

Research Article

Adverse Effects of In-Vitro Cadmium Exposure on Quality and Antioxidant Enzyme Status of Boar Spermatozoa

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Abstract

This study was aimed to evaluate the reproductive toxicity of cadmium chloride ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) in Boar spermatozoa in vitro. Boar spermatozoa obtained from the caudal epididymis of freshly slaughtered boars and dispersed in semen incubation medium (containing tris-hydroxymethyl-aminomethane, citric acid and fructose) were incubated at four different concentrations (0, 0.5, 1.0 and 2.0mM) for 3 hours at 37°C. Sperm viability, motility and percentage of abnormal spermatozoa were assessed by microscopy every one hour during the 3-hour incubation period, using aliquots from the incubated samples. Samples thus treated with cadmium chloride were centrifuged and the supernatant was used in the assessment of biochemical parameters of oxidative stress including hydrogen peroxide (H_2O_2), reduced glutathione (GSH) and Lipid peroxidation. The activities of antioxidant enzymes, catalase (CAT), superoxide dismutase (SOD), Glutathione peroxidase (GPX) as well as transaminases (ALT and AST) and alkaline phosphatase (ALP) were also assessed. The percentage of motile and viable spermatozoa decreased significantly ($p < 0.05$) after exposure of spermatozoa to CdCl_2 in a concentration- and time-dependent manner. Cadmium significantly increased ($p < 0.05$) the levels of H_2O_2 and malondialdehyde (MDA) in the spermatozoa with significant reductions ($p < 0.05$) in the activities of SOD, GPX, and CAT. Slight but insignificant increase in GSH concentration was accompanied with a slight increase in GST activity. ALT, AST and ALP activities were differentially modified. The results of this study revealed that cadmium chloride caused reductions in sperm motility and viability, induction of oxidative stress and impairment of antioxidant enzyme activities.

Keywords; Cadmium chloride, boar, spermatozoa, antioxidant, toxicity

INTRODUCTION

Infertility, a reduced or complete loss capacity to reproduce, can be caused by several environmental factors, including heavy metals (Hodgson, 2004). Earlier studies have suggested that at least half of the cases of male infertility of unknown etiology may be attributable to various environmental and occupational exposures to toxic metals (Steen and Pangkahila, 1984; Gagnon, 1988 Colban et al, 1993). Cadmium is a widespread industrial and environmental pollutant, arising primarily from battery, electroplating, pigment, plastic and fertilizer industries (Waisberg, 2003; Thompson and Bannigan, 2008). In several species, long-term exposure to cadmium produces organ damage or functional deficiency (Sharara et al, 1998). Reports indicate that both the male and female reproductive systems are susceptible to cadmium intoxication resulting in decreased fertility (Saygi et al, 1991; Lymperopoulos et al, 2000).

Cadmium naturally occurs in the earth crust combined with other elements including oxygen, sulphur, chloride and carbon (Ige et al, 2012). Heavy metal contamination of soil is believed to pose risks and hazards to humans and the

ecosystem through direct ingestion or contact with contaminated soil, the food chain (soil-plant-human or soil-plant-animal-human), drinking of contaminated ground water, reduction in food quality (safety and marketability) via phytotoxicity, etc. (McLaughlin et al, 2000; Ling et al, 2007). Soils may become contaminated by the accumulation of heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals, and atmospheric deposition (Khan et al, 2008; Zhang et al, 2010).

The main mechanism of cellular toxicity by cadmium appears to be the generation of reactive oxygen species. Several studies have demonstrated that cadmium stimulates free radical production, resulting in oxidative deterioration of lipids, proteins and DNA (Manca et al, 1991; Shen and Sangiah, 1995, Shaikh et al, 1999, Thevenod et al, 2000; Almazan et al, 2000). It is thought that cadmium itself is unable to generate free radicals directly, but indirect generation of various radicals involving the superoxide radical, hydroxyl radical and nitric oxide has been reported (Galan et al, 2001). Watanabe (2003) has confirmed the generation of the non-radical, Hydrogen peroxide, due to cadmium exposure which itself may be a significant source of radicals via Fenton Chemistry. Moreover, cadmium is believed to attach to sulfhydryl groups of enzymes involved

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in antioxidant mechanisms, such as superoxide dismutase, peroxidase and catalase, thus inhibiting their activities (Xiao et al, 2002; Bertin and Averbek, 2006). It is also thought that cadmium forms Cd-selenium complexes in the active centre of glutathione peroxidase, thus inhibiting the activity of this enzymic antioxidant (Gambhir and Nath, 1992). Lipid peroxidation is a very significant consequence of oxidative stress induced by generation of reactive oxygen species. The process has been associated with morphological abnormalities, decreased sperm motility and viability all of which impair the ability of spermatozoa to fertilize the ovum (Griveau and Le Lannou, 1997).

