Effects of Vinclozolin on Nrf2 Testicular Expression in Sexually Mature Boars

Anna Anandan, Tim Evans, Tom Reilly, Susan Schommer.
Veterinary Medical Diagnostic Laboratory, College of Veterinary Medicine, University of Missouri, Columbia, MO

Endocrine Disruptor Background Information

Many chemicals can interfere with normal endocrine function (i.e., endocrine disrupting chemicals or EDCs) and cause adverse reproductive effects, especially in experimental rodent models. Swine are more similar to humans than rodents, so the porcine model might be a more accurate predictor of the reproductive risks associated with human EDC exposures. Vinclozolin (VCZ), a dicarboximide fungicide, has been associated with antiandrogenic effects in animal models. Preliminary data suggest that adult boars are more susceptible than adult male rodents to the adverse reproductive effects of VCZ and its metabolites.

OVERALL RESEARCH OBJECTIVE

- Further development and refinement of a porcine model for human risk assessment involving exposures to endocrine disrupting chemicals (EDCs).

In Vivo Experimental Approach

- Thirty-two post-pubertal boars were dosed orally with 0, 0.25, 5 or 100 mg of VCZ (technical grade; 95% pure)/kg BW /day (four replicates of n = 2 in each treatment group; total of eight animals per replicate) for 15 days (Days 0-14).
- Blood samples were collected on Day 0 prior to the beginning of treatment and on Days 8 and 15 for analyses for immunoreactive testosterone and total estrogen concentrations.
- Semen samples were collected prior to Day 0 and on Days 3, 7, 10 and 14 for computer-assisted sperm analysis (CASA) and sperm ubiquitin tag immunoassay (SUTI).
- Boars were humanely euthanized and necropsied on Day 15. Organ weights were determined for selected tissues, portions of which were flash-frozen in liquid nitrogen or fixed in 10% neutral buffered formalin or modified Davidson's fixative.
- Sperm were collected from the right cauda epididymidis, and CASA, SUTI, cryopreservation, and IVF were performed.
- Histologic processing and evaluation (including counting of testicular interstitial cells and staging of the seminiferous tubules) were performed on selected PAS- and/or H&E-stained tissue sections.
- Statistical analyses were performed using ANOVA and, where indicated, repeated measures ANOVA.
- Selected flash-frozen tissues were archived for future analyses evaluating the expression of various genes.

Nrf2 Background Information

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor which regulates endogenous antioxidant responses. Under normal conditions, Nrf2 is localized in the cytoplasm. Under conditions of oxidative stress, Nrf2 translocates to the nucleus to induce the transcription of genes regulating cellular responses to oxidative stress. Nrf2 localization and expression within rodent testes change in response to oxidative stress. Oxidative stress is one mechanism by which EDCs damage testes, and VCZ and its metabolites can induce oxidative damage.

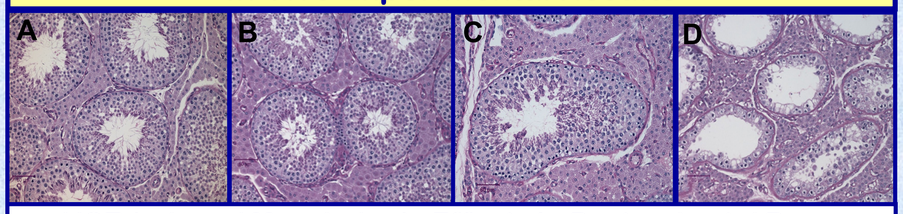
RESEARCH HYPOTHESES FOR TESTICULAR EXPRESSION OF Nrf2

- Subacute oral exposure of sexually mature boars to VCZ will affect the testicular expression as well as localization of Nrf2, in a dosage-dependent fashion.

Specific Aims

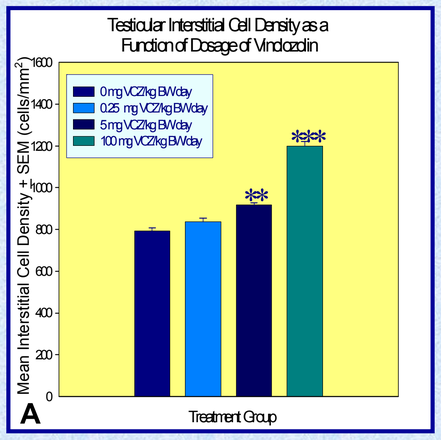
- Evaluate of the effects of VCZ dosage on oxidative stress within the testes of sexually mature boars.
- Characterize VCZ-related changes in Nrf2 expression using immmunohistochemical (IHC) staining.

