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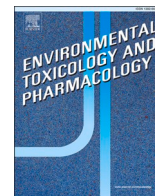
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In vitro exposure to triazoles used as fungicides impairs human granulosa cells steroidogenesis

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ABSTRACT

Triazoles are the main components of fungicides used in conventional agriculture. Some data suggests that they may be endocrine disruptors. Here, we found five triazoles, prothioconazole, metconazole, difenoconazole, tetraconazole, and cyproconazole, in soil or water from the Centre-Val de Loire region of France. We then studied their effects from 0.001 μM to 1000 μM for 48 h on the steroidogenesis and cytotoxicity of ovarian cells from patients in this region and the human granulosa line KGN. In addition, the expression of the aryl hydrocarbon receptor (AHR) nuclear receptor in KGN cells was studied. Overall, all triazoles reduced the secretion of progesterone, estradiol, or both at doses that were non-cytotoxic but higher than those found in the environment. This was mainly associated, depending on the triazole, with a decrease in the expression of CYP51, STAR, CYP11A1, CYP19A1, or HSD3B proteins, or a combination thereof, in hGCs and KGN cells and an increase in AHR in KGN cells.

1. Introduction

Triazoles are the main active products of fungicides in conventional agriculture. They act on lanosterol 14 α -demethylase (CYP51), which produces ergosterol in fungi, an important component of the fungal plasma membrane (Chambers et al., 2014). CYP51 is highly conserved through evolution and is present in many human tissues (EFSA, 2009), including the testis and ovaries (Stromstedt et al., 1996). In mammals, CYP51 enables the *de novo* synthesis of cholesterol, converting lanosterol and dihydrolanosterol into follicular fluid meiosis-activating

sterol (FF-MAS), a precursor of cholesterol (Keber et al., 2013). In ovaries, CYP51 is upregulated by follicle-stimulating hormone (FSH) and has a protective effect on granulosa cells during the maturation of follicles. Moreover, the invalidation of the gene coding for CYP51 in the granulosa cells of mice leads to the inhibition of the secretion of estradiol (E2) and progesterone (Pg) induced by FSH (Liu et al., 2017).

Prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro) are all triazoles used in France. Tauchnitz et al. (2020) have shown that PT is the most-used agricultural pesticide in Germany after tebuconazole and glyphosate.

Abbreviations: AHR, aryl hydrocarbon receptor; Ahnt, AHR nuclear translocator; BCA, bicinchoninic acid; CYP19A1, cytochrome P450 family 19 subfamily A member 1; CYP51, lanosterol 14 α -demethylase; Cypro, cyproconazole; Dif, difenoconazole; DMEM, Dulbecco's Modified Eagle's Medium; E2, estradiol; ER, estrogen receptor; FBS, fetal bovine serum; FF, follicular fluid; FSH, follicular stimulating hormone; FSHR, follicular stimulating hormone receptor; GAPDH, human Glyceraldehyde-3-phosphate dehydrogenase; hGCs, human primary granulosa cells; HSD3B, human 3 beta-hydroxysteroid dehydrogenase; HSP, heat shock protein; LC50, lethal concentration 50; JEG-3, placental cells; LHR, luteinizing hormone receptor; LOEC, lowest observed effect concentration; MC, metconazole; MVLN, human breast carcinoma cells; NOAEL, non-observable adverse effect level; P450 scc or CYP11A1, human Cytochrome P450 family 11 subfamily A member 1; Pg, progesterone; PT, prothioconazole; ROS, reactive oxygen species; RT, Room temperature; RT, reverse transcription; SEM, standard error of the mean; STAR, human Steroidogenic acute regulatory; TT, tetraconazole; UGW, underground water; XAP2, hepatitis B virus X-associated protein; XRE, xenobiotic-responsive element.

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In humans, the estimated daily intake was reported at 0.00002 mg/kg body weight/day (bw/d), and the acceptable daily intake was placed at 0.001 mg/kg bw/d in Australia (Australian Pesticides and Veterinary Medicines Authority, 2010). Prothioconazole has been considered as a cytotoxic component at 115 μ M in human breast carcinoma cells (MVLN cells) and placental cells (JEG3 cells) (Kjeldsen et al., 2013) and also in embryo culture in rats (Dimopoulou et al., 2017). At a lower dose (5 μ M), PT is considered an endocrine disruptor, causing the induction of the estrogen receptor (ER) in MVLN and aromatase (CYP19A1) production in JEG3 cells (Kjeldsen et al., 2013). In human extravillous trophoblast primary cells, PT decreased cell proliferation and migration at 5 μ M (Dong et al., 2022). The effects of MC on human health have not yet been explored. In zebrafish, the median lethal concentration was placed at 3.17 mg/L (He et al., 2022). MC was cytotoxic at 6 mg/kg bw/d in rodents (EFSA, 2009). Testosterone secretion decreased in mouse Leydig cells exposed *in vitro* to 1 μ M of MC (Botteri Principato et al., 2018). A 28-day *in vivo* exposure study in mice at 287 mg/kg bw/d led to a decrease in the weight of ovaries and the presence of cysts (BASF we create chemistry, 2015). According to our knowledge, no studies have been yet performed on human reproduction.

