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Effect of 4-nonylphenol on the sperm dynamic parameters, morphology and fertilization rate of *Bufo raddei*

Minyi Feng, Peng Chen, Xue Wei, Yingmei Zhang*, Wenya Zhang and Yongmei Qi

School of Life Sciences, Lanzhou University, Lanzhou, China.

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4-Nonylphenol (NP) is a compound that causes endocrine disruption and affects sperm quality of mammals and fish. However, the effects of NP on the sperm and fertilization rate of amphibians remain unknown. This study investigates the *in vivo* and *in vitro* effects of NP on the sperm dynamic parameters and fertilization rate of *Bufo raddei* during the period of amplexus and fertilization, and proposes the induction of these effects. In *in vivo* assay, male *B. raddei* were exposed to 3 concentrations of NP (50, 200, or 400 µg/l) or alcohol (0.04%, control) for 1-3 days. The results suggested that effects on NP on the sperm dynamic parameters, sperm integrity and fertilization rate were not significant ($p > 0.05$). In *in vitro* assay, the sperm of *B. raddei* was directly exposed to NP. Based on the results, NP significantly affected the sperm dynamic parameters and integrity ($p < 0.05$). Meanwhile, the sperm reactive oxygen species (ROS) level in the sperm increased significantly ($p < 0.05$), and a negative correlation was recorded between sperm motility and its corresponding ROS level ($R = -0.90$). Besides, fertilization rate was significantly reduced compared with that of control ($p < 0.01$). The sperm membrane was impaired as well. However, a risk that NP can disrupt the reproduction behavior of *B. raddei* exists, and the ROS induced by NP and NP itself would be associated with the reduction of fertilization.

Key words: 4-Nonylphenol, *Bufo raddei*, sperm, morphology, fertilization.

INTRODUCTION

Previous studies have shown that habitat degradations such as water pollution might play an important role in the decline of amphibians (Houlahan et al., 2000). 4-Nonylphenol (NP) was one of the predominant organic pollutants in the Lanzhou Region of Yellow River (Northwest, China), and its concentration fluctuate from 0.065 to 83 µg/l at different sites (Wang et al., 2006). Whether NP in aquatic environment in Lanzhou Region would affect the reproduction of amphibian like that of the native common

species *Bufo raddei*, is unknown. Sperm dynamic parameters, sperm morphology and fertilization rate could serve as valid indexes to access the effects of pollutants on toad reproduction, and the information would be useful to access the decline of amphibian.

NP is estrogenic to aquatic organisms (Servos, 1999), and studies concerning amphibians have mainly focused on its estrogenic effects. NP could induce vitellogenin in the gonads of male *Xenopus laevis* (Naoko et al., 2007), disrupt the development of the embryo and larvae of *Bombina orientalis* (Chan et al., 2010), and cause an extremely skewed sex ratio in amphibians by disrupting sex hormones (Mosconi et al., 2002). The estrogen effects of NP are associated with the estrogen receptors. Currently, estrogen receptors have been identified on rat sperm membranes (Saberwal et al., 2002). However, whether or not the estrogen receptors are present on the sperms of amphibians or other aquatic organisms is still unclear.

*Corresponding author. Email: ymzhang@lzu.edu.cn. Tel: 0086 13919123067.

Abbreviation: NP, 4-Nonylphenol; CASA, computer assisted sperm analysis system; ROS, reactive oxygen species; DCFH-DA 2, 7-dichlorofluorescein-diacetate; VSL, velocity of strain line; VAP, sperm velocity of average path.

The information regarding the effects of NP on the sperm and fertilization of amphibians is unclear. Whereas, there have been some studies regarding the effects of NP on the sperm of fish. Research shows that NP significantly reduced sperm motility, fertility and its swimming velocity of rainbow trout ($p < 0.05$) in a non-estrogenic way, and NP would not impair the morphology of sperm in the short-period of fertilization (Lahnsteiner, 2005; Yutaka et al. 2007). It is worth noting that the fertilization between fishes and toads is different. The oviposition and ejaculation of toads is not synchronous. The spawning of *B. raddei* lasts for 20-40 min; thus, its sperm would be directly exposed to the water environment for several minutes or more before insemination. The effects of NP on sperm and fertilization rate in toads are thus more complicated than its effects on fish. Moreover, the period during amplexus and fertilization is an appropriate assessment time compare with other periods with unpredictable exposure time.

Considering the inadequate information and the specificity of the fertilization of amphibians, this study investigates the *in vivo* and *in vitro* effects of environmentally relevant concentrations of NP (0.065-83 $\mu\text{g/l}$) on the dynamic sperm parameters, sperm morphology, and fertilization rate of the toad *B. raddei* during the period of amplexus and fertilization. This work also explores the potential induction of the effects. Such information would be valuable for better estimating the risks of NP in relation to the decline of wild amphibian population.

MATERIALS AND METHODS

Animal

Mature and healthy *B. raddei* (25.32 \pm 3.04 g) were purchased from the Medical Experimental Animal Center of Lanzhou University [China, certification number: SCXK (Gan) 2009-0004] and kept in glass tanks for 7 days accommodation with dechlorinated tap water (pH 7.3), under 16-8 h light-dark cycles at 20 \pm 1°C; these were fed with newly hatched *Tenebrio molitor* larvae.

Chemicals, apparatus and solutions

NP (99.70%) and Fluor chrome 2, 7-Dichlorofluorescein-diacetate (DCFH-DA) were purchased from Sigma-Aldrich. All other reagents were of the highest analytical grade. The Computer Assisted Sperm Analysis System (CASA) was from Hamilton Thorne Biosciences (version 12 TOXIVOS). Ultraviolet spectrophotometer for ROS detection was from PerkinElmer (LS55).

