The effect of conazoles on reproductive organs structure and function – a review

Milan Maretta[1], Elena Marettová[1], Jaroslav Legáth[2]

University of Veterinary Medicine and Pharmacy Košice, [1]Department of Morphological Disciplines, [2]Department of Toxicology and Pharmacology, Košice, Slovak Republic

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Abstract

Conazoles are azole antifungals used in agricultural and pharmaceutical products. Exposure to conazole fungicides leads to several toxic endpoints, including reproductive and endocrine. The results of animal experiments have shown that various conazole fungicides at high doses affect the structure and functions of reproductive organs. In males, adverse effects of conazole fungicides are manifested in the testes, prostate, sperm viability, fertility and sexual behaviour. Reduced testis weight, testis atrophy and reduced or absent sperm production were frequently observed. In female genitalia, structural changes in the ovaries and uterus have been observed. The extent of the changes depends on the dose and duration of treatment. Triazoles affected the expression of multiple genes involved in steroid hormone metabolism and modulate enzyme activity of multiple cytochrome P450 (CYP) and other metabolic enzymes in mammalian liver and other tissues. Conazole fungicides act as endocrine disruptors. Conazoles have been reported to reduce oestradiol and testosterone production and to increase progesterone concentration, indicating the inhibition of enzymes involved in the conversion of progesterone to testosterone. The reproductive effects are consistent with impairment of testosterone homeostasis. The disruption in steroid homeostasis is a common mode of action, leading to abnormal reproductive development and diminished reproductive function. At high doses, azole fungicides affect reproductive organs and fertility in several species.

Conazoles, toxicity, reproduction, pesticides

Conazole pesticides are a class of imidazole- or triazole-containing fungicides used as an antifungal agent in agriculture but also as a therapeutic agent in human and veterinary medicine. Conazoles are widely used to protect cultivated plants by preventing the growth of fungi on fruits, vegetables, cereals and seeds. Pharmaceutically, they are used in the treatment of various fungal diseases, either to treat local or systemic fungal infections, primarily for the treatment of candidiasis, cryptococcosis and coccidiomycosis. These fungicides have various toxicological effects in mammals, including carcinogenicity (Georgopapadakou and Walsh 1996; Menegola et al. 2005; Wolf et al. 2006; Nesnow et al. 2009; Hamdi et al. 2019). Of the 27 known conazoles, 15 were predicted as positive (carcinogenic) and 12 negative (non-carcinogenic) (Tully et al. 2006). Some of them have been shown to be hepatotoxic and to induce hepatocellular tumours and/or thyroid follicle tumours (Hester et al. 2006; Juberg et al. 2006; Peffer et al. 2007; Nesnow et al. 2009). They can also act as inducers and inhibitors, depending on the tissue and the particular conazole in question.

Azole fungicides have the ability to act through multiple mechanisms and to induce various endocrine-disrupting effects (Zarn et al. 2003; Vinggaard et al. 2005a; Vinggaard et al. 2005b; Trösken et al. 2006). In mammals, conazoles modulate many CYP enzymes involved in the metabolism of xenobiotics, sterols, steroids, vitamin D and various endobiotics. Triazole fungicides are designed to inhibit the fungal cytochrome P450 (CYP) enzyme and other metabolic enzymes but may also modulate the expression and function of mammalian CYP genes. Triazoles have affected the expression of many

Address for correspondence:
Dr. Elena Marettová
Department of Morphological Disciplines
University of Veterinary Medicine and Pharmacy
Komenského 68/73, 041 81 Košice, Slovak Republic
E-mail: elena.marettova@uvlf.sk