Residues of Dif have been found in water in India at around 4 μ g/L (Ganaie et al., 2023), in wheat grain in Canada at around 0.012 mg/kg (Thabit et al., 2021) and in blood plasma in birds (about 44.27 pg/g; Angelier et al., 2023). In HepG2 cells, the median lethal concentration was placed at 24.72 μ M with increased ROS production from 10 μ M after 24 h of exposure (Wang et al., 2021). However, at high doses (600 μ M) in rat embryo culture, Dif was less toxic than PT (Dimopoulou et al., 2017). As with PT, Dif is considered an endocrine disruptor, inhibiting aromatase in H295R cells at 4 μ M (Sanderson et al., 2002). Dif also has an agonist activity toward ER α (Wang et al., 2022), and at 2.77 μ M, it decreases 3 β -hydroxysteroid dehydrogenase (HSD3B) in human primary placental cells (Zhai et al., 2023). In some species, such as zebrafish, this endocrine disruption can decrease the fertilization rate (Chen et al., 2022). Tetraconazole is applied in agriculture, viticulture, horticulture, and home gardening (EFSA, 2008). Residues of TT have been found in fruit juices consumed in Spain at μ g/kg levels (Plana et al., 2014) and have been detected in farm workers' hair at 0.42 μ g/kg after the application season (Polledri et al., 2021) and in blood plasma in birds (around 1194.16 pg/g, Angelier et al., 2023). On epithelial cells from bovine mammary glands, TT induced apoptosis, autophagy, and reduced cell viability and proliferation (Jeong et al., 2023). Cyproconazole is used on cereal crops, coffee, sugar beet, fruit trees, and grapes (EFSA, 2009). Residues were found in French fruits and wheat at μ g/kg levels (Nougadère et al., 2012; Thabit et al., 2021). Moreover, it was found in human urine at concentrations above 10.38 μ g/L (Machado et al., 2019). Cyproconazole decreased progesterone production in JEG3 cells (Rieke et al., 2014), and in MA-10 Leydig cells, it inhibited cytochrome P450 family 11 subfamily A member 1 (CYP11A1), CYP17, steroidogenic acute regulatory protein (STAR), and the production of testosterone (Roelofs et al., 2014). At the molecular level, several studies have shown that triazoles can modulate nuclear receptors such as the aryl hydrocarbon receptor (AHR) (Ghisari et al., 2015; Knebel et al., 2018a; Knebel et al., 2018b; Serra et al., 2023; Tian et al., 2022). AHR is constitutively expressed in granulosa cells (Baba et al., 2005). When it is not activated, AHR is sequestered in the cytosol. When it is activated by binding with a toxic ligand or endogen ligand, it is translocated to the nucleus and dimerizes with the AHR nuclear translocator (Arnt), then it recognizes the xenobiotic-responsive element (XRE) (Ohtake et al., 2009). AHR is also upregulated by FSH and luteinizing hormone (LH) during the maturation of the antral follicles. Interestingly, AHR can also bind the promotor of the FSH receptor and directly regulate its expression (Abbott et al., 1999; Matvere et al., 2019) as well as ER (Ohtake et al., 2009).

The aim of our study was firstly to determine the concentration of five triazoles, prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro) in soils and waters

of the French Centre-Val de Loire area. Secondly, we investigated *in vitro* the dose effect of PT, MC, Dif, TT, and Cypro in terms of cytotoxicity and steroidogenesis in ovarian cells of patients from this French area and on the human granulosa cell line, KGN. Indeed, triazoles from blood plasma could be found in follicular fluid and affect the ovarian cell function.

2. Material and methods

2.1. Triazole analyses of French soil and water

The characterization of agricultural soils close to where the patients lived in France's *Région Centre-Val de Loire* area was carried out at the INRAE soil analysis laboratory in Arras, France, and accredited by the French Committee of Accreditation (COFRAC). The analysis of soils sieved at 2 mm was performed by LC-QTOF-MS (liquid chromatography quadrupole time-of-flight mass spectrometry). The quantification limits determined for each triazole in soil were 10 μ g/kg for prothioconazole, 35 μ g/kg for difenoconazole, and 0.1 μ g/kg for tetraconazole and cyproconazole. The results were expressed in ng/kg of soil dried at 105 °C.

Triazole concentrations for surface and underground water were obtained from the ADES (Accès aux Données sur les Eaux Souterraines, www.ades.eaufrance.fr) (Winckel et al., 2022) and NAIADES (https://www.brgm.fr/en/website/naiades-data-surface-water-quality) databases for different cities in the Centre-Val de Loire region near the patients' residences. The results were expressed in μ g/L of water.

2.2. Ethical issues

The study was carried out in accordance with the Declaration of Helsinki principles, and free, informed consent was obtained from all participants. The study protocol was previously approved by the Ethics Committee of the University Hospital of Tours as part of the "HAPO-FERTI" projects (authorization number 2016_075).

2.3. Isolation of human granulosa cells (hGCs)

A total of 159 women undergoing *in vitro* fertilization procedures for male infertility reasons at the reproductive medicine center of Tours Hospital between March 16, 2021, and May 27, 2023, were included in this study. Follicular fluid was recovered after isolation of luteinized cumulus-oocyte complexes and centrifuged at 1800 rpm for 15 min to pellet cell remnants. Human primary granulosa cells (hGCs) from this pellet were then isolated, Percoll purified, and cultured as described in Cloix et al. (2014) for 24 h before *in vitro* treatments. Human primary granulosa cells from three patients were pooled together for each experiment.

