

Ameliorative effect of aqueous extract of *Allium sativum* on the acrosome status of spermatozoa in cadmium induced male infertility in Wistar rats

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Abstract

Background

Male infertility is a common disorder affecting approximately 50% of couples. The causes are multifactorial and some environmental toxicants such as cadmium have been implicated in some cases. Garlic (*Allium sativum*) is reported to have some antioxidant and therapeutic properties, and it is used as herbal medicine for the treatment of several disease conditions. This study seeks to investigate effect of cadmium chloride administration on acrosome status of spermatozoa and the possible amelioration by garlic in Wistar rats.

Materials and Methods

A total of 20 adult male Wistar rats were used for this study. Five rats were assigned to four groups (A-D). Group A, the control group was administered normal feed and water, Group B was administered 20mg/kg of cadmium, Group C was administered 20mg/kg of cadmium and 750mg/kg of garlic simultaneously, while Group D was administered 20mg/kg of cadmium and thereafter treated with 750mg/kg of garlic. All administration was done for twenty-eight (28) days, then semen was collected for analysis. Multiple comparisons were done using analysis of variance (ANOVA).

Results and Discussion: The acrosome-intact spermatozoa in animals in group B 62.67 ± 1.45 , C.I 56.42-68.92), group C (71.33 ± 2.91 , C.I 58.83-83.84) and group D (64.50 ± 2.50 , C.I 32.73-96.27) were significantly lower ($P < 0.025$) than the control group A (79.33 ± 3.48 , C.I 64.36-94.31). Conversely, the depleted acrosome numbers were significantly higher ($P < 0.025$) in animals in group B (37.33 ± 1.45 , C.I 31.08-43.58), group C (28.67 ± 2.91 , C.I 16.16-41.17) and group D (35.50 ± 2.50 , C.I 3.73-67.27) when compared with the control group A (20.67 ± 3.48 , C.I 5.69-35.64). In conclusion, the data from this study revealed that *Allium sativum* at 750mg/kg was able to ameliorate cadmium toxicity on acrosome status of spermatozoa in Wistar rats when administered simultaneously. However, treatments with 750mg *Allium sativum* after the induction of subfertility with cadmium were unable to improve the acrosome status of spermatozoa in Wistar rats.

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Introduction

Cadmium is a toxic metal to which humans are exposed either occupationally or environmentally. It has been recognized as an endocrine disruptor by binding to androgen and estrogen receptors thereby inhibiting steroidogenesis and spermatogenesis, which may affect semen quality (Benoff et al., 2009;

Yeung et al., 2011). Exposure to cadmium in the environment can accumulate in the body over a lifetime (Ashok et al., 2015). Toxic metals have the potential to induce oxidative stress, damage lipid membranes, and spermatozoa rich in poly-unsaturated fatty acids are readily susceptible to oxidative damage. Some authors have recently

reported that cadmium has the potential to cause severe structural damage to the seminiferous tubules, Sertoli cells, and blood-testis barrier, thus leading to the loss of sperm (Zhu et al., 2020). Cadmium may prevent Leydig cell function, and disrupts the vascular system of the testis. It may also induce DNA damage, thus epigenetically regulating somatic cell and germ cell function, leading to male subfertility/infertility (Zhu et al., 2020), but the effect of cadmium on acrosome status has not been investigated in in-vivo study to the best of our knowledge. Human beings are exposed to Cd via food intake, drinking water, contaminated soil, inhalation of tobacco smoke, or particulate matter from ambient air (ATSDR, 2012).

The causes of male infertility are not completely understood and the etiology of about half of male infertility remains unclear. Although genetic factors can explain some of these causes, exposure to environmental pollutants is increasingly implicated in male infertility (Nordkap et al., 2012; Gao et al., 2015).

Nigeria lies within the so-called infertility belt of sub-Saharan Africa where the incidence of male factor infertility is on the rise (Okonofua, 2003; Emokpae and Ikuejamoye, 2020). About 30%-50% of the causes of infertility are related to male problems (Roozbeh et al., 2016). It was previously reports that except drastic measures are taken, the burden of male infertility is unlikely to decline in Nigeria (Emokpae et al., 2009; Uadia and Emokpae, 2015).

