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Effects of *in vivo* and *in vitro* Zinc and Cadmium Treatment on Sperm Steroidogenesis of the African Catfish *Clarias gairepinus*

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Abstract: The aim of present research to study the effects of *in vitro* and *in vivo* exposure of catfish to heavy metals to determine whether the steroidogenic activity in sperm would be affected by two heavy metals contaminants, zinc and cadmium. Three groups of six male African catfish were fed from the sexually immature juvenile stage, with diet containing 1000 ppm zinc, cadmium or zinc + cadmium for 110 days and 20αHSD activity in milt of these fish were compared with six other control fish fed with normal diet. The 20αHSD enzyme activity was also measured in *in vitro* incubation of milt from six control fish with different concentrations (0, 0.1, 1, 3, 10, 30, 100, 1000 and 3000 ppm) of zinc, cadmium or zinc + cadmium. A very high 20α hydroxy steroid dehydrogenase enzyme activity was found in all sperm incubations of African catfish. This enzyme converts 17-hydroxy progesterone (17P) substrate to 17,20α-dihydroxy progesterone (17, 20αP) product and the rate of enzyme activity is related to substrate (17P) concentrations. Significant differences ($p<0.05$) in enzyme activity in converting 17P to 17,20αP were found between *in vitro* incubations of sperm with different concentrations of zinc, cadmium or zinc + cadmium and control group (0 ppm). Significant differences ($p<0.05$) in enzyme activity and 17,20αP production were found between fish fed with diet containing 1000 ppm zinc or cadmium and the group fed with diet containing 1000 ppm zinc + cadmium and control groups. The results showed that 20αHSD enzyme activities in fish sperm may be used as indicator of water contamination with heavy metals and their bioaccumulations in testis of aquatic animals.

Key words: Zinc, cadmium, steroidogenesis, catfish, sperm, pollution

INTRODUCTION

For an assessment of the toxic effects of different pollutants in the aqueous environment, fish could be very important indicator organisms, due both to their position in the food chain and their requirement for great volumes of water in respiration, making their exposure to pollutants very intensive and so may provide the first warning signs of gross pollution and aquatic ecosystem catastrophy (Kime, 1995). The obvious sign of highly polluted water, dead fish, is readily apparent, while low-level pollution may have no apparent impact on the fish itself, but it may decrease the fecundity of fish populations, leading to a long-term decline and eventual extinction of this important natural resource. Such low-level pollution could impact on reproduction, either indirectly via accumulation in the reproductive organs (Liu *et al.*, 2005; Mathur *et al.*, 2005; Vogel-Mikus *et al.*, 2005; Lagorio *et al.*, 2006), or directly on the free gametes (sperm or ovum) which are released into the water (Takeda *et al.*, 2004).

Pituitary damage (Baos *et al.*, 2006), testicular degeneration (da Cruz *et al.*, 2006; Salmore *et al.*, 2006) and decrease in fry numbers (Paraszkiewicz *et al.*, 2006;

Has-Schon *et al.*, 2007) after cadmium exposure have already been reported. Spermatogenesis may also be inhibited, with only a few spermatids and mature sperm remaining after cadmium pollution (Haffor and Abou-Tarboush, 2004; McClusky, 2006). Decreased spermatogenesis resulting from zinc toxicity has also been shown (Sheweita *et al.*, 2005). Sperm may expose to pollutants through bioaccumulation of toxicants in the testis, or may directly be exposed to aquatic pollutants after ejaculation (Kime, 1995). Disruptive effects of pollutants could be due to disruption of the endocrine system and the inhibition of hormone production. The deleterious effects of toxicants may be exerted on the hypothalamic-pituitary system (Kime, 1995) or could be directed at gonadal steroid production. Plasma estrogen decreased after exposure of Atlantic croaker (Marine environment) to lead (Martynowicz *et al.*, 2005) and *Monopterus albus* to cadmium (Suzuki *et al.*, 2006; Rodriguez *et al.*, 2007), although it has been shown that cadmium contamination stimulates both vitellogenesis, plasma levels and *in vitro* synthesis of estradiol in the Atlantic croaker (Thomas and Trant, 1989). It has also been shown that cadmium increased the transcription of the progesterone receptor but decreased that of the

estrogen receptor gene in humans (Garcia Morales *et al.*, 1994). Plasma androgens in females are also depressed by lead (Thomas, 1988) or cadmium exposure.