CYP genes in rat liver and other tissues, including several Cyp2c and Cyp3a isoforms, as well as other xenobiotic metabolising enzyme and transport genes (Ronis et al. 1994; Nelson et al. 2004; Sun et al. 2005; Allen et al. 2006; Barton et al. 2006; Geotz et al. 2006; Tully et al. 2006). Inducing CYP activity may also lead to metabolic activation of chemicals whose metabolites may have toxic effects (Nesnow et al. 2009). Their ability to block the synthesis of ergosterol, an essential component of the fungal cell wall, is conditioned by the inhibition of lanosterol oxidative conversion by binding to cytochrome P450 isoenzymes (Zhang et al. 2008; Heeres et al. 2010). The inhibition of specific CYP steroidogenic enzymes is associated with adverse effects on development and reproduction (Cummings et al. 1997; Zarn et al. 2003; Rocket et al. 2006; Roelofs et al. 2013). Due to the widespread use of conazoles, there is a potential risk that humans and wildlife are often – probably chronically – exposed to these substances in the environment. New data show a high incidence of their distribution. Conazole fungicides are among the most frequently detected pesticides in the arable lands of Central Europe (Šudoma et al. 2021). The aim of this study was to indicate the reproductive toxicity of frequently used conazole fungicides and to present the effects on reproductive tissue and hormonal changes in the male and female reproductive organs of animals.

Materials and Methods

We searched the PubMed database for all relevant articles until using the main search terms "conazoles" combined with the following terms: "reproduction, male, female, reproductive organs". The references from relevant articles were also checked. Only articles in English that were related to human and veterinary studies were included.

Mechanism of toxicity

Azole pesticides have competitive inhibitory effects on a number of cytochrome P450 enzymes and reversible inhibition of two cytochrome P450 enzymes, sterol-14α-demethylase (CYP51) (Ronis et al. 1994; Debeljak et al. 2003) and aromatase (CYP19) (Strushkevich et al. 2010). The protein components of the CYP51 cytochrome system are deeply embedded in the membrane to provide a hydrophobic environment suitable for the metabolism of most lipophilic substrates. The fungal lanosterol-14 α-demethylase (CYP51), a key enzyme in the synthesis of ergosterol, the main sterol in most fungal cells (Van den Bossche et al. 1990), catalyses the early stages of sterol biosynthesis to form functional fungal cell membranes (Como and Dismukes 1994; Ghannoum and Rice 1999; Zarn et al. 2003; Trösken et al. 2006; Strushkevich et al. 2010). Sterol 14α-demethylase is critical for the production of meiosis-activating sterols, which have recently been shown to modulate germ cell development in both sexes of mammals. Aromatase is responsible for the physiological balance of androgens and oestrogens. These two enzymes are also present in mammals, and their inhibition can affect mammalian steroidogenesis (Zarn et al. 2003).

Some conazole fungicides have been shown to affect the activity of other members of the CYP450 family, including key enzymes involved in steroid biosynthesis and metabolism in humans and animals (Dreisig et al. 2013). Conazoles also cause catalytic inhibition of the CYP17 enzyme, which is responsible for converting pregnenolone and progesterone to androgen precursors (Roelofs et al. 2013). An *in vitro* pattern of hormone production suggests that CYP17 activity was inhibited in the testes of neonate and adult rats. In combination with a decrease in testosterone secretion, an increase in progesterone biosynthesis has been observed after prochloraz exposure, suggesting a role for the CYP17 enzyme (Laier et al. 2006). These effects strongly suggest that one of the major underlying mechanisms of the endocrine-disrupting effects of azole fungicides is the disruption of key enzymes, such as CYP17, which are involved in the synthesis of steroid hormones. Some results suggest that CYP17 can be a target of endocrine-disrupting compounds (Roelofs et al. 2013).

Conazoles have been found to affect also steroidogenesis and reproduction in fish. Propiconazole at different doses (0, 5, 50, 500, or 1000 μg propiconazole/l) during 3-weeks study reduced plasma oestradiol (E2) and vitellogenin levels. Other authors observed compensatory increased gonad weight with up-regulation of genes coding steroidogenic enzymes (CYP19, CYP17, CYP11A) (Skolness et al. 2013). Several other widely used azol compounds (prochloraz and tebuconazole) lead to significant reduction of female egg numbers and oestradiol level in zebrafish. Additionally, tebuconazole reduced the expression of *cyp19a*, which correlated with the drop in oestradiol levels (Li et al. 2019; De Oliveira et al. 2020). The line of evidence suggests that several conazoles inhibit brain and ovarian aromatase activity in a dose-dependent manner, leading to disruption of oestrogen production (Hinfray et al. 2006).

