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Review
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Review

# Effects of the endocrine disruptor vinclozolin in male reproduction: a systematic review and meta-analysis†

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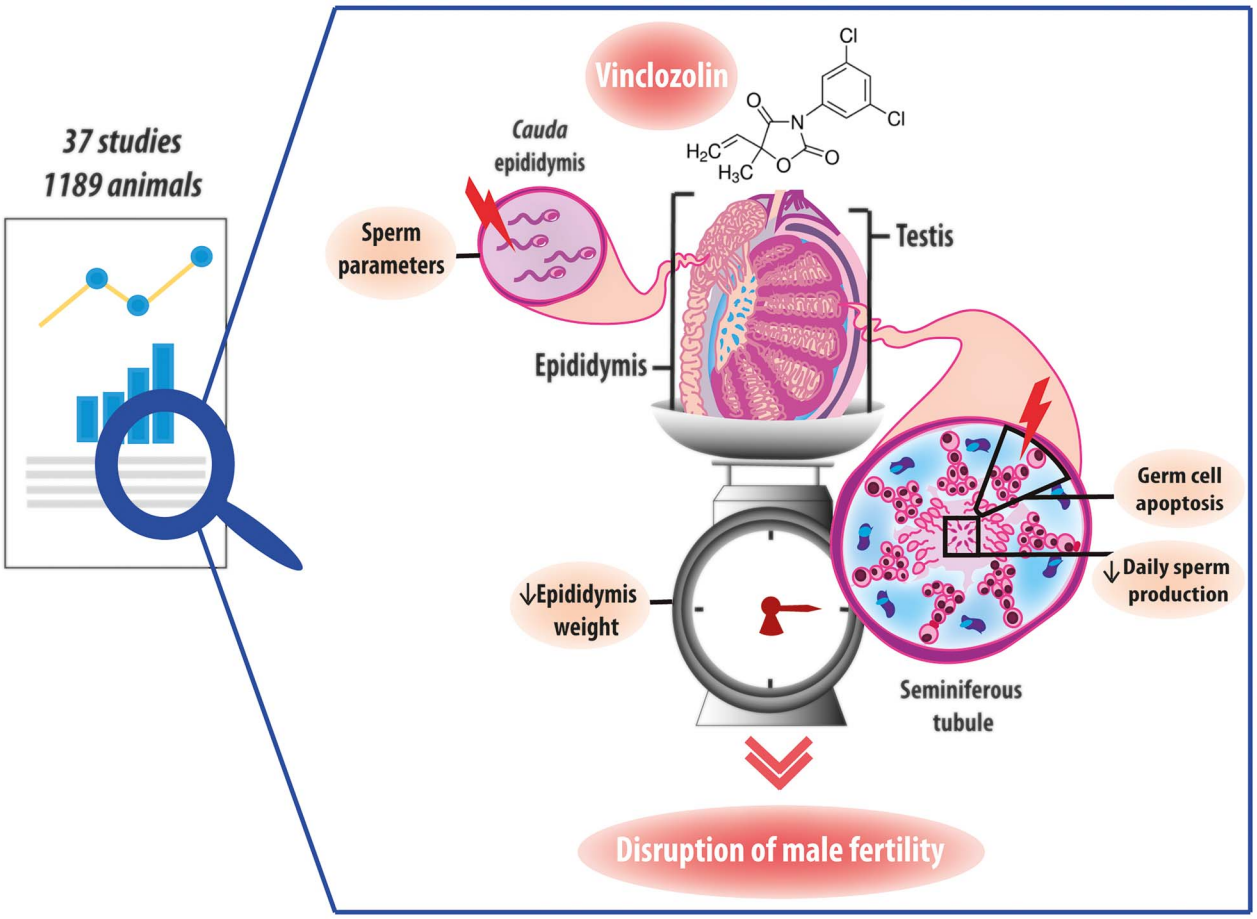
## Abstract

Endocrine-disrupting chemicals have become an issue of scientific and public discussion. Vinclozolin (VNZ) is a fungicide that competitively antagonizes the binding of natural androgens to their receptor, disturbing the function of tissues that are sensitive to these hormones, as is the case of the male reproductive organs. A systematic review with meta-analyses of rodent studies was conducted to answer the following question: Does exposure to VNZ affect sperm parameters and testicular/epididymal weight? The methodology was prespecified according to the Cochrane Handbook for Systematic Reviews and PRISMA recommendations. Sixteen articles met the inclusion criteria, comprising a total of 1189 animals. The risk of publication bias was assessed using the Trim and Fill adjustment, funnel plot, and Egger regression test. Heterogeneity and inconsistency across the findings were tested using the $Q$-statistic and $I^2$ of Higgins, respectively. Sensitivity was also analyzed. Statistical analysis was performed on Comprehensive Meta-Analysis software (Version 2.0), using random models and weighted mean differences along with a 95% confidence interval. Sperm motility, counts, daily sperm production (evidence of publication bias), and epididymis weight were decreased in VNZ-treated animals. Exposure length and dose, as well as the time point of exposure, influenced the obtained results. Despite the moderate/high heterogeneity observed, the sensitivity analysis overall demonstrated the robustness of the findings. The quality scores of the included studies were superior to 4 in a total of 9, then classified as good. The obtained data corroborate the capability of VNZ exposure to disrupt spermatogenic output and compromise male fertility.

## Summary sentence

A systematic review with meta-analysis was conducted to understand how the exposure to VNZ affects fertility-related outcomes such as sperm quality parameters and testicular/epididymal weight.

## Graphical Abstract




**Key words:** epididymis, male reproduction, sperm parameters, testis, vinclozolin.

## Introduction

Biomonitoring data from all over the world provided evidence of human exposure to a range of xenobiotic substances [1–4]. Extensive detection of industrial chemicals in human serum and seminal plasma has led the scientific community to hypothesize that these compounds may act as endocrine-disrupting chemicals (EDCs), leading to a vast array of physiological impairments. In fact, in recent years, EDCs have become an issue of scientific and public discussion. Vinclozolin (VNZ, 3-(3,5-dichlotophenyl)-5-ethenyl-5-methyl-2, 4-oxazolidinedione, CAS Number 50471-44-8) is a dicarboximide fungicide that inhibits spore germination, being useful in several crops such as oilseed, vines, fruits, vegetables, ornamental plants, and turf [5–7]. VNZ was widely used in Europe until 2007, when it was banned from some countries, such as Denmark, Finland, Norway, and Sweden, whereas USA remains a major use area with restrictions in some crops [7]. These restrictions have led to a decrease in the quantities emitted to the environment through the last years. However, VNZ is moderately persistent in agricultural soils with half-lives for this compound and its dichloroaniline-containing metabolites of 179 to more than 1000 days [7]. Furthermore, VNZ has high capability to enter the human food chain. In general, fungicides have been shown to circulate through the water and air. Therefore, contamination of untreated foods is likely to occur [8]. Also, consumers alone cannot easily reduce their exposure because fungicides are not removed from food with tap water and commercial processing increases their concentrations [9, 10].

In the environment and after administration, VNZ is readily degraded in its two main antiandrogenic dichlorophenyl carbamate-related metabolites by cleavage of the oxazolidine ring [5]. VNZ and its active metabolites bind competitively to the androgen receptor (AR), thereby antagonizing the binding of natural androgens to this receptor and disturbing androgen-dependent gene expression [5, 6, 11]. Thus, VNZ may disturb the function and development of tissues that are sensitive to testosterone or other androgens, as is the case of the male reproductive organs.