Previous studies have investigated the testicular and/or spermatozoa toxicity of cadmium in different species (Yang et al, 2003; Gil-Guzman et al, 2004; Yousef et al, 2007; Arabi and Mohammadpour, 2006; Leoni et al, 2002). This present work was aimed to determine the effects of cadmium chloride in vitro on boar spermatozoa using three concentrations of cadmium chloride.

MATERIALS AND METHODS

Chemicals: Cadmium chloride ($\text{CdCl}_2 \cdot 2.5 \text{ H}_2\text{O}$), epinephrine, glutathione, 5, 5'-dithio-bis-2-nitrobenzoic acid, hydrogen peroxide, thiobarbituric acid and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade and were obtained from the British Drug Houses (Poole, Dorset, UK).

Collection and incubation of spermatozoa: Semen samples used in this study were obtained from the epididymis of freshly slaughtered mature boars collected from the municipal abattoir in Ibadan, Nigeria. The caudal epididymis were excised and cut open with scalpel to release the semen which was extruded and dispersed into semen incubation medium containing tris-(hydroxymethyl)-aminomethane (37.85g/litre), citric acid anhydrous (21.15g/litre) and D (-) fructose (10 g/litre) (Roca et al., 2000). The chemicals were dissolved in 900 ml of distilled water. The pH was adjusted to 7.0 and then made up to 1 litre with distilled water. A 5000 μM stock solution of Cadmium chloride was prepared and mixed with the semen incubation medium at 500 μM (0.5mM), 1000 μM (1.0mM) and 2000 μM (2.0mM) concentrations. Sperm samples were then incubated with the different cadmium concentrations for 3 hours at 37°C.

Sperm motility assay: Sperm motility was assessed by the method described by Zemjanis (1970). The epididymal spermatozoa that were in rapid forward movement (progressive motility) were evaluated microscopically within 2–4 min of their isolation from the cauda epididymis and data were expressed as percentages.

Morphological abnormalities and percentage viability assay: Aliquots of spermatozoa placed on a slide glass was

smear out with another slide and stained with Wells and Awa's stain (0.2 g of eosin and 0.6 g of fast green dissolved in distilled water and ethanol in the ratio 2:1) for morphological examination and 1% eosin and 5% nigrosine in 3% sodium citrate dehydrate solution for live/dead ratio for according to the method described by Wells and Awa (1970).

Biochemical assays: The supernatant was collected for the estimation of catalase (CAT) activity using hydrogen peroxide as substrate according to the method of Clairborne (1995). Superoxide dismutase (SOD) was assayed by the method described by Misra & Fridovich (1972). Glutathione-S-transferase (GST) was assayed by the method of Habig et al. (1974). Protein concentration was determined by the method of Lowry et al. (1951). Reduced glutathione (GSH) was determined at 412 nm using the method described by Jollow et al. (1974). Hydrogen peroxide generation was assessed by the method of Wolff (1994). Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Farombi et al. (2000) and expressed as micromoles of MDA per gram tissue. Testicular activities of aspartate amino-transferase (AST) and alanine aminotransferase (ALT) according to Reitmann and Frankel (1957) and alkaline phosphatase (ALP) according to Rec Gsc (DGKC) (1972) were all determined using the Randox kit (Randox Laboratories Limited, Crumlin, UK).

RESULTS

Effects of CdCl_2 on spermatozoa quality characteristics in boar spermatozoa incubated in vitro with CdCl_2 .