Recent studies have shown that in addition to the gonads, sperm itself has enough enzymes to produce steroid hormones and is capable of producing 20α or 20β-reduced progestogen metabolites (Kime *et al.*, 1993; Asahina *et al.*, 1994; Ebrahimi *et al.*, 1995; Tan *et al.*, 1995; Ebrahimi *et al.*, 1996) The main aim of the present research is to study the effects of *in vitro* and *in vivo* exposure of catfish to heavy metals to determine whether the steroidogenic activity in sperm would be affected by two heavy metals contaminants, zinc and cadmium. Different substrate concentrations (1 and 10 μg mL^{-1}) have also been added to these incubations as an indicator of enzyme capacity. The model used may provide a suitable tool for monitoring the low level effects of different pollutants on fish reproduction (Kime, 1995). This method can future use as an bioindicator of water low level pollution and also it can be used to look at the fish steroidogenesis during exposure to heavy metals pollution.

MATERIALS AND METHODS

Chemicals: [^{3}H]17P (Radiolabelled-17-hydroxy progesterone) (Sp. Act. 2.41 TBq/mmol) was purchased from Amersham International and its radiochemical purity confirmed by Thin Layer Chromatography (TLC). Reference steroids (17P, 17,20αP, Progesterone, Testosterone) were purchased from Sigma (Poole, UK.) and Steraloids (Wilton, USA).

Fish: Three groups of six male African catfish originating from a stock held at the Zoological Institute, Catholic University of Leuven (in 1996 in Belgium), were fed from the sexually immature juvenile stage (initial mean individual weight: 211.9±52.5 g), with diet containing 1000 ppm zinc, cadmium or zinc + cadmium for 110 days. They were killed by decapitation and milt (intratesticular sperm) was collected directly from the testes. Since catfish do not readily release milt by hand stripping, milt was also collected from six other control fish (1018±73 g) fed with ordinary diet without adding 1000 ppm zinc, cadmium or zinc + cadmium.

Incubations:

• Twenty microlitres of milt from each of six control male catfish was incubated with gentle shaking for 3 h at 20°C in 2 mL carp incubation medium (Jalabert, 1976) containing [^{3}H]17P (74 kBq, 10 ng) plus 0, 0.1, 1 or 10 μg mL^{-1} unlabelled 17P. Twenty microlitres of

milt taken from each of 3 individual catfish treated *in vivo* with zinc, cadmium or zinc + cadmium (1000 ppm for 110 days) was incubated with gentle shaking under the same conditions.

• Twenty microlitres of milt from the same six control catfish were incubated with different concentrations of zinc, cadmium or zinc + cadmium (0.1, 1, 3, 10, 30, 100, 300, 1000 and 3000 ppm) in the same medium as above containing only [^{3}H]17P (74 kBq, 10 ng) for 3 h at 20°C. After incubation, the medium was frozen at -20°C until steroid extraction.

Steroid hormone extraction: Free steroid fractions and gluconates were extracted as previously described (Ebrahimi *et al.*, 1996) and sulphates were extracted using a modification of previously described procedure (Scott and Canario, 1992). Aliquots from the free, glucuronate and sulphate fractions and of the aqueous residues after the butanol and ethyl acetate extraction were counted for radioactivity.

Separation and identification of steroids: The procedure was used to identify products from *in vitro* incubation with radiolabelled precursor as previously described (Ebrahimi *et al.*, 1996). Fraction 4 (product fraction) was further resolved as necessary by HPLC. A Philips PU4100 system with a PU6030 data capture unit, a PU4110 detector set at 242 nm and a Radiomatic Flo-one/Beta detector using a Monoflow 5 scintillator (National Diagnostics, New Jersey) was used to identify fractions. Metabolites and standards were eluted from a 25 cm ×4.6 mm column (Apex Octadecyl 5U, Jones Chromatography, Hengoed, Mid-Glamorgan, UK) with 35% acetonitrile, 65% water for 10 min, followed by a gradient of acetonitrile increasing from 35 to 45% over 20 min at a flow rate of 1 mL min^{-1}. Retention times were: 11-KT, 7.9 min; 11-deoxycortisol, 9.25 min; 17,20αP, 14.35 min; 17,20βP, 16.75 min; 11β-hydroxytestosterone, 10.5 min; testosterone, 19,0 min androstenetrione, 10.9 min androstenedione, 23.4 min and 17P, 24.8 min. A standard mixture containing these steroids (50 μg mL^{-1}) was injected with each radioactive fraction. The criteria for identification of metabolites were in accord with Sandor and Idler (1972). Comparable TLC fractions from all incubations were combined for identification. Metabolites were identified by chemical reaction followed by crystallization described by Ebrahimi *et al.* (1995 and 1996).