The goal of our search was to identify and systematize all articles that reported primary data on the effects of VNZ in male reproduction. Several xenobiotics are tested in different model animals for potential toxic effects on human health including reproduction and fetal development. Hereupon, studies performed in male rodents showed the reprotoxicity of VNZ, since its antiandrogenic activity lowered testosterone levels [12]; increased the number of apoptotic germ cells [13–17]; reduced testis and epididymis weight [18–26]; decreased sperm number, daily sperm production (DSP) [12, 15,

16, 18–21, 24, 27], and sperm motility [15–17, 22, 28]; increased sperm head abnormalities [18, 27, 29], hypospadias [12, 21, 26], and cryptorchidism [12, 26]; and reduced penis size and anogenital distance [14, 18, 21]. The choice of an animal model (rodents) instead of human exposure reports is justified by the fact that most of the available information about human environmental exposure is not directed to the aim of the study (male reproductive toxicity), possibly due to the ethical issues related to sample collection. Thus, in the scarcity of human data, animal research is the most reliable mean of detecting important toxic properties of chemical substances and to estimate risks to human and environmental health. Inasmuch as human present much lower sperm production than other animals [30], reduction in human semen quality and production caused by VNZ would be more serious in humans when compared with other species. Moreover, in vivo exposure studies can present a great variability of study designs (e.g., length of exposure and dose, stage of development at the time of exposure, etc.), which, consequently, can be translated in distinct outcomes. Therefore, the need of an integrative and segmented analysis to minimize the heterogeneity of data and understand if and which condition is influencing the results is evident.

This background has elicited a call for a rigorous and complete systematic review and meta-analysis of a comprehensive range of studies on the effects of VNZ in male reproduction. Focusing on sperm parameters and testicular/epididymal morphological changes, the present work reviews the relevant supporting evidence, summarizes the current knowledge, identifies gaps and limitations in the interpretation of published data, and defines future directions in research.

## Methods

### Search strategy, inclusion criteria, and study selection

The literature search for this systematic review was performed on the electronic databases PubMed and Scopus, during January 2020. Following the recommendation of the Cochrane Handbook for Systematic Reviews [31], search was performed in all fields for both index (MeSH) terms and keywords. The MeSH terms "male," "reproduction," "testis," "Leydig cells," and "germ cells," with "vinclozolin" as supplementary concept, which includes three additional terms ("ronilan," "vinclozoline," and "50471-44-8") were used. To increase search sensitivity, "toxicity" was added as a subheading term. The databases were queried using the Boolean operator tools, with the following strategy: "vinclozolin" AND ("male reproduction" OR "testis" OR "Leydig cells" OR "germ cells" OR "toxicity"). All publications between 1 January 1994 (all MeSH terms were added to MEDLINE prior this year) and 31 December 2019 (the last full year at the time we began our search) were included.

The list of references of the articles reviewed was also checked to find additional studies. Following the PRISMA recommendations [32, 33], titles and abstracts of records retrieved were initially screened, and the full texts of those considered relevant were then downloaded and analyzed in detail. The question that motivated our search was "what are the effects of VNZ on male reproduction?" The PICO process [34] was used in order to frame a specific question, providing more specificity to study selection. The recent worrisome trends in sperm quality [35], together with the well-defined and standardized protocols to analyze sperm parameters, render these outcomes the focus of our study. Although secondarily, the assessment of the inherent cellular alterations in testis (germ cell apoptosis) and organ weights was also considered for data extraction since most of the studies performing sperm analysis also include these parameters. Therefore, the initial question was reformulated to "what are the effects of VNZ on male rodent's sperm parameters and epididymal/testicular weight?"

Two authors independently conducted the literature selection process, with a third being consulted in case of discrepancies. To be included in this systematic review, studies must fulfill the following criteria: (1) to be an original article; (2) to be a full-text article in English; (3) to focus on the chemical compound VNZ, exclusively or not; (4) to use rodents as the animal model; (5) to report primary data on sperm quality and/or testis/epididymis weight; and (6) unambiguously describe the methods of analysis for each parameter. First, based on the title and abstract, the publication was either excluded or advanced to full-text screening. Secondly, the full text was reviewed to confirm study eligibility or assigned it to exclusion within a specific justification.

### Data extraction and synthesis

After the phase of selection, the included studies were carefully analyzed, and the following data were extracted and summarized: first author's last name, year of publication, outcomes analyzed, animal model used, dose of VNZ, type of administration, and type/duration of exposure. According to the PRISMA methodology [32, 33], two authors independently reviewed and extracted the data using a prespecified protocol. In cases of discordance, a third reviewer was consulted to analyze discrepancies in data extraction. The extracted results were both initial and post-intervention mean values of the outcomes with the corresponding standard deviation (SD) and sample size ($n$). Some results were reported in figures in the original studies, and for that reason, the Inkscape program (Version 0.92.4) was used to obtain the numerical values to perform the statistical analysis.

### Statistical analysis

For the outcomes of interest, an assessment was performed on the pooled effect of the treatment with VNZ in terms of weighted difference in means weighted mean difference (WMD) between post-treatment mean values of the intervention and control groups. Statistical analysis was performed using Comprehensive Meta-Analysis software (Version 2.0) by introducing the number of animals, the mean values, and SD values of the outcomes for intervention and control groups, being the random effects model employed [36]. Forest plots were generated to illustrate the study-specific effect sizes along with a 95% confidence interval (CI). Heterogeneity between trial results was tested using the $Q$-statistic (also referred to as "Cochrane $Q$"). Under the hypothesis that homogeneity exists (null hypothesis), $Q$ will follow a $\chi^2$ distribution. A $P$-value of less than 0.05 leads to the rejection of the null hypothesis, and therefore, some (undetermined) degree of heterogeneity exists. The statistic $I^2$ of Higgins was used as a measure of inconsistency across the findings of the included studies. The scale of $I^2$ has a range of 0–100%, and values on the order of 25, 50, and 75% are considered low, moderate, and high heterogeneity, respectively [37]. $I^2$ reflects the proportion of observed dispersion, which was due to the heterogeneity. Subgroup analysis was performed on the outcomes under study, per the animal model used, dose and method of VNZ administration, and type and duration of exposure, in order to evaluate the impact of these experimental factors on the VNZ outcomes and to explore potential sources of

heterogeneity. The sensitivity analysis was performed by removing each study one at a time to evaluate the stability of the results.

Meta-regression analysis was also undertaken to evaluate the association between calculated WMD in sperm parameters and testis/epididymis weight, and the length of exposure [36].

### Risk of bias assessment

Three different analyses were used to assess the potential impact of publication bias on the present meta-analysis: (1) Funnel plot [38, 39], (2) Egger regression test [36, 40], and (3) Duval and Tweedie's Trim and Fill approach [41, 42], which allows the best estimate of the unbiased pooled effect size to be obtained and lends itself an intuitive visual display as it creates a funnel plot that includes both the observed and the necessary imputed studies to obtain the absence of bias.

The methodological quality of the included studies was evaluated by a 9-item quality checklist adapted from the risk of bias assessment tool provided by the SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE RoB tool) [43], comprising: (1) peer-review publication, (2) standardized age of animals at the day of necropsy, (3) randomized allocation of animals to treatment and control groups, (4) blinded assessment of outcome, (5) adequate address of incomplete outcome data, (6) sample size calculation performed, (7) compliance with animal welfare regulations, (8) statement of potential conflicts of interest, and (9) reported the explanation of any treated animals excluded from analysis.

