The Effects of Oral Exposure to Vinclozolin on Postpubertal Boars

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ABSTRACT

While there is increasing awareness of the potential adverse effects of endocrine-disrupting chemicals (EDCs) on male reproductive function, the ability to assess the impact of these compounds on male fertility has been hindered by the limitations of available animal models and the relative insensitivity of traditional research end points. Similarities between humans and swine make the porcine model an attractive, non-rodent alternative to accurately define EDC exposures which are potentially harmful to humans and large domestic animals. The results of preliminary experiments suggested that postpubertal swine are more susceptible than sexually mature rodents to the adverse effects of an antiandrogenic fungicide, vinclozolin (VCZ), and its metabolites. Flow cytometric evaluation of sperm chromatin integrity and computer-assisted sperm analysis (CASA) are likely to be more sensitive indicators of toxic insult to the male reproductive tract than routine sperm counts and conception rates. Using VCZ as a model EDC, we hypothesized that exposure of postpubertal boars to VCZ and its metabolites will alter the integrity of sperm chromatin, as well as sperm morphology and motility, in a dosage- and duration of exposure-dependent manner. Archived sperm samples collected from postpubertal boars exposed to varying dosages of VCZ and its metabolites for 15 days (n=4 per treatment) will be evaluated using the sperm chromatin structure assay (SCSA®). In addition, sperm from sexually mature boars treated with either 0 or 5 mg VCZ/kg/day over the entire 8-week duration of porcine spermatogenesis and epididymal sperm maturation and transport will be evaluated using both CASA and SCSA® methodologies.

Background

- There is increasing awareness of the potential adverse effects of exposures to endocrine disrupting chemicals (EDCs) on reproductive function in male animals
- The ability to assess the impact of EDCs on male fertility is limited by currently available animal models and the relative insensitivity of traditional research end points, such as total sperm counts and conception rates.
- The similarities in size, anatomy, and physiology between humans and swine make the porcine model an attractive alternative to rodent models to identify EDC exposures which are potentially harmful to humans, as well as other non-rodent mammals.
- The results of preliminary studies suggest that postpubertal boars are more susceptible, than sexually mature rodents, to the adverse effects associated with exposure to vinclozolin (VCZ), and its antiandrogenic metabolites.
- Flow cytometric evaluations of the integrity of sperm chromatin (SCSA®) and computer-assisted sperm analyses (CASA) are likely to be more sensitive antemortem indicators of toxic insult to the testes than routine sperm counts and conception rates.

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RESEARCH OBJECTIVE

Development of a postpubertal porcine model for risk assessment procedures involving environmental exposures of non-rodent mammals to suspected endocrine disrupting chemicals (EDCs).

EXPERIMENTAL HYPOTHESIS

Using Vinclozolin (VCZ) as a model EDC, we hypothesized that exposure of postpubertal boars to VCZ and its antiandrogenic metabolites will alter the integrity of the sperm chromatin, as determined by the sperm chromatin structure assay (SCSA®), in a manner dependent on the VCZ dosage and duration of VCZ exposure.