Data analysis: The effects of *in vivo* and *in vitro* treatments with zinc, cadmium and zinc + cadmium were compared with control fish, using the paired sample t-test by using SPSS 10 for Windows software (SPSS Inc., 444 N. Michigan Avenue, Chicago, Illinois 60611, USA).

RESULTS

In all incubations, recovery of initial radioactivity in the media varied from 67 to 97.3%, but showed no obvious correlation with either substrate concentration or metal treatments. Less than 5% of the radioactivity remained in the aqueous residue after the extraction of free steroids and was not further analyzed. This indicates that no detectable and significant amount of conjugates was produced in incubations of milt.

Incubation of milt from six control catfish with different concentrations of 17P (0, 0.1, 1, 10 µg mL⁻¹): Two fractions were eluted from the thin-layer chromatogram of the free fraction, corresponding to the major peaks of radioactivity and to carriers 17P (F2) and 17,20αP/17,20βP/deoxycortisol/11-KT (F4). There was no significant activity in the other regions which were not eluted. In the control fish, F2 area, which corresponded to 17P substrate, increased from $64.9\pm14.1\%$ of recovered activity with radiolabelled substrate alone to over $89.3\pm4.5\%$ in the presence of $10\ \mu g\ mL^{-1}$ substrate (Fig. 1). Yields of 17,20αP, the sole metabolite of F4 decreased from $35.1\pm20.4\%$ at $0\ \mu g\ mL^{-1}$ substrate to $10.7\pm4.5\%$ at the highest concentration. F4 was further chromatographed by HPLC and shown to contain only 17,20αP in all incubations (Fig. 1).

Incubation of milt from six catfish fed with diet containing 1000 ppm zinc, cadmium or zinc + cadmium with different concentrations of 17P (0, 0.1, 1 and 10 µg mL⁻¹): The yields of 17,20αP decreased from 15.2 ± 0.8, 16.9 ± 1.1 and $28.7\pm2.7\%$ with radioactive substrate alone to 8.5 ± 1.7, 8.9 ± 1.1 and 13.3 ± 1.2 at $10\ \mu g\ mL^{-1}$ substrate in zinc, cadmium and zinc + cadmium treated groups, respectively. There were significant differences ($p<0.05$) between groups treated with zinc or cadmium and the control group and between zinc or cadmium treated groups and the zinc + cadmium group at all substrate concentrations.

***In vitro* incubation of milt from control fish with zinc, cadmium or zinc + cadmium:** Two peaks of activity were eluted from TLC-VI corresponding to 17P (F2) and 17,20αP/17,20βP (F4). HPLC of F4 gave a single peak corresponding to 17,20αP in all incubations. No significant differences ($p>0.05$) were found between sperms incubated with zinc, cadmium or zinc + cadmium and their controls (0 ppm) or among different metal concentrations (Fig. 3).
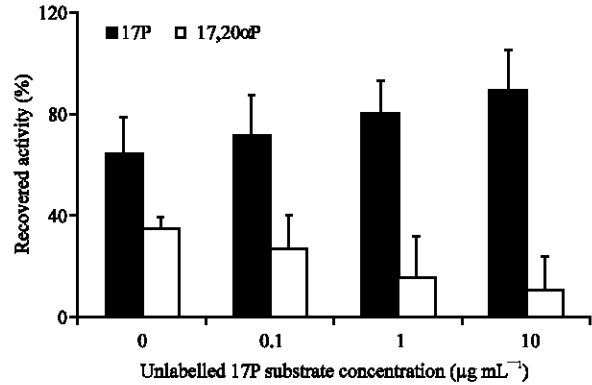

