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Effect of heavy metals on oxidative markers and semen quality parameters in HF crossbred bulls

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ABSTRACT

The study was conducted to assess the effect of toxic heavy metals on seminal antioxidants, trace minerals and semen quality parameters in crossbred breeding bulls. Semen samples were collected from breeding bulls (82) maintained at bull rearing unit of the institute. Toxic heavy metals (Pb and Cd), trace minerals (Zn, Cu, Co and Fe) and oxidative stress markers (SOD, catalase and MDA) were determined in semen samples of breeding bulls. Sperm motility and concentration were measured in fresh ejaculates. Significant levels of Pb (0.23±0.006 µg/ml) and Cd (0.11±0.005 µg/ml) were detected in semen samples of breeding bulls. On analysis of the data between good and poor bull categories, significantly higher concentration of Pb and Cd was present in semen samples of poor bulls as compared to good bulls. There was significantly higher malondialdehyde (MDA) and low superoxide dismutase (SOD) and catalase in seminal plasma of poor bulls compared to good bulls. A significant decrease in Zn and Cu was observed in poor bulls as compared to good bulls. Cobalt and iron values did not show any significant variation between good and poor quality bulls. On correlation analysis, lead and cadmium showed significant negative correlation with Zn, Cu, SOD, catalase, motility and sperm concentration while significant positive correlation was seen with MDA respectively. The study concludes that increased Pb and Cd in bull semen may increase the risk of seminal oxidative stress development and a subsequent reduction in male fertility.

Key words: Bulls, Heavy metals, Oxidative stress, Semen

The modern agricultural practices and unrestricted developmental activities carried out during the past few decades have lead to serious problems of environmental pollution with heavy metals (Rajaganapathy *et al.* 2011). Metals contaminate the environment through natural weathering of the earth's crust, industrial effluents, pest or disease control agents applied to plants and animals, urban domestic sewage, mining, soil erosion, air pollution fallout etc. Exposure to heavy metals occurs through ingestion of contaminated feed and water, air and dirt that may have negative impact on reproductive potential of semen in animals and humans (Tvrda *et al.* 2013a, Zhao *et al.* 2017). Lead (Pb) and cadmium (Cd) are two most abundant toxic metals present in the environment. Significant levels of lead (0.06–0.57 µg/ml) and cadmium (0.09–0.11 µg/ml) have been recorded in bovine seminal plasma (Massanyi *et al*. 2003, Tvrda *et al.* 2012).

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As the male infertility is on the rise worldwide (Kumar and Singh 2015), reproductive hazards from metal exposure in males are one of the fastest growing areas of concern in toxicology. Pb and Cd have been reported to accumulate in male reproductive organs and induce reprotoxicity in animals as well as in humans (Arabi and Mohammadpour 2006, Jeng *et al.* 2015). Both metals have been shown to be associated with an overproduction of reactive oxygen species (ROS) and a subsequent impairment of antioxidant defense systems. The unique cellular structure of spermatozoa and low concentrations of antioxidants makes them sensitive to oxidative stress (Tvrda *et al.* 2013a). The objective of the study was to determine the status of environmentally relevant toxic metals (lead and cadmium) in seminal plasma and their effect on antioxidant markers and semen quality parameters in crossbred breeding bulls.

MATERIALS AND METHODS

Sample collection and processing: Semen samples were collected from adult crossbred (Holstein Friesian × Sahiwal) breeding bulls (82) maintained at bull rearing unit of the institute. The semen samples from each bull were collected twice in a week using an artificial vagina. The bulls were kept under a loose housing system in individual pens and maintained on standard feeding schedule and management conditions. Neat semen (2 ml) was collected for heavy metal and trace mineral analysis. Seminal plasma was collected for measurement of oxidative stress parameters. The semen samples were centrifuged (10 min, 8000×g, 4°C) and seminal plasma was separated and transferred into 1.5 ml tubes and kept frozen (–80°C) until analysis.

Semen quality analysis: The fresh ejaculates (24 from each bull) were subjected to evaluation for volume, sperm concentration and initial motility as per Chenoweth and Mcpherson (2016). The concentration of spermatozoa was measured with Accucell bovine photometer (IMV, France). To appreciate the sperm motility, 20 µl diluted semen was placed on a microscope slide under a coverslip and directly observed using an Olympus BX40 phase contrast microscope (Olympus, Tokyo, Japan). The mobile and immobile spermatozoa were counted randomly in five fields, and the percentage of the mobile spermatozoa was calculated.