Fig. 1 shows a significant decline ($p < 0.05$) in motility (%) of boar spermatozoa exposed to CdCl_2 at 0, 0.5, 1.0 and 2.0mM and for 0, 1, 2 and 3 hours. The decline in sperm motility was concentration and time-dependent. Spermatozoa were no longer observed to be motile at the end of three hours of incubation with the three different concentrations of CdCl_2 . The viability (%) of boar spermatozoa was not significantly affected in the first hour of incubation (Fig. 2).

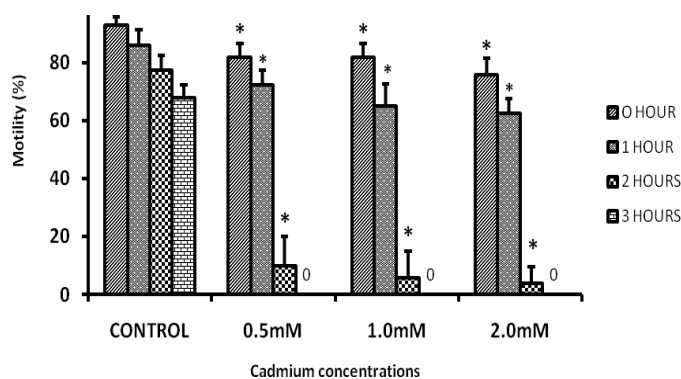


Fig. 1: Effects of different concentrations of CdCl_2 on Motility of boar spermatozoa. Each bar represents the mean \pm s.d. of three replicates. * indicates that values differ significantly from control ($p < 0.05$).

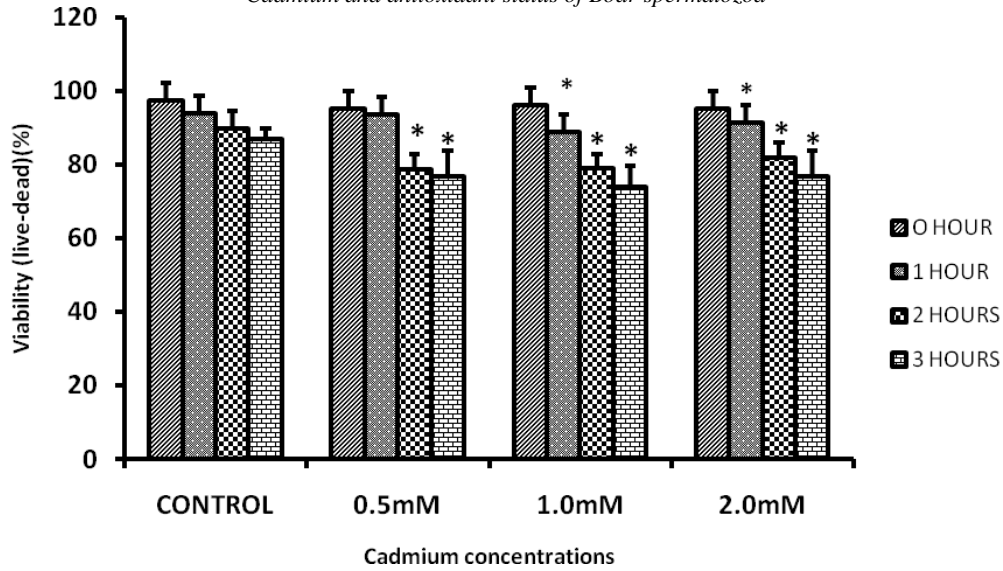


Fig. 2:

Effects of different concentrations of CdCl₂ on Viability of boar spermatozoa. Each bar represents the mean±s.d. of three replicates. * indicates that values differ significantly from control (p<0.05).

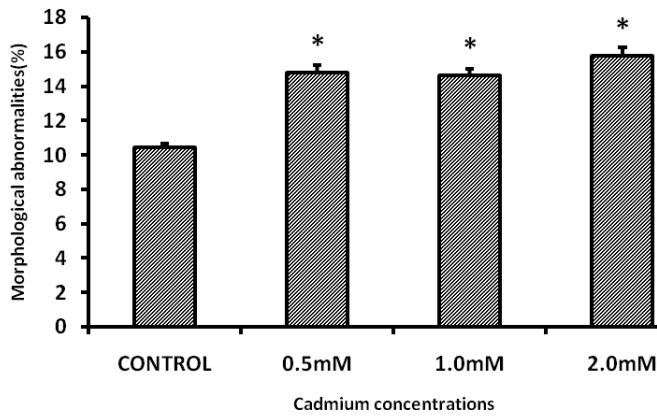


Fig. 3:

Effects of different concentrations of CdCl₂ on percentage of morphological abnormalities of boar spermatozoa. Each bar represents the mean±s.d. of three replicates. * indicates that values differ significantly from control (p<0.05).