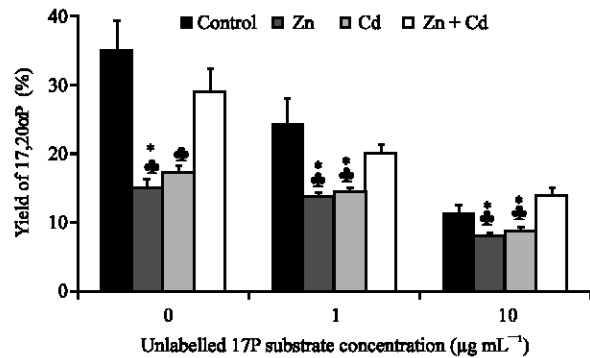Fig. 1: Conversion of $[^3H]17P$ to 17,20αP in incubations of milt from control catfish

Fig. 2: Conversion of $[^3H]17P$ to 17,20αP in the presence of different concentrations of unlabelled 17P by catfish milt treated *in vivo* with zinc, cadmium or zinc + cadmium. *indicates significant difference ($p<0.05$) between Zn or Cd groups and controls and ♣: indicates a significant difference ($p<0.05$) between zinc or cadmium groups and the zinc + cadmium groups

Statistical analysis:

- Very high 20α hydroxy steroid dehydrogenase (20αHSD) enzyme activity found in all sperm incubations of African catfish which converts 17P substrate to 1720αP product. The enzyme activity was checked with different concentration of substrate (17P) in the presence of different concentrations of heavy metals (Fig. 1).

- The results showed that a significant differences ($p<0.05$) in 20αHSD enzyme activity and 17,20αP production were detectable between milt incubation of groups treated with zinc, cadmium or zinc + cadmium and control groups (Fig. 2).

- Significant differences ($p<0.05$) in 17,20αP production were observed by African catfish fed
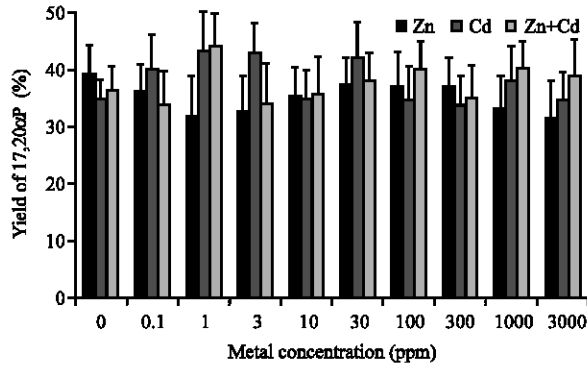
Fig. 3: Conversion of [^{3}H]17P to 17,20αP by catfish milt incubated with different concentrations of zinc, cadmium or zinc + cadmium (no significant difference (p>0.05) was found

with diet containing 1000 ppm zinc or cadmium with group fed with diet containing 1000 ppm zinc + cadmium (Fig. 2).

• No significant differences (p>0.05) were found in 20αHSD enzyme activity between milt from control groups and those incubated with zinc, cadmium or zinc + cadmium or among different metal concentrations (Fig. 3).

DISCUSSION

Zinc as a trace element and is necessary for normal growth of the reproductive organs (Stanwell-Smith *et al.*, 1983), but its excessive uptake is harmful to spermatozoa (Samanta and Pal, 1986) and at high concentrations may seriously affect sperm motility of fish (Kime and Ebrahimi, 1996; Kime and Van_Look, 2001). Cadmium is extremely toxic to spermatozoa and even at very low concentrations (50 ppm) affected sperm motility (Kime, 1995; Kime and Ebrahimi, 1996; Kime and Van_Look, 2001). The aim of this work was to investigate the possible effects of such pollutants on the enzymes involved in the steroidogenic process of sperm.

Although *in vitro* exposure of sperm to both toxicants have shown dramatic effects on sperm motility and structure (Kime, 1995; Kime and Ebrahimi, 1996; Kime and Van_Look, 2001), it did not elicit any obvious effects on 20αHSD enzyme activity in sperm (Fig. 3). This might be due to the short duration of exposure of the enzyme to heavy metals in only 3 h incubation time, although zinc and cadmium exposure affect sperm motility in just only a few seconds. But in fish fed with different concentrations of zinc, cadmium and zinc + cadmium for 110 days, a significant differences (p<0.05) found in enzyme activity

(Fig. 2). The findings suggest that long time exposure inside the testis may be needed for heavy metals to alter enzyme capabilities.