2.4. KGN cell line

KGN derived from luteinized granulosa tumoral cells collected from a 63-year-old woman in 1994 (Nishi et al., 2001) was used. Cells were cultivated with Dulbecco's Modified Eagle's Medium (DMEM; PAA, Thermo Fisher, Waltham, USA) with 10% fetal bovine serum (FBS) (Sigma-Aldrich; Saint-Louis, USA) and 1% of penicillin 10,000 UI/ml/-streptomycin 10,000 μ g/ml (Eurobio, Les Ulis, France). For viability and proliferation assay, 50,000 cells/well were incubated for 24 h at 37 °C, CO₂ 5% in 96-well plates or 250,000 cells/25cc-plates for steroidogenesis analysis before stimulation with triazoles.

2.5. Cell treatments

Difenoconazole (reference: PESTANAL™ 119446-68-3), metconazole (PESTANAL™ 125116-23-6), prothioconazole (PESTANAL® 178928-70-6), tetraconazole (PESTANAL™ 112281-77-3) and cyproconazole (PESTANAL™ 94361-06-5) were purchased from Sigma

Aldrich and diluted with 100% methanol (final concentration: 100 mM). Serial concentrations of triazole were prepared from 0.001 μM to 1000 μM . Non-observed adverse effect levels (NOAEL) of each triazole are described in Table 1. The dilutions were prepared with McCoy's 5 A medium containing FBS (10%) for hGCs or DMEM containing FBS (10%) for KGN, and cells were treated for 24 h or 48 h.

2.6. Cell viability

Cells (KGN or hGCs) were treated in 96-well plates with PT, MC, Dif, TT, or Cypro (from 0.001 to 1000 μM) for 24 h or 48 h (for KGN cells) in ranges associated with NOAEL. A Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, Saint-Louis, USA) was used for the cell viability assay, which was performed following the manufacturing protocol. The absorbances were measured with a spectrophotometer at the 450 nm wavelength.

2.7. RNA isolation and real-time quantitative PCR (qPCR)

KGN cells were treated for 48 h with the triazoles PT, MC, Dif, TT, or Cypro at non-cytotoxic concentrations (from 0 to 100 μM). Total RNA from the KGN cells was extracted with QIAzol® Lysis Reagent (Qiagen Sciences, Maryland, USA) following the manufacturing recommendations. DNase treatment (NucleoSpin® RNA Plus, Macherey-Nagel, France) was applied to the samples, and the quantity and the quality of RNA were determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). The cDNA was obtained by reverse transcription (RT) of total RNA (2 μg). Briefly, a mix of RT buffer (2 M), deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; 0.5 mM each), 15 $\mu\text{g}/\mu\text{L}$ of Oligo(dT) primer, 0.125 U of ribonuclease inhibitor, and 0.05 U of Moloney murine leukemia virus reverse transcriptase (MMLV) was prepared and mixed with samples before incubation for 1 h at 37 °C. The efficiency of primers was tested before the qPCR, as explained by Estienne et al. (2020). The couple primers used, for KGN cells, are for reference genes: *human β -actin* (F: 5'-AAGAGAGGCATCCTCACCCCT-3'; R: 5'-TACATGGCTGGGGTCTTGAA-3'), *human glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (F: 5'-ATGGAATCCCATCACCATCTT-3'; R: 5'-CGCCCCACTTGATTTTGG-3'), *human peptidylprolyl isomerase A (PPIA)* (F: 5'-CGCGTCTCCTTTGAGCTGT-3'; R: 5'-ATTTCTGTCTCTTGGGACC-3') and for targeted genes: *human steroidogenic acute regulatory protein (STAR)* (F: 5'-AAACTTACGTGGCTACTCAGCATC-3'; R: 5'-GACCTGGTTGATGCTCTTG-3'), *human cytochrome P450 family 11 subfamily A member 1 (CYP11A1)* (F: 5'-CAGGAGGGGTGGACACGAC-3';

R: 5'-AGGTTGCGTGCCATCTCATAC-3'), *human 3 beta-hydroxysteroid dehydrogenase (HSD3B)* (F: 5'-GCCTTCCAGACCAGAATTGAGAGA-3'; R: 5'-TCCTTCAAGTACAGTCAGCTTGGT-3'), *human Cytochrome P450 Family 19 subfamily A member 1 (CYP19A1)* (F: 5'-CCATAAAGACCCGATTCCACCA-3'; R: 5'-GCTGAGGCATAAATCGACAGAC-3'), *Cytochrome P450 14 α -sterol demethylase (CYP51)* (F: 5'-AAGCGCAGGCTGTCAAAGA-3'; R: 5'-CAGTCCGATGAGCATCCCTG-3') and *human aryl hydrocarbon receptor (AHR)* (F: 5'AGAGTTGACCGTTTGGCTA-3'; R: 5'-AGTTATCCTGGCCTCCGTT-3'). A new mixture of the cDNA of samples (3 μL) diluted ten times with SYBR Green Supermix 1X reagent (Bio-rad, Marnes la Coquette, France), 250 nM of specific human primers, and distilled water was prepared before performing the PCR procedure. After incubation for 2 min at 50 °C and a denaturation step of 10 min at 95 °C, the samples were subjected to 40 cycles (30 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) followed by the acquisition of the melting curve. The efficiency (E) of the primers was determined from a serial dilution of cDNA and was calculated as $E = 10 - 1/\text{slope value}$, and was between 1.9 and 2. Relative abundance for each gene was calculated according to primer efficiency (E) and quantification cycle (Cq), where expression = $E - Cq$. Melting curve analysis was performed to verify the presence of a single amplicon. The expression of the target gene was expressed relative to the geometric mean of three specific reference genes (*beta-actin*, *PPIA*, and *GAPDH*). The geometric mean of housekeeping genes has been reported as an accurate normalization factor (Vandesompele et al., 2002).