Nowadays, various methods are used to treat infertility, including: hormone therapy, surgical procedures, assisted reproductive technology (ART) (Baniaghil et al., 2016). These treatment methods enumerated above are usually expensive and the stress and complications that come with them are not pleasant. Because of the high costs of medical interventions, some people have turned to complementary medicine (Anbari and Ghanadi, 2015).

Among the different therapeutic methods, herbs are used in many countries to treat male infertility (Mohammadi et al., 2013; Amidi et al., 2016). Plants are more affordable and accessible than invasive and chemical treatments. Medicinal herbs such as *Allium sativum* with high antioxidant properties are

used to treat several illnesses (Amidi et al., 2014).

Allium sativum (Garlic) is an aromatic herbaceous annual spice and one of the oldest authenticated and most important herbs that have been used from ancient times as traditional medicine (Ayaz and Alposy, 2007). It is considered the second broadly used *Allium* species with onion (*Allium cepa*), which is used as a remedy against several common diseases such are cold, influenza, snake bites, diabetes mellitus, cardiovascular diseases and hypertension (Badal et al., 2019). The need to investigate the effect of cadmium exposure on acrosomal status of spermatozoa and possible ameliorative potential of *Allium sativum* on spermatozoa is imperative due to the increasing incidence of male infertility in Nigeria. It may present a modifiable target for public health intervention. This study seeks to investigate the ameliorative effect of *Allium sativum* on the acrosome status of spermatozoa in cadmium induced infertility in Wistar rats.

Materials and Methods

Plant Collection and Extraction

Fresh garlic bulbs were obtained from Uselu market, Benin City, Edo State, Nigeria. It was authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Benin-City, Nigeria. The bulbs were screened of bad ones, washed and air-dried for 48hrs and thereafter, pulverized into smooth powder using the British Grinding Machine. The pulverized sample was weighed and suspended in 1L of distilled water with regular agitation for 24hrs. The solution obtained was filtered and the resulting filtrate was concentrated over water bath at 40°C and yielded crude extract. The dried crude extract was stored in the refrigerator prior to use. All extraction and preparations of the aqueous extract of *Allium sativum* was performed at the Department of Pharmacology, University of Benin, Benin-City.

Sample Size Determination

The sample size for this study was determined using the sample size calculation in Animal studies (Resource Equation Approach) by Arifin and Zahiruddin,(2017). For a study that compares pretreatment with treatment groups, the sample size per group (for three groups

beside the control): $\text{Min } n=10/3+1=4.3$ (round up to 5 animals per group. $\text{Max } n=20/3+1=7$ (round down to 7 animals per group). For the purpose of this study, five animals per group were used to keep the DF within the group range of 10 to 20.

Animal Care and Management.

Twenty (20) adult Wistar rats weighing approximately 220-300grams were used for the experiment. The animals were purchased and bred in well-ventilated conventional cages in the Animal Holdings of the Department of Anatomy, School of Basic Medical Sciences, University of Benin, Benin-city. The animals were acclimatized for two weeks before the commencement of treatment. During this period, they were fed with growers mash livestock feed with clean water and weighed. The animals were raised at room temperature with a reverse natural light/dark cycle in the animal house. The animals were housed and cared for in accordance with the guidelines of the Animal Research Ethics Committee.

Study Design

This is experimental study involving 20 Wistar rats, assigned into four groups using completely randomized design with five (5) rats in each group. The rats in group A were fed with feeds and water ad libitum without treatment. Group B was treated with 20mg/kg cadmium, group C was administered with 20mg/kg cadmium and 750mg/kg garlic extract combination while rats in group D were initially administered with 20mg/kg cadmium for 14days followed by treatment with 750mg/kg garlic extract for 14days. At the end of the experiment period, the animals were weighed and sacrificed under slight chloroform anaesthesia. After anaesthetizing the rat for about 2minutes, the rat was placed in a supine position in a dissection table and abdominal incision was made with sterilized surgical blade to expose the internal genital organs. The sperm were collected for semen analysis and acrosome status.