The accumulation of fungi and yeasts in the cell membrane is disrupted by blocking the synthesis of the basic component of the ergosterol membrane. The inhibition of ergosterol biosynthesis coincides with lanosterol

accumulation. Accumulated lanosterol causes changes in permeability, membrane leakage, changes in nutrient transport and inactivity of membrane-bound enzymes, growth inhibition, increased susceptibility to host defense mechanisms and possibly cell death (Hof 2006). The fungicidal mode of action of the triazole involves disruption of cell membranes and fungal walls. This basic biochemical mechanism is the basis for the use of azole fungicides in agriculture and in human and veterinary antifungal therapies (Zarn et al. 2003).

Conazole fungicides and sex steroid hormones

Studies on azole fungicides suggest that they have the ability to induce various endocrine-disrupting effects (Zarn et al. 2003; Vinggaard et al. 2005b). Several triazoles have been described that alter the concentration or transcription of genes involved in steroid homeostasis, and thirteen triazoles have been identified as endocrine disruptors (Goetz et al. 2007; Hester and Nesnow 2008; Nesnow et al. 2009). Disruption of steroid homeostasis is considered a common mode of action that leads to abnormal reproductive development and decreased reproductive function. Conazoles cause similar disorders of hormonal homeostasis and reproduction in fish and mammals (Goetz et al. 2009; Ankley et al. 2012; Skolness et al. 2013; Chu et al. 2016).

Azole fungicides can cause endocrine disruption by inhibiting steroid biosynthesis (Nellemann 2010) in a number of ways, including direct interaction with steroid hormone receptors or altered steroidogenesis (Kjærstad et al. 2010; Kjeldsen et al. 2013). This has been demonstrated by several imidazole compounds such as ketoconazole (0–2000 μM), propiconazole (0, 5, 50, 500, or 1000 μg) or myclobutanil and triadimefon (1, 10, or 100 μM) in humans and various animal species (Engelhardt et al. 1991; Goetz et al. 2009; Skolness et al. 2013). Conazoles inhibit the steroidogenic enzyme aromatase (CYP19) in several tissues and cell lines involved in the conversion of androgens to oestrogens (Vinggaard et al. 2000; Sanderson et al. 2002; Zarn et al. 2003; Trösken et al. 2004; Villeneuve et al. 2007; Kjærstad et al. 2010). The ability of conazoles to inhibit the CYP enzymes involved in steroid hormone biosynthesis can potentially cause endocrine-related side effects, such as testosterone depletion and an increased risk of adverse effects during pregnancy (Schurmeyer and Nieschlag 1984; Narotsky and Kavlock 1995; Menegola et al. 2005; Nellemann 2010).

The endocrine-disrupting effect varied between the conazoles used in the experiments. Imidazoles (econazole, ketoconazole, miconazole, prochloraz) were more effective than triazoles (epoxiconazole, propiconazole, tebuconazole) (Kjeldsen et al. 2013). Although myclobutanil, propiconazole and triadimefon were weak inhibitors of testosterone production *in vitro*, exposure of rats to triazole experiments *in vivo* resulted in increased serum testosterone and intratesting levels (Goetz et al. 2009). A study in Wistar Han rats exposed to myclobutanil (500, 2000 ppm), propiconazole (500, 2500 ppm) or triadimephon (500, 1800 ppm) showed that serum testosterone levels were increased after birth by post natal day (PND) 50 by triadimefon and at PND92/99 by all three triazole treatments (Goetz and Dix 2009). The combination of elevated serum testosterone levels by all triazoles suggest impaired testosterone homeostasis as the cause of triazole toxicity (Goetz et al. 2007). Tebuconazole treatment (10, 25, and 50 mg/kg) reduced serum testosterone levels, inhibited testicular P450 and glutathione S-transferase activities and induced antiandrogenic effects in male rats (Yang et al. 2018).

