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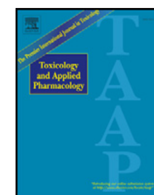

# Triazine herbicides inhibit relaxin signaling and disrupt nitric oxide homeostasis

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ABSTRACT

Triazines are herbicides that are widely used worldwide, and we previously observed that the maternal exposure of mice to simazine (50 or 500 μg/kg) resulted in smaller ovaries and uteri of their female offspring. Here, we investigated the underlying mechanism that may account for the reproductive dysfunction induced by simazine. We found that following maternal exposure, simazine is transmitted to the offspring, as evidenced by its presence in the offspring ovaries. Analyses of the simazine-exposed offspring revealed that the expression of the relaxin hormone receptor, relaxin-family peptide receptor 1 (RXFP1), prominently decreased in their ovaries and uteri. In addition, downstream target genes of the relaxin pathway including nitric oxide (NO) synthase 2 (*Nos2*), *Nos3*, matrix metallopeptidase 9 (*Mmp9*), and vascular endothelial growth factor (*Vegf*) were downregulated in their ovaries. Moreover, AKT and extracellular signal-regulated kinases (ERK) levels and their phosphorylated active forms decreased in simazine-exposed ovaries. *In vitro* exposure of the human ovarian granulosa cells (KGN) and uterine endometrium cells (Hec-1A) to very low concentrations (0.001 to 1 nM) of triazines including atrazine, terbuthylazine, and propazine repressed NO production with a concurrent reduction in RXFP1, NOS2, and NOS3. The inhibitory action of triazines on NO release was dependent on RXFP1, phosphoinositol 3-kinase (PI3K)/AKT, and ERK. Radioligand-binding assay also confirmed that triazines competitively inhibited the binding of relaxin to its receptor. Therefore, the present study suggests that triazine herbicides act as endocrine disrupters by interfering with relaxin hormone signaling. Thus, further evaluation of their impact on human health is imperative.

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## 1. Introduction

The triazine family of chemicals, which includes simazine, atrazine, propazine, and terbuthylazine, is widely used as herbicides worldwide to control the growth of broad-leaved and grassy weeds as well as algae (Abbas et al., 2008; Herranz et al., 2008). Owing to their effectiveness, simazine and atrazine have been frequently used in quantities of approximately 5 to 7 and 64 to 76 million pounds, respectively, for both agricultural and nonagricultural purposes annually in the US (USEPA, 2006b; USEPA, 2006a). Owing to herbicide runoff, they are the most commonly detected pesticides in surface and groundwater in regions worldwide including the US, Europe, China, and Australia (Ma et al., 2003; Gunasekara et al., 2007). Human exposure to environmental triazine herbicides is evident by their presence in humans at up to 1 μg/L of simazine, which has been detected in the urine of pregnant women (Chevrier et al., 2011), while 0.02 mg/kg of atrazine has been found in breast milk (Balduini et al., 2003). Moreover, epidemiologic evidence indicates that maternal exposure to atrazine and simazine is associated with fetal growth restriction (Ochoa-Acuna et al., 2009; Chevrier et al., 2011).

Endocrine disruptors (EDs) are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones, and elicit adverse reproductive, developmental, neurological, and immunological effects in both humans and wildlife (Brevini et al., 2005; WHO, 2012). Simazine and atrazine were included in the final list of chemicals in the initial Tier 1 screening in the EPA's ED screening program (EDSP) in 2009. In previous mouse studies, we provided *in vivo* evidence that simazine is an ED based on the significantly smaller reproductive organs including the ovaries, uterus, testes, Cowper's glands, and epididymis of mouse offspring maternally exposed to simazine during gestational and lactational periods (Park and Bae, 2012; Park et al., 2014).

Relaxin is a heterodimeric peptide hormone that belongs to the insulin superfamily (Bryant-Greenwood and Schwabe, 1994). It is predominantly produced by the ovaries, and its physiological functions during pregnancy and the female reproductive cycle are well characterized (Halls et al., 2015). Recent studies have extended its pleiotropic roles to other systems such as the heart, kidney, lung, and liver; suggesting that it controls vasodilation, angiogenesis, tissue fibrosis, and would healing (Halls et al., 2015). The relaxin/insulin-like family peptide receptor 1 (RXFP1), the cognate receptor for relaxin, is a G-protein-

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coupled seven-transmembrane receptor (Hsu et al., 2002) expressed in reproductive organs including the ovary, uterus, mammary gland, placenta, testis, and prostate, as well as other tissues (Hsu et al., 2000; Bathgate et al., 2013). The association of relaxin with RXFP1 induces coupling to the $G\alpha_s$ subunit that activates adenylyl cyclase to increase cyclic adenosine monophosphate (cAMP) production (Hsu et al., 2002; Halls et al., 2006). Its coupling to $G\alpha_{i3}$ dissociates $G\beta\gamma$, which triggers phosphoinositol 3-kinase (PI3K), subsequently activating AKT (protein kinase B; PKB) and leading to endothelial nitric oxide synthase (eNOS/NOS3) activation, resulting in nitric oxide (NO) production (Bathgate et al., 2013). The activated PI3K also stimulates extracellular signal-regulated kinases 1 and 2 (ERK1/2), resulting in NO production by neuronal NOS (nNOS/NOS1) induction (Chow et al., 2012). In addition, ERK-mediated activation of inducible NOS (iNOS/NOS2) by relaxin has been demonstrated (Alexiou et al., 2013). In a previous study, we reported downregulated RXFP1 expression in the testes of simazine-exposed mice (Park and Bae, 2012). However, the influence of environmental toxicants on relaxin signaling in female reproductive organs is presently unknown to the best of our knowledge.