A solution consisting of 10 $\text{g}\cdot\text{l}^{-1}$ NP was prepared with alcohol. The stock solution was diluted with dechlorinated tap water to make final concentrations of 50, 200 or 400 $\mu\text{g/l}$ NP to represent about 0.6 to 5 times the highest environmentally relevant concentrations reported so far (from 0.065-83 $\mu\text{g/l}$) (Wang et al., 2006); these also cover the median lethal dose in most of the aquatic organisms (60-770 $\mu\text{g/l}$) (US EPA 2005). Half of the liquid in each tank was renewed daily during the experiments. The control group contained 0.04% alcohol at the same concentration as that for the 400 $\mu\text{g/l}$ NP group.

In vivo experiment

Male toads were randomly selected and exposed to NP (50, 200 or 400 $\mu\text{g/l}$) or 0.04% alcohol (control) for 3 days, with 3 independent triplicates in each group. Sperm motility was analyzed once every day during the 3-day exposure. The toads were then euthanized by pithing (American Veterinary Medical Association 2001), and their gonads were removed and cut with surgical scissors to prepare sperm suspensions. The sperm concentration was adjusted to 5.00 \times 10⁶ sperm/ml by cell counting. After about 10 min activation, the sperm suspensions were analyzed by CASA.

For the fertilization rate test, the male toads were exposed to NP (50, 200 or 400 $\mu\text{g/l}$) or the control for 3 days and the female toads that were not subjected to NP or solvent exposure were given intraperitoneal injections of chorionic gonadotropin (males: 400 IU; females: 600 IU). The toads were then kept in the glass tanks with NP-free dechlorinated tap water and maintained at 24 \pm 1°C. They would be in amplexus and spawn one day later. Fertilization rate calculation began 4 h after spawning. The eggs were pooled and randomly divided into 3 parts (egg number is 100) to calculate the fertilization rate (Krupa, 1988).

Analysis of sperm exposed to NP directly, reactive oxygen test (ROS) test and fertilization

As mentioned above, after pithing, the gonads of *B. raddei* were removed to prepare the sperm suspensions of 5.00 \times 10⁶ sperm/ml with 0.04% alcohol (control), 50 $\mu\text{g/l}$ H₂O₂ (positive control), after which 50, 200 or 400 $\mu\text{g/l}$ NP and the sperm suspensions were immediately analyzed using CASA. The experiment was repeated at 3 replicates for each sample.

For the ROS test, the sperm was stained and activated with 10 μM DCFH-DA and incubated in the dark for 10 min at 25°C before NP or H₂O₂ treatment. The fluorescence intensity was detected using a PerkinElmer fluorescence spectrometer LS55 at 488 nm excitation and 525 nm emission filter settings (Myhre et al., 2003). Each sample was repeated for up to 3 replicates. The ROS levels were examined separately after 1, 3, 6, 9, 12 or 25 min.

For the fertilization rate test, male and female toads without prior NP exposure were given intraperitoneal injections of chorionic gonadotropin. After the injection, the male and female toads were divided into 5 groups (negative control, positive control, 50, 200 or 400 $\mu\text{g/l}$ NP) with 3 replicates in each group and 3 pairs of toads in each replicate. Fertilization rate calculations were conducted as previously mentioned (Krupa, 1988).

Morphological observation of sperm

All sperm samples were treated with NP or the positive-control for 12 min, with each group has 3 replicates. Afterwards, the samples were fixed in 2% glutaraldehyde and observed under electron microscope.

Statistical analysis

The statistical software, Statistical Package for the Social Sciences (SPSS) 16.0 for Windows, was used for the interpretation of data with $p < 0.05$ for significance. Analysis of variance (ANOVA) and Student's *t*-test was used to determine significant differences between the control, the positive-control and treatment groups.