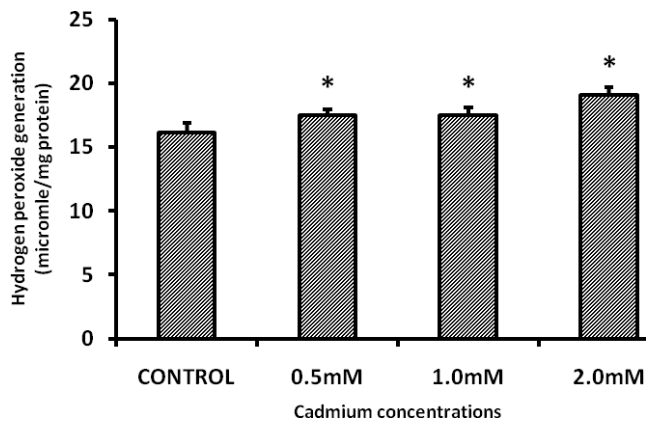


Fig. 4:

Effects of different concentrations of CdCl₂ on hydrogen peroxide generation in boar spermatozoa. Each bar represents the mean±s.d. of three replicates. * indicates that values differ significantly from control (p<0.05).

However, in the second and third hours, the viability of the spermatozoa was significantly reduced (p<0.05) when compared with the control. The percentage of morphological abnormalities in the incubated sperm samples assessed at the end of 3 hours of incubation revealed a concentration-dependent increase in morphological abnormalities of spermatozoa, when compared to control (Fig. 3).

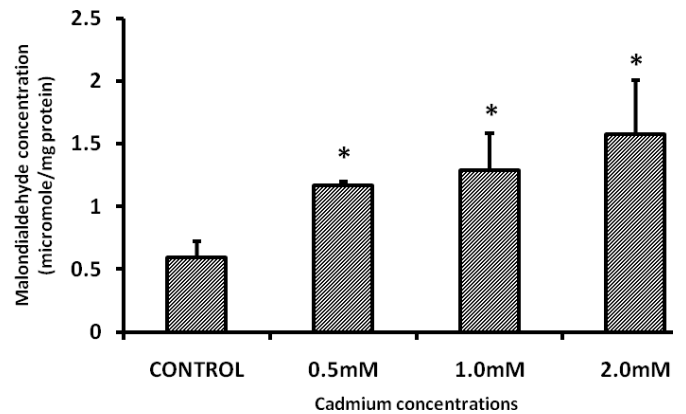


Fig. 5:

Effects of different concentrations of CdCl₂ on Malondialdehyde (MDA) concentration of boar spermatozoa. Each bar represents the mean±s.d. of three replicates. * indicates that values differ significantly from control (p<0.05).

Effects of CdCl₂ on antioxidant systems of boar spermatozoa incubated in vitro with CdCl₂

Results of the effects of CdCl₂ on antioxidant systems in the different samples are presented in Table 1. Levels of reduced glutathione in the spermatozoa were not significantly altered with any of the concentrations of CdCl₂ tested when compared with the control. Glutathione S-transferase activity also was not significantly affected when compared to the control values.

Table 1:

Effects of Cadmium chloride on antioxidant systems of boar spermatozoa incubated with cadmium chloride in vitro

	CONTROL	0.5mM	1.0mM	2.0mM
GSH	12.85±1.68	11.25±0.91	12.78±1.15	12.85±0.24
GPX	13.15±2.47	13.65±0.83	8.98±1.48*	7.76±0.59*
GST	1.08±0.29	1.07±0.07	1.60±0.42	1.60±0.04*
SOD	0.17±0.03	0.12±0.01*	0.08±0.05*	0.01±0.03*
CAT	70.43±5.31	67.67±0.50	57.08±3.12*	52.26±1.51*

Data are expressed as mean±standard deviation. * indicates values that differ significantly from control (p<0.05). GSH: Reduced glutathione; GPX: Glutathione peroxidase; GST: Glutathione S-transferase; SOD: Superoxide dismutase; GSH (micromole per g tissue) CAT: catalase; GPX activity (units per mg protein); GST activity (micromole CDNB-GSH complex formed per minute per mg protein); SOD (units per mg protein), CAT (micromole H₂O₂ consumed per minute per mg protein).