The results showed that short-term exposure does not change the structure or efficiency of the steroidogenic enzymes in reproductive tissues. Long term pre-incubation of sperm in extender with heavy metals and then 3 h *in vitro* incubation with [^{3}H]17P might exert deleterious effects on the enzyme's activity and would be of great concern. Long term exposure of sperm to heavy metals may have deleterious effects on the whole sperm rather than a specific enzyme since a study currently in progress revealed that at concentrations as low as 10 ppm of cadmium, caused structural deformities in carp sperm after 5 h as shown by scanning electron microscopy (Ebrahimi, 2004). Other possible inhibitory effects of cadmium on sperm motility may be due to destabilization of the sperm chromatin via removal of essential zinc (Bianchi *et al.*, 1994; Martin Ponthieu *et al.*, 1994; Suzuki *et al.*, 1995). The other possible toxic effect of heavy metals on sperm might be through respiratory activity inhibition (ATP depletion) (Christen *et al.*, 1987). None of these routes is applicable to steroidogenic enzyme activity in sperm. Therefore it is not surprising if no effect of short-term exposure of sperm to heavy metals was found.

20αHSD enzyme activity was significantly decreased (p<0.05) in incubations of sperm from fish fed and treated *in vivo* with zinc or cadmium compared to untreated control fish for 110 days (Fig. 2). Unlike direct contamination of sperm with pollutants, indirect contamination of sperm via bioaccumulation of toxicants in the testis over a long period dramatically decreased enzyme efficiency. This destructive effect of pollutants on enzyme activity might be exerted either a) at the level of the gene by disturbing transcription, translation or protein synthesis, since cadmium can cause genotoxic damage in somatic and germ cells (Mukherjee *et al.*, 1988), or b) it may destroy the special configuration of the enzyme or cause structural damages within the cell which may destroy the effectiveness of the enzymes (Gazdzik and Kaminski, 1985; Earnshaw *et al.*, 1986; Au *et al.*, 2000; Rho *et al.*, 2002).

As shown in Fig. 2, the toxicities of zinc and cadmium in *in vivo* exposure were similar, while zinc had a lower spermiotoxic effect on sperm than cadmium in *in vivo* treatment (fish fed with diet containing 1000 ppm zinc, cadmium and zinc + cadmium). This may be due to the fact that zinc tends to concentrate in the male reproductive system and its concentration is much higher in sperm and seminal plasma than in other body fluids (Saito *et al.*,

1988; Xu *et al.*, 2001), while the liver accumulates cadmium and hence the concentration of cadmium in liver is much higher than other organs.

The present results confirm our previous findings in which cadmium toxicity was alleviated by an equal amount of zinc (Kime and Ebrahimi, 1996). In fish exposed to zinc + cadmium, the 20αHSD was significantly (p<0.05) higher than each metal exposure alone and no significant (p>0.05) difference was observed between this group and control fish. This interaction between zinc and cadmium is in accord with the previous findings (Kime and Ebrahimi, 1996).

It is important that growth of the male catfish fed with contaminated food also showed the protective effect of zinc against cadmium and fish fed 1000 ppm zinc + cadmium had weights comparable to controls (weight at harvest time after 4 months), while fish fed 1000 ppm cadmium had significantly (p<0.05) lower weights than control or zinc + cadmium exposed fish. This compensatory effect of zinc against cadmium was even significant at 2000 ppm of zinc + cadmium (p<0.05). The gonadosomatic indexes (GSIs) of *in vivo* treated catfish showed similar results and was higher in zinc + cadmium contaminated fish than in those treated with cadmium alone.

ACKNOWLEDGMENTS

REFERENCES

Asahina, K. and J.G.D. Lambert *et al.*, 1994. Bioconversion of 17-alpha-hydroxyprogesterone into 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one and 17-alpha,20-beta-dihydroxy-4-pregnen-3-one by flounder (*Platichthys flesus*) spermatozoa. Zool. Sci., 11: 859-863.

Au, D.W. and M.W. Chiang *et al.*, 2000. Effects of cadmium and phenol on motility and ultrastructure of sea urchin and mussel spermatozoa. Arch. Environ. Contam. Toxicol., 38: 455-463.

Baos, R. and J. Blas *et al.*, 2006. Adrenocortical response to stress and thyroid hormone status in free-living nestling white storks (*Ciconia ciconia*) exposed to heavy metal and arsenic contamination. Environ. Health Perspect., 114: 1497-501.