RESULTS

In vivo exposure

When sperm motility was activated in dechlorinated tap

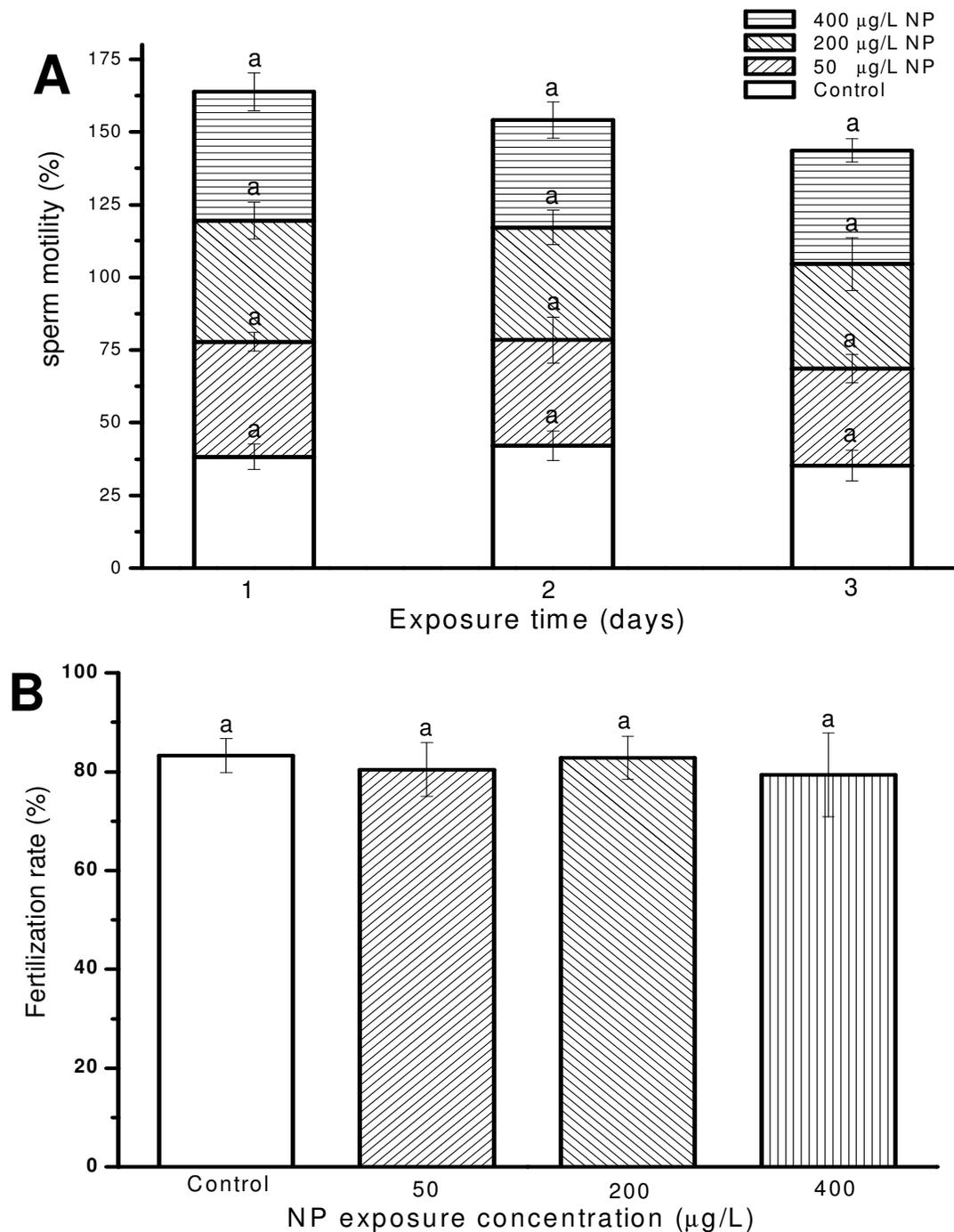


Figure 1. Motility of sperm in the gonad and fertilization rate of toad exposed to NP. *B. raddei* were exposed to 0.04% alcohol, 50, 200 or 400 µg/l NP for 3 days. A. Sperm motility; B. Fertilization rate. Sperm motility was measured once a day, about 10 min after being activated in dechlorinated tap water without NP, pH 7.3. In fertilization test, HCG was used as a stimulant for spawning, *B. raddei* mating and spawning in dechlorinated tap water without NP, pH 7.3. All values are means \pm S.D.

water for 10 min, significant differences in sperm motility were not observed between the control and NP treatments ($p > 0.05$; Figure 1A). The fertilization rate of *B. raddei* exposed to NP for 1, 2 or 3 days were not signifi-

cantly different from that of the control ($p > 0.05$; Figure 1B). Sperm velocity of strain line (VSL), sperm velocity of average path (VAP), and sperm integrity in the NP treatments were similar to those of control ($p > 0.05$; Table

Table 1. Dynamic parameters and integrity of sperm in the gonad of toad exposed to NP.

Group	VSL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	Sperm integrity (%)
0.04‰ alcohol (Control)	8.26 \pm 0.38 ^a	16.84 \pm 0.31 ^a	82.43 ^a
50 $\mu\text{g/l}$ NP	8.45 \pm 0.77 ^a	17.52 \pm 0.26 ^a	80.16 ^a
200 $\mu\text{g/l}$ NP	7.96 \pm 0.43 ^a	16.37 \pm 0.93 ^a	81.30 ^a
400 $\mu\text{g/l}$ NP	8.08 \pm 0.25 ^a	17.08 \pm 0.31 ^a	85.04 ^a

VSL: Velocity straight line; VAP: average path velocity. *Bufo raddei* exposed to NP for 3 days. After pithing, gonad of *B. raddei* was removed to prepare the sperm suspensions of 5.00×10^6 sperm/ml for the analysis by CASA. Sperm integrity according to the morphology observation under the electron microscope, $n=20$, 3 replicates. Values are means \pm S.D. Values with different letters are significantly different, $p < 0.05$.

1); about 81-85% integral sperm were observed in control and treatment groups after the 3 days NP exposure of *B. raddei*.

In vitro exposure

Significant reduction in the sperm motility exposed for 9 min to 400 $\mu\text{g/l}$ NP and the positive-control was recorded relative to the control ($p < 0.05$; Figure 2A). The sperm motility of 400 $\mu\text{g/l}$ NP and positive-control at 9 min was decreased by about 73 and 77% compared to control. After 12 min exposure, the reduction of sperm motility was more significant in NP ($\geq 200 \mu\text{g/l}$) group or positive-control ($p < 0.01$; Figure 2A). This indicates that direct NP exposure had an evident effect on sperm motility.

The VSL of sperm in the positive-control and the 400 $\mu\text{g/l}$ NP treatment groups ($12.94 \mu\text{m}\cdot\text{s}^{-1}$ and $11.75 \mu\text{m}\cdot\text{s}^{-1}$, respectively) decreased dramatically with the prolonged treatment time, which was significantly faster than those of control ($7.83 \mu\text{m}\cdot\text{s}^{-1}$) and 50 $\mu\text{g/l}$ NP group ($8.06 \mu\text{m}\cdot\text{s}^{-1}$) short time after spermiation (2 min) ($p < 0.01$; Table 2).