2.8. Progesterone and estradiol assays

Progesterone and estradiol concentrations in GC culture medium were measured employing the ELISA described by Canépa et al. (2008) and an ELISA kit provided by Cayman Chemicals, respectively, and normalized to the protein concentration of each well. For both tests, intra- and inter-assay coefficients of variation averaged < 10%.

2.9. Western blot

Proteins were extracted from hGCs and stimulated or not with triazoles PT, MC, Dif, TT, or Cypro at non-cytotoxic concentrations (from 0.1 μM to 500 μM), in lysis buffer (Tris 1 M (pH 7.4), NaCl 0.15 M, EDTA 1.3 mM, EGTA 1 mM, VO43 – 23 mM, NaF 0.1 M, NH2PO41%, Triton 0.5%). The lysates were centrifuged for 10 min at 13,500 g and 4 °C, and the supernatants containing proteins were collected and kept on ice. Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay (Interchim, Montluçon, France). Lysates protein (80 μg) were mixed with Laemli buffer 5X, and proteins were denatured for 5 min at 95 °C. Proteins were loaded in an electrophoresis sodium dodecyl sulfate-polyacrylamide gel (12%) before the transfer onto a nitrocellulose membrane. Membranes were blocked with Tris-Buffered Saline with Tween buffer containing 0.05% Tween 20% and 5% skim milk for 45 min at room temperature. Membranes were then incubated overnight at 4 °C with the appropriate primary antibodies: HSD3B (ThermoFisher Scientific, Illkirch-Grattenstaden, France, reference: PA5–106895), CYP11A1 (ThermoFisher Scientific, reference: PA5–37359), STAR (ThermoFisher Scientific, reference: PA5–21687), CYP19A1 (Serotec (Varilhes, France), AHR (Agro-Bio, La Ferté Saint Aubin, France), and vinculin (Sigma, Saint-Quentin-Fallavier, France, reference: V9131)) at a dilution of 1/1000. After five washings of 5 min with Tris-Buffered Saline with Tween buffer containing 0.05% of Tween 20, membranes were incubated for 90 min at room temperature with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Bio-Rad Laboratories, Marnes-la-Coquette, France) at a dilution of 1/5000. Proteins of interest were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, Villebon-sur-Yvette, France) with a G-box SynGene (Ozyme, St-Quentin-en-Yvelines, France) and GeneSnap software (Ozyme, St-Quentin-en-Yvelines, France). Then, proteins were quantified with GeneTools software

Table 1

Non-observable adverse effect level (NOAEL) of triazoles.

Triazoles	NOAEL (mg/kg bw/d)	Description	NOAEL (μM) ^a	References
Prothioconazole (344.26 g/mol)	1.1	Chronic and carcinogenic study in rats	3.2	(Australian Pesticides and Veterinary Medicines Authority, 2010)
Metconazole (319.83 g/mol)	8	Reprotoxicology in two generations of rats	25	(EFSA, 2006)
Difenoconazole (406.26 g/mol)	1	One year study in rats	2.5	(EFSA, 2011)
Tetraconazole (372.15 g/mol)	3.6	Reprotoxicology in two generations of mice	9.6	(EFSA, 2008)
Cyproconazole (291.78 g/mol)	6.76	Reprotoxicology in two generations of rats	23.2	(ECHA, 2016)

^a((NOAEL*10³)/Molar mass)* 10⁶

(version 4.01.02; Syngene). The results were expressed as the intensity signal in arbitrary units after normalization with vinculin.

2.10. Statistical analysis

For statistical analysis, we used GraphPad Prism 6® (San Diego, California, USA) with data presented as mean \pm standard error of the mean (SEM). The means of independent and random replicates were used, and the Bartlett and Shapiro-Wilk tests were run to test the homogeneity of variance and normal distribution, respectively. Unpaired Student or one-way ANOVA tests followed by Holm-Šidák's multiple comparisons test were applied for parametric values, and the Mann-Whitney test or the Kruskal-Wallis test followed by Dunn's multiple comparisons test were applied for non-parametric values. Means were considered different at $p < 0.05$.