Bianchi, F. and R. Rousseaux Prevost *et al.*, 1994. Interaction of mammalian sperm nuclear protamines and peptides derived there of with immobilized zinc. Int. J. Peptide Protein Res., 43: 410-416.

Christen, R. and J.L. Gatti *et al.*, 1987. Trout sperm motility: The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur. J. Biochem., 166: 667-672.

da Cruz, A.C. and B.C. Couto *et al.*, 2006. Estimation of the critical effect level for pollution prevention based on oyster embryonic development toxicity test: The search for reliability. Environ. Int.

Earnshaw, M.J. and S. Wilson *et al.*, 1986. The action of heavy metals on the gametes of the marine mussel, *Mytilus edulis*: III. The effect of applied copper and zinc on sperm motility in relation to ultrastructural damage and intracellular metal localization. Mar. Environ. Res., 20: 261-278.

Ebrahimi, M. and P.B. Singh *et al.*, 1995. Biosynthesis of 17,20alpha-dihydroxy-4-pregnen-3-one, 17,20beta-dihydroxy-4-pregnen-3-one and 11-ketotestosterone by testicular fragments and sperm of the roach, Rutilus rutilus. Gen. Comp. Endocrinol., 100: 375-384.

Ebrahimi, M. and A.P. Scott *et al.*, 1996. Extragonadal production of 17,20-dihydroxy-4-pregnen-3-ones *in vitro* in cyprinid fish. Gen. Comp. Endocrinol., 104: 296-303.

Garcia Morales, P. and M. Saceda *et al.*, 1994. Effect of cadmium on estrogen receptor levels and estrogen-induced responses in human breast cancer cells. J. Biol. Chem., 269: 16896-16901.

Gazdzik, T. and M. Kaminski, 1985. Ultrastructural study of development of the rat testis: II. After injecting cadmium chloride. Folia Morphol., 33: 218-222.

Haffor, A.S. and F.M. Abou-Tarboush, 2004. Testicular cellular toxicity of cadmium: Transmission electron microscopy examination. J. Environ. Biol., 25: 251-258.

Has-Schon, E. and I. Bogut *et al.*, 2007. Heavy metal concentration in fish tissues inhabiting waters of Busko Blato reservoir (Bosnia and Herzegovina). Environ. Monit. Assess.

Jalabert, B., 1976. *In vitro* oocyte maturation and ovulation in rainbow trout (*Salmo gairdneri*), northern pike (*Esox lucius*) and goldfish (*Carassius auratus*). J. Fish. Res. Bd. Can., 33: 974-988.

Kime, D.E. and S. Bhattacharya *et al.*, 1993. Steroidogenesis by ovaries and testes of the European catfish, the wels (*Silurus glanis*), *in vitro*. Fish Physiol. Biochem., 10: 389-398.

Kime, D.E., 1995. The effects of pollution on reproduction in fish. Rev. Fish Biol. Fish., 5: 52-96.

Kime, D.E. and M. Ebrahimi *et al.*, 1996. Use of Computer Assisted Sperm Analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; application to effects of heavy metals. Aqua. Toxicol.

Kime, D.E. and K.J. Van_Look *et al.*, 2001. Computer Assisted Sperm Analysis (CASA) as a tool for monitoring sperm quality in fish, 130: 425-433.

Lagorio, S. and F. Forastiere *et al.*, 2006. Air pollution and lung function among susceptible adult subjects: A panel study. Environ. Health, 5: 11.

Liu, H. and X.R. Wang *et al.*, 2005. Effects of long-term exposure of low level zinc and zn-EDTA complex on zinc accumulation and antioxidant defense system in liver of *Carassius auratus*. Huan Jing Ke Xue, 26: 173-176.

Martin Ponthieu, A. and D. Wouters Tyrou *et al.*, 1994. Isolation and characterization of a small putative zinc finger protein from cuttlefish epididymal sperm cells. Eur. J. Biochem., 220: 463-468.

Martynowicz, H. and R. Andrzejak *et al.*, 2005. The influence of lead on testis function. Med. Pr., 56: 495-500.

Mathur, N. and P. Bhatnagar *et al.*, 2005. Mutagenicity assessment of effluents from textile/dye industries of Sanganer, Jaipur (India): A case study. Ecotoxicol. Environ. Saf., 61: 105-113.