The VSL in positive-control and 400 $\mu\text{g/l}$ NP treatment group decreased continuously for up to 25 min; at 12 min, the VSL values of this group and positive-control were about 52 and 56% that of control, respectively. However, the VSL values of control and the 50 $\mu\text{g/l}$ NP treatment groups increased gradually from 2-12 min (Table 2). The VAP had the same trend as the VSL.

Morphology observations showed that NP ($\geq 200 \mu\text{g/l}$) and H_2O_2 impaired the sperm membrane. After being subjected to exposure for 6 min, the sperm integrity values of the positive-control, 200 and 400 $\mu\text{g/l}$ NP treatment groups were significantly lower than those of control and 50 $\mu\text{g/l}$ NP treatment group. At 25 min, only about 31 and 25% intact sperm were observed in the positive-control and 400 $\mu\text{g/l}$ NP group (Table 2; Figure 3 P1-P3; Figure 3 T31-T33).

During the exposure that lasted from 1-25 min, sperm ROS levels increased gradually from 7.82-11.21 in the control. They were 22.82-37.68 in the positive-control, 10.82-15.90 in the 50 $\mu\text{g/l}$ NP treatment group, 15.37-27.94 in the 200 $\mu\text{g/l}$ NP treatment group, and 18.47-

35.20 in the 400 $\mu\text{g/l}$ NP treatment group (Figure 2B). The sperm ROS in the NP-treated groups was significantly higher than that of control at any time points ($p < 0.01$). The sperm motility has a significant negative correlation with the mean ROS level within 25 min, $R = -0.90$ (Figure 2C).

NP and H_2O_2 exposure decreased the fertilization rate significantly ($p < 0.01$; Figure 2D). The fertilization rate of 400 $\mu\text{g/l}$ NP treatment group was about 53% that of the control. As for the positive-control, the fertilization rate was about 61% that of control.

DISCUSSION

This study demonstrates that NP ($\leq 400 \mu\text{g/l}$) exposure for 3 days would not significantly affect sperm motility, VSL, VAP, and its viability in the gonads of *B. raddei* ($p > 0.05$). However, direct NP exposure during fertilization impairs sperm membranes and reduces sperm motility, VSL, VAP, and viability. If only the short period during amplexus and fertilization is considered, this study suggests that the key period in which NP can affect the sperm is the period of fertilization.

Previous study showed that NP ($\leq 0.75 \mu\text{g/l}$) did not affect sperm motility, egg viability, or fertilization rate of *Oncorhynchus mykiss* within 60 days of exposure (Franz et al., 2005). While Mackenzie et al. (2003) and Weber et al. (2002) found that NP could go through the blood-testis barrier and 100 $\mu\text{g/l}$ NP exposures for six weeks could induce apoptosis of sperm mother cells, supporting cells, nucleus interstitial cells in male *Oryzias latipes*. Our study has revealed that *B. raddei* exposed to NP ($\leq 400 \mu\text{g/l}$) during amplexus (1-3 days) dose not affect its sperm dynamic parameters or the fertilization rate. We proposed that the efficient dosage of NP in the body of *B. raddei* was not enough to significantly affect the sperm in the gonad. Reproductive behaviors of amphibian (that is, amplexus, ovulation or ejaculation activities) are regulated by hormones (Gobbetti and Zerani, 1999; Licht 1979); hence, NP could disturb the reproductive behaviors of amphibian. Actually, we had found that more clasping toads would divorce after 3 days exposure to NP

