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Sperm of rosy barb (*Puntius conchonius*) as an *in vitro* assay system of nonylphenol cytotoxicity

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Nonylphenol (NP) is the final degradation product of alkylphenol polyethoxylates (APEs), which are widely present in plastics, papers, pulp, detergents, pesticides, herbicides, paints and cosmetics. It is estimated that annual global production of APEs is about 500000 t, 60% of which eventually ends up in aquatic ecosystems primarily via wastewater (Solé *et al.*, 2000). NP is resistant to biodegradation and persistent in the environment (Arukwe *et al.*, 2000).

The toxicity of NP on reproductive biology of organisms has been extensively studied by *in vivo* bioassays, which require live animals. NP has been demonstrated to possess estrogenic activity and exert adverse effects on species including fish and mammals (Kobayashi *et al.*, 2005). In addition, it is shown to elicit depletion of antioxidant defense system in epididymal sperm in rats (Chitra *et al.*, 2002). Cell lines and primary cell cultures have also been utilized in evaluating the toxicity of NP. Aoki *et al.* (2004) exhibited that NP induces apoptosis in PC12 cells, a cell line of rat pheochromocytoma cells, while Lamche and Burkhardt-Holm (2000) show that NP provokes vesiculation of the Golgi apparatus in fish epidermis cultures. However, information concerning the *in vitro* toxicity of NP to sperm of aquatic animals is very limited.

The rosy barb, *Puntius conchonius*, a member of family Cyprinidae, is native to rivers and fast-flowing streams of Afghanistan, Bangladesh, India, Pakistan and Nepal, and is now very popular with aquarists throughout the world. It has been widely used in ecotoxicological studies. Recently, Xu *et al.* (2005) and Hu *et al.* (2005) have suggested that rosy barb sperm are a suitable candidate for rapidly evaluating *in vitro* cytotoxicities of abamectin and isocarbophos. The aims of this study were thus to examine the effects of NP on motility and ultrastructures of rosy barb sperm, to relate the observed changes in sperm motility to changes in ultrastructures of sperm after exposure to NP (Sigma-Aldrich, USA), to explain the impairment of sperm motility from a cytopathological point of view, and to test the applicability of rosy barb sperm in evaluating the *in vitro* acute toxicities of NP.

A total of 30 sexually matured male rosy barbs (*Puntius conchonius*) with a body length of 3—5 cm and a body weight of 2—3 g were procured from a local fish dealer. They were acclimatized in the laboratory in constantly aerated water at 26±1℃ with natural light/dark cycle (14-h L/10-h D) for 2 weeks prior to use. The fishes were fed with live bloodworms and fish flakes (Tetramin, Germany) twice a day.

Rosy barb semen was squeezed out of the cloaca by gently compressing the genital area of the sexually matured male fish. Great care was taken to avoid contamination with faeces and water. The semen squeezed was immediately diluted 100 fold in fish physiological solution (FPS) (Márián *et al.*, 1997). The diluted suspensions were stored on ice for the following experiments, which were all conducted within 120 min after collecting the semen.

The motility of sperm was immediately observed under a BX51 Olympus microscope (100 X) by mixing 10 μl of the diluted semen with hypo-osmotic sodium buffer (HSB) (Márián *et al.*, 1997) on a slide, and the concentration of sperm was calculated by counting sperm number in a Neubauer cell counter. Only the semen containing over 95% motile sperm were used in the experiments below.

Preliminary experiments were performed to determine the concentration range of NP in which the sperm motility of rosy barb changed but was not inhibited completely. On the basis of the results of the preliminary experiments, five concentrations of NP, 2, 4, 8, 16 and 32 μmol/L were chosen for the sperm motility assay. Briefly, an aliquot of 10 μl of the diluted semen was mixed on a slide with 90 μl HSB into which various concentrations of NP were added. The percentage of motile sperm was determined every 5 min at a magnification of 10 ×10 at room temperature. All experiments were repeated at least three times. Data obtained were expressed as mean ± standard deviation (mean±*SD*).