Heavy metals analysis: Semen samples (2 ml) were digested with nitric and perchloric acid mixture (Kolmer *et al.* 1951). The resulting solution was diluted to 10 ml with demineralized water. The toxic heavy metals (lead and cadmium) and trace elements (zinc, copper, cobalt and iron) concentrations in the digested samples were estimated using atomic absorption spectrophotometer (GBC Scientific) at the wavelength of 217.0, 229.5, 213.9, 324.7, 240.7, 248.3 with 6, 6, 7, 6, 7, 5 mA current, respectively. The standards procured (Sisco Research Laboratory, Mumbai, India) for each element were used to calibrate the equipment. The values were expressed in µg/ml of semen.

Measurement of antioxidant markers: All the parameters were measured based on a colorimetric reaction of the target substance and a subsequent UV/VIS spectrophotometric detection at a specific wavelength. The levels of lipid peroxidation (LPO) were determined by measuring the levels of malondialdehyde (MDA) which is an end product of lipid peroxidation. MDA was estimated using thiobarbituric acid (TBA) assay (Sanocka and Kurpisz 2004). Superoxide dismutase (SOD) was estimated as per Marklund (1976). Catalase activity was determined as per Bergmeyer (1983).

Statistical analysis: The data were analyzed by applying independent t-test to find out the statistical difference between the mean values of different parameters of good and poor bulls using SPSS 16 for Windows software (SPSS Incorporated Chicago, IL, USA). The bulls were categorized into two groups according to their initial sperm motility (Good bulls: >60% motile, Poor bulls: <60% motile). A parametric (Pearson) correlation between toxic heavy metals, trace elements, antioxidant markers and semen quality parameters was analyzed using standard statistical methods (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

Significant levels of Pb (0.23±0.006 µg/ml) and Cd (0.11±0.005 µg/ml) were detected in semen samples of crossbred breeding bulls. Analysis of the data between good and poor bull categories revealed, significantly ($P<0.05$) higher concentration of Pb and Cd in semen samples of poor bulls as compared to good bulls. There were significantly ($P<0.05$) higher MDA and low SOD and catalase in seminal plasma of poor bulls compared to good bulls ($P<0.01$) (Fig. 1). The correlation analysis revealed a strong positive correlation between the lead and cadmium concentrations ($r=0.805$; $P<0.01$). Both metals showed significant negative correlation with Zn, Cu, SOD, catalase, motility and sperm concentration while significant positive correlation was observed with MDA (Table 1). Animals are often exposed to toxic heavy metals which are dispersed into the environment from some sources like distilleries, fertilizer, automobile emissions, oil refineries, petro-chemicals, pesticides, tanneries, paint industries, discarded batteries (Rajaganapathy *et al.* 2011). Toxic metals are accumulated in the fodder grown in a contaminated environment and reach to the animal body through ingestion of contaminated fodder and water. The higher blood lead

Table 1. Correlation between semen heavy metals, trace elements, antioxidant markers and quality parameters in crossbred breeding bulls (n=82)

	Pb	Cd	Zn	Cu	Co	Fe	MDA	SOD	Catalase	Motility	Sperm concentration
Pb	1										
Cd	.805**	1									
Zn	–.578**	–.481**	1								
Cu	–.610**	–.542**	.419**	1							
Co	.196	.194	.029	.058	1						
Fe	–.095	–.056	.139	.003	.055	1					
MDA	.726**	.640**	–.510**	–.480**	.193	–.052	1				
SOD	–.300**	–.395**	.347**	.359**	.146	–.136	–.477**	1			
Catalase	–.248*	–.326**	.266*	.300**	.181	–.079	–.414**	.816**	1		
Motility	–.218*	–.247*	.279*	.273*	.190	.077	–.307**	.443**	.430**	1	
Sperm concentration	–.313**	–.300**	.294**	.282*	.056	–.110	–.266*	.279*	.333**	.331**	1

*P<0.05; **P<0.01; Pb, lead (µg/ml); Cd, cadmium (µg/ml); Zn, zinc (µg/ml); Cu, copper (µg/ ml); Co, cobalt (µg/ ml); Fe, iron (µg/ml); SOD, superoxide dismutase (U/mg protein); catalase (U/mg protein); MDA, malondialdehyde (µM); motility (%), sperm concentration (million/ml).