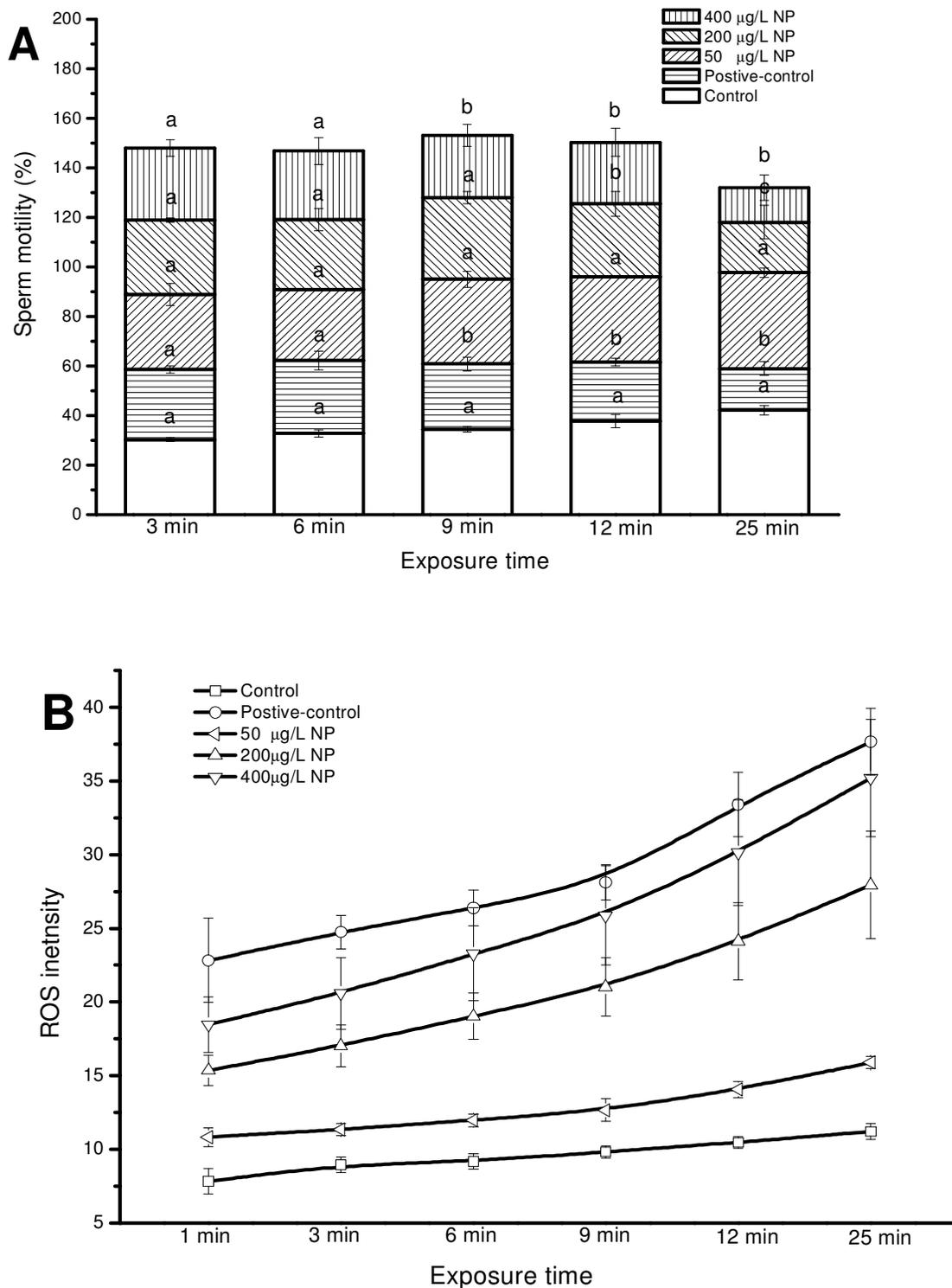


Figure 2. Motility, ROS level of sperm exposed to NP directly and the fertilization rate. A. Sperm motility; B, ROS level; C, Relationship analysis between sperm motility and ROS mean intensity within 25 min; D, Fertilization rate. B, raddei accommodated in dechlorinated tap water without NP for 7 days, pH 7.3. After pithing, gonad of *B. raddei* was removed to prepare the sperm suspensions of 5.00×10^6 sperm/ml with 0.04% alcohol (control), 50 $\mu\text{g/l}$ H_2O_2 (positive-control), 50 $\mu\text{g/l}$, 200 $\mu\text{g/l}$ and 400 $\mu\text{g/l}$ 4-nonylphenol respectively. Before ROS level examined, sperm suspensions were activated 10 ± 1 min in 10 μM fluorescence DCFH-DA, 25°C (Myhre, 2003); fluorescence intensity would be checked immediately after being treated by solvent. In fertilization, HCG was used as a stimulant for spawning. All values are means \pm S.D.; values with different letters are significantly different.