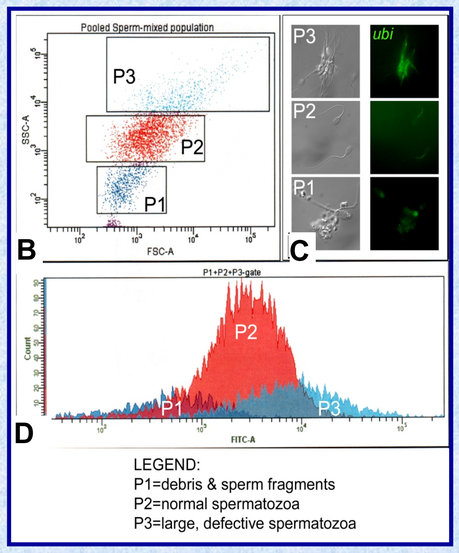
In Vivo Experimental Results



VCZ-induced Morphologic Effects in Post-pubertal Boars

Figure 1. Sections of PAS-stained testes from boars treated with 0, 0.25, 5 and 100 mg VCZ/kg BW are shown in A, B, C and D, respectively (200X magnification). Histological changes were noted in the 5 and 100 mg VCZ treatment group, and there here were increased mean percentages of abnormal seminiferous tubules in boars treated with 5 or 100 mg VCZ/kg BW/day (20.8 ± 1% and 33.9 ± 12.3%, respectively).

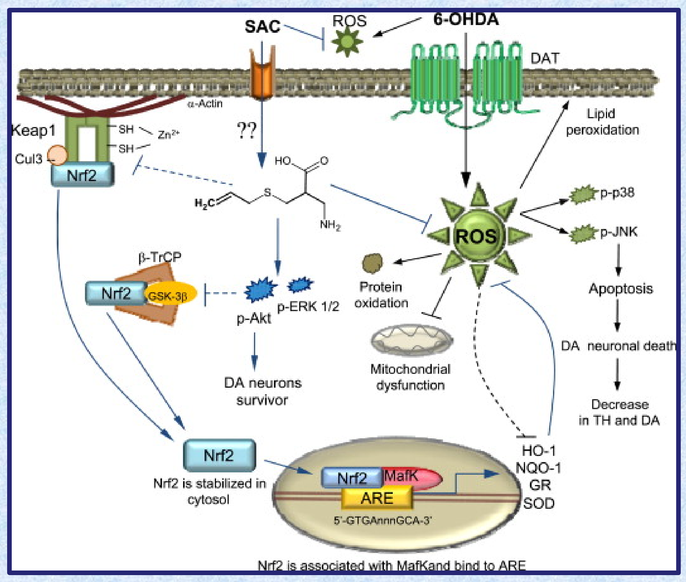




Effects of VCZ and Metabolites on Interstitial Cell Density, Hormone Concentrations, and Sperm Parameters

Figure 2. The effects of VCZ on testicular interstitial cell density in this study are shown in A (** and *** denote $P < 0.05$ and $P < 0.001$ with respect to pairwise comparisons between treatment groups), and are reflective of Interstitial Cell hyperplasia and associated changes in the mean serum concentrations of immunoreactive testosterone and total estrogens. Boars treated with either 5 or 100 mg VCZ/kg BW/day had increased mean percentages of abnormal sperm on Day 14 (29 ± 16.6 % and 54.5 ± 9.9%, respectively), as compared to pre-exposure and Day 7 sperm, as reflected by ubiquitination of abnormal sperm (B, C, and D).