The sperm exposed to 8 μmol/L NP for 15 and 30 min were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer solution (pH 7.3). They were then post-fixed in 1% osium tetroxide, dehydrated

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with graded ethanol, dried by the critical point method and coated with gold. Observation was made under a JSM-840 scanning electron microscope. In addition, the osium tetroxide-post-fixed sperm were also dehydrated, embedded in Epon 812, and sectioned. The sections were stained with uranyl acetate and lead citrate, and observed and photographed under a JEM-1200EX electron microscope. Control samples were similarly prepared and observed, respectively. Any consistent changes observed in the treated sperm (with > 50% occurrence) but not in the control (with < 5% occurrence) were considered an abnormality induced by NP.

Rosy barb sperm are quiescent in the fresh semen squeezed out of the gonads. The concentration of sperm in the semen is about $(3.1 \pm 1.1) \times 10^{11}$ cells/ml, which is very close to the concentration reported by Xu *et al.* (2005). The motility of rosy barb sperm activated by HSB decreased with time, but 76% sperm remained motile at 30 min after activation. In contrast, the motility of sperm exposed to NP declined markedly with time. The motility of sperm exposed to 2, 4, 8, 16 and 32 μmol/L NP for 15 and 30 min were reduced to 80.57%, 66.75%, 55.21%, 47.08%, and 35.42%, and to 60.86%, 44.75%, 30.71%, 21.15% and 5.46%, respectively (Fig.1). It was clear that the toxicity of NP to rosy barb sperm was both dose-dependent and exposure time-dependent.

Rosy barb sperm had a round head with 1.45 ± 0.13 μm, a mid-piece surrounded by several mitochondria and a tail consisting of a 27.73 ±2.59 μm flagellum, and their cell membranes were pretty smooth (Fig. 2a and 2b), which are similar to those described previously by Xu *et al.* (2005). Transmission electron microscopy examination showed that rosy barb sperm head consisted of a round homogenous nucleus covered by a thin layer of cell membrane, but no acrosomal vesicle was found in it (Fig.2c). In addition, the mitochondria around the mid-piece contained numerous cristae.

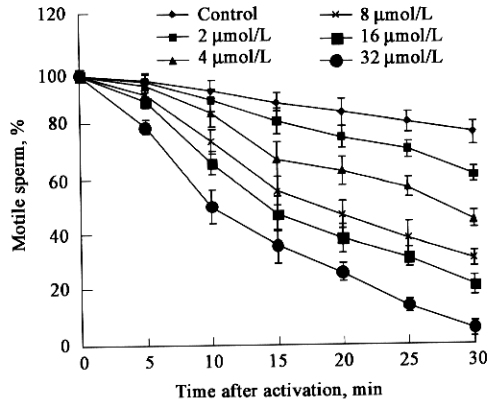

Fig.1 Percentage of motile sperm exposed to different concentrations of NP
Data were obtained from three experiments and expressed as means±*SD*