3. Results

3.1. Environmental triazoles in the French Centre-val De Loire area

Environmental triazole residues in soil and water (underground and surface) of the French Centre-Val de Loire area are represented in [Supplemental Data 1–5](#). PT residues were not found in soils, but the residue of its metabolites, prothioconazole-desthio, was found at doses up to around 7 $\mu\text{g}/\text{kg}$ (around 20 nM^1). PT residues in surface and underground waters were found at up to 0.1 $\mu\text{g}/\text{L}$ (around 0.29 nM^2) and 0.135 $\mu\text{g}/\text{L}$ (around 0.39 nM^3) ([Supplemental Data 1](#)). MC residues were found at 10 $\mu\text{g}/\text{kg}$ (around 31 nM^4), 0.006 $\mu\text{g}/\text{L}$ (around 0.02 nM^5), and 0.36 $\mu\text{g}/\text{L}$ (around 1.1 nM^6) in soil, surface water, and underground water, respectively ([Supplemental Data 2](#)). Residues of Dif were found at 6 $\mu\text{g}/\text{kg}$ (around 15 nM^7), 0.04 $\mu\text{g}/\text{L}$ (around 0.1 nM^8), and 0.2 $\mu\text{g}/\text{L}$ (around 0.4 nM^9) ([Supplemental Data 3](#)) in soil, surface water, and underground water, respectively. TT was found at up to 10 $\mu\text{g}/\text{kg}$ (around 27 nM^{10}), 0.011 $\mu\text{g}/\text{L}$ (around 0.03 nM^{11}), and 0.12 $\mu\text{g}/\text{L}$ (around 0.3 nM^{12}) in soil, surface water, and underground water, respectively ([Supplemental Data 4](#)). Cypro was found at 30 $\mu\text{g}/\text{kg}$ (around 0.1 μM^{13}), 4.8 $\mu\text{g}/\text{L}$ (around 16 nM^{14}), and 1 $\mu\text{g}/\text{L}$ (around 3 nM^{15}) in soil, surface water, and underground water, respectively. ([Supplemental Data 5](#)).

3.2. Viability of KGN cells after in vitro triazole exposure

In KGN cells, after 24 h of exposure, PT decreased cell viability from 1000 μM ($p < 0.05$) ([Fig. 1A](#)), Dif from 500 μM ($p < 0.0001$) ([Fig. 1C](#)), and TT from 100 μM ($p < 0.05$) ([Fig. 1D](#)). MC and Cypro increased cell viability from 0.001 μM , but they decreased it at 1000 μM ($p < 0.0001$) ([Figs. 1B and 1E](#)). After 48 h of exposure, no variation of PT exposure from 0 to 1000 μM was significant ([Supplemental Data 6A](#)). Cell viability was increased at 1 and 10 μM of MC and decreased at 1000 μM of MC ($p < 0.0001$; [Supplemental Data 6B](#)). Dif decreased cell viability from 10

μM ($p < 0.001$; [Supplemental Data 6C](#)). TT first increased cell viability at 0.1 μM ($p < 0.05$) and then decreased it from 500 μM ($p < 0.0001$; [Supplemental Data 6D](#)). For Cypro, the same pattern seen at 24 h of exposure was observed at 48 h for concentrations between 0.001 and 100 μM ($p < 0.0001$); Cypro then decreased cell viability from 500 μM ($p < 0.05$; [Supplemental data 6E](#)).

3.3. Effect of in vitro triazole exposure on steroidogenesis in KGN cells

The secretions of Pg and E2 content in the conditioned medium of KGN cells after triazole exposure are illustrated in [Fig. 2](#). The results for only non-cytotoxic doses are shown. The mean basal secretions of Pg and E2 in KGN cells were 2.26 ± 0.08 ng/ml and 3.11 ± 0.09 ng/ml, respectively, and for hGCs, they were 6.00 ± 0.27 ng/ml and 9.56 ± 0.48 ng/ml, respectively. A tendency for decreased Pg secretion was noticed after PT exposure (1000 μM ; $p = 0.0927$), and a reduction of E2 production was observed at 1000 μM ($p < 0.0001$; [Fig. 2A and B](#)). No significant difference in Pg and E2 content was observed after MC and Dif exposure ($p < 0.05$) ([Fig. 2A–F](#)). TT reduced both Pg and E2 secretion at 10 μM ($p < 0.05$) ([Fig. 2G and H](#)). Cypro decreased only E2 secretion at 100 μM ($p < 0.001$) ([Fig. 2J](#)).

We next investigated whether these negative effects of triazoles on Pg and E2 production were a result of a reduction in three key enzymes of steroidogenesis (CYP11A1, HSD3B, and CYP19A1), a cholesterol carrier (STAR), and an enzyme involved in *de novo* cholesterol synthesis (CYP51). As shown in [Table 2](#), PT inhibited STAR and CYP19A1 mRNA expression at 1000 μM . No variations were observed after Dif exposure at a non-cytotoxic concentration. MC inhibited the expression of STAR from 10 μM ($p < 0.05$) and CYP19A1 at 100 μM ($p < 0.01$). Tetracozazole reduced STAR from 0.1 μM ($p < 0.01$) and at 10 μM reduced the expression level of HSD3B ($p < 0.0001$), CYP19A1 ($p < 0.0001$), and CYP51 ($p < 0.0001$) mRNAs. Finally, at 10 μM , Cypro has a positive effect on the expression of STAR ($p < 0.01$) and CYP11A1 ($p < 0.01$).

3.4. Effect of triazole exposure on the AHR mRNA expression and AHR protein concentrations in KGN cells

In KGN Cells, the expression of AHR mRNA was increased at 1000 μM of PT, 100 μM of MC ($p < 0.0001$), and 10 μM of TT ($p < 0.0001$). No variation of AHR expression was observed at non-cytotoxic concentrations for Dif and Cypro. The results are illustrated in [Table 3A](#). At non-cytotoxic concentrations, none of the triazoles showed variation for AHR protein levels. At cytotoxic concentrations, MC (5000 μM ; $p < 0.05$) and TT (1000 μM ; $p < 0.05$) increased AHR content in KGN cells, and a tendency for increase was observed for Dif (at 1000 μM ; $p = 0.0931$). The results are illustrated in [Table 3B](#) and [Supplemental Data 10](#).