Table 2:

Effects of Cadmium chloride on transaminases and alkaline phosphatase in boar spermatozoa incubated with cadmium chloride in vitro.

	CONTROL	0.5mM	1.0mM	2.0mM
ALT	48.87±0.74	54.29±0.82*	53.63±2.60*	55.30±0.91*
AST	15.99±0.30	13.35±0.36*	13.13±0.53*	12.64±0.96*
ALP	700.43±5.31	239.81±38.42*	141.07±10.13*	85.26±1.51*

Data are expressed as mean±standard deviation in U/L. * indicates values that differ significantly from control (p<0.05). ALT: alanine transaminase; AST; aspartate transaminase; ALP: alkaline phosphatase.

The activities of Superoxide dismutase, Glutathione peroxidase and Catalase in the spermatozoa were significantly (p<0.05) reduced in the CdCl₂-incubated samples compared to the control. This reduction in the activities of these antioxidant enzymes was concentration-dependent. Incubation of boar spermatozoa with CdCl₂ caused a significant (p<0.05) increase in the generation of Hydrogen peroxide (Fig. 4) and the concentration of malondialdehyde (Fig. 5) in a concentration-dependent manner.

Effects of CdCl₂ on activities of aminotransferases and alkaline phosphatase in boar spermatozoa incubated in vitro with CdCl₂

The activities of alanine transaminase was increased significantly (p<0.05) in the sperm samples exposed to CdCl₂ in a concentration-dependent manner, when compared to control (Table 2). However, the activities of aspartate transaminase and alkaline phosphatase were significantly (p<0.05) reduced in a concentration-dependent manner (Table 2).

DISCUSSION

Cadmium is known to exert adverse effects on reproductive structures and functions directly at the testicular level or by altering post-testicular events such as sperm progress motility and/or viability, all of which may result in infertility (Akinloye et al, 2006). In this study, exposure of rats to Cadmium chloride resulted in decreased sperm motility and viability with increased sperm abnormalities. In vitro studies performed by Leoni et al (2002) showed that the viability of ram spermatozoa was significantly affected by exposure to 2

and 20µM cadmium. The decrease in sperm motility may be due to the effects of cadmium on microtubules. Previous studies suggest that cadmium inhibits microtubule sliding in bovine sperm (Kanous, 1993). Microtubule sliding is the basis of flagella motion that propels the spermatozoa in progressive linear motion. Moreover, other studies also showed that cadmium inhibits microtubule assembly in pig (Brunner et al, 1991) and bull brain (Wallin and Hartley-Asp, 1993).

In addition, spermatozoa being uniquely rich in polyunsaturated fatty acids (Vernet et al, 2004) are very susceptible to oxidative insult. Generally, the generation of reactive oxygen species and consequent oxidative damage to macromolecules as a mechanism of Cadmium toxicity has been established in many investigations (Shaikh et al, 1999; Stohs et al, 2000). Oxidative stress is known to play a crucial role in the aetiology of defective sperm function via mechanisms involving the induction of peroxidative damage to the plasma membrane (Sharma and Agarwal, 1996; Agarwal and Saleh, 2002; Vernet et al, 2004). Increased sperm lipid peroxidation has been shown to impede sperm progressive motility and increase percent total sperm abnormalities as well as cause a dramatic loss in the fertilizing potential of sperm (Sharma and Agarwal, 1996; El-Demerdash et al, 2004).

In this study, the concentration of malondialdehyde as an index of lipid peroxidation was significantly increased, implying the generation of reactive oxygen species. This effect was substantiated by the increased levels of Hydrogen peroxide observed. According to Waisberg et al (2003), cadmium induces oxidative stress due to reactive oxygen species (ROS) accumulation, mostly superoxide anion radical (O₂[•]), hydrogen peroxide (H₂O₂) and hydroxyl radical

(•OH). The extent to which oxidative damage affects spermatozoa highly depends on their antioxidant defense status (Sikka, 2001).