Garlic Extract Preparation

The extract was prepared on daily basis from the stock and administered orally using an orogastric tube. The rats were weighed at 24hour interval and also subjected to thorough observation for mortality and behavioural pattern during the 28days experimental period.

Semen Collection

Sperm cells were collected from the vas deferens of the sacrificed rats; the rats were sacrificed and the vas deferens located and ligated with a minimum of 36mm length, both extremities of the vas deferens was ligated, cut and placed in a sterile petri dish. To the petri dish, 6 μ l of normal saline already adjusted to $37\pm 2^{\circ}\text{C}$ was added. The vas deferens was teased to allow the sperm cell diffuse out of it. A drop of the semen from the petri dish was placed on a grease free clean slide and covered with a transparent cover slip and examined to ascertain adequate collection was made. The semen analysis was done according to World Health Organization (2010) criteria.

Determination of Acrosome Number

To discriminate between viable and dead spermatozoa, 4 mM calcein acetoxymethyl ester (CAM: Molecular Probes Inc., OR, USA) dissolved in dimethyl sulfoxide and 2 mM ethidium homodimer-1 (EthD-1: Molecular Probes Inc., OR, USA) dissolved in a mixture of dimethyl sulfoxide and distilled water were used in this study. CAM and EthD-1 staining solution were added to the diluted sperm suspension and incubated with α -chlorohydrin in a CO_2 incubator (5% CO_2 in air at 37°C). The final concentration of CAM and EthD-1 were 4 $\mu\text{mol/mL}$ and 2 $\mu\text{mol/mL}$, respectively.

Spermatozoa labeled with CAM and EthD-1 displaying green fluorescence on the mid-piece without red fluorescence on the head was identified as live sperm, and those spermatozoa displaying red fluorescence on the head were identified as dead sperm. This procedure helps to discriminate the viable spermatozoa from dead spermatozoa in the rat as previously described (Kato et al., 1999).

The procedure for evaluating the acrosomal status of rat spermatozoa in this study was adapted from that of Holden et al., (1990). To examine the acrosomal status, 5mg/mL fluorescein isothiocyanate-conjugated concanavalin A (FITC-ConA) lectin (Vector Laboratories Inc., CA, USA) dissolved in 0.08% sodium azide solution was used in this study. One, 3, and 5 h after the start of incubation with α -chlorohydrin and with CAM and EthD-1 staining solution, the sperm sample was washed with phosphate-buffered saline (PBS) 3 times

and labeled with 20 µg FITC-ConA/mL in PBS for 30 min at room temperature. After vortex mixing, a drop of 4 µL labeled sperm sample was placed on a slide glass and covered with a cover slip. In each sample, the acrosomal status of 100 spermatozoa was examined by fluorescence microscopy (Olympus BH2-RFL, Olympus Optical Co. Ltd., Tokyo, Japan) set on the B excitation unit excited at between 460 and 490 nm wavelength and the O530 emission filter passed over 530 nm wavelength. Spermatozoa labeled with FITC-ConA were classified as "acrosome lost sperm" with bright green fluorescence on the acrosomal region of the head, and "acrosome intact sperm" with no fluorescence in the acrosomal region.

Statistical Analysis

Data generated was analyzed using the statistical package for social sciences (SPSS), version 17. Comparison between groups was done using one way analysis of variance (ANOVA) and values obtained were presented as mean $\pm$ Standard error of mean (SEM) for test and control group. A value of $p < 0.05$ was considered as statistically significant.