## Results

### Search and selection of studies

The employed search strategy and subsequent analyses complied with the PRISMA statement [32, 33]. Application of search protocol allowed the identification of 514 publications meeting the established criteria for abstract screening. Among the 514 articles initially identified, 164 duplicate records were removed and 155 were excluded based on title or abstract screening. Full texts of the remaining 197 articles were reviewed for eligibility and 179 studies were excluded. All original articles recovered from search are listed in the end of the "Supplemental File." Studies regarding other VNZ effects than the ones explored on the present work were excluded under the reason "other methods/results" ($n = 95$), mostly because not allowing to withdraw solid conclusions due to: (1) the insufficient number of reports to generate statically significant results, fetal effects ($n = 3$), RNA studies ($n = 3$), sexual behavioral studies ($n = 2$), accessory glands ($n = 2$), malformations of male reproductive tract ($n = 2$), bioinformatics ($n = 1$), hormonal biotransformation ($n = 1$), toxicological effects ($n = 1$), and oxidative stress ($n = 1$); (2) the high heterogeneity of the results, epigenetics ($n = 14$), genetics ($n = 11$), proteomics ($n = 5$), hormonal receptors ($n = 5$), and levels ($n = 4$); (3) not fitting our area of interest, other than male reproductive outcomes ($n = 23$), methodological testing ($n = 6$), physical alterations ($n = 5$), nonsexual behavioral studies ($n = 4$); (4) not exploring the individual effects of VNZ ($n = 1$); and (5) article retraction ($n = 1$). From the remaining articles that met all the inclusion criteria for the systematic review ($n = 18$) [13–15, 17–24, 27–29, 44–47], two were excluded from the quantitative analysis during data extraction because the outcome data were incomplete [13, 28]. Figure 1 shows the detailed steps of the article selection process described above, and Supplementary Figure S1 schematizes the differences between studies. Ten of the 16 included studies were divided into different experiments, which give a total of 37 studies included in the present review with meta-analysis. The works done by Flick et al., Eustache et al., Schneider et al., Yu et al., Monosson et al., Ostby et al., and Liu et al. were separated into different trials as the intervention groups were treated with more than one dose of VNZ (3, 2, 2, 3, 2, 6, and 2 different used, respectively). The work of Elzeinova et al. was divided because the authors studied two different types of exposure to VNZ (only intrauterine (IU) and IU + Direct). The study of Uzumcu et al. was divided because the authors euthanized the animals at two different time-points for analysis (animals with 20 and 60 days of age). In the Matsuura et al. report, data were divided into six different trials because the authors analyzed different doses and types of exposure to VNZ (only IU and IU + Direct). Finally, the work of Kubota et al. was divided since it presented the results obtained after two different times of convalescence from exposure to VNZ (8 and 36 days).

To the best of our knowledge, no previous systematic review with meta-analysis was performed regarding the reproductive toxicity of VNZ.

### Included studies and characteristics

The principal characteristics of the included studies are outlined in Table 1 and Supplementary Figure S1, particularly, year of publication, number of animals, type of study, animal model (strain), dosage of VNZ, type of exposure, type and duration of administration, and animal age during exposure and at the time of analysis. VNZ was administered to the studied animals (rats and mice) through different methods (food, water, oral gavage, or intraperitoneal injection) at different stages of development (embryonic, youth, and adulthood). Also, distinct windows of exposure (from 5 to 165 days) and dosages (acceptable daily intake (ADI, 0.005 mg/kg/day); no observed adverse effect level (NOAEL, 1–12.5 mg/kg/day); low observed adverse effect level (20 mg/kg/day); low dose (25–50 mg/kg/day); high dose (100–1000 mg/kg/day)) were observed in these studies. Such variables were included in this meta-analysis to explore potential sources of heterogeneity.

### Effects of VNZ on sperm parameters, testis, and epididymis weight

The present meta-analysis was performed to clarify the effects of VNZ on male fertility by summarizing the results of sperm quality and testicular/epididymal weight analysis after VNZ administration to rodents. The 37 included studies totalize 1189 animals, 558 exposed and 631 unexposed to VNZ, and the overall results are presented in Table 2. The meta-analysis results of the effects of VNZ on sperm motility, sperm counts, DSP, sperm morphological abnormalities, germ cell apoptosis, and testis/epididymis weight are graphically reported in Figures 2 and 3. It is possible to verify that VNZ significantly reduced sperm motility (WMD: −3.28%; 95% CI: −5.07 to −1.49; $P < 0.0001$), sperm counts (WMD: $-11.93 \times 10^6$ spermatozoa/g epididymis; 95% CI: −22.49 to −1.37; $P = 0.027$), the DSP (WMD: $-3.52 \times 10^6$ spermatozoa/g testis; 95% CI: −6.97 to −0.07; $P = 0.046$), and the epididymis weight (WMD: −6.30%; 95% CI: −8.92 to −3.69; $P < 0.0001$), indicating that VNZ administration seems to decrease sperm quality and numbers, inducing epididymal morphological changes. Nevertheless, it should be noted that moderate/high heterogeneity was observed (motility: $I^2 = 60.67\%$; sperm counts: $I^2 = 64.59\%$; DSP: $I^2 = 12.25\%$; epididymis weight: $I^2 = 77.53\%$). It is important to notice that high heterogeneity is common in meta-analysis dealing with data

**Table 1.** Characteristics of 37 included studies in this systematic review with meta-analysis

| Study | Year | Animal model (strain) | Sample size (VNZ/Control) | Type of study | Type of exposure | Dose (mg/kg/day) | Administration | Intervention length (days) | Outcomes analyzed |
|---|---|---|---|---|---|---|---|---|---|
| [47] | 2019 | Mouse (NMRI) | 19/28 | Animal research | IU + Direct | 40 | Food | 21 + 144 | DSP |
| | | | 17/28 | | | 300 | | | |
| [46] | 2016 | Rat (Wistar) | 10/10 | Animal research | IU + Direct | 0.005 | Gavage | 15 + 83 | Motility; sperm counts; abnormal sperm |
| | | | 10/10 | | | 4 | | | |
| | | | 10/10 | | | 20 | | | |
| [27] | 2012 | Rat (Sprague Dawley) | 10/10 | Animal research | Direct | 100 | Gavage | 15 | motility; sperm counts; abnormal sperm; DSP; testis weight; epididymis weight |
| [29] | 2011 | Rat (Wistar) | 25/25 | Animal research | Direct | 100 | Food | 84 | Motility; sperm counts; abnormal sperm |
| [14] | 2010 | Rat (Sprague Dawley) | 15/15 | Animal research | IU | 100 | Gavage | 5 | Germ cell apoptosis |
| [22] | 2009 | Rat (Wistar) | 10/10 | Animal research | IU + Direct | 1 | Gavage | 21 + 58 | Motility; sperm counts; abnormal sperm; epididymis weight |
| | | | 10/10 | | | 30 | | | |
| [45] | 2009 | Rat (Sprague Dawley) | 5/5 | Animal research | IU | 100 | Intraperitoneal | 7 | Motility; abnormal sperm; testis weight; epididymis weight; germ cell apoptosis |
| [18] | 2008 | Mouse (CD1) | 16/10 | Animal research | IU | 1 | Water | 7 | Abnormal sperm; testis weight; epididymis weight |
| | | | 14/10 | | IU + Direct | 1 | | 7 + 21 | |
| [15] | 2008 | Rat (Sprague Dawley) | 9/9 | Animal research | IU | 100 | Intraperitoneal | 6 | Motility |
| [44] | 2008 | Rat (Wistar) | 50/50 | Animal research | IU | 4 | Gavage | 9 | Motility; sperm counts; abnormal sperm; testis weight; epididymis weight |
| | | | 50/50 | | | 100 | | | |
| [23] | 2005 | Rat (Sprague Dawley) | 20/20 | Animal research | Direct | 40 | Food | 70 | Motility; testis weight; epididymis weight; |
| | | | 20/20 | | | 200 | | | Motility; testis weight; epididymis weight; |
| | | | 20/20 | | | 1000 | | | Motility; testis weight; epididymis weight; sperm counts; abnormal sperm |
| | | | 18/17 | | IU + Direct | 40 | | 21 + 69 | Motility; testis weight; epididymis weight; |
| | | | 18/17 | | | 200 | | | Motility; testis weight; epididymis weight; |
| | | | 15/17 | | | 1000 | | | Motility; testis weight; epididymis weight; sperm counts; abnormal sperm |
| [24] | 2004 | Rat (Sprague Dawley) | 10/10 | Animal research | Direct | 100 | Gavage | 9 | Sperm counts |
| | | | 10/10 | | | 200 | | | |
| | | | 10/10 | | | 400 | | | |
| [17] | 2004 | Rat (Sprague Dawley) | 10/10 | Animal research | IU | 100 | Intraperitoneal | 6 | Testis weight |
| | | | 10/10 | | | 100 | | | Motility; sperm counts; testis weight |
| [19] | 2003 | Rat (Holtzman) | 8/8 | Animal research | Direct | 100 | Gavage | 6 | DSP; testis weight |
| | | | 8/8 | | | 100 | | | |
| [20] | 1999 | Rat (Long Evans) | 10/10 | Animal research | Direct | 30 | Gavage | 33 | Epididymis weight |
| | | | 10/10 | | | 100 | | | Sperm counts; epididymis weight |
| [21] | 1999 | Rat (Long Evans) | 19/24 | Animal research | IU | 3.125 | Gavage | 10 | Sperm counts; testis weight; epididymis weight |
| | | | 23/24 | | | 6.25 | | | |
| | | | 18/24 | | | 12.5 | | | |
| | | | 13/24 | | | 25 | | | |
| | | | 6/24 | | | 50 | | | |
| | | | 2/24 | | | 100 | | | |