The antioxidant enzyme system of spermatozoa is a very important factor for normal sperm function as they play different roles in detoxifying reactive oxygen species. Impairment of the antioxidant enzymatic activities could promote oxidative damage to plasma membrane lipids, structural proteins and enzymes as well as oxidation of bases in DNA (O'Flaherty, 2014). SOD prevents the formation of hydroxyl radical that occurs when superoxide radicals and hydrogen peroxide reacts in the Haber-Weiss reaction. Hydroxyl radicals particularly promote lipid peroxidation in spermatozoa membranes. The spermatozoa is known to contain an important isoform of GPX, called GPX4, a structural protein which is important for normal formation of the myelin sheath, making up about 50% of the sperm mid-piece and protein content. Male mice, for example, lacking the mitochondrial GPX4 are infertile possessing abnormal and less motile spermatozoa (Imai et al, 2009; Schneider et al, 2009). CAT detoxifies both intracellular and extracellular hydrogen peroxide to water and oxygen and it also causes activation of nitric-oxide-induced capacitation which is essential for spermatozoa function (Lamirande et al, 1997). The activities of antioxidant enzymes in spermatozoa exposed to cadmium in this study showed significant decreases in SOD, CAT and GPx, while GSH levels and GST activities were not significantly altered. These results point to an induction of oxidative stress with exposure to cadmium and further supports the evidence provided by increases in H₂O₂ and MDA.

Cadmium is known to bind to cysteine in GSH resulting in the inactivation of GPX which therefore, fails to metabolize hydrogen peroxide to water. Evidence from other investigations also indicated that cadmium interacts with Selenium and disrupts GPx activity (Ursini, 1995). GSH functions as a free radical scavenger, a co-substrate for GPx activity and a co-factor for many enzymes (Sies, 1999). It is known that the level of GSH decreases in tissues exposed to cadmium, and a complex is formed between cadmium and GSH via a reaction catalyzed by GST (Singhal et al, 1987). A deficiency of GSH can lead to defective motility by alterations in the mid-piece (Lenzi et al, 1994)

Transaminases are located primarily in the mid-piece of spermatozoa. AST and ALT are essential for metabolic processes which provide energy for survival, motility and fertility of spermatozoa and their activities are good indicators of semen quality as they provide a measure of sperm membrane stability (Perumal et al, 2013). Seminal ALP activity is observed on sperm head, mid-piece, and tail fragments, and it is known to regulate phosphorylation of proteins by the cAMP-dependent protein kinase necessary for spermatozoal motility (Juyena and Stelletta, 2012).

Further spermiotoxic effects of cadmium obtained in this study included the reductions in the activities of AST and ALP. This finding is similar to the reports of El-Demerdash et al (2004) who observed decreased ALP levels in the testes. It was believed that the decrease in ALP activity may be due to Cd-induced disturbances in the balance between synthesis

and degradation of the enzyme (Rana et al., 1996). Moreover, Cd is thought to inhibit ALP by competing with and displacing zinc, which is an essential part for the enzyme activity (Suzuki et al., 1989). The activity of ALT evaluated in boar sperm showed a concentration-dependent increase with exposure to cadmium.

In conclusion, the results of this study suggest that cadmium stimulated oxidative damage in boar spermatozoa as indicated by increased lipid peroxidation, hydrogen peroxide generation and reduction in the activities of anti-oxidative enzymes, CAT, SOD and GPX. The accompanying reductions in sperm motility, viability and increased morphological abnormalities in the spermatozoa all indicate adverse effects promoted by cadmium. The metal therefore, may contribute to reproductive toxicity in the boar.