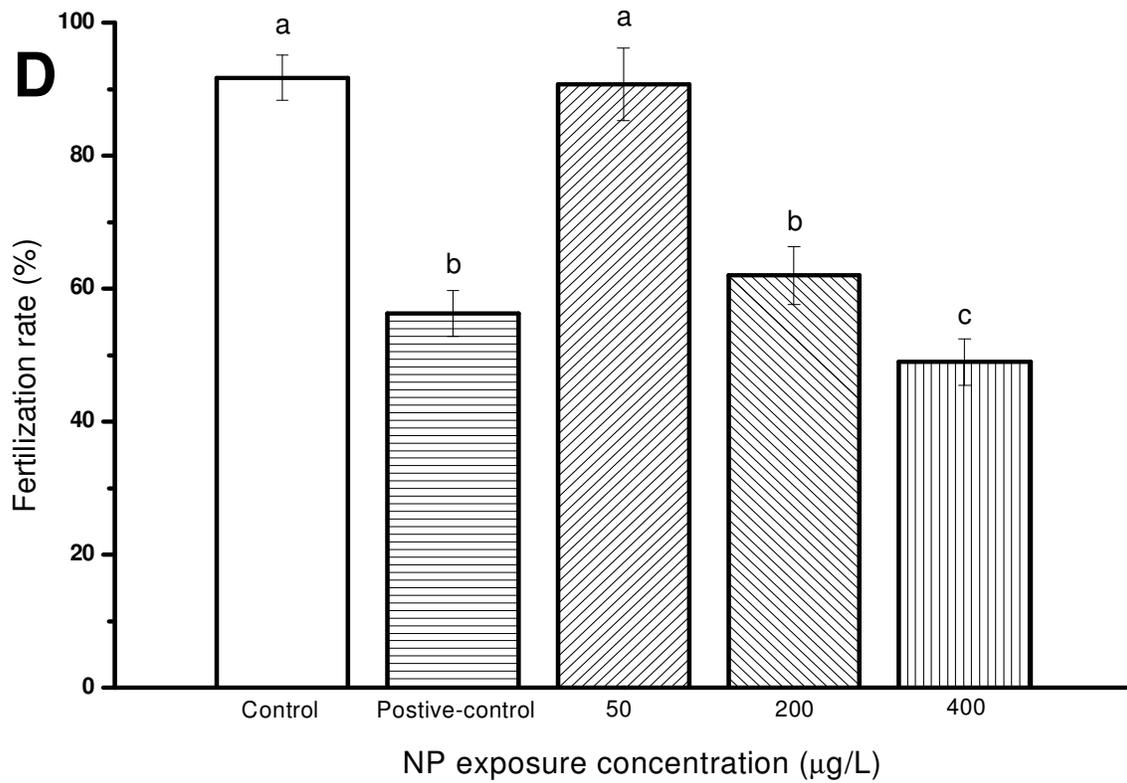
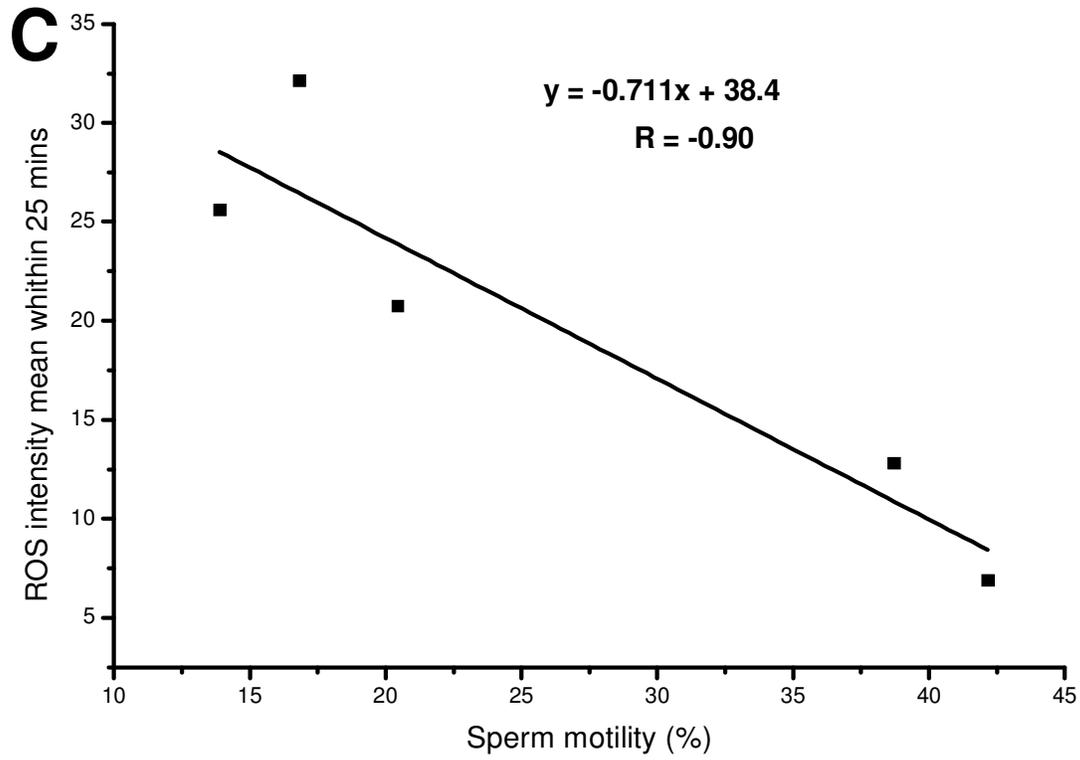


Figure 2. Contd.

Table 2. Dynamic parameters and integrity of sperm exposed to NP directly during fertilization.

Time (min)	Group	VSL ($\mu\text{m/s}$)	VAP ($\mu\text{m/s}$)	Sperm integrity (%)
2	0.04% alcohol (Control)	7.83 \pm 0.31 ^a	9.74 \pm 2.04 ^a	77.33 ^a
	50 $\mu\text{g/l}$ H ₂ O ₂	12.94 \pm 0.04 ^b	19.24 \pm 0.76 ^b	73.06 ^a
	50 $\mu\text{g/l}$ NP	8.06 \pm 0.25 ^a	17.24 \pm 1.00 ^c	81.85 ^b
	200 $\mu\text{g/l}$ NP	10.25 \pm 0.16 ^b	14.43 \pm 1.23 ^d	76.14 ^a
	400 $\mu\text{g/l}$ NP	11.75 \pm 0.82 ^b	20.86 \pm 2.36 ^b	77.47 ^a
6	0.04% alcohol (Control)	8.25 \pm 0.68 ^a	14.16 \pm 2.55 ^a	75.22 ^a
	50 $\mu\text{g/l}$ H ₂ O ₂	7.87 \pm 1.22 ^b	10.87 \pm 0.27 ^b	54.27 ^c
	50 $\mu\text{g/l}$ NP	8.34 \pm 0.78 ^a	13.43 \pm 0.63 ^a	72.46 ^a
	200 $\mu\text{g/l}$ NP	9.86 \pm 1.26 ^a	14.85 \pm 1.04 ^a	66.38 ^b
	400 $\mu\text{g/l}$ NP	7.24 \pm 0.56 ^b	11.98 \pm 0.01 ^b	50.25 ^d
12	0.04% alcohol (Control)	11.26 \pm 0.36 ^a	17.25 \pm 0.84 ^a	76.43 ^a
	50 $\mu\text{g/l}$ H ₂ O ₂	6.35 \pm 0.44 ^b	9.13 \pm 0.25 ^b	36.38 ^c
	50 $\mu\text{g/l}$ NP	10.73 \pm 0.68 ^a	14.40 \pm 0.44 ^c	74.22 ^a
	200 $\mu\text{g/l}$ NP	6.37 \pm 0.25 ^b	10.36 \pm 0.76 ^d	61.43 ^b
	400 $\mu\text{g/l}$ NP	5.89 \pm 0.41 ^b	8.15 \pm 0.99 ^b	31.29 ^d
25	0.04% alcohol (Control)	10.32 \pm 0.18 ^a	16.31 \pm 0.18 ^a	70.56 ^a
	50 $\mu\text{g/l}$ H ₂ O ₂	4.30 \pm 0.44 ^b	7.98 \pm 0.58 ^b	31.03 ^b
	50 $\mu\text{g/l}$ NP	8.82 \pm 0.03 ^c	12.16 \pm 0.82 ^c	69.04 ^a
	200 $\mu\text{g/l}$ NP	4.77 \pm 0.56 ^b	7.54 \pm 0.93 ^b	51.26 ^c
	400 $\mu\text{g/l}$ NP	2.43 \pm 0.17 ^d	5.40 \pm 0.31 ^d	25.75 ^d