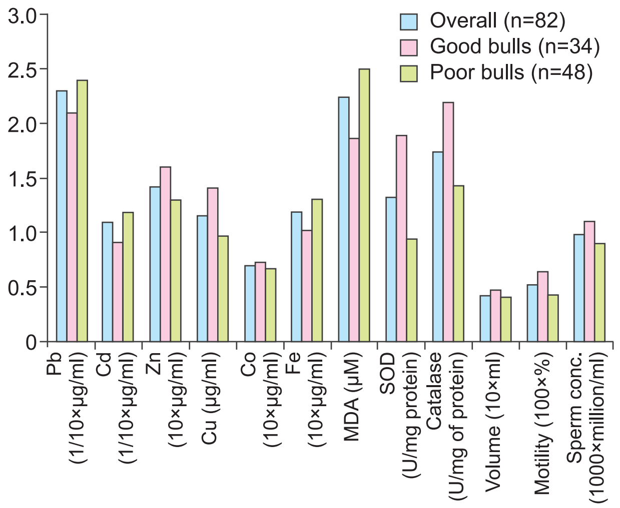

Fig. 1. Semen heavy metals, trace elements, antioxidant markers and quality parameters of crossbred breeding bulls. Quality groups are based on the initial sperm motility values: Good bulls, >60% motile, Poor bulls, <60% motile.

and cadmium level in animals around different industrial areas in India have been reported (Rajaganapathy *et al.* 2011).

Lead and cadmium are common and persistent environmental contaminants, which have been reported to cause reproductive toxicity in males through disturbances in the pro-oxidant-antioxidant balance in the body, with subsequent development of oxidative stress (Tvrda *et al.* 2013a). Both metals tend to accumulate in the testes, epididymis, vas deferens, seminal vesicles and ejaculate (Roy Chowdhury 2009). The coexposure to Pb and Cd usually has synergistic cytotoxicity, which supports the strong positive correlation (r=0.805; P<0.01, Table 1) between the two metals detected in our study. A high positive correlation between seminal lead and cadmium in ram (r=0.976) and boar (r=0.973) has been detected earlier by Massanyi *et al*. (2003). Pb and Cd exposure causes irreversible toxic insults to male reproductive system in animals and humans, that has been documented by various authors (Roy Chowdhury 2009, Jeng *et al*. 2015, Zhao *et al*. 2017) and same was confirmed by results of our study, since the Pb and Cd contents of the semen samples were negatively associated with sperm motility (r=–0.247, P<0.05; r=–0.218, P<0.05) and concentration (r=–0.300, P<0.05; r=–0.313, P<0.01) respectively (Table 1).

Our study also demonstrated that lower Pb and Cd concentrations were negatively correlated with higher motility and sperm concentration in the good quality bulls (Fig. 1). Pb intoxication can delay spermiation, and release of immature spermatogenic cells in the tubules of the testis (Corpus *et al.* 2002). Massanyi *et al.* (2009) demonstrated that excessive Cd intake could decrease spermatozoa production and motility with subsequent reproductive alterations. Furthermore Arabi and Mohammadpour (2006) documented that Cd intoxication induces membrane impairments, low spermatozoa motility and decreased rate of acrosome reactions. *In vitro* incubation of human or mouse sperms with cadmium for a long time (up to 24 hours) significantly decreases sperm motility in a concentration and time dependent manner (Zhao *et al*. 2017).

Significantly (P<0.05) low SOD and Catalase activities were recorded in seminal plasma of poor bulls when compared with good bulls (*P*<0.01; Fig. 1). Oxidative stress is an imbalance of oxidants and antioxidants in favor of the oxidants leading to cell damage (Tvrda *et al.* 2011). Antioxidants present in the seminal plasma protect spermatozoa against reactive oxygen species and it has been documented that low seminal antioxidant capacity is related to male infertility (El-Tohamy *et al*. 2010). Pb and Cd greatly influence the proper function of antioxidants. SOD as a typical metal-enzyme has a prosthetic group that may be replaced by these heavy metals leading to an inhibition of enzyme activity (El-Tohamy *et al*. 2010). Pb and Cd can replace zinc in its sites, suggesting interactions of these metals with the Cu/Zn SOD isoenzymes (Martin *et al.* 2017). Such interactions support our significantly negative correlations between the Pb and Cd content and SOD activity (r=–0.395, P<0.01; r=–0.300, P<0.01, Table 1) respectively, as well as the decreased activity of SOD with an increased concentration of Pb and Cd in the poor quality bulls (Fig. 1). Catalase plays important role in seminal protection against H_2O_2, as demonstrated by its significant positive correlation with sperm motility(r=0.430, P<0.01; Table 1) and sperm concentration (r=0.333, P<0.01; Table 1) in our study. A significant difference of the catalase activity in the quality groups (*P* < 0.01; Fig. 1) observed in the present study is consistent with the results of Zelen *et al.* (2009) who documented that the catalase activity was significantly lower in infertile groups compared to the fertile controls. Furthermore, catalase is directly dependent on various essential trace elements, which render it a potential target for Pb and Cd toxicity. Both metals have a non-competitive inhibitory and deactivating effect on catalase either by antagonistically displacing metal ions from the active center of the enzyme or by inhibiting the heme synthesis pathway (Flora *et al.* 2008).