3.5. Cell viability of human primary granulosa cells (hGCs) after in vitro triazole exposure

Regarding hGCs, after 24 h, PT decreased cell viability from 100 μM ($p < 0.01$; [Fig. 3A](#)). No significant difference was observed after MC exposure ([Fig. 3B](#)). Dif increased cell viability at 0.1 μM , whereas it decreased it from 500 μM ($p < 0.05$; [Fig. 3C](#)). Finally, TT decreased cell viability from 500 μM ($p < 0.0001$) ([Fig. 3D](#)), and Cypro decreased cell viability from 1000 μM ($p < 0.05$) ([Fig. 3E](#)).

3.6. Effect of in vitro triazole exposure on steroidogenesis in human granulosa cells (hGCs)

A tendency for decreased Pg secretion at 1 μM of PT was observed ($p = 0.0562$) ([Fig. 4A](#)). PT exposure decreased E2 secreted by hGCs at only 0.1 μM ($p < 0.05$) ([Fig. 4B](#)). MC exposure inhibited the secretion of Pg from 100 μM ($p < 0.001$) and the secretion of E2 at only 500 μM ($p < 0.0001$) ([Fig. 4C and D](#)). Dif reduced Pg content from 1 μM

¹ $(0.007 \cdot 10^{-3}) / 312.19$ (g/mol) = 20 nM

² $(0.1 \cdot 10^{-6} \text{ g/L}) / 344.26$ g/mol = 0.29 nM

³ $(0.135 \cdot 10^{-6} \text{ g/L}) / 344.26$ g/mol = 0.39 nM

⁴ $(0.01 \cdot 10^{-3}) / 319.83$ (g/mol) = 31 nM

⁵ $(0.006 \cdot 10^{-6} \text{ g/L}) / 319.83$ g/mol = 0.02 nM

