect on GPx in lead-induced oxidative stress in rats. The result of our study was similar to previous findings. One of the possible mechanisms for the effect of garlic on GPx is via detoxification of glutathione S-transferase or N-fructosyl glutamate (whose antioxidant activity is comparable to ascorbic acid; Mansour**i** and Abdennour, 2011). Based on complex interactions between ROS and various antioxidants, perhaps, seminal GPx is not a potent factor to investigate sperm damage (Hsieh *et al*., 2006). Further studies are needed to clarify possible mechanisms as how antioxidants act on GPx.

Individual antioxidants can promote biological fluid´s antioxidant power by assessing their aggregate known as TAS (Fingerova *et al*., 2007). According our data, sole administration of $CrCl_3$ significantly lessened TAS levels compared to control animals. Interestingly, garlic juice treatment attenuated $CrCl_3$ effects on semen TAS in rat. Also, it was able to restore TAS in corresponding levels. There is a positive correlation between oxidative stress, TAS and apoptosis (Bu *et al*., 2012). In conclusion, it seems that garlic juice may be useful in infertility treatment. Obviously, more studies should be undertaken to investigate the potential level of garlic juice as antioxidant in spermatogenesis. In addition, merit studies need to clarify direct interaction of antioxidants and semen MDA, SOD, GPx and TAC activity in the physiology of reproduction. Also, we suggest further studies are needed to distinguish garlic juice potential for clinical use in clinical trials.

Conflicts of interest

The author declares that there are no conflicts of interest.

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