Imidazoles inhibit testicular and male reproductive function by inhibiting testosterone secretion, testicular interstitial fluid production and luteinising hormone (LH) secretion regulatory systems (Adams et al. 1998). At high doses (0, 6.25, 25 or 100 mg/kg/day), ketoconazole blocks testicular and adrenal biosynthesis. A repeated 28-day oral toxicity study of ketoconazole in rats showed a decrease in testosterone and an increase in oestradiol, luteinising hormone, and follicle stimulating hormone (Shin et al. 2006). The specific sites of ketoconazole inhibition in testicular steroidogenesis confirm the observation that ketoconazole is a potent inhibitor of androgen biosynthesis in several species. A relatively potent inhibitory effect of ketoconazole on CYP17A1 activity in Leydig cells from human testes, stallions, and pigs was observed. Ketoconazole (30 or 300 μg/l) in fish consistently suppressed *ex vivo* testosterone gonadal synthesis in both sexes and 17β-oestradiol (E2) in females during the exposures, and ketoconazole exposure also reduced plasma T concentrations in men and E2 in women (Ankley et al. 2012). Ketoconazole has been shown to cause gynaecomastia in humans (Thompson and Carter 1993).

The inhibition of testosterone synthesis by hexaconazole, flusilazole, and ketoconazole was used to explain the increased incidence of Leydig cell tumours. Ketoconazole inhibited the catalytic component of the adenylate cyclase holoenzyme in MA-10 mouse Leydig tumour cells (Chang and Fung 2006). Measurement of ovarian progesterone production *in vitro* in ovarian cells removed from ketoconazole-treated rats *in vivo* indicated a decrease in progesterone production, suggesting a direct effect of ketoconazole on ovarian steroidogenesis (Cummings et al. 1997). The drug may have potential use as an acute and reversible modulator of ovarian steroidogenesis in pathological circumstances (Gal and Orly 2014a).

Conazole antifungals have also been tested for endocrine-disrupting effects using a panel of *in vitro* tests. All have shown the potential to disrupt the endocrine system and the ability to act through several different mechanisms. Disruption of steroid biosynthesis appears to be a critical mechanism. In the H295R cell assay, conazoles reduced oestradiol and testosterone production and increased progesterone levels, suggesting inhibition of enzymes involved in the conversion of progesterone to testosterone (Kjærstad et al. 2010). Some triazole fungicides, such as propiconazole and triadimefon, inhibit CYP19 aromatase (Vinggaard et al. 2000) and in steroidogenesis aromatase converts androgens to the corresponding oestrogens. Aromatase is responsible for the physiological balance of androgens and oestrogens (Zarn et al. 2003). Azole fungicides are known to disrupt normal aromatase function at the transcriptional level, affecting gene expression (Saitoh et al. 2001; Benachour et al. 2007). The inhibition of aromatase by azole fungicides could disrupt the androgen/oestrogen ratio and

the classical endocrine functions of sex steroids (Egbuta et al. 2014). The potential (anti-)androgenic effects of ten conazoles (cyproconazole, fluconazole, flusilazole, hexaconazole, myconazole, penconazole, prochloraz, tebuconazole, triadimefon and triticonazole) were evaluated and compared with existing data. Six conazoles caused a decrease in basal testosterone secretion, and nine conazoles were found to reduce T-induced androgen receptor (AR) activation. Flusilazole was the most potent (effect potencies 3.61) and myconazole (effect potencies 0.03) the least potent AR antagonist (Roelofs et al. 2014).

Imidazoles may also have important indirect effects on testicular steroidogenesis. The precise mechanisms involved in the suppressive effects of imidazoles on testicular steroidogenesis are not known; direct inhibitory effects on testicular steroidogenic enzymes are possible (Adams et al. 1998). Antifungal imidazoles, such as ketoconazole, inhibit cytochrome P450-containing steroidogenic enzyme systems that bind to heme and show clinically undesirable side effects on endocrine systems and inhibit testosterone (Oftebro et al. 1994). CYP51 is highly expressed in the testes and plays an important role in cholesterol biosynthesis and ultimately in testosterone production (Debeljak et al. 2003). Data on the reproductive toxicity of triazole fungicides in male rats also point towards the disruption of testosterone homeostasis as the key event in the effect on triazole-induced reproductive toxicity (Goetz and Dix 2009). Because cholesterol is a substrate for subsequent steps in the production of other sterols, such as sex steroid hormones, disruption of this pathway can lead to endocrine changes and abnormalities in reproduction, development and fertility (Georgopapadakou and Walsh 1996; Zarn et al. 2003).