VSL: Velocity straight line; VAP: average path velocity. n=20, 3 replicates. After pithing, gonad of *B. raddei* was removed to prepare the sperm suspensions of 5.00×10^6 sperm/ml with 0.04% alcohol (control), 50 $\mu\text{g/l}$ H₂O₂ (positive-control), 50 $\mu\text{g/l}$, 200 $\mu\text{g/l}$ and 400 $\mu\text{g/l}$ NP respectively for the analysis by CASA. Sperm integrity was calculated according to the morphology observation under the electron microscope, n=20, 3 replicates. Values are means \pm S.D. Values with different letters are significantly different, p<0.05. The 0 min data: analyzed immediately after gonad broken in DW, 0.04% alcohol or NP solvent.

(≥ 200 $\mu\text{g/l}$) compare to the control in our other reproductive behavior observation experiments. A risk that NP can disrupt the reproduction behavior of *B. raddei* exists.

During fertilization, the *B. raddei* sperm were exposed to NP directly. Whether or not estrogen receptors are present on sperm of amphibian or fish is unclear. However, Tributyltin, another estrogen-like compound, impairs sperm motility as an aromatase or mitochondrial respiration inhibitor other than estrogen (McAllister and Kimer, 2003; Rurangwa et al., 2002), and NP reduces the sperm viability in Japanese medaka by the nongenomic effects of NP (Yutaka et al., 2007). Similarly, this study reveals that NP could alter *B. raddei* sperm dynamic parameters during the period of ejaculation and insemination.

Appropriate level of ROS would facilitate the capaci-

tation of human sperm, but excessive ROS level can lead to sperm inactivation (Aitken et al., 1993; De Lamirande and Gagnon, 1992; De Lamirande and Gagnon, 1993). In this study, ROS levels were also examined, and based on the results, both NP and H₂O₂ significantly increased the ROS level in sperm during exposure; these also changed the sperm dynamic parameters (motility, VSL, VAP). Meanwhile, sperm motility was negatively correlated with the mean values of ROS value within 25 minutes ($R = -0.90$). It is likely that the ROS level induced by NP (≥ 200 $\mu\text{g/l}$) greatly exceeded the appropriate level for *B. raddei* sperm capacitation. The excessive ROS level may have induced the change in sperm dynamic parameters.

Yutaka et al. (2007) found that NP causes a reduction in sperm viability in teleost fish in a short period (≤ 60 s)

