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Effect of Nonyphenol on the Expression of Hepatic Vitellogenin mRNA in Male *Bombina orientalis* (Boulenger)

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A number of studies have assessed endocrine disruption as a contributing factor in the global decline of amphibians (Renner 2002). Evidence for abnormalities such as feminization of wildlife animals implies contamination by estrogenic compounds at concentrations high enough to be of concern (Vos et al. 2000; Hutchinson et al., 2000). In amphibians, xenoestrogens can induce the expression of estrogen-responsive genes and proteomes and is associated with adverse effects in amphibian development (Purdom et al. 1994; Palmar and Palmar 1995; Hayes and Menendez 1999; Kloas et al. 1999; Lutz and Kloas 1999; Mann and Bidwell 2000; Hurter et al. 2002; Mosconi et al. 2002; Bevan et al. 2003; Mackenzie et al. 2003; Gye 2004; Kohno et al. 2004). A number of alkylphenolic compounds such as 4-octylphenol, 4-nonylphenol, 4-nonylphenoxycarboxylic acid, and 4-nonylphenoldiethoxylate used in a variety of commercial products are estrogenic in vertebrates. Of these, 4-nonylphenol (NP), one of the weak estrogenic endocrine disrupters is associated with adverse reproductive and health effects in wildlife and laboratory animals (Chapin et al. 1999). NP has been found in sewage treatment plant effluents, river systems, estuaries, sediments and tissues and may already be ubiquitous (Kuch and Ballschmiter 2001; Khim et al., 2001; Li et al. 2004). Accordingly, NP in the aquatic environment could induce toxic effects by coming into direct contact during embryonic as well as larval life in amphibians inhabiting a contaminated aquatic environment.

Because of its structural similarity to estrogen, NP can induce the estrogen-responsive transcription of genes and is associated with adverse effects in amphibian development (Kloas et al. 1999; Lutz and Kloas 1999). Vitellogenin (Vg), an egg yolk precursor protein is physiologically synthesized in female liver by estrogen in oviparous vertebrates. In amphibian, several xenoestrogens, as well as natural estrogen, stimulate synthesis of estrogen responsive including Vg proteins *in vivo* as well as *in vitro* (Wangh and Knowland 1975; Carnevali and Mosconi 1992; Carnevali et al. 1995; Bogi et al. 2003; van Wyk et al. 2003; Gye 2004; Gye and Kim 2005). Therefore, the plasma Vg protein or hepatic Vg mRNA has been used as a biomarker for feminization of the male frog and became method to characterize the estrogenic potential of environmental media (Rotchell and Ostrander 2003).

Bombina orientalis (Boulenger) is one of the most common amphibians in the world and comprising a large proportion of their total number in Korea. However,

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little is known about the endocrine disruption effect of NP on this frog species. Recently, quantitative analysis of hepatic Vg mRNA level was established in *Bombina orientalis* (Gye and Kim 2005). In the present study we examined the effect of NP on the expression of hepatic Vg mRNA in adult male *B. orientalis*