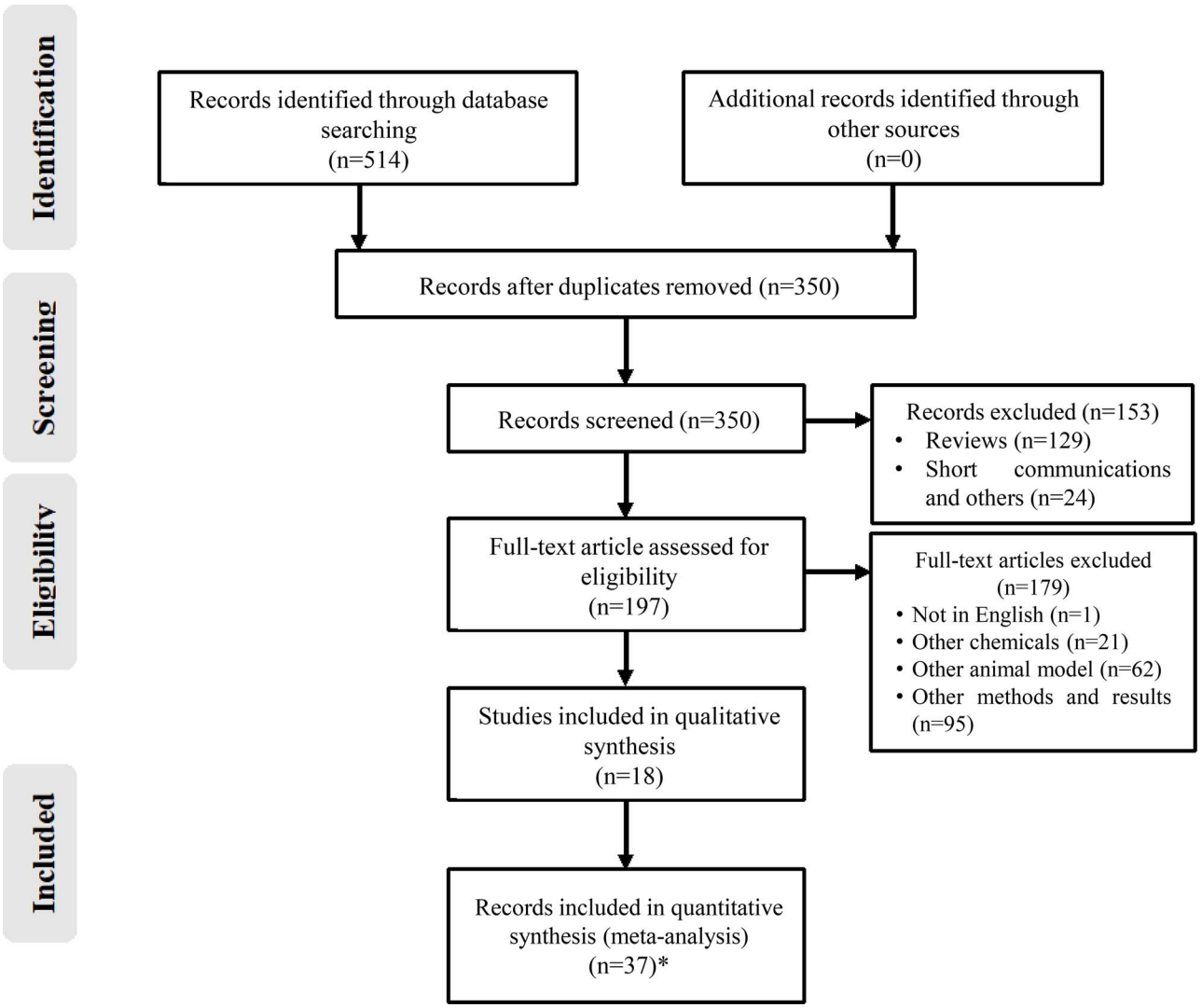



**Figure 1.** Flow diagram of database search, trial selection, and articles included in this meta-analysis. *The works (Eustache et al., 2009; Elzeinova et al., 2008; Schneider et al., 2008; Uzumcu et al., 2014; Kubota et al., 2003; Monosson et al., 1999; Liu et al., 2019) were divided into two different trials. The works (Flick et al., 2010; Yu et al., 2004) were divided into three different trials. The works (Ostby et al., 1999; Matsuura et al., 2005) were divided into six different trials. The works (Schneider et al., 2013; Stouder et al., 2009) were excluded from quantitative analysis since the outcome data were incomplete.

obtained using animal models [43], being difficult to identify the cause of that heterogeneity and thus to prevent it in future animal studies.

## Subgroup and sensitivity analysis

A subgroup analysis was also undertaken (Table 4) to evaluate the influence of the animal model, type of administration, dosage, and type and duration of exposure.

Regarding the animal model, for rats, it was obtained a significant reduction in sperm motility (WMD: −2.97%; 95% CI: −4.81 to −1.13; $P = 0.002$), sperm counts (WMD: $-11.23 \times 10^6$ spermatozoa/g epididymis; 95% CI: −22.49 to −1.37; $P = 0.0027$), DSP (WMD: $-5.38 \times 10^6$ spermatozoa/g testis; 95% CI: −08.99 to −1.82; $P = 0.003$), and epididymis weight (WMD: −6.53%; 95% CI: −9.46 to −3.60; $P < 0.0001$). For mice, only two studies were considered, and only a significant increase on the percentage of abnormal sperm was observed (WMD: 15.23%; 95% CI: 12.9–17.6; $P < 0.0001$). The low number of studies ($n = 2$) made it difficult to understand if the animal model has influence on the results. In order to explore if there are some finding changes with the removal of mice studies, the results obtained with both animal models, only with rats, and only with mice were compared (Supplementary Table S2). After removing mice studies, the level of inconsistency across the findings ($I^2$) was decreased for most of the analyzed outcomes (abnormal sperm, testis, and epididymis weight), being only increased in the case of DSP analysis. As expected, the $I^2$ of sperm motility and counts remained similar since the first was only assessed in one study using mice and for the second any report was found. Considering the statistically significant results, no substantial changes were found.