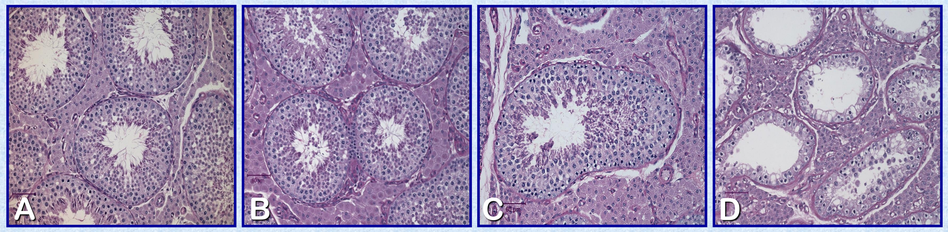
Specific Aims

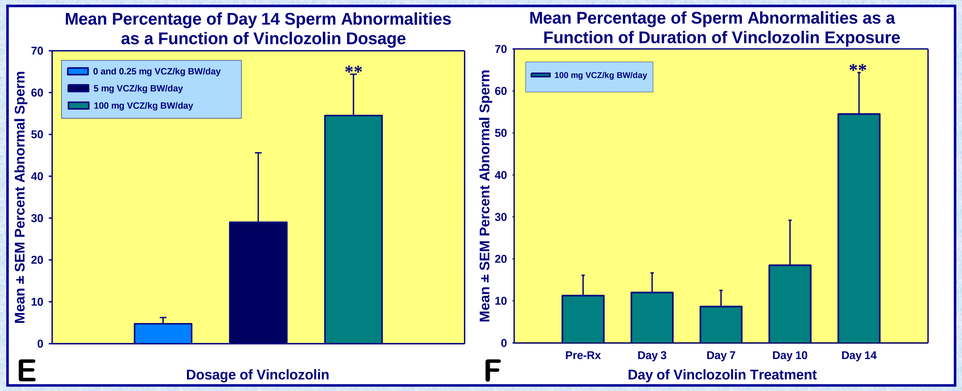
- Using the SCSA® procedure, demonstrate the effects of different dosages of VCZ and duration of VCZ exposure on the integrity of the sperm chromatin of postpubertal boars.
- The generation of preliminary data for the design of future experiments evaluating the effects of EDCs on male reproductive morphology and function in boars.

Experimental Design

- A total of 32, postpubertal (12- to 36- month-old) boars were dosed orally with either 0, 0.25, 5 or 100 mg/kg BW of VCZ (n = 8 per treatment) for 15 days (Days 0-14).
- It was possible to collect antemortem semen samples from 13 of the boars (n = 5 for boars dosed with 0 or 0.25 mg VCZ/kg BW; n = 4 for boars dosed with 5 mg VCZ/kg BW; n = 4 for boars dosed with 100 mg VCZ/kg).
- Semen was collected from boars prior to treatment and on Days 0, 4, 7, 11, and 14.
- Aliquots of the collected sperm-rich fraction of the semen were frozen on dry ice (-40 C) immediately after collection and stored at -80 C until analyzed using SCSA®.
- The remainder of the collected sperm were extended and analyzed by CASA for motility and normal morphology.
- Samples of testis, epididymis, prostate, and seminal vesicle were fixed in either modified Davidson's solution (MDS) or 10% neutral buffered formalin (NBF) and processed for histological examination.
- Archived antemortem samples of sperm were thawed and stained with Acridine Orange (AO) and evaluated using the flow cytometric SCSA® procedure (See Materials and Methods and References).
- Flow cytometric data were analyzed to determine the percentage of AO-stained sperm exhibiting red fluorescence.
- Statistical analyses of the effects of VCZ dosage and duration of VCZ exposure on sperm chromatin integrity in postpubertal boars will be performed using ANOVA for non-repeated (comparison between dosages) and for repeated variables (comparisons between durations of exposure).

Preliminary Experimental Results

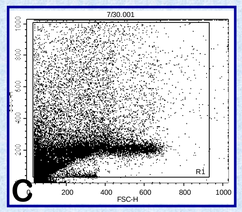


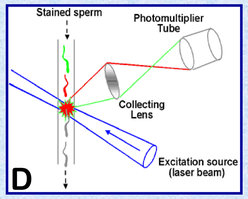


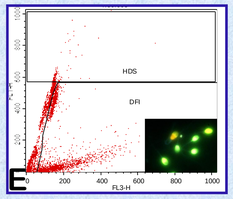
Subacute Vinclozolin Exposure-associated Effects on Postpubertal Boars

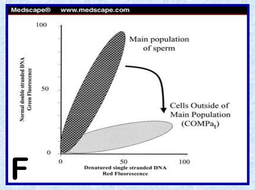
Figure 1: Sections of PAS-stained testes from boars treated with 0, 0.25, 5 and 100 mg VCZ/kg BW are shown in **A**, **B**, **C**, and **D** (most severe effects shown), respectively (200X magnification). While there were no significant VCZ-associated effects on sperm motility, the apparent effects of VCZ and its metabolites on sperm morphology are shown in **E** and **F** (** denotes P < 0.01 compared to controls or Pre-RX values).

MATERIALS AND METHODS FOR SCSA®









SCSA® (Sperm Chromatin Structure Assay) Standard Protocol

Figure 2:

A. Thawed sperm samples, diluted to 1 to 2 X 10^6 sperm/mL, are treated with an acid detergent solution for 30 seconds and then stained with Acridine Orange (AO).