McClusky, L.M., 2006. Stage-dependency of apoptosis and the blood-testis barrier in the dogfish shark (*Squalus acanthias*): Cadmium-induced changes as assessed by vital fluorescence techniques. Cell Tissue Res., 325: 541-553.

Mukherjee, A. and A.K. Giri *et al.*, 1988. Relative efficacy of short-term tests in detecting genotoxic effects of cadmium chloride in mice *in vivo*. Mutat. Res., 206: 285-296.

Paraszkiewicz, K. and A. Frycie *et al.*, 2006. Enhancement of emulsifier production by *Curvularia lunata* in cadmium, zinc and lead presence. Biometals.

Rho, G.J. and S. Kim *et al.*, 2002. Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. Mol. Reprod. Dev., 63: 464-470.

Rodriguez, E.M. and D.A. Medesani *et al.*, 2007. Endocrine disruption in crustaceans due to pollutants: A review. Comp. Biochem. Physiol. A Mol. Integr. Physiol., 146: 661-671.

Saito, S. and K. Ohno *et al.*, 1988. Studies of human seminal zinc and acid phosphatase: Studies of human seminal plasma: Report 3. Jap. J. Fertil. Steril., 33: 219-224.

Salmore, A.K. and E.J. Hollis *et al.*, 2006. Delineation of a chemical and biological signature for stormwater pollution in an urban river. J. Water Health, 4: 247-262.

Samanta, K. and B. Pal, 1986. Zinc feeding and fertility of male rats. Int. J. Vitamin Nutr. Res., 56: 105-108.

Sandor, T. and D.R. Idler, 1972. Steroid Methodology. Steroids in Nonmammalian Vertebrates. Idler, D.R. (Ed.), New York, Academic Press, pp: 6-36.

Scott, A.P. and A.V.M. Canario, 1992. 17-alpha,20-beta-Dihydroxy-4-pregnen-3-one 20-sulphate: A major new metabolite of the teleost oocyte maturation-inducing steroid. Gen. Comp. Endocrinol., 85: 91-100.

Sheweita, S.A. and A.M. Tilmisany *et al.*, 2005. Mechanisms of male infertility: Role of antioxidants. Curr. Drug Metab., 6: 495-501.

Stanwell-Smith, R. and S.G. Thompson *et al.*, 1983. A comparative study of zinc, copper, cadmium and lead levels in fertile and infertile men. Fertil. Steril., 40: 670-677.

Suzuki, T. and K. Nakajima *et al.*, 1995. Metallothionein binding zinc inhibits nuclear chromatin decondensation of human spermatozoa. Andrologia, 27: 161-164.

Suzuki, N. and M.J. Tabata *et al.*, 2006. Tributyltin inhibits osteoblastic activity and disrupts calcium metabolism through an increase in plasma calcium and calcitonin levels in teleosts. Life Sci., 78: 2533-2541.

Takeda, K. and N. Tsukue *et al.*, 2004. Endocrine-disrupting activity of chemicals in diesel exhaust and diesel exhaust particles. Environ. Sci., 11: 33-45.

Tan, A.M.C. and S.T.L. Lee *et al.*, 1995. 17-alpha,20-alpha-dihydroxy-4-pregnen-3-one, not its 20-beta isomer, is produced from 17-alpha-hydroxyprogesterone by spermatozoa of secondary male groupers (*Epinephelus tauvina*) derived from females implanted with 17-alpha-methyltestosterone. J. Exp. Zool., 271: 462-465.

Thomas, P., 1988. Reproductive endocrine function in female Atlantic croaker exposed to pollutants. Mar. Environ. Res., 24: 179-183.

Thomas, P. and J.M. Trant, 1989. Evidence that $17\alpha,20\beta,21$-trihydroxy-4-pregnen-3-one is a maturation inducing steroid in spotted sea trout. Fish Physiol. Biochem., 7: 185-191.

Vogel-Mikus, K. and D. Drobne *et al.*, 2005. Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonisation of pennycress *Thlaspi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. Environ. Pollut., 133: 233-242.

Xu, L.C. and S.Y. Wang *et al.*, 2001. Effects of cadmium on rat sperm motility evaluated with computer assisted sperm analysis. Biomed. Environ. Sci. Bes., 14: 312-317.