MATERIALS AND METHODS

Bombina orientalis were collected from Kapyung Cheon, Kyonggi-Do in Korea during April 2005. Frogs were put in aquaria maintained at a diurnal 14:10h light: dark cycle at 20-22℃ and fed mealworm three times a week. Adult males with a mean body weight (BW) of 10.0 ± 0.5g were selected (n = 4) and given an intraperitoneal injection of NP (0.01, 0.1, 1, 10 and 100 mg/kg BW) dissolved in 100 μL of sesame oil. Vehicle control males (n = 4) were given sesame oil only. Frogs were returned to the aquaria, and sampled 48 h after the vehicle or NP injection. Frogs were anesthetized by inhalation of ether to minimize pain, and livers were dissected. Liver total RNA was isolated using TRI reagent (Moleular Research Center, Inc., Cincinati, Ohio) as directed by the manufacturers. Amounts of RNA were determined and then stored at –85℃ until use. RNA (1 μg) was reverse transcribed for 60 min at 42℃ in a 20 μL reaction with 50 units of MuLV reverse transcriptase and 2.5 μM oligo $d(T)_{16}$ primer by the standard protocol from the supplier (Applied Biosystems, Foster City, CA). Semiquantitative RT-PCR was performed according to Gye and Kim (2005). Briefly, 25 μL of a PCR mixture containing 0.5 μL of the RT product, 1.25 units of Ex Taq™ polymerase (Takara, Japan), 1X Ex PCR™ Buffer II (Mg^{2+}-free), 2.5 mM $MgCl_2$, 0.4 mM of each dNTP mixture, and 0.4 μM of each primer. A pair of specific primers for Vg were designated 5'- TGC TGA TCC ATC TGT CCT GA-3' (forward) and 5'- AAC AGG CTG TGT GAG CTT GA-3' (reverse). ß actin mRNA was amplified as an internal control using preimer set 5'-GAG AGG TAT CCT GAC CCT GAA GTA-3' (forward) and 5'-ATA ACC TTC ATA GAT GGG CAC AGT-3' (reverse). PCR were performed with temperature gradient mode using i-Cyler (BioRad, CA). Each cycle consisted of the following: 95℃, 30s; 56℃, 30s; 72℃, 45s. RT-PCR was conducted at 30 or 34 cycles for Vg and 26 cycles for ß actin. PCR products were resolved on 2 % agarose gels containing 0.5 μg/mL ethidium bromide and photographed under UV light. The amounts of RT-PCR products were quantified by analyzing the band intensity with imaging software (Scion Corporation, Frederick, MD). The relative density of RT-PCR product for Vg mRNA vs. that of the ß actin mRNA was presented. Statistical significance was analyzed by the Student's *t*-test and accepted at $p < 0.05$.

RESULTS AND DISCUSSION

The NP dosing at 100 mg/kg evoked neither death nor narcosis at 2 days after injection, suggesting that NP dose examined in this study did not evoke a systemic acute toxic effect on *B. orientalis*. RT-PCR analysis revealed the expression of very high level of hepatic Vg mRNA in female liver following 30 cycle of PCR amplification. In males only, Vg mRNA was hardly detected and apparently induced following 2 days after single injection of NP (100 mg/kg) (Fig. 1). This suggests that NP has an estrogen mimicking effect on adult male *B. orientalis*.

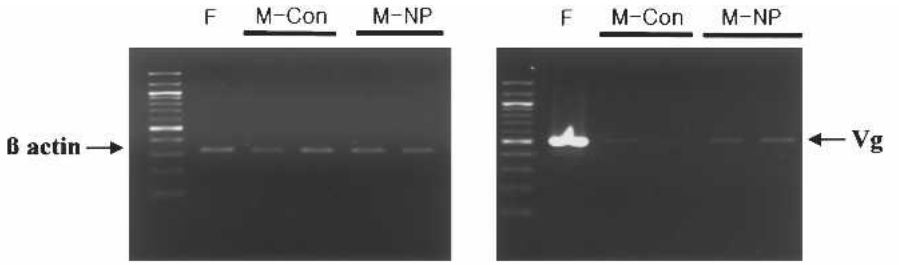

Figure 1. RT-PCR of Vg and ß actin mRNA in liver from *B. orientalis*. F, female; M-Con, un-injected male; M-NP, male 48 hour after a single injection of NP (100 mg/kg). Thirty cycles of PCR amplification was conducted for Vg.