Regardless of the type of exposure, VNZ led to a significant reduction on epididymis weight (direct: WMD: −8.87%, 95% CI: −13.33 to −4.04, $P < 0.0001$; IU: WMD: −4.34%, 95% CI: −8.05 to −0.63, $P = 0.022$; IU + Direct: WMD: −6.50%, 95% CI: −11.38 to −1.62, $P = 0.009$), whereas directly exposed animals showed reduced sperm counts (WMD: $-14.74 \times 10^6$

**Table 2.** Effects of vinclozolin (VNZ) on the outcomes of this meta-analysis

| Outcomes analyzed | Number of trials | Number of animals (VNZ/Control) | WMD observed (95% CI) | *P*-value | $I^2$ (%) | Model used | WMD adjusted (95% CI) |
|---|---|---|---|---|---|---|---|
| Motility (%) | 20 | 320/320 | −3.28 (−5.07; −1.49) | <0.0001* | 60.67 | Random | −3.39 (−4.35; −2.32) |
| Sperm counts (×10⁶/g epididymis) | 22 | 351/416 | −11.93 (−22.49; −1.37) | 0.027* | 64.59 | Random | −11.93 (−22.49; −1.37) |
| Abnormal sperm (%) | 12 | 255/247 | 3.37 (−1.03; 7.76) | 0.133 | 99.65 | Random | −3.86 (−0.347; 8.07) |
| DSP (×10⁶/g testis) | 3 | 62/82 | −3.52 (−6.97; −0.07) | 0.046* | 12.25 | Random | −3.52 (−6.97; −0.07) |
| Germ cell Apoptosis (×10⁷/μm²) | 2 | 20/20 | 1,10 (−1.48; 2.82) | 0.543 | 87.68 | Random | - |
| Testis weight (% relative to control) | 24 | 368/426 | −0.08 (−2.23; 2.08) | 0.994 | 57.02 | Random | −0.08 (−2.23; 2.08) |
| Epididymis weight (% relative to control) | 22 | 377/430 | −6.30 (−8.92; −3.69) | <0.0001* | 77.53 | Random | −7.65 (−8.70; −6.61) |

Effect sizes are expressed as the WMD with 95% CIs calculated using random effects models. $I^2$ is a measure of heterogeneity. Positive WMDs represent an increase in the outcome measure after exposure. Negative WMDs represent a decrease in the outcome measure after exposure. The *P*-values are relative to comparisons between control- and VNZ treated-group. WMD, weighted mean differences.

*A significant result.

spermatozoa/g epididymis; 95% CI: −28.99 to −0.49; $P = 0.043$) and DSP (WMD: $-5.38 \times 10^6$ spermatozoa/g testis; 95% CI: −8.99 to −1.82; $P = 0.003$) and IU exposure resulted in decreased sperm motility (WMD: −5.94%; 95% CI: −9.52 to −2.35; $P = 0.001$). Thus, despite having dissimilar effects, the different types of exposure culminate in epididymal morphological changes.

Sperm motility was shown to be diminished when VNZ was administered by oral gavage (WMD: −2.40%; 95% CI: −4.29 to −0.50; $P = 0.013$) and intraperitoneal injection (WMD: −10.73%; 95% CI: −14.65 to −6.80; $P < 0.001$), while a reduction in DSP (WMD: $-5.38 \times 10^6$ spermatozoa/g testis; 95% CI: −8.94 to −1.82; $P = 0.003$) and epididymis weight (WMD: −6.99%; 95% CI: −10.65 to −3.33; $P < 0.0001$) was only observed with administration by oral gavage. The latter observation was also seen in VNZ administration through food (WMD: −6.16%; 95% CI: −11.39 to −0.93; $P = 0.021$), and an augmented frequency of sperm abnormalities was observed when animals were exposed to VNZ in water (only two studies, WMD: 15.24%; 95% CI: 12.37–18.11; $P < 0.0001$). Here, the observed differences are, most likely, due to the discrepancies in the number of studies between categories and not necessarily demonstrate the impact of the route of administration. As expected, higher doses of VNZ had a more accentuated impact on the evaluated parameters: sperm motility (WMD: −3.98%; 95% CI: −6.82 to −1.14; $P = 0.006$), sperm counts (WMD: $-16.98 \times 10^6$ spermatozoa/g epididymis; 95% CI: −29.10 to −4.85; $P = 0.006$), DSP (WMD: $-4.48 \times 10^6$ spermatozoa/g testis; 95% CI: −8.02 to −0.94; $P = 0.013$), and epididymis weight (WMD: −8.54%; 95% CI: −12.71 to −4.37; $P < 0.0001$) were decreased. Low dose treatment also reflected a decrease in epididymis weight (WMD: −7.15%; 95% CI: −12.14 to −2.16; $P = 0.005$). Curiously, an increase in sperm abnormalities was observed at NOAEL dose (WMD: 10.85%; 95% CI: 7.19–14.50; $P < 0.0001$), accompanied by a decrease in epididymis weight (WMD: −6.92%; 95% CI: −13.21 to −0.64; $P = 0.031$).

This systematic review showed that VNZ was administered to the animals at different intervention durations. Therefore, a meta-regression analysis (Figure 4) was conducted to evaluate the association between sperm parameters, testis weight and epididymis weight, and the study duration. The results of meta-regression suggest a significant correlation between the intervention duration and motility (Figure 4A; $P = 0.02$), sperm counts (Figure 4B; $P = 0.0003$), abnormal sperm (Figure 4C; $P < 0.00001$), and DSP (Figure 4D; $P < 0.0022$).

The sensitivity analysis was also performed by excluding one or more studies from the analysis to see how this affected the results. The results showed that the pooled effects of VNZ on the sperm parameters, testis weight, and epididymis weight did not change substantially if a single or a few studies were omitted (Supplementary Figures S2 and S3). Overall, the sensitivity analysis demonstrated the robustness of the findings obtained with this meta-analysis.

## Publication bias

To analyze the publication bias, a funnel plot was generated considering the Trim and Fill adjustment (Supplementary Figure S4). It was observed that for some outcomes there are more studies on one side than on the other. For that reason, studies were inputted on the adequate side to adjust the funnel plot to the absence of publication bias. Both observed and adjusted WMD were reported in Table 2. In addition to the visual inspection of the funnel plot, the presence of publication bias was explored using Egger regression test. This test indicates evidence of publication bias for the impact of VNZ administration on DSP (Table 3).

Ultimately, Supplementary Table S1 shows the study quality scores assessed using the checklist adapted from SYRCLE RoB tool. All the included studies are peer-reviewed publications and referred the randomization of the animals for both treatment and control groups. However, none of the studies reported the blind of outcome assessment and have calculated the sample size. Overall, the global quality of the included studies is good (quality scores superior to 4 in a total of 9).