⁶ $(0.36 \cdot 10^{-6} \text{ g/L}) / 319.83$ g/mol = 1.1 nM

⁷ $(0.006 \cdot 10^{-3}) / 406.26$ (g/mol) = 15 nM

⁸ $(0.04 \cdot 10^{-6} \text{ g/L}) / 406.26$ g/mol = 0.1 nM

⁹ $(0.2 \cdot 10^{-6} \text{ g/L}) / 406.26$ g/mol = 0.4 nM

¹⁰ $(10,000 \cdot 10^{-6}) \cdot 10^{-3} / 372.15$ = 27 nM

¹¹ $(0.01 \cdot 10^{-6}) / 372.15$ = 0.03 nM

¹² $(0.11 \cdot 10^{-6}) / 372.15$ = 0.3 nM

¹³ $(30,000 \cdot 10^{-6}) \cdot 10^{-3} / 291.78$ = 0.1 μM

¹⁴ $(4.8 \cdot 10^{-6}) / 291.78$ = 16 nM

¹⁵ $(1 \cdot 10^{-6}) / 291.78$ = 3 nM

KGN cells

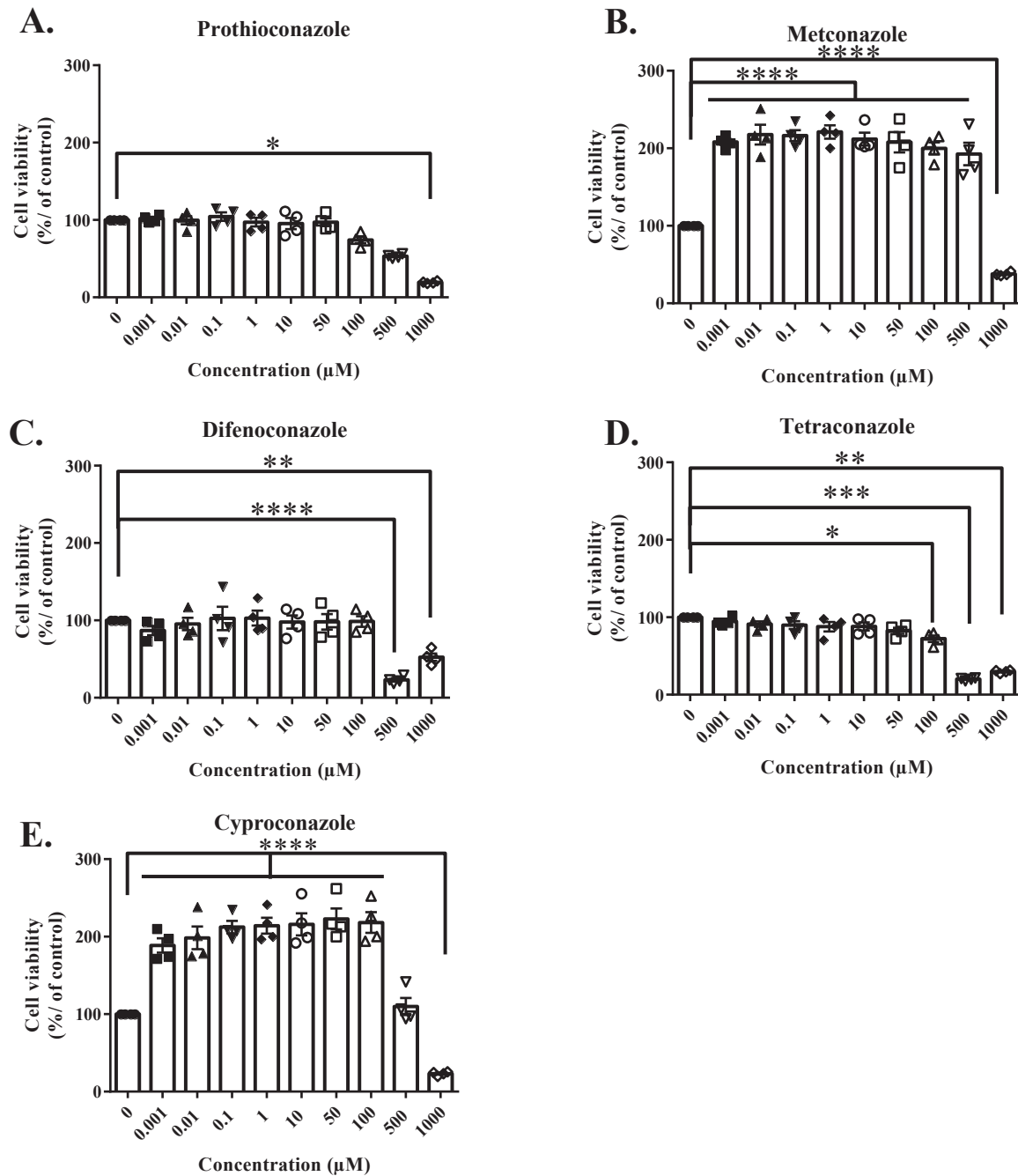


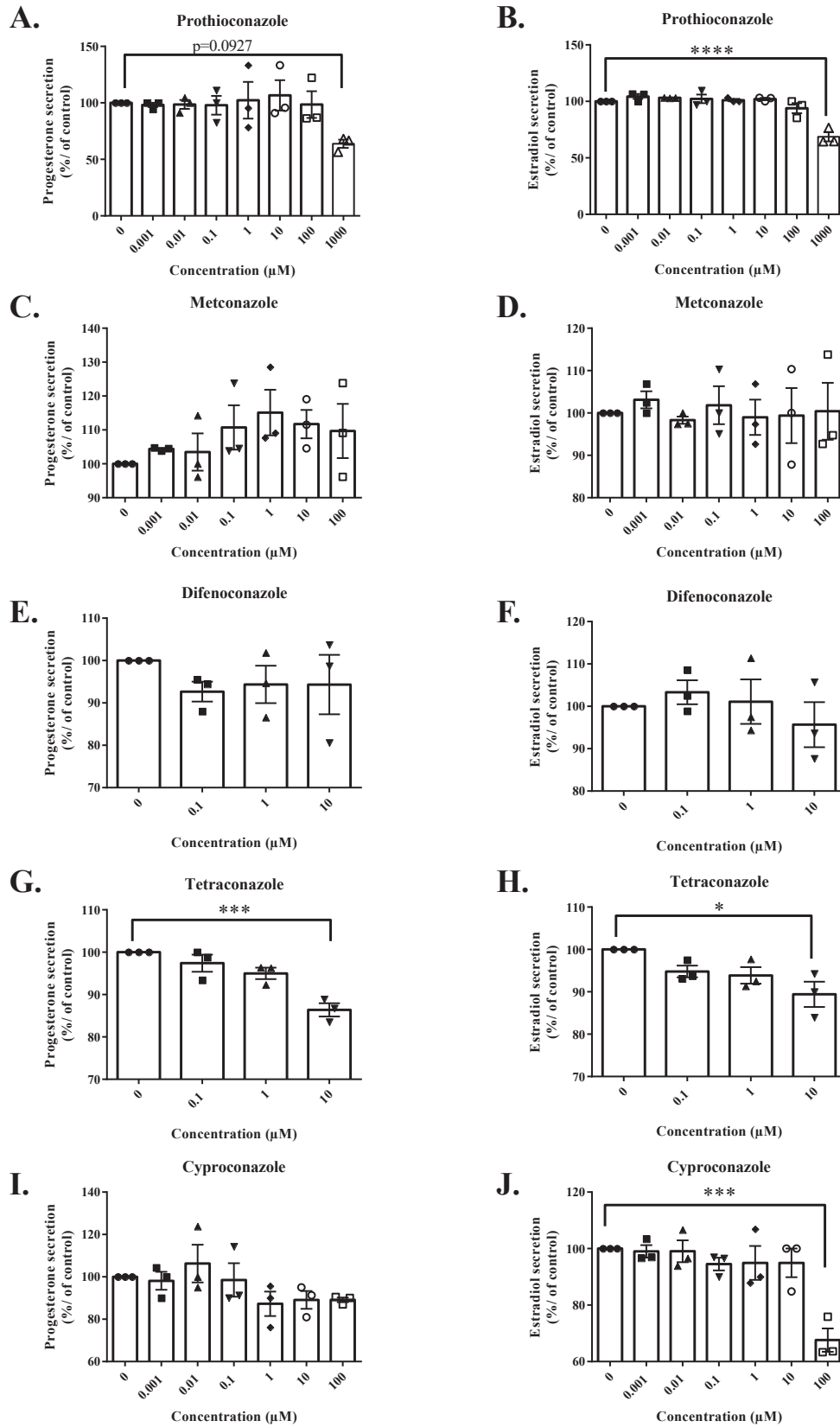
Fig. 1. Effect of triazoles after 24 h of exposure on KGN cell viability. Dose effect (0.001–1000 μM) of five triazoles (Prothioconazole (A), Metconazole (B), Difenoconazole (C), Tetraconazole (D) and Cyproconazole (E)) for 24 h on cell viability of KGN cell line. Data are means \pm SEM of 4 independent experiments. The values were normalized with the control (without triazole exposure). Stars (*) correspond to the one-way ANOVA significance followed by the Dunnett's multiple comparison test or the Kruskal Wallis significance followed by the Dunn's multiple comparison corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