Nrf2 Mechanism of Action



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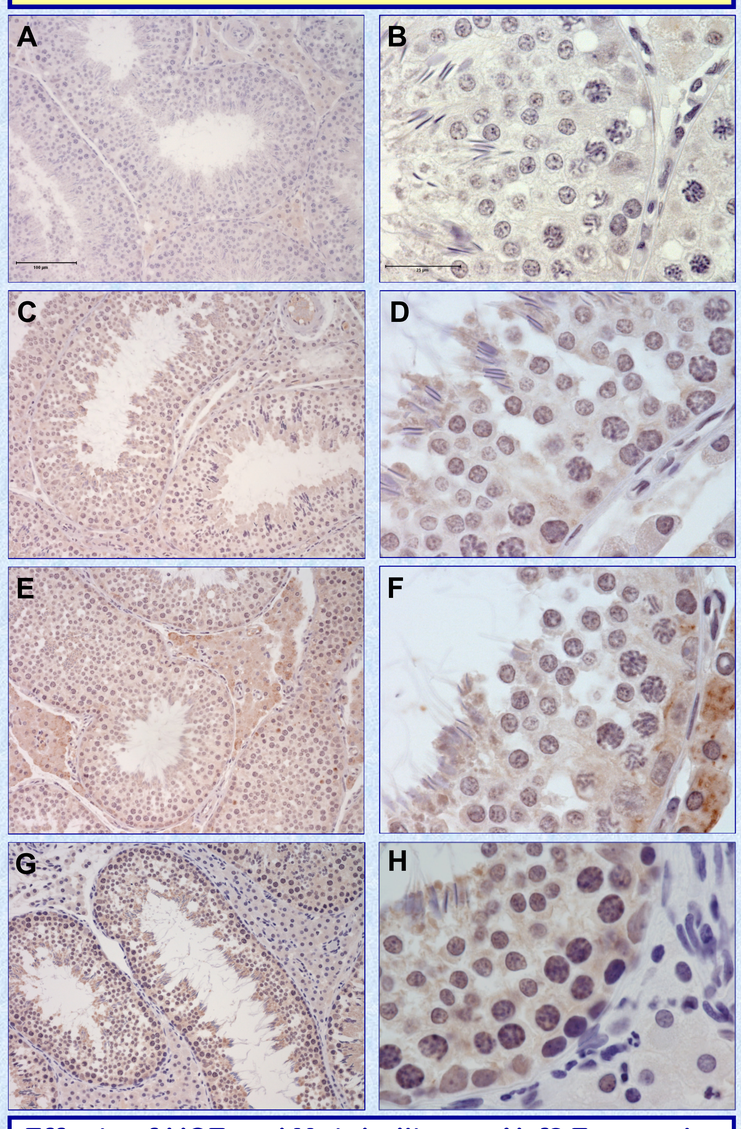
Mechanism of Action of Nrf2

Figure 3. Without oxidative stress, Nrf2 is kept in the cytoplasm by Kelch like-ECH-associated protein 1 (KEAP1) and degraded by ubiquitination. In the presence of reactive oxygen species (ROS), more Nrf2 protein is produced, and Nrf2 translocates into the nucleus. Nuclear Nrf2 forms a heterodimer with a small Maf protein and binds to the Antioxidant Response Element (ARE) in the upstream promoter region of many genes involved in cellular responses to oxidative stress.

Nrf2 IHC Experimental Approach

- Paraffin-embedded testicular tissues from one boar in each treatment group were sectioned into 4 µm-thick slices and mounted onto positively-charged barrier slides.
- The mounted slides were heated, deparaffinized, and hydrated using sequential treatments of xylene, absolute 100% ethanol, 95% ethanol, and distilled water.
- The samples were incubated with 3% H_2O_2 buffer block to reduce non-specific background staining and were pretreated with BORG (BG1000; pH = 9).
- After rinsing with de-ionized water, the slides were loaded into the Intellipath FIX for automated IHC staining.
- A Snyper (BS966H) incubation was performed to further reduce non-specific background staining.
- The sections were treated with rabbit polyclonal anti-Nrf2 (SC-722; Santa Cruz Biotechnology) diluted 1:200, with muscle and kidney sections stained as positive controls.
- After rinsing with deionized water, the sections were treated with the secondary anti-rabbit antibody, RABBIT ENV (K4003).
- Sections were washed, treated with Romulin Red (RAE810) chromogen to detect the antigen, and counterstained with IP FLX Hematoxylin (IPCS5006).
- Sections were dehydrated and cleared using sequential treatments with 95% ethanol, absolute 100% ethanol, and xylene, followed by application of cover slips.
- IHC-stained testicular sections were evaluated using a Olympus BX41 microscope, and images of IHC-stained testicular sections were captured using an Insight Firewire Color Mosaic video camera (Spot Diagnostic Instruments).

PRELIMINARY Nrf2 IHC EXPERIMENTAL RESULTS



Effects of VCZ and Metabolites on Nrf2 Expression

Figure 4. IHC-stained sections of testes from four boars treated orally with 0 (A & B), 0.25 (C & D), 5 (E & F), or 100 mg VCZ/kg BW/day (G & H) are shown above. Expression of Nrf2 appeared to increase in a VCZ dosage-dependent manner in the seminiferous tubules. Nuclear translocation of Nrf2 was noted in Sertoli cells, pachytene spermatocytes, and round spermatids of the boar in the 100 mg VCZ/kg BW treatment group. For these selected boars, interstitial cell cytoplasmic expression of Nrf2 appeared to be greatest in the 5 mg VCZ/kg BW treatment group.

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SUMMARY OF PRELIMINARY FINDINGS

- Porcine testicular Nrf2 expression and localization appear to be dependent on the dosage of Vinclozolin.
- Nrf2 appears to be a useful biomarker for porcine testicular responses to EDC-induced oxidative stress.