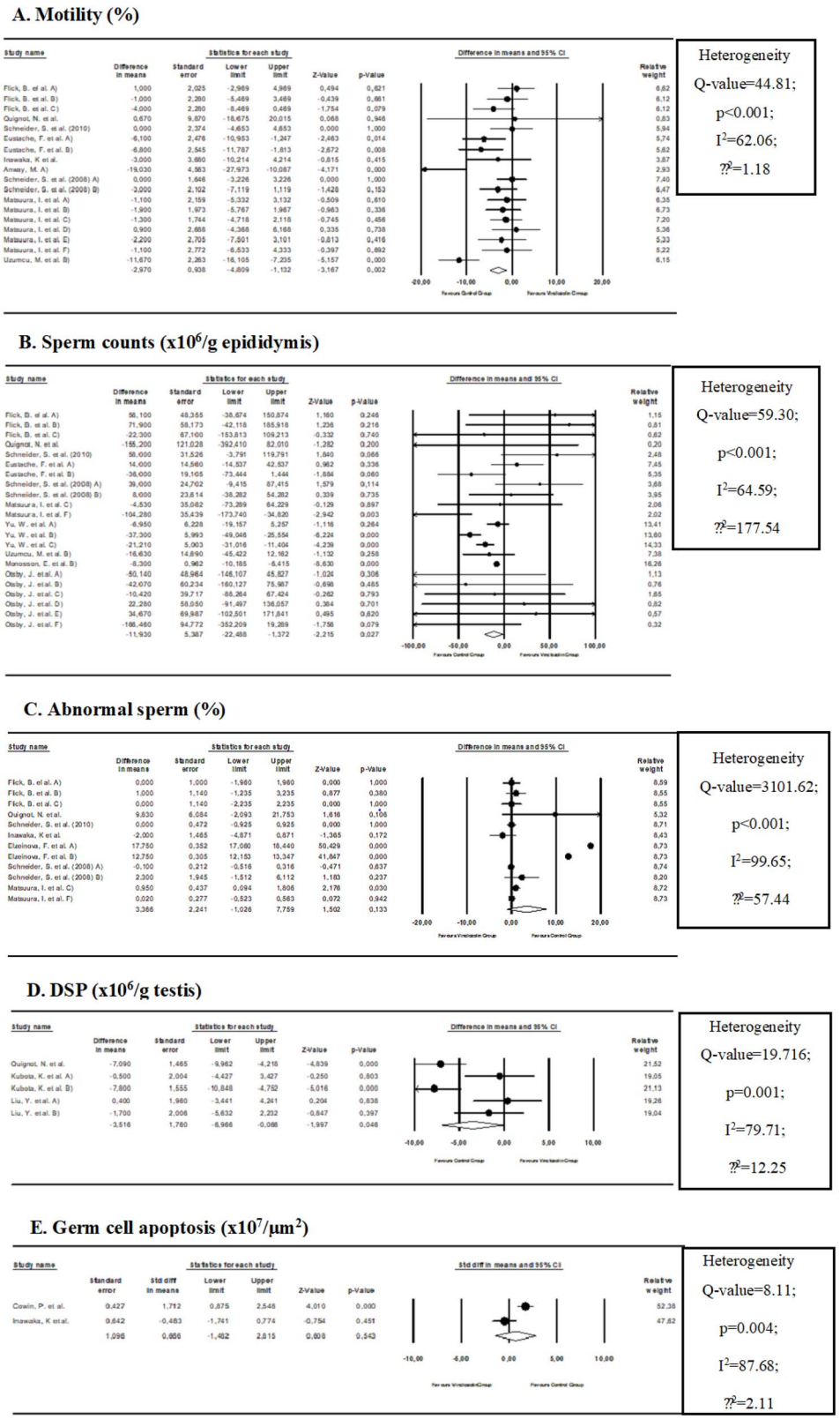



**Figure 2.** Forest plot for comparisons of the effects of VNZ on sperm parameters. (A) Sperm motility, (B) sperm counts, (C) abnormal sperm, (D) DSP, and (E) germ cell apoptosis.

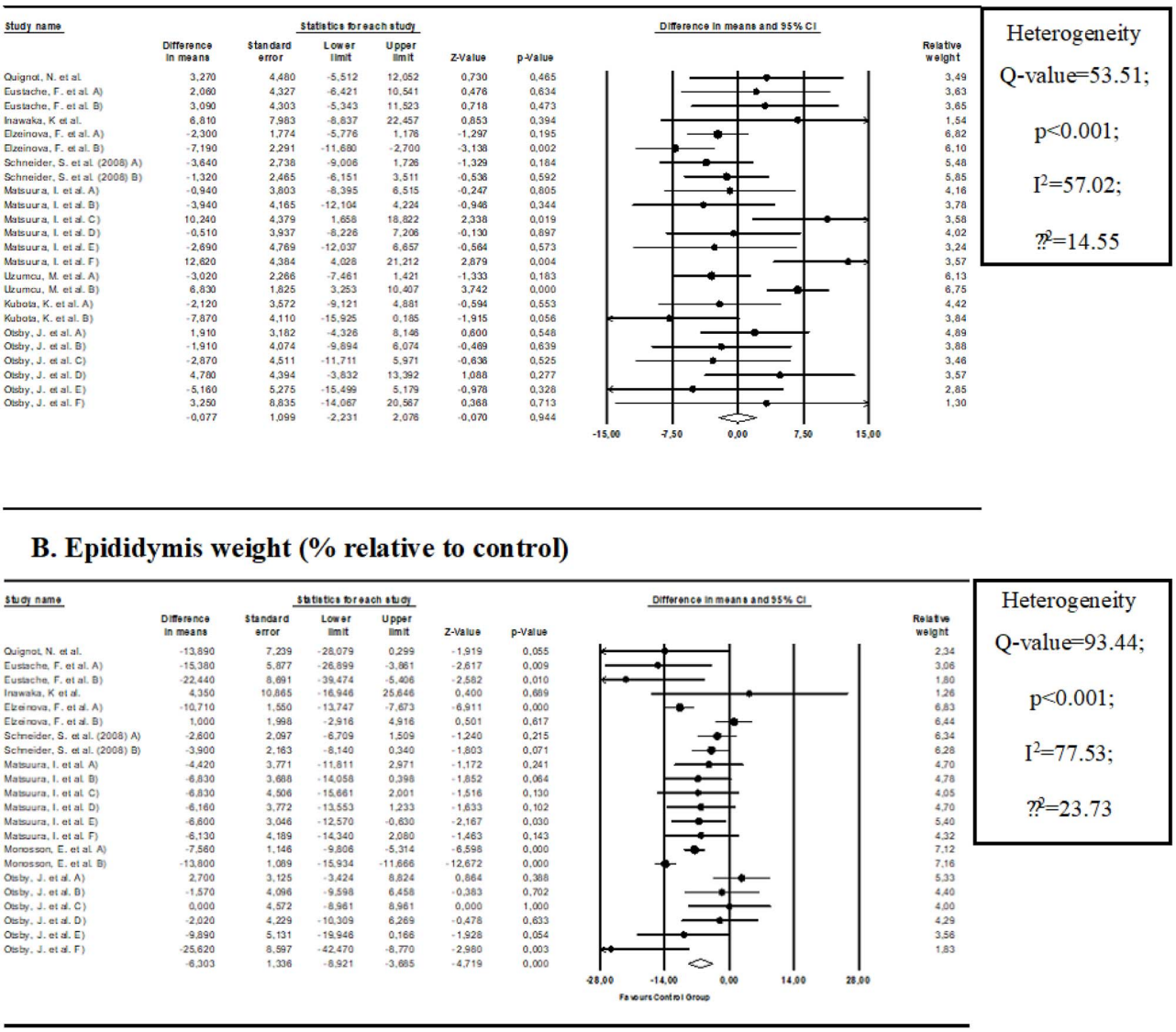



**Figure 3.** Forest plot for comparisons of the effects of VNZ on organ weight. (A) Testis and (B) epididymis.

## Discussion

Man-made chemicals have become a part of everyday life, and it is now clear that some of these chemical pollutants can affect the endocrine system. Certain of these EDCs may also interfere with the developmental processes of humans and wildlife species [7]. Here, we focused on the effects of VNZ, an antiandrogenic fungicide, in male reproductive health. VNZ is an AR antagonist, which means that this chemical competes with endogenous androgens for receptor binding preventing the downstream activation of androgen-dependent gene transcription. Male gonadal development occurs during a relatively narrow time window and is strictly dependent on sex steroid hormones, mainly androgens. Spermatogenesis is also strongly regulated by these hormones, since puberty and through lifetime [48]. Knowledge of the modes of action of VNZ as an antagonist of AR allows to make some predictions of its capability to disrupt the same reproductive tissues and processes that are regulated by androgens. The results obtained in this first systematic review and meta-analysis about the effects of the EDC VNZ in male reproduction corroborate this prediction. Sperm parameters and reproductive organs weight were shown to be affected. Statistically significant differences were found between exposed and nonexposed groups. Sperm motility, sperm counts, DSP, and epididymis weight, all decreased in VNZ-treated animals (Table 2). Noteworthy, almost no differences were found between the observed and adjusted WMD, enforcing the robustness of the results.