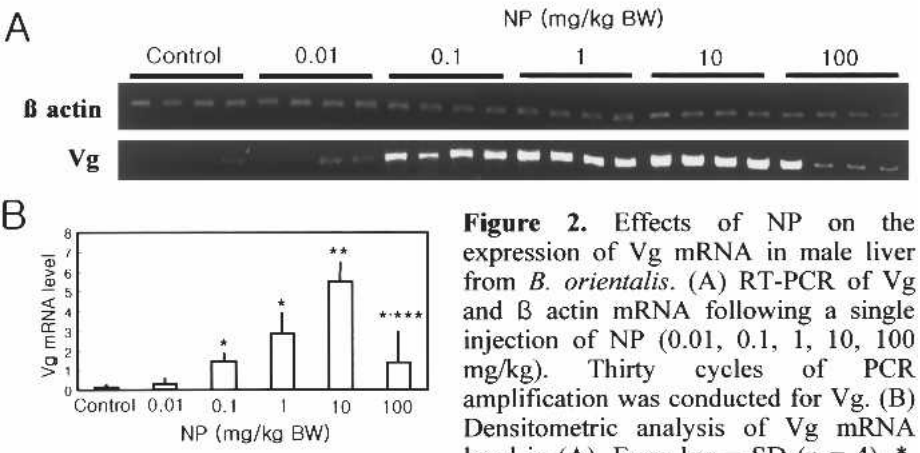

Figure 2. Effects of NP on the expression of Vg mRNA in male liver from *B. orientalis*. (A) RT-PCR of Vg and ß actin mRNA following a single injection of NP (0.01, 0.1, 1, 10, 100 mg/kg). Thirty cycles of PCR amplification was conducted for Vg. (B) Densitometric analysis of Vg mRNA level in (A). Error bar = SD (n = 4). *, **, and ***, significantly different from vehicle control, others, and 10 mg/kg NP group, respectively by Student's *t* test.

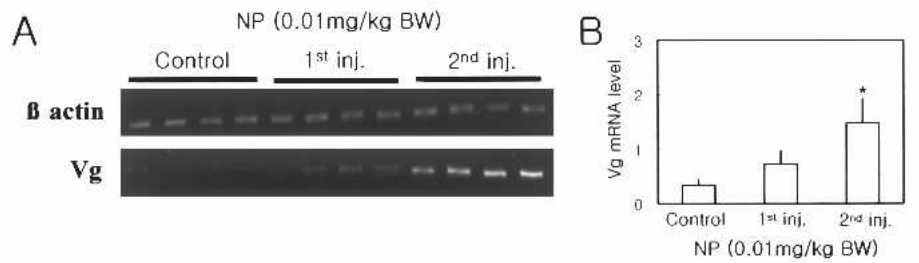

Figure 3. Effects of repeated exposure to NP on the expression of Vg mRNA in male liver from *B. orientalis*. (A) RT-PCR of Vg and ß actin mRNA following twice injections of NP (0.01 mg/kg) with a two days interval. Thirty cycles of PCR amplification was conducted for Vg. (B) Densitometric analysis of Vg mRNA level in (A). Error bar = SD (n = 4). *, significantly different from vehicle control by Student's *t* test.

In 0.01 mg/kg NP-treated males, Vg mRNA level was a little increased but not statistically different from vehicle controls. At 0.1 – 10 mg/kg dose NP significant increased Vg mRNA level in dose-dependent manner. In 100 mg/kg NP dosing group, Vg mRNA level was significantly higher than vehicle control but significantly lower than 10 mg/kg NP (Fig. 2). Therefore it is suggested that the single lowest effective dose level for NP to induce Vg mRNA is between 0.01 - 0.1 mg/kg in male *B. orientalis*. In NP (0.01 mg/kg BW)-primed males, a second injection of the same dose of NP with a two day interval resulted in a marked increase in Vg mRNA (Fig. 3). This suggests that the primary injection of low concentration of NP did not evoke observable Vg expression but it may sensitize the transcription mechanism for hepatic Vg expression, allowing for a marked increase in Vg mRNA in response to second injection of NP in male frogs.

According to the guideline from the National Toxicology Program (NTP), it can be calculated that an acceptable exposure value for NP lacking overt toxic effects on this frog species is 0.01 μg/kg/day. Because repeated exposure to NP together with other xenoestrogens, such as BPA and octylphenol is most likely unavoidable to frogs in the contaminated habitat, it should be emphasized that NP even at a no observable effect concentration (NOEC) may elicit endocrine disruption in this frog species when other xenoestrogens are also present in environment (Rajapakse et al. 2002). In fresh water systems in Korea, the environmentally relevant concentration of NP ranged 23.2 to 187.6 ng/L (Li et al. 2004). In fish, NP can exert endocrine disruption at 0.17 - 0.79 μg/L (Sumpter and Jobling 1993). Taken together, our data suggests that aquatic contamination by NP in fresh water systems in Korea reached the threshold level to cause endocrine disruption in this frog species.

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