Results

Table 1 shows the comparison of acrosome-intact spermatozoa among groups of animals treated with cadmium and aqueous extract of garlic. The acrosome-intact spermatozoa in animals in group B 62.67 ± 1.45 , C.I 56.42-68.92), group C (71.33 ± 2.91 , C.I 58.83-83.84) and group D (64.50 ± 2.50 , C.I 32.73-96.27) were significantly lower ($P < 0.025$) than the control group A (79.33 ± 3.48 , C.I 64.36-94.31).

Table 2 shows the comparison of depleted acrosome numbers of spermatozoa among groups of animals treated with cadmium and aqueous extract of garlic. The depleted acrosome numbers were significantly higher ($P < 0.025$) in animals in group B (37.33 ± 1.45 , C.I 31.08-43.58), group C (28.67 ± 2.91 , C.I 16.16-41.17) and D (35.50 ± 2.50 , C.I 3.73-67.27) when compared with the control group A (20.67 ± 3.48 , C.I 5.69-35.64).

Table 3 shows that the total sperm count of rats in group B ($p < 0.001$) was significantly lower than groups A, C and D. The normal percentage morphology of rats in group B ($p < 0.001$) and D

($p < 0.05$) were significantly reduced when compared with control group A. The abnormal percentage morphology was increased in groups B, C and D ($p < 0.001$). The percentage progressive motility was significantly reduced among rats in groups C and D ($p < 0.001$). Conversely, percentage immobile sperm cells was decreased among rats in groups C and D ($p < 0.001$). Sperm cell liveability was increased among rats administered with cadmium and 750mg garlic extract combination (group C) and those treated with 750mg garlic extract (group D). The pH and sperm cell volume were not significantly different in the entire experimental group when compared with the control group ($P > 0.05$).

Discussion

Semen analysis is an important procedure during clinical investigation of infertile couples. The acrosomal status of spermatozoa is not routinely assessed during semen analysis. Only human spermatozoa that have intact acrosome have the ability to undergo acrosome reaction and are able to penetrate the zona pellucida (Liu and Baker, 2003; Vogiatzi et al., 2013). Evidence has shown that relationship exists between acrosome-intact spermatozoa and fertilization rates (Krausz et al., 1996; Liu and Baker, 1998). In cases of teratozoospermia and oligozoospermia, the semen analysis may be normal but with abnormal acrosome number (Soderlund and Lundin, 2001). Therefore, the assessment of the acrosomal status in cadmium induced infertility and the ameliorative effect of *Allium sativum* may present a modifiable target for public health intervention.

The animals exposed to cadmium chloride had significantly lower sperm characteristics than animals not exposed to cadmium. Cadmium is an environmental toxicant capable of inducing subfertility/infertility in males (Zhou et al., 2020). Cadmium has been reported to cause infertility via several mechanisms such as structural damage to the seminiferous tubules, Sertoli cells, and breakdown of blood-testis barrier, thus leading to the loss of sperm (Zhu et al., 2020). Cadmium may prevent Leydig cell function, sperm cell development and disrupts the vascular system of the testis. It is not very clear whether acrosome status is altered in cadmium toxicity and this has not been sufficiently

Table 1: comparison of intact acrosome numbers of spermatozoa among groups of animals treated with cadmium and aqueous extract of garlic.

Groups & Treatment	N	Acrosome number	95% Confidence interval
A (control)	5	79.3±3.48	64.36-94.31
B (20mg/kg cadmium only	5	62.7±1.45	56.42-68.92
C (20mg/kg cadmium + 750mg/kg garlic combination)	5	71.3±2.91	58.83-83.84
D (20mg/kg cadmium +750mg/kg garlic treatment)	5	64.5±2.50	32.73-96.27
F-value		4.026	
p-value		0.025	

Table 2: Comparison of depleted acrosome number of spermatozoa among groups of animals treated with cadmium and aqueous extract of garlic.