Therefore, in this study, we analyzed the ovaries and uterus of offspring of mice exposed to simazine maternally and found downregulated expression of critical molecules involved in the relaxin signaling pathway. In addition, we provide biochemical and molecular evidence that demonstrates triazine herbicides act as potent antagonists of binding of relaxin to its receptor, leading to disruption of relaxin-mediated NO homeostasis.

## 2. Materials and methods

### *2.1. Chemicals*

Simazine (CAS No. 122-34-9; 99.9% pure), atrazine (CAS No. 1912-24-9; 98.8% pure), terbuthylazine (CAS No. 5915-41-3; 98.8% pure), propazine (CAS No. 139-40-2; 99.0% pure), and human relaxin-1 (CAT No. R2156) were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). The U0126 was obtained from Calbiochem (San Diego, CA, USA) while all other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

### *2.2. Animal study*

The *in vivo* simazine-exposure experiment with mice was performed as described previously (Park et al., 2014). Briefly, 11-week-old virgin C57BL/6 female mice and 18-week-old DBA/2 male mice (SLC, Tokyo, Japan) were mated after acclimatization. The pregnant female mice were administered simazine (0, 5, 50, or 500 μg/kg body weight per day) daily by gavage in 0.1 mL corn oil starting from gestation day 12 to postnatal day 20. The 9-week-old female offspring mice (F1) were sacrificed, their tissues were collected in RNase-free water, and then snap-frozen in liquid nitrogen gas or stored at 4 °C in 10% formaldehyde (Sigma, Steinheim, Germany). The mice were given water *ad libitum* and AIH-76A rodent feed (Research Diets, New Brunswick, NJ, USA). The animals were treated humanely according to the experimental protocol approved by the CHA University Institutional Animal Care and Use Committee.

### *2.3. Simazine extraction*

Extraction of simazine from ovaries of 9-week-old F1 offspring was performed as described by Brandhonneur et al. (2015) with modifications. The ovarian tissues were homogenized with magnesium sulfate ($MgSO_4$, Agilent Technologies, Santa Clara, CA, USA). The high-performance liquid chromatography (HPLC)-grade acetonitrile and hexane (Fisher Scientific, Pittsburgh, PA, USA) were added, and the samples were incubated for 10 min at room temperature with shaking followed by incubation for 30 min at −20 °C and centrifugation for 5 min at 25,000 × *g*. The organic layer was added to MgSO4, primary and secondary amine (PSA) (Agilent Technologies), and C18 (Agilent Technologies) and the samples were incubated for 10 min at room temperature with shaking and then centrifuged for 5 min at 25,000 × g. The supernatant was transferred to a clean vial, evaporated to dryness under nitrogen, and the sample was reconstituted in acetone and the internal standard (IS, 2-chloronaphtalene) for gas chromatography–mass spectrometry (GC–MS) analysis.

### *2.4. GC–MS analysis*

A 7890A Agilent GC (Agilent Technologies) model equipped with a 5975C MSD detector (Agilent Technologies), autosampler (7683 B series, Agilent Technologies), split/splitless injector, injection module, and Chemstation software (Agilent Technologies) were used for the GC–MS analysis. The inlet temperature was set to 25075C MSD detector (Agilent Technologies, and helium was used as the carrier gas. The auxiliary, MS source and MS quad temperatures were set to 280, 230, and 150quad temperatures were set to 280, 230ilent Technologies), autosampler (7683 B series, Agilent Technologies), split/splitless injector, injection module and the splitless mode was used. The mass range was 70–400 Da and data were obtained in the SIM mode with the selection of two *m*/*z* values (*m*/*z* 186 and 201). The initial oven temperature was set at 100 nt Agilent Technologies), split/splitless injector, injection n. The analysis was performed with six replicates (biological and experimental replicates = 3 and 2, respectively). For the quantitative analysis of simazine in mouse ovary, internal standard calibration was used, which was carried out using GC–MS under the same conditions. The calibration standard of simazine was analyzed in triplicate, and the intensities were *m*/*z* 201 and 162 were for the quantification of simazine and IS, respectively. The ratios of ion fragments (ion fragments of simazine standard/ion fragments of internal standard) *versus* concentrations were used to obtain the calibration curve. The amount of simazine was calculated using the regression equation of the calibration curve. The regression equation, correlation coefficient, limit of detection (LOD), and limit of quantitation (LOQ) are listed in Supplementary Table 1.

### *2.5. Cell culture*

The human KGN ovarian granulosa (provided by Drs. Nishi and Yanase, Kyushu University, Fukuoka, Japan) and human Hec-1A endometrial (American Type Culture Collection, ATCC, Manassas, VA, USA) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Caisson Laboratories, North Logan, UT, USA) and McCoy's 5A (Caisson Laboratories) media, respectively, containing 10% fetal bovine serum (FBS, Caisson Laboratories) and 1% penicillin-streptomycin (Caisson Laboratories).

### *2.6. RNA preparation*

Total RNA was isolated from the ovaries of 9-week-old F1 mice, as well as the KGN and Hec-1A cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

### *2.7. qRT-PCR*

The concentration and quality of RNA isolated were determined using an ND-1000 spectrophotometer (NanoDrop, Waltham, MA, USA). The RNA was reverse-transcription to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. All the cDNAs used in the qPCR were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The qPCRs were performed using an iQ™ SYBR Green Supermix (Bio-Rad Laboratories) while the gene expression was quantified using the Δ-Δ-CT method, and the qPCRs were performed using a CFX-96™ thermal cycler and detection system (Bio-Rad Laboratories). The primer

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