Changes in testis, sperm morphology and fertility

The reproductive toxicity of the three conazoles after exposure from pregnancy to adulthood has been demonstrated in rodents. When Wistar Han rats were fed myclobutanil (500, 2000 ppm), propiconazole (500, 2500 ppm) or triadiminone (500, 1800 ppm) from day 6 of gestation to PND120, the absolute testicular weights were increased on PND1 by myclobutanil, on PND22 by myclobutanil and triadimefon, and on PND50 by propiconazole and triadimefon treatment (Goetz and Dix 2009). Myclobutanil was evaluated in a two-year study in rats and many effects on the male reproductive system were found. At a dose of 10 mg/kg body weight, testicular weight loss, testicular atrophy, decreased or absent sperm production were observed (Zarn et al. 2003). A critical effect of myclobutanil was testicular atrophy in Sprague-Dawley rats after chronic exposure to 9.84 mg/kg/day. Treatment at 46.4 mg/kg/day also resulted in testicular atrophy and correlated with reproductive failure (US EPA 2005; Tully et al. 2006). In a reproduction study in rats, a decrease in testicular and epididymal weights was also observed after administration of propiconazole at a dose ≥ 21 mg/kg body weight. However, rat testicular weight increased to 256 mg/kg body (Tully et al. 2006).

Chronic administration of ketoconazole (50 mg/kg, orally) leads to a reduction in testicular tissue spermatogenesis indices in male mice (Dabagh et al. 2013). Ketoconazole exposure caused an increase in the gonadosomatic index in both sexes, and in males the fungicide caused marked proliferation of interstitial (Leydig) cells in the thorn (Ankley et al. 2007). Three days of oral administration of ketoconazole to rats at 200 mg/kg reduced fertility, and at 400 mg/kg, complete fertility loss was observed, while motility decreased in the high dose group and progression decreased (Waller et al. 1990). Wistar rats were gavaged daily with propiconazole (4 mg/kg and 20 mg/kg) from postnatal day 50 to 120, and an increase in abnormal tail morphology sperm, seminal vesicle and vas deferens weight was found (Costa et al. 2015). Exposure of rats to different doses (5, 10, 50 mg/kg bw/day) of difenoconazole may impair sperm quality. Progressive motility, acrosomal integrity and percentage of morphologically normal sperm were reduced after difenoconazole treatment. Sperm decline was observed in the epididymis in all groups exposed to difenoconazole (Pereira et al. 2019). Sperm count in the testes and caput/corpus epididymis and daily sperm production were reduced in the three exposed groups. A decrease in sperm count in the cauda epididymis (11%, 14%, and 21%) has also been reported following exposure to tebuconazole in a dose-dependent manner (10, 25, and 50 mg/kg) (Yang et al. 2018).

Propiconazole exposure indicated a reduction in sperm count in the testes of exposed rats (Costa et al. 2015). Ketoconazole (300 mg/kg) reduced likewise epididymal sperm motility (*in vivo*) in male mice 4 h after administration. The exposure of the epididymis *in vitro* to ketoconazole also led to a significant reduction in sperm motility (Heckman et al. 1992). Analysis of sperm morphology in rats indicated a slight reduction in normal sperm after fluconazole exposure (Tully et al. 2006). Cauda sperm motility showed a declining trend in rats exposed to triadimefon and myclobutanil. When adult male Sprague-Dawley rats were probed with myclobutanil for 14 days, sperm motility decreased (Tully et al. 2006). Pairs of males treated with myclobutanil or triadimefon had a significantly reduced insemination index and associated reduced fertility. Decreased fertility and complete loss of fertility were observed after high doses.

Changes in the accessory genital glands

In addition to changes in the testicles, insemination and fertility, changes in the genital organs were also found. In Wistar Han rats, the relative ventral prostate weight increased after treatment with myclobutanil, propiconazole or triadimefon, while the absolute weight of the epididymis and seminal vesicles decreased after exposure to triadimefon (Goetz et al. 2007). Relative ventral prostate weights increased at PND 92 by treatment with myclobutanil and triadimefon. An increase in seminal vesicles and the vas deferens has been observed in rats exposed to 4 mg/kg body weight of propiconazole (Costa et al. 2015). Additional genital weight loss was also observed in male rats treated with ketoconazole, and prostate atrophy was confirmed in a two-generation

reproduction study in rats given myclobutanil (US EPA 2005). A loss of the epididymis and accessory genitals has also been observed in male rats treated with ketoconazole (Shin et al. 2006).