Groups	N	Acrosome number	95 % confidence interval
A (control)	5	20.7±3.48	5.69-35.64
B (20mg/kg cadmium	5	37.3±1.45	31.08-43.58
C (20mg/kg cadmium + 750mg/kg garlic combination)	5	28.7±2.91	16.16-41.17
D (20mg/kg cadmium + 750mg/kg garlic treatment)	5	35.5±2.50	3.73-67.27
F-value		4.026	
p-value		0.025	

Table 3: Comparison of sperm indices in Wistar Rats fed with different concentrations of cadmium and aqueous extracts of *Allium sativum* with controls. (n=5)

PARAMETERS	GROUP A (control)	GROUP B (20mg Cd only)	GROUP C (20mg Cd +750mg/kg garlic combination)	GROUP D (20mg/kg Cd+ 750mg/kg garlic treatment)
Total Sperm cell count ($\times 10^6$ cells/mm ³)	443.3 $\pm$ 23.33*	253.3 $\pm$ 31.80 ^a	416.7 $\pm$ 12.02*	414.7 $\pm$ 43.33*
Normal Morphology (%)	86.7 $\pm$ 3.33*	73.3 $\pm$ 3.33 ^a	80.0 $\pm$ 0.00*	76.7 $\pm$ 23.33 ^b
Abnormal Morphology (%)	13.3 $\pm$ 3.33*	26.7 $\pm$ 3.33 ^a	20.0 $\pm$ 0.00 ^a	23.3 $\pm$ 3.33 ^{ab}
Progressive Motility (%)	40.0 $\pm$ 15.28*	33.3 $\pm$ 13.33 ^b	63.3 $\pm$ 3.33 ^a	50.0 $\pm$ 5.77 ^{ab}
Non Progressive Motility (%)	20.0 $\pm$ 5.77*	23.3 $\pm$ 3.33*	16.7 $\pm$ 3.33 ^a	26.7 $\pm$ 3.33 ^a
Immotile sperm(%)	40.0 $\pm$ 20.00*	43.3 $\pm$ 12.02*	20.0 $\pm$ 0.00 ^a	23.3 $\pm$ 6.67 ^{ab}
Sperm Cell Liveability (%)	60.0 $\pm$ 20.00*	56.7 $\pm$ 12.02*	80.0 $\pm$ 0.00 ^a	76.7 $\pm$ 6.67 ^a
pH	7.4 $\pm$ 0.00*	7.4 $\pm$ 0.00*	7.4 $\pm$ 0.00*	7.4 $\pm$ 0.00*
Sperm cell volume	8.0 $\pm$ 0.00*	8.0 $\pm$ 0.00*	8.0 $\pm$ 0.00*	8.0 $\pm$ 0.00*

a=p<0.001; b=p<0.05 *p>0.05

investigated in cadmium toxicity. If it does, then the ability of *Allium sativum* to prevent or ameliorate the impact of cadmium needs to be evaluated since the use of herbal medicine is also increasingly popular.

In this study, the acrosome-intact spermatozoa were significantly reduced among group B rats administered with 20mg cadmium/kg (p<0.001), and group D treated with 750mg/kg *Allium sativum* after the induction of subfertility (p<0.05). The difference in number of the acrosome-intact spermatozoa among group C rats treated with 20mg cadmium/kg and 750mg *Allium sativum* simultaneously was not significant. This is an indication that *Allium*

sativum extract may have significantly prevented cadmium toxicity when administered simultaneously but after the toxic damage it was unable to sufficiently repair the damage done by cadmium. Some authors have implicated reduced acrosome-intact spermatozoa with male infertility (Cooper et al., 2010; Egeberg et al., 2013; Dorte et al., 2018). Cadmium is a toxic metal with a severe risk to human health and its serious toxicity via oxidative damage has been reported (Godt, et al., 2006). However, treatment of the animals with *Allium sativum*, which is rich in antioxidant that scavenges free radicals (that can damage cell membranes and DNA), demonstrated a significant restoration of the intact acrosome number when administered

simultaneously. The group C Wistar rats treated with cadmium and 750mg *Allium sativum* had a significant increase ($p < 0.05$) in the number of intact acrosome in comparison to group D administered with cadmium and then treated with aqueous extract of garlic. This finding is consistent with previous study (Ola-Mudathir, 2008). The author reported that garlic may confer a measure of protection against Cd-induced testicular oxidative damage and spermotoxicity by possibly reducing lipid peroxidation and increasing the antioxidant defense mechanism in rats (Ola-Mudathir et al., 2008). This statement was only true among Wistar rats in group C but not group D since garlic extract couldn't mitigate the damage done by cadmium administration.