The outcomes of our study remained substantially unchanged after controlling some preselected variables (Table 4, Figure 4), whereas dose, duration, and window of exposure appear to have more influence on the results. Thus, leading to believe that VNZ can differently impair androgen-dependent mechanisms depending on these variables.

Few limitations were noticed while performing this systematic review and meta-analysis. First, some parameters needed to be normalized to allow their inclusion in meta-analysis: organs weight

**Table 3.** Assessment of publication bias for the impact of VNZ on the outcomes

| Variables | Motility (%) | | | | Sperm counts ($\times 10^6$/g epididymis) | | | | Abnormal sperm (%) | | | | DSP ($\times 10^6$/g testis) | | | | Testis weight (% relative to control) | | | | Epididymis weight (% relative to control) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) |
| Dose | | | | | | | | | | | | | | | | | | | | | | | | |
| ADI | 1 | 1.0 (−6.96; 8.96) | 0.806 | 0 | 1 | 56,1 (−42.2; 154.4) | 0.263 | 0 | 1 | 0 (−6.48; 6.48) | 1 | 0 | 0 | - | - | - | 0 | - | - | - | 0 | - | - | - |
| NOAEL | 2 | −3.48 (−9.37; 2.41) | 0.246 | 56.43 | 2 | 19.71 (−17.03; 56.46) | 0.293 | 0 | 3 | 10.85 (7.19; 14.50) | <0.0001* | 99.225 | 0 | - | - | - | 3 | −3.17 (−8.32; 1.99) | 0.228 | 57.47 | 3 | −6.92 (−13.21; −0.64) | 0.031* | 91.572 |
| LOAEL | 2 | −1.85 (−7.44; 3.74) | 0.517 | 50.57 | 5 | 3.06 (−34.64; 40.75) | 0.874 | 2.080 | 2 | −0.05 (−4.56; 4.45) | 0.982 | 0 | 0 | - | - | - | 4 | −1.58 (−6.59; 3.44) | 0.538 | 0 | 4 | −0.46 (−6.15; 5.24) | 0.875 | 0 |
| Low | 3 | −2.33 (−7.19; 2.53) | 0.347 | 58.79 | 3 | −22.92 (−63.6; 17.77) | 0.270 | 0 | 0 | - | - | - | 1 | 0.40 (−6.88; 7.68) | 0.914 | 0 | 5 | 0.42 (−4.54; 5.38) | 0.867 | 0 | 6 | −7.15 (−12.14; −2.16) | 0.005* | 10.20 |
| High | 10 | −3.98 (−6.82; −1.14) | 0.006* | 70.27 | 11 | −16.98 (−29.1; −4.85) | 0.006* | 78.000 | 6 | 0.65 (−2.20; 3.50) | 0.655 | 47.2 | 4 | −4.48 (−8.02; −0.94) | 0.013* | 76.92 | 12 | 1.35 (−1.71; 4.41) | 0.386 | 67.22 | 9 | −8.54 (−12.71; −4.37) | <0.0001* | 71.37 |
| Administration | | | | | | | | | | | | | | | | | | | | | | | | |
| Food | 7 | −1.03 (−3.02; 0.95) | 0.309 | 0 | 3 | −11.77 (−53.28; 29.73) | 0.578 | 82.98 | 3 | 0.27 (−2.04; 2.68) | 0.789 | 43.37 | 2 | −0.64 (−5.24; 3.36) | 0.785 | 0 | 6 | 2.32 (−1.94; 6.63) | 0.292 | 62.69 | 6 | −6.16 (−11.39; −0.93) | 0.021* | 0 |
| Gavage | 8 | −2.40 (−4.29; −0.50) | 0.013* | 37.28 | 18 | −11.31 (−23.09; 0.46) | 0.06 | 64.05 | 6 | 0.08 (−1.29; 2.74) | 0.480 | 0 | 3 | −5.38 (−8.94; −1.82) | 0.003* | 78.76 | 13 | −0.79 (−3.64; 2.06) | 0.588 | 0 | 13 | −6.99 (−10.65; −3.33) | <0.0001* | 82.05 |

Continued

**Table 3.** Continued

| Variables | Motility (%) | | | | Sperm counts (×10⁶/g epididymis) | | | | Abnormal sperm (%) | | | | DSP (×10⁶/g testis) | | | | Testis weight (% relative to control) | | | | Epididymis weight (% relative to control) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) | Number of trials | 95% CI | *P*-value | $I^2$ (%) |
| Intraperitoneal | 3 | −10.73 (−14.65; −6.80) | <0.001* | 74.66 | 1 | −16.63 (−56.41; 23.15) | 0.413 | 0 | 1 | −2.00 (−6.93; 2.93) | 0.43 | 0 | 0 | - | . | - | 3 | 2.66 (−2.55; 7.79) | 0.320 | 82.90 | 1 | 4.35 (−19.35; 28.05) | 0.719 | 0 |
| Water | 0 | - | - | - | 0 | - | - | - | 2 | 15.24 (12.37; 18.11) | <0.0001* | 99.133 | 0 | - | - | - | 2 | −4.58 (−10.00; 0.84) | 0.098 | 64.90 | 2 | −5.04 (−12.76; 2.75) | 0.206 | 95.34 |
| Exposure | | | | | | | | | | | | | | | | | | | | | | | | |
| Direct | 5 | −1.06 (−4.63; 2.52) | 0.563 | 0 | 7 | −14.74 (−28.99; −0.49) | 0.043* | 82.731 | 3 | 0.54 (−8.28; 14.11) | 0,610 | 55.780 | 3 | −5.38 (−8.99; −1.82) | 0.003* | 78.76 | 6 | −0.43 (−5.06; 4.20) | 0.855 | −53.85 | 6 | −8.87 (−13.33; −4.04) | <0.0001* | 75.25 |
| IU | 5 | −5.94 (−9.52; −2.35) | 0.001* | 85.9 | 9 | −3.34 (−27.30; 20.63) | 0.785 | 8.981 | 4 | −0.14 (−4.73; 13.82) | 0.34 | 99.84 | 2 | −0.64 (−5.24; 3.96) | 0.785 | 0 | 12 | −0.12 (−3.24; 2.99) | 0.938 | 53.16 | 10 | −4.34 (−8.05; −0.63) | 0.022* | 70.71 |
| IU + Direct | 8 | −2.38 (−5.12; 0.35) | 0.088 | 34.12 | 6 | −11.13 (−37.08; 14.82) | 0.401 | 67.743 | 5 | 0.11 (−5.49; 11.03) | 0.51 | 99.61 | 0 | - | - | - | 6 | 0.47 (−4.05; 5.02) | 0.841 | 72.92 | 6 | −6.50 (−11.38; −1.62) | 0.009* | 68.35 |
| Animal model | | | | | | | | | | | | | | | | | | | | | | | | |
| Rat | 18 | −2.97 (−4.81; −1.13) | 0.002* | 62.06 | 22 | −11.23 (−22.49; −1.37) | 0.0027* | 64.59 | 10 | 0.29 (−0.94; 1.52) | 0.645 | 20.46 | 3 | −5.38 (−8.99; −1.82) | 0.003* | 78.76 | 22 | 0.59 (−1.60; 2.78) | 0.597 | 48.70 | 20 | −6.53 (−9.46; −3.60) | <0.0001* | 72.98 |
| Mouse | 0 | - | - | - | 0 | | | | 2 | 15.23 (12.90; 17.60) | <0.0001* | 99.13 | 2 | −0.64 (−5.24; 3.96) | 0.785 | 0 | 2 | −4.58 (−10.10; 0.94) | 0.104 | 64.90 | 2 | −5.01 (−12.61; 2.59) | 0.196 | 95.34 |

Publication bias is expressed as *t* with 95% CIs and df. The *P*-values are relative to comparisons between control- and VNZ treated-group with untreated controls. df, degrees of freedom.

* A significant result.