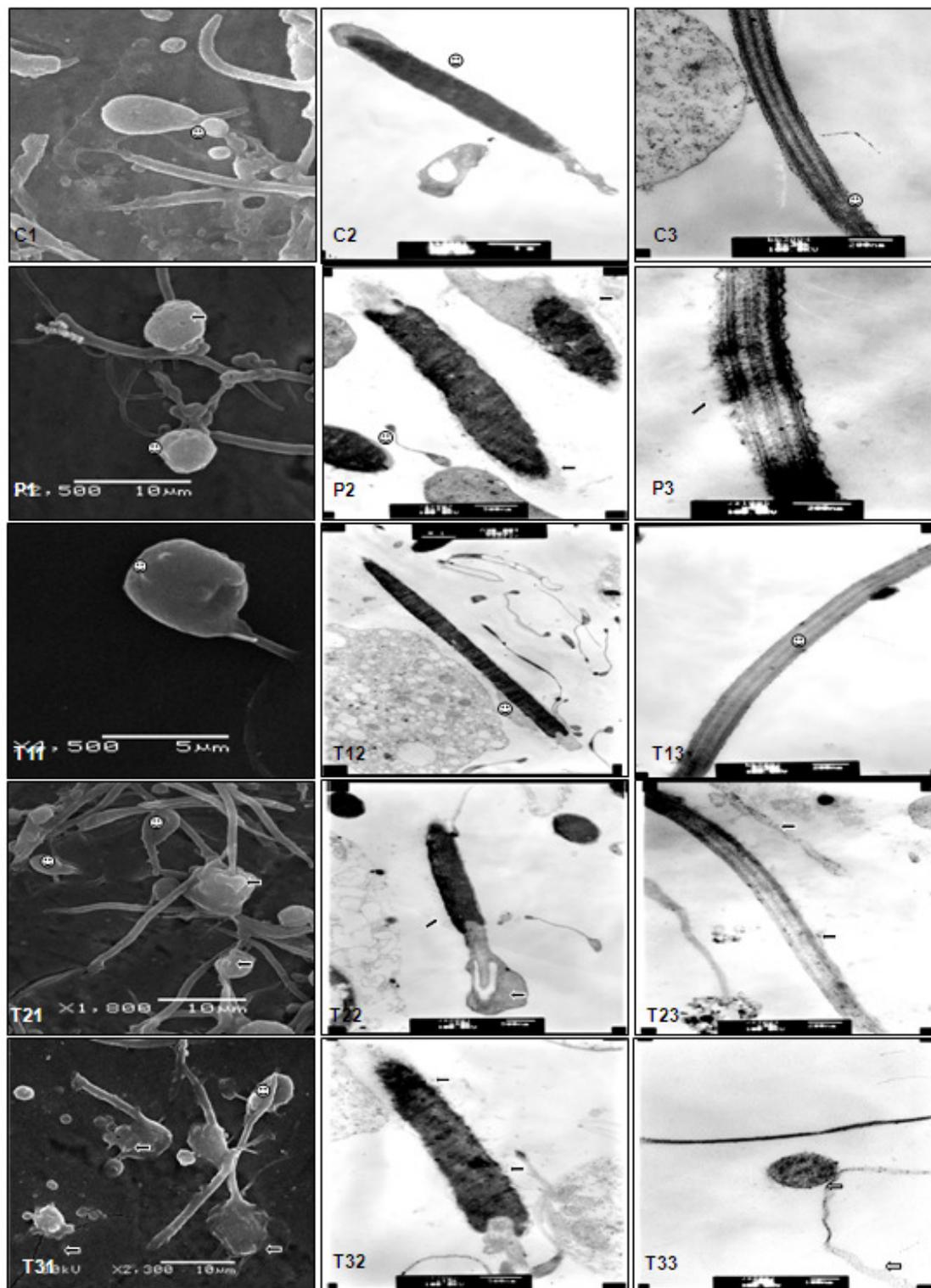


Figure 3. Morphology observation of sperm exposed to NP directly. *B. raddei* accommodated in dechlorinated tap water without NP, pH 7.3 for 7 days. After pithing, gonad of *B. raddei* was removed to prepare the sperm suspensions of 5.00×10^6 sperm/ml with 0.04% alcohol, 50 $\mu\text{g/l}$ H_2O_2 , 50, 200, or 400 $\mu\text{g/l}$ NP respectively. After 12 min exposure, sperm was fixed in 2% glutaraldehyde. The first column showed the photo of sperm obtained from scanning electron microscope. The second and third column displayed photo of sperm head and tail taken from transmission electron microscopy. [☺]: good sperm; [☹]: impaired sperm head or tail. C1-3: 0.04% alcohol (Control). P1-3: 50 $\mu\text{g/l}$ H_2O_2 (Positive-control). T11-T13: 50 $\mu\text{g/l}$ NP. T21-T23: 200 $\mu\text{g/l}$ NP. T31-T33: 400 $\mu\text{g/l}$ NP.

without impairing sperm membrane. However, compared with fish, the sperm of *B. raddei* would be exposed to NP for several minutes or more before insemination; in this case, the fertilization was different and more complicated than that of fish. Morphologic observation showed that NP impaired the sperm structure within 12 min of NP exposure. A similar effect was observed in the positive-control (H_2O_2), indicating that ROS would be responsible for the results above. Generally, ROS causes the lipid peroxidation of the cell membrane and increase membrane permeability (McLaughlin et al., 1998), which would be a way to cause the impairment of sperm in water. ROS level was higher in 50 $\mu\text{g/l}$ (positive-control) H_2O_2 than 400 $\mu\text{g/l}$ NP treatment group, whereas more impaired sperms and more serious impairment were observed in 400 $\mu\text{g/l}$ NP treatment group (Figure 2B, Figure 3, Table 2). This indicates that ROS is not the only inducer for the impairment of sperm membrane. Further investigation is needed to address this issue.

In this study, the sperm has been directly exposed to NP to evaluate the effect of the latter on *B. raddei* sperm viability. Our study has shown that NP ($\geq 200 \mu\text{g/l}$) could significantly decrease fertilization rate ($p < 0.01$). Glogowski et al., (2005) found that the fertilization rate and hatching of *Siberian sturgeon* embryos are highly positively correlated with sperm motility and VSL. Meanwhile, Martinez and Cabada (1996) have found that sperm capacitation and inactivation are gradual processes during fertilization. In this study, the sperm motility and sperm VSL of control and 50 $\mu\text{g/l}$ NP treatment group showed a similar manner as that described in the work of Martinez and Cabada (1996). In other treatment groups, the excessive ROS level induced by NP or H_2O_2 disrupted the motility and VSL of sperm which was unfavorable to fertilization. Meanwhile, NP ($\geq 200 \mu\text{g/l}$) impaired the sperm structure, which resulted in the reduction of fertilization rate. According to the data, the changes in sperm dynamic parameters and the impairment of morphology were compatible with the reduction of fertilization.

In this study, the effects of NP on eggs could not be excluded; however, Andrea et al. (2007) had reported that the entry of pollutants into eggs through the jelly coat is a slow process, wherein the jelly coat reduces the chemical absorption. Compared with the egg, the sperm was exposed to NP without any protection, thus the adverse effect of NP in fertilization should mainly be attributed to its effects on the sperm. Further information is needed to explore whether or not such a difference exists between sperm and egg exposed to NP.

The concentration of NP that clearly affected sperm in this study (200 $\mu\text{g/l}$) is about 2.5 times of highest NP concentration found in natural aquatic environments (0.065-83 $\mu\text{g/l}$) (Wang et al., 2006). The NP in the aquatic environments of Lanzhou Region has potentially negative effects on the fertilization of *B. raddei*. Besides, there would be a risk of NP on *B. raddei* reproductive behavior. Moreover, there would be a greater risk for the effects of

NP on the reproduction of *B. raddei* considering the longer NP exposure time during the complete life cycle. The ROS induced by NP and the membrane impairment by NP in an unknown way maybe the way to affect sperm dynamic parameters and the fertilization rate. The effects of NP on *B. raddei* sperm and fertilization rate presented in this study indicate that NP exposure of amphibian is complex. Therefore, the risks of amphibian or other aquatic animals exposed to pollutants in fertilization period need to be adequately evaluated.

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