B. Flow cytometry is performed using a Beckman-Dickinson FACS Scan.

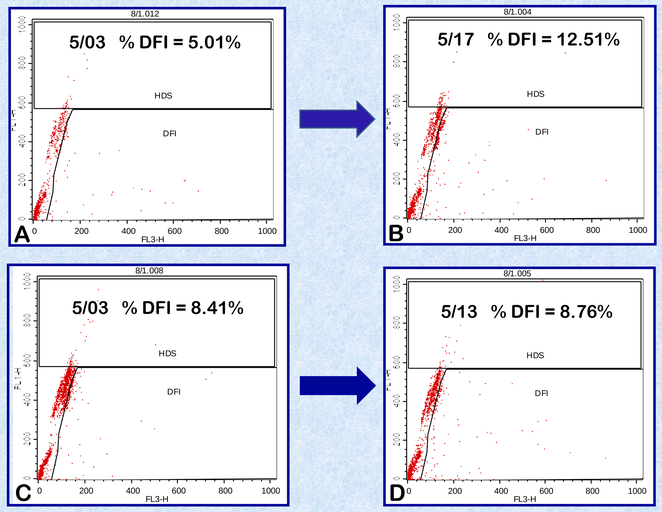
C. Prior to running the AO-stained samples, the gating and the forward and side scatter parameters are adjusted to develop an appropriate template for the flow cytometric analyses.

D. The flow cytometer emits a laser beam with a wavelength of 488 nm to "excite" the fluorescent AO dye, which differentially stains double- stranded DNA (green fluorescence 515-530 nm wavelength) versus single-stranded DNA (red fluorescence >630nm).

E. This cytogram of AO-stained, hydrogen peroxide-treated sperm from a control boar shows how sperm with green fluorescence are associated with the Y-Axis and sperm with red fluorescence correspond to the X-Axis. The inset shows human AO-stained sperm fluorescing green with native, double-stranded DNA and red with denatured, single-stranded DNA (http://www.um.es/grupo-fisiovet/human%20sperm2.jpg).

F. Similar to E, this stylized cytogram shows how green fluorescence (Y-Axis) corresponds to native, double-stranded DNA and red fluorescence (X-Axis) to denatured, single-stranded DNA. The portion of sperm cells outside the main population ($COMPa_t$) or the DNA fragmentation index (DFI) is calculated for each sample, and means are compared between treatments.

PRELIMINARY SCSA® RESULTS



Subacute Vinclozolin Exposure-associated Effects on % DFI

Figure 3: Alterations in sperm chromatin integrity in postpubertal boars associated with subacute exposure to vinclozolin, as shown by SCSA® analyses of changes in % DFI, are shown. In one instance, sperm from a VCZ-treated boar showed more than a two-fold increase in % DFI after 14 days of VCZ exposure (A & B). Sperm from another VCZ-treated boar did not show a significant change in % DFI (C & D) after 10 days of exposure, even though there were many abnormal sperm in that sample. Unfortunately it was not possible to collect another sample from that boar, but it should be noted that the the top boar had a % DFI of only 7.59% after 10 days VCZ exposure.

DISCUSSION & CONCLUSIONS

- Preliminary data from a small number of treated boars suggest that exposure to vinclozolin and its antiandrogenic metabolites affected the integrity of chromatin in ejaculated, postpubertal boar sperm only after at least 14 days of oral exposure.
- This is not necessarily surprising, given that vinclozolin exposure-associated increases in sperm morphological abnormalities were noted most consistently at the very end of the study (Figure 1).
- The relatively short duration of exposure to vinclozolin in this study was just slightly longer than epididymal sperm maturation and transport (10 to 12 days in the boar), so that only the sperm ejaculated at the end of the study would have first been exposed to vinclozolin during the very last stages of spermatogenesis (Figure 3).
- Additional SCSA® analyses of ejaculated sperm samples from boars in this study treated for 14 days, as well as boars exposed for longer durations in future studies, should help to differentiate the stages of spermatogenesis and sperm maturation, when germ cell precursors and sperm are most susceptible to alterations in sperm chromatin integrity associated with the adverse effects of vinclozolin and its antiandrogenic metabolites.

FUTURE EXPERIMENTS

Sperm from sexually mature boars, treated with either 0 or 5 mg Vinclozolin/kg/day over the entire 8-week duration of porcine spermatogenesis and epididymal sperm maturation and transport, will be collected and evaluated using both CASA and SCSA® methodologies.

References

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2. Evenson DP, Wixon R: Environmental toxicants cause sperm DNA fragmentation as detected by the Sperm Chromatin Assay (SCSA®). *Toxicology and Applied Pharmacology* 2005; 207:S532-S537.