REFERENCES

- Almazan, G., H.N. Liu, A. Khorchid, S. Sundarajan, A.K. Martinez and S. Bermudez. 2000. Exposure of developing oligodendrocytes to cadmium causes HSP72 induction, free radical generation reduction in glutathione levels and cell death. *Free radic. Biol. Med.* 29: 858-869
- Bertin G., and D. Averbeck. 2006. Cadmium cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). *Biochimie.* 88: 1549-1559.
- Colborn, T., F.S. Vom Saal, and A.M. Soto. 1993. Developmental effects of endocrine disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* 101: 378-84
- El-Demerdash, F.M., M.I. Yousef, F.S. Kedwany, H.H. Bahadadi. 2004. Cadmium-induced changes in lipid peroxidation, blood hematology, biochemical parameters and semen quality of male rats: protective role of vitamin E and b-carotene. *Food Chem. Toxicol.* 42, 1563-1571
- Gagnon, C. 1988. The role of environmental toxins in unexplained male infertility. *Sem. Reprod. Endocrinol.* 6: 369-376.
- Galan A., L. Garcia-Bermejo, A. Troyano, N.E. Vilaboa, C. Fernandez, E. de Blas, and P. Aller. 2001. *Europ.J. Cell. Biol.* 80: 312-320
- Gambhir J. and R. Nath. 1992. Effect of cadmium on tissue glutathione and glutathione peroxidase in rats: influence of selenium supplementation. *Indian J. Exp. Biol.* 30: 597-601.
- Gil-Guzman E., M. Ollero, M.C. Lopez, R.K. Sharma, J.G. Alvarez and A.J. Jr.2001. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum. Reprod.* 16: 1922-1930.
- Hodgson, E. 2004. *A Textbook of Modern Toxicology.* Third Edition, John Wiley and Sons, Inc.
- Ige, S.F., S.B. Olaleye, R.E. Akhigbe, O.A. Oyekunle, and U.S. Udoh. 2012. Testicular toxicity and sperm quality following cadmium exposure in rats: ameliorative potentials of *Allium cepa*. *J. Hum. Rep. Sci.* 5(1):37-42.