Significantly (P<0.05) higher MDA was recorded in seminal plasma of poor bulls when compared with good bulls (*P* < 0.01; Fig. 1). Both Pb and Cd have pro-oxidant catalytic activity with respect to lipid peroxidation (LPO). The plasma membranes of spermatozoa contain large quantities of polyunsaturated fatty acids, which are susceptible to the damage induced by excessive ROS production (Tvrda *et al*. 2011). Our significant negative correlations between MDA, sperm motility(r=–0.307, P<0.01) and antioxidant markers (Catalase: –0.414** P<0.01; SOD: –0.477** P<0.01) of seminal plasma along with the increase of MDA in the poor quality bulls demonstrate that LPO could be the key mechanism of ROS-induced sperm damage leading to infertility(Tvrda *et al.* 2011). Significant negative correlations between MDA and sperm motility was found in caprine (Bucak *et al.* 2009) and boar (Cerolini *et al.* 2000) semen. Bucak *et al.* (2009) reported that excessive ROS impaired the motility and fertilization capacity of ram semen. Significant positive correlations between both heavy metals and MDA in the present study (P<0.05; P<0.01; Table 1) further suggest that LPO could be one of the primary mechanisms of heavy metals induced seminal oxidative stress with a subsequent decrease in the antioxidant capacity and spermatozoa viability. Also, our findings agree with Tvrda *et al.* (2013a) demonstrating strong negative correlations between antioxidant enzymes and MDA concentration.

Significantly (P<0.05) low Zn and Cu were observed in poor bulls as compared to good bulls. Cobalt and iron values did not show any significant variation between good and poor quality bulls. Our analysis revealed a significant positive effect of Zn on both spermatozoa motility (r= 0.279, P<0.05) and antioxidant markers (SOD: r = 0.347, P<0.01; catalase: r = 0.266, P< 0.05; Table 1). The cytosolic Cu/Zn SOD is the major SOD isoenzyme found in seminal plasma (Tvrda *et al.* 2011), which explains the antioxidant property of Zn and Cu. Negative correlations between Zn and MDA (r=-.510, P<0.01) (Table 1), as well as a significant decline of Zn in the poor quality bulls (Fig. 1) as observed in our study, further support the antioxidant capacity of seminal zinc. Zn levels in seminal plasma have been positively associated with sperm motility (Tvrda *et al.* 2013b). Significant positive associations between the seminal plasma Cu and SOD (r= 0.359, P<0.01) confirm that Cu is essential for proper activation of the Cu/Zn SOD isoenzyme increasing the number of normal and healthy sperm cells. Positive effects of Cu on the sperm count and motility as well as on the prevention of lipid peroxidation via the activity of SOD and CAT have been reported (Tvrda *et al.* 2013b). Lower Zn and Cu concentrations in the seminal plasma of poor quality bulls and the significant negative correlation between heavy metals (Pd and Cd) and these trace elements observed in present study suggested an antagonistic effect of lead and cadmium on Zn and Cu status adversely affecting the antioxidant status of bovine semen. Tissue specific changes in the distribution of zinc and copper have been reported in rats after experimental administration of lead and cadmium (Patra and Swarup 1998). Swarup *et al.* (2006) observed lower blood copper contents in goats reared around lead-zinc smelter and revealed a negative correlation between blood lead and copper.

In conclusion, lead and cadmium were negatively correlated with motility, sperm concentration and antioxidant markers of bovine seminal plasma. Significant positive correlations were observed between MDA and both heavy metals, indicating their influence on oxidative damage to seminal lipids. The increased Pb and Cd in semen may increase the risk of seminal oxidative stress development and a subsequent reduction in male fertility. Further, the occurrence of toxic heavy metals in the semen samples of bulls and their associated ill effects on antioxidant status and semen quality suggested that breeding bulls can be utilized as a sentinel to monitor the level of environmental pollution with heavy metals and their possible association with increasing human male fertility problems during present times.

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