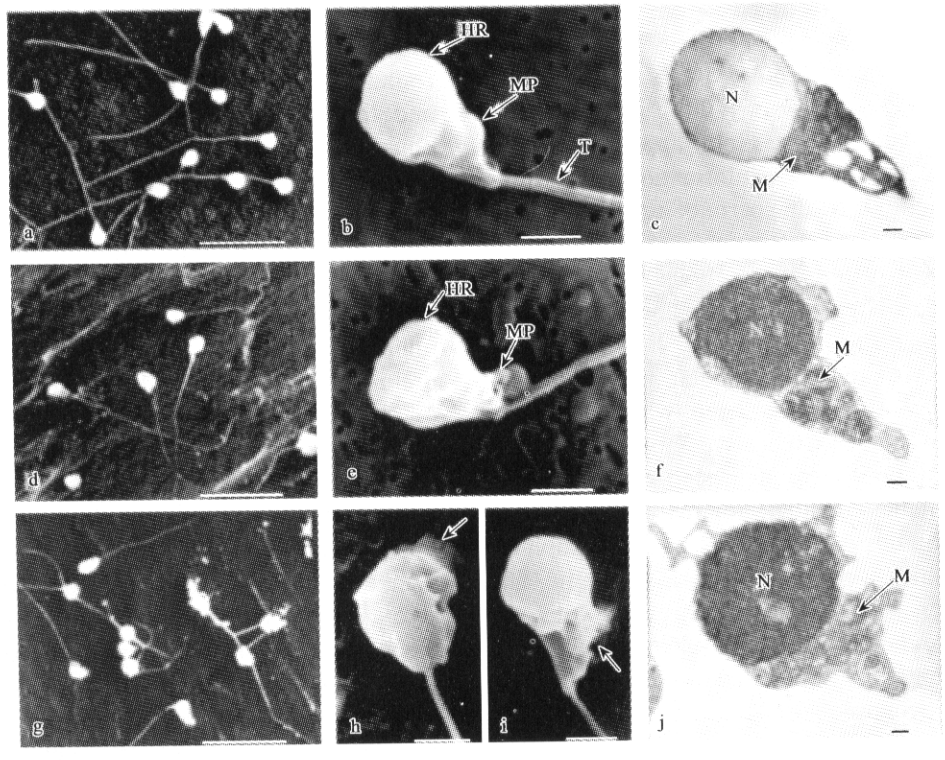

Fig.2 Electronic microscopic micrographs of normal and NP-treated sperm
a, b and c: micrographs of normal rosy barb sperm; d, e and f: micrographs of rosy barb sperm exposed to 8 μmol/L NP for 15 min; g, h, i and j: micrographs of rosy barb sperm exposed to 8 μmol/L NP for 30 min; scale bars =10 μm in a, d and g; scale bars = 1 μm in b, e, h and i; scale bars = 200 nm in c, f and j; HR: head region; MP: mid-piece; T: tail; N: nucleus; M: mitochondrion

The ultrastructures of rosy barb sperm were impaired by exposure to NP. Most of the sperm exposed to 8 μmol/L NP for 15 min remained morphologically normal, but some of them had their cell membranes in the head wrinkled, and their mitochondria around the mid-piece damaged and with fewer cristae (Fig. 2d, 2e and 2f). When the sperm were treated with 8 μmol/L NP for 30 min, most sperm cell membranes surrounding the head were dissolved, and the cristae in mitochondria swelled up markedly (Fig. 2g, 2h, 2i and 2j).

This study demonstrates that NP exerts deleterious effects on rosy barb sperm. NP is capable of impairing the sperm motility at 2 μmol/L. This concentration is quite close to the NP concentration, 1.5 μmol/L, in the wastewaters discharged into the British River Aire (Blackburn and Waldock, 1995), and much lower than the NP concentration, 28.6 μmol/L, in some sewage effluents in USA (Hale *et al.*, 2000). It is clear that rosy barb sperm are fairly sensitive to NP, which makes rosy barb sperm a suitable candidate for rapid evaluating the acute toxicities of NP.

Compared with Dinnel's (1981) sperm assays, the sperm cell assay described here only needs sperm, and is not disturbed by egg quality and the ratio of sperm to egg. It is thus a simpler and quicker assay system applicable all year round in many areas over the world by using closely related species. Moreover, it can also provide information on sperm damage caused by toxicants from a cytopathological point of view.

It has been shown that NP has multiple toxic effects on organisms and their cells via endocrine disruption (Sweeney, 2002), depletion of antioxidant defense system (Chitra *et al.*, 2002), induction of apoptosis (Aoki *et al.*, 2004) and vesiculation of Golgi apparatus (Lamche and Burkhardt-Holm, 2000). Here we showed that NP caused damage to the mitochondria of rosy barb sperm. Mitochondria are organelles that produce energy that the sperm can use for their motility. It is thus proposed that the ultrastructural alteration caused by NP correlates with the impairment of the motility of rosy barb sperm. It is highly likely that mitochondria might be the most prominent site of NP toxicity in rosy barb sperm. This apparently agrees with the idea that the preferential target of NP in living organisms is mitochondria (Bragadin *et al.*, 1999).

NP is a lipophilic chemical, and its hydrophobic alkyl residue can destroy cell membrane integrity (Argese *et al.*, 1994). This has been confirmed in the present study by the fact that NP caused dissolution of the plasma membrane of rosy barb sperm at higher concentration. It also indicates that direct contact with NP may have adverse effects on organisms including humans.

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