- Imai, H., Hakkaku, R., Iwamoto, R. et al. Depletion of selenoprotein GPX4 in spermatocytes causes male infertility in mice. *Journal of Biological Chemistry*. 284(47): 32522-32532.
- Juyena, NS., and Stelletta, C. 2012. Seminal Plasma: An Essential Attribute to Spermatozoa *Journal of Andrology*, 33 (4): 536-552.
- Khan, S., Q. Cao, Y. M. Zheng, Y. Z. Huang, and Y. G. Zhu. 2008. Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. *Environmental Pollution*, vol. 152, no. 3, pp. 686–692.
- Lamirande, E., Leclerc, P., Gagnon C. 1997. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* 3(3): 175-194.
- Lenzi, A., Picardo, M., Gandini, L. et al. 1994. Glutathione treatment of dyspermia: effect on the lipid peroxidation process. *Hum Repro.* 9(11): 2044-2050
- Leoni, G., L. Bogliolo, G. Deiana, F. Berlinguer, I. Rosati, P.P. Pintus, et al. 2002. Influence of cadmium exposure on in vitro ovine gamete dysfunction. *Reprod Toxicol.* 16:371–7.
- Ling, W., Q. Shen, Y. Gao, X. Gu, and Z. Yang. 2007. Use of bentonite to control the release of copper from contaminated soils. *Australian Journal of Soil Research*, vol. 45, no. 8, pp. 618–623.
- Lymberopoulous A.G., A.V. Kotsaki-Kovatsi, A. Taylor, N. Papaioannou, and P. Brikas. 2000. Effects of cadmium chloride administration on the macroscopic and microscopic characteristics of ejaculates from chios ram-lambs. *Theriogen.* 54: 1145-57.
- Manca, D., A. Richard, B. Trottier, and G. Chevallier. 1991. Studies on lipid peroxidation in rat tissues following administration of cadmium chloride. *Toxicology* 67: 303-323
- McLaughlin, M.J., B.A. Zarcinas, D.P. Stevens, and N. Cook. 2000. Soil testing for heavy metals. *Communications in Soil Science and Plant Analysis*, vol. 31, no. 11–14, pp. 1661–1700.
- Mostafa, T., G. Tawadrous, P. Edward, M.M. Roaia, M.K. Amer, R.A. Kader, and A. Aziz. 2006. Effect of smoking on seminal plasma ascorbic acid in infertile and fertile males. *Andrologia* 38: 221-224.
- O’Flaherty, C. 2014. The enzymatic antioxidant system of Human spermatozoa. *Advances in Andrology*. Epub ahead of print <http://dx.doi.org/10.1155/2014/626374>
- Perumal, P., Khate, K., Rajkhowa, C. 2013. Effect of foot and mouth disease vaccination on seminal and biochemical profiles of mithun (*Bos frontalis*) semen. *Asian Pacific journal of Reproduction.* 2(3): 178-184
- Rana, S.V., S. Rekha, and V. Seema, 1996. Protective effects of few antioxidants on liver function in rats treated with cadmium and mercury. *Indian J. Exp. Biol.* 34, 177– 179.
- Roca, J., S. Martinez, J.M. V’azquez, X. Lucas, I. Parrilla, and E.A. Martinez. 2000. Viability and fertility of rabbit spermatozoa diluted in Tris-buffer extenders and stored at 15°C. *Anim. Reprod. Sci.* 64: 103-112.
- Saygi, S., G. Deniz, O.Kutsal, and N.Vural. 1991. Chronic effects of cadmium on kidney, liver, testis and fertility of male rats. *Biol. Trac. Elem. Res.* 31: 209-14.
- Schneider, M., Forster, H., Boersma, A, et al. 2009. “Mitochondrial glutathione peroxidase 4 disruption causes male infertility” *The FASEB journal.* 23(9): 3233-3242
- Shaikh, Z.A., T.T. Vu and K. Zaman (1999) Oxidative stress as a mechanism of chronic cadmium-induced hepatotoxicity and renal toxicity and protection by antioxidants. *Toxicology and Applied Pharmacology* 154: 256-263
- Sharara F.I., D.B. Seifer, and J.A. Flaw. 1998. Environmental toxicants and female reproduction. *Fert. Ster.* 70: 613-22
- Shen Y., and S. Sangiah. 1995. Na⁺-K⁺ ATPase, glutathione and hydroxyl free radicals in cadmium chloride-induced testicular toxicity in mice. *Archives of environmental contamination and Toxicology* 27: 174-179
- Steen, O.A. and A. Pangkahila. 1984. Occupational influences on male fertility and sexuality. *Andrologia*, 16: 5-22
- Suzuki, Y., I. Morita, Y. Yamane, and S. Murota, 1989. Preventive effects of zinc on cadmium-induced inhibition of alkaline phosphatase activity in osteoblast-like cells, MC3T3-E1. *J. Pharmacobiodyn.* 12, 94–99
- Thevenod F., J.M. Friedmann, A.D. Katsen, I.A. Hauser. 2000. Upregulation of multidrug resistance p-glycoprotein via nuclear factor-kappa B activation protects kidney proximal tubule cells from cadmium and reactive oxygen species-induced apoptosis. *J. Biol. Chem.* 275: 1887-1896.
- Thompson, J., and J. Bannigan. 2008. Cadmium: Toxic effects on the reproductive system and the embryo. *Reprod. Toxicol.* 25: 304-15.
- Waisberg, M., P. Joseph, B. Hale, and D. Beyersmann. 2003. Molecular and Cellular mechanisms of cadmium carcinogenesis: a review. *Toxicology.* 192: 95-117
- Watanabe, M., K. Henmi, K. Ogawa and T. Suzuki. 2003. *Compar. Biochem. Physiol. C. Toxicol. Pharmacol.* 134: 227-234.
- Xiao, L., D.R. Pimentel, J. Wang, K. Singh, W.S. Colucci, D.B. Sawyer. 2002. Role of reactive oxygen species and NAD (P) H oxidase in alpha (1)-adrenoceptor signaling in adult rat cardiac myocytes. *Am. J. Physiol. Cell Physiol.* 282: 926-834.
- Yang J.M., M. Arnush, Q.Y. Chen, X.D. Wu, B. Pang, and X.Z. Jiang. 2003. *Reprod. Toxicol.* 17: 553-560
- Yousef, M.I., K. I. Kamel, M.I. El-Guendi, and F.M. El-Demerdash. 2007. An in vitro study on reproductive toxicity of aluminium chloride on rabbit sperm: the protective role of some antioxidants. *Toxicology* 239: 213-223.
- Zhang, M. K., Z.Y. Liu, and H. Wang. 2010. Use of single extraction methods to predict bioavailability of heavy metals in polluted soils to rice. *Communications in Soil Science and Plant Analysis*, vol. 41, no. 7, pp. 820–831.