A deleterious effect of cadmium on sperm membrane integrity has been reported. The presence of cadmium in the environment may exert adverse effect on sperm cell membrane (Arabi and Mohammadpour, 2006) including the acrosome status. Similarly, acrosome breakage with formation of various sized microvesicles and a round hole and numerous folds in acrosome membrane as a result of cadmium toxicity were also reported among male rabbits (Castelli et al., 2009).

The mechanism of cadmium toxicity is not completely understood, but some have reported oxidative stress due to the generation of free radicals. In male reproductive system, reactive oxygen species disrupts the integrity of sperm DNA and its biological membrane. Excess exposure could damage the sperm membrane, spermatogenesis dysfunction and spermotoxicity. Some authors have observed that, treatment with 400mg/kg of garlic caused increase in testicular weight, sperm count, percentage of motile sperm and spermatogenic density (Noh et al., 2020). Biomonitoring evaluations in the last 4 decades have shown increased levels of environmental contaminants thus exposing the population to toxicity. These toxic elements tend to bio-accumulate and induce multiple organ alterations. These exogenous compounds are usually present in the environment either in the air, water, soil or food. Sadly, environmental and occupational exposure of the population to toxicants has been implicated as one of the risk factors for the increased prevalence of male infertility and the

continuous decrease in human semen quality observed in the last decades (Levine et al. 2017, Mínguez-Alarcón et al. 2018). Some authors have reported increased risk of reproductive disorders arising from prenatal and postnatal persistent exposure to environmental chemicals (Bonde et al. 2016, Wang et al. 2016, Sifakis et al. 2017). Reproductive systems are especially sensitive to the deleterious effects of these toxic elements which may result to changes in gonadal function and other male reproductive accessories and negatively affect gamete functions (Sifakis et al., 2017). Recent study has shown that cadmium chloride toxicity may damage the sperm fertilization potential (Marchiani et al., 2019).

Alternatively, in a study of in-vitro exposure of human spermatozoa to cadmium, it was observed that the spermatozoa treated with CdCl_2 had an increase percentage of spontaneous acrosome reaction which was interpreted as acrosome stability (Marchiani et al., 2019). The increased acrosome reaction may suggest an alteration of the acrosomal structure due to the cadmium exposure. In-vitro exposure of rabbit spermatozoa to toxic metals, the formation of macrovesicles or large holes in the acrosome of sperm membrane were reported (Castellini et al. 2009). Also, a premature sperm acrosome reaction was reported in cadmium in-vivo exposure studies in mice (Oliveira et al. 2009, Wang et al. 2017).

Others have reported that the administration of garlic to rats caused decrease in membrane integration, sperm quality, functionality and sperm viability. Their data indicated that garlic had a deleterious effect on male reproductive system (Qian et al., 1986). Conversely, another study revealed that garlic administration to rats caused improvement in sexual dysfunction and sperm quality (Valente et al., 2014). The reason for the differences could be the lack of standardization and the different doses of garlic administered. In this study, garlic at 750mg/kg administered together with cadmium effectively ameliorated cadmium induced acrosome damage of spermatozoa.

Conclusion

In conclusion, the data from this study revealed that *Allium sativum* at 750mg/kg was able

mitigate cadmium toxicity on acrosome status of spermatozoa in Wistar rats when administered simultaneously. However, treatments with 750mg *Allium sativum* after the induction of subfertility with cadmium was unable improve the acrosome status of spermatozoa in Wistar rats. To ascertain the effects of this plant and its compounds, clinical studies with a larger sample size as well as an increase in the duration of its administration, comparison with safe drugs and the determination of the exact molecular mechanism are suggested.

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