Changes in female reproductive organs

Exposure to high concentrations of the three triazole fungicides used – propiconazole, myclobutanil, and triadimephon – had an adverse effect on reproductive development in female rats, though the effects appeared to be short-lived or reversible. The relative ovarian weight was significantly higher in rats exposed to food from PND 6 to day PND 98 at high doses of myclobutanil (2,000 ppm) and high doses of triadimephon (1,800 ppm) (Rockett et al. 2006). Ovarian weight was thought to be affected by swelling or the number of corpora lutea present on the ovary. Propiconazole, myclobutanil, and triadimefon impaired the oestrous cyclicity. At a medium dose (500 ppm) this can be attributed to the high incidence of animals with abnormal cycles and at the high dose (2,500 ppm) to the high incidence of animals with prolonged cycles (Rockett et al. 2006).

Several studies on female reproductive organs have been performed with ketoconazole. Single administration of ketoconazole gel at a dose of 6, 12, and 24 mg/rat resulted in a reduction in ovulated eggs. Ketoconazole anovulation was caused by arrest of follicle development at the 800–840 μm stage of the Graafian follicles compared to the 920 μm periovulatory follicles observed in the control group (Gal and Orly 2014b). In addition, the absence of CYP11A1 expression in the growth-granulated follicle cell layers was evident, and they also lacked mucosally mature complexes of accumulated cells. These results suggest that inhibition of ketoconazole-mediated follicular maturation is likely due to impaired steroidogenesis at an early stage of follicular development toward ovulation. Thus, attenuation of folliculogenesis can be used to modulate gonadotropin and ovarian stimulation in the treatment of infertility (Gal and Orly 2014b). Exposure to ketoconazole or perchloraz caused a significant reduction in Japanese medaka fertility and decreased expression of oestrogen receptor-α precursors (Zhang et al. 2008).

Both tebuconazole and epoxiconazole affected reproductive development in offspring after *in utero* exposure. Following peri- and postnatal exposure, females reduced uterine weight (Moser et al. 2001). Both compounds virilised female offspring, as evidenced by the increased anogenital distance on postnatal day 0. High doses of epoxiconazole had significant foetotoxic effects. The exposure of pregnant female rats to tebuconazole at doses around the lowest level of effect observed resulted in damage to the reproductive system of the offspring. Common properties of azole fungicides are to increase gestational age, virilise female pups and affect steroid hormone levels in foetuses and/or females (Taxvig et al. 2007). These effects strongly suggest that one of the major underlying mechanisms of the endocrine-disrupting effects of azole fungicides is the disruption of key enzymes involved in the synthesis of steroid hormones. Common features of azole fungicides are that they prolong pregnancy, virilise female pups, and affect steroid hormone levels in foetuses and/or females.

Conclusion

Azole fungicides and other azole compounds may affect the reproductive organs, fertility, and development in several species. Propiconazole, myclobutanil, and triadimephon have been shown to have reproductive toxicity in rodents after exposure from pregnancy to adulthood. *In vivo* results have shown that many commonly used azole fungicides act as endocrine disruptors. Azole fungicides interact with several P450 enzymes in various species and have the potential to affect the endocrine system by interacting with steroidogenesis. Imidazoles (econazole, ketoconazole, miconazole, prochloraz) had a stronger effect than triazoles (epoxiconazole, propiconazole, tebuconazole). *In vitro* studies have shown the antiandrogenic effects of several conazole fungicides. Antiandrogenic effects suggest that potential testicular toxicity may arise from two mechanisms: inhibition of T secretion and AR antagonism. Disruption of steroid homeostasis reduces reproductive organ function. It has been suggested that azole-mediated disorder of hormonal homeostasis alters developmental and reproductive processes. The potential toxic effects of conazoles on both male and female reproductive organs depend on the dose level and duration of treatment of the animals.

Conflict of Interest

The authors declare no conflict of interest.

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