**Table 4.** Subgroup analysis of the effects of vinclozolin (VNZ) on sperm parameters, testis weight and epididymis weight

| Outcomes | Egger regression test | | | |
|---|---|---|---|---|
| | 95% CI | *t* | df | *P*-value |
| Motility (%) | (−4.75; 0.949) | 1.41 | 16 | 0.175 |
| Sperm counts ($\times 10^6$/g epididymis) | (−1.06; 0.60) | 0.58 | 20 | 0.571 |
| Abnormal sperm (%) | (−19.51; 19.06) | 0.03 | 10 | 0.980 |
| DSP ($\times 10^6$/g testis) | (5.67; 21.55) | 5.46 | 3 | 0.012* |
| Testis weight (% relative to control) | (−1.27; 2.24) | 0.57 | 22 | 0.572 |
| Epididymis weight (% relative to control) | (−0.73; 2.61) | 1.17 | 20 | 0.254 |

Effect sizes are expressed as the WMD with 95% CIs calculated using random effects models. From each study, we considered each analysis with a specific dose, method of administration, type of exposure, and animal model as a separate individual comparison. $I^2$ is a measure of heterogeneity. Positive WMDs represent an increase in the outcome measure after exposure. Negative WMDs represent a decrease in the outcome measure after exposure. The *P*-values are relative to comparisons between control- and VNZ treated-group with untreated controls. LOAEL, low observed adverse effect level; NA, not available; WMD, weighted mean differences.

*A significant result.

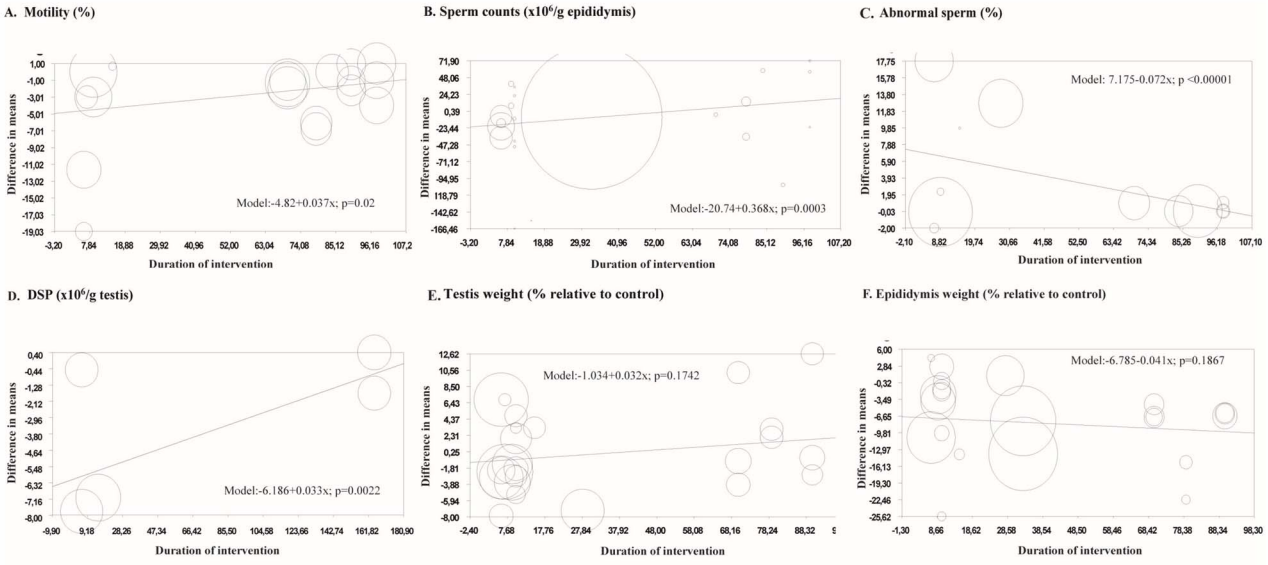



**Figure 4.** Meta-regression plots of the association between calculated weighted mean differences (WMD). (A) Sperm motility, (B) sperm counts, (C) abnormal sperm, (D) DSP, (E) testis weight, and (F) epididymis weight and the study duration. The circles dimension represents sample size.

was calculated as percentage relative to control values, and sperm counts and DSP were normalized considering the weight of the organs, where final values were calculated per gram of epididymis and testis, respectively. During the analysis of the selected articles, the research methodology was compared within each parameter and only similar procedures were considered in order to maintain consistency. However, we do not exclude that slight differences could exist on the execution of the procedures, even with the same methodology, and that these differences could be reflected in the results. Lastly, when data were divided into subgroup analysis, some of the parameters were left with a small sample size and differences between the experimental conditions may have been masked for this reason. The main limitation that hampers us to do other type of analysis was the low number of studies; in order to overcome this limitation, other animal species must be used or even a generalist analysis with different species could be performed allowing the increase of the number of studies and comparison between species. These analyses must be considered of major priority, followed by the study of the reversibility of the VNZ effects. In other words, it would have been interesting to study if VNZ effects were either permanent or transient, considering the developmental stage of the animals during exposure, but there was not enough data to perform such analysis. Despite of the well-documented transgenerational inheritance of VNZ, in the present study, the primary goal was to understand the effects of this EDC on the first generation (F1), considering different time-points of exposure (embryonic, youth, and adulthood). Thus, the effects on the subsequent generations must be considered as the next step and would be interesting in the context of a meta-analysis that could concomitantly evaluate the genetic and epigenetic effects of VNZ.

Large proportions (up to 40%) of young men in some countries have low semen quality, which reduces their ability to father children [7]. The incidence of genital malformations, such as nondescending testes (cryptorchidism) and penile malformations (hypospadias), in baby boys has increased over time or leveled off at unfavorably high rates [7]. A recent meta-regression analysis showed that sperm counts declined significantly among men from North America, Europe, and Australia during 1973–2011, with an astonishing 50–60% decline

[35]. These findings strongly suggest a significant decline in male reproductive health, and further research efforts on the causes and implications of this decline are urgently needed. Nevertheless, it is highly likely that this decline in sperm quality over the past few years is associated with multiple environmental influences, both prenatally and in adult life. In particular, endocrine disruption driven from chemicals exposure during critical windows of male reproductive development can be importantly damaging. Therefore, this rationale brings pertinence to the present systematic review and meta-analysis. In the future, other meta-analysis needs to be performed to cover the wide panoply of VNZ effects in reproduction. In fact, this is a matter far from finished at the present time. Noteworthy, there are several studies about the effects of VNZ in female reproduction as well, bringing the pertinence of carrying out a meta-analysis also in this context.

## Conclusions

This rigorous and comprehensive meta-analysis explored the effects of VNZ in sperm quality and the conditions that can modulate these effects. Sperm motility, sperm counts, DSP, and epididymis weight were decreased in VNZ-treated animals compared to their control counterparts. Also, the exposure length and dose, as well as the animal stage of development during exposure, revealed themselves as conditions with great influence on the results. Thereby, the obtained data provided robust and sensitive indication for the impact that VNZ exposure might have disrupting the spermatogenic output and compromising male fertility.

Future research should make an integrative evaluation and measure multiple endpoints in order to address if and in what circumstances the effects of VNZ could be reversed. The next step could be the use of well-parameterized studies, as it can provide a better understanding of the mechanisms of action and better prediction of toxicity, which would consider the influence of the variables studied in this systematic review with meta-analysis.

## Supplementary material

Supplementary material is available at *BIOLRE* online.

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## Conflict of interest

The authors declare they have no actual or potential conflicts of interest.

## Author contributions

MF has conducted the literature selection process, analyzed the data, and wrote the manuscript. RVLM has conducted the literature selection process. SS critically revised the manuscript. LP collaborated in statistically analysis and critically revised the manuscript. SC was consulted in case of discrepancies in the literature selection procedure and contributed to critical reading and edition of the manuscript and final approval of manuscript.

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