($p < 0.001$) and E2 content from 10 μM ($p < 0.05$) (Figs. 4E and 4F). TT decreased Pg secretions from 10 μM and E2 secretions from 100 μM ($p < 0.01$) (Figs. 4G and 4H). Cypro reduced Pg production from 10 μM ($p < 0.0001$), but a tendency ($p = 0.0656$) was observed at 1 μM (Fig. 4I). Cypro also decreased E2 production from 10 μM ($p < 0.01$) (Fig. 4J).

As secretions of Pg and E2 were lower in KGN cells and protein content was poor in cell lysates, only proteins from hGCs were analyzed by western blot. The results are summarized in Table 4. No significant

difference was observed for STAR, CYP11A1, HSD3B, and CYP19A1 protein levels after exposure to PT. MC reduced the level of HSD3B protein after 100 μM ($p < 0.05$), but no variation was observed for STAR, CYP11A1, and CYP19A1. Dif tended to decrease HSD3B concentration in primary cells from 1 μM ($p = 0.0557$) and CYP11A1 from 100 μM ($p < 0.05$); no significant difference was observed for STAR and CYP19A1. TT exposure tended to reduce CYP19A1 levels at 0.1 μM ($p = 0.0880$) and STAR and CYP19A1 levels at the non-cytotoxic concentration 100 μM ($p < 0.05$). Cypro exposure reduced the levels of

KGN cells



(caption on next page)

Fig. 2. Effect of triazoles exposure on steroid production by KGN cells Dose effect (0.001–1000 μM) of five triazoles (Prothioconazole (A-B), Metconazole (C-D), Difenoconazole (E-F), Tetraconazole (G-H) and Cyproconazole (I-J) for 48 h on progesterone (A-C-E-G-I) and estradiol (B-D-F-H-J) production of KGN. Data are means \pm SEM of 3 independent experiments. The values were normalized with the control (without triazole exposure). Stars (*) correspond to the one-way ANOVA significance followed by the Dunnett's multiple comparison test or the Kruskal Wallis significance followed by the Dunn's multiple comparison test corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Table 2

Relative mRNA expressions of STAR, CYP11A1, HSD3B, CYP19A1, and CYP51 in the KGN cell line after exposure to the triazoles prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro) for 48 h. For each triazole compound, the triazole concentration was compared to the control (without exposure). Data are means \pm SEM of three independent experiments. Stars (*) correspond to ordinary one-way ANOVA significance followed by Dunnett's multiple comparison test or Kruskal-Wallis significance followed by Dunn's multiple comparison test corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Genes	Triazoles	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM	1000 μM	P-values
STAR	PT	0.8182 \pm 0.0322	0.7652 \pm 0.0325	0.8262 \pm 0.0163	0.7965 \pm 0.0213	0.7975 \pm 0.046	0.8217 \pm 0.0142	0.2646 \pm 0.0060 ****	< 0.0001
	MC	0.7898 \pm 0.0081	0.8 \pm 0.0138	0.7733 \pm 0.0311	0.7242 \pm 0.019	0.6726 \pm 0.02 *	0.4447 \pm 0.02 ****	/	< 0.0001
	Dif	0.3446 \pm 0.0216	0.389 \pm 0.0162	0.3484 \pm 0.0062	0.385 \pm 0.0372	0.3516 \pm 0.0163	/	/	0.4042
	TT	14.6731 \pm 1.0609	12.4276 \pm 0.3568	10.3668 \pm 0.5255 **	11.0935 \pm 0.087 *	6.1783 \pm 0.4414 ****	/	/	< 0.0001
	Cypro	0.0685 \pm 0.0067	0.0694 \pm 0.0222	0.078 \pm 0.0218	0.0938 \pm 0.0203	0.1673 \pm 0.0174 **	0.097 \pm 0.0034	/	0.0126
	CYP11A1	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM		P-values
PT	0.21 \pm 0.0122	0.2276 \pm 0.0092	0.2114 \pm 0.0022	0.1949 \pm 0.0072	0.2204 \pm 0.0086	0.2386 \pm 0.0016	0.2030 \pm 0.0123	0.0444	
MC	0.2018 \pm 0.0197	0.2024 \pm 0.0067	0.1872 \pm 0.0105	0.188 \pm 0.0126	0.1932 \pm 0.0008	0.2001 \pm 0.0096	/	0.8742	
Dif	0.0957 \pm 0.0076	0.0975 \pm 0.0069	0.1001 \pm 0.0023	0.1033 \pm 0.0034	0.104 \pm 0.0058	/	/	0.7500	
TT	0.7944 \pm 0.0337	0.8499 \pm 0.0341	0.7597 \pm 0.0151	0.829 \pm 0.0679	0.8142 \pm 0.0562	/	/	0.1167	
Cypro	0.0149 \pm 0.0029	0.0168 \pm 0.0044	0.0118 \pm 0.0014	0.0172 \pm 0.0045	0.0387 \pm 0.007 **	0.0203 \pm 0.0017	/	0.0077	
HSD3B	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM		P-values	
PT	0.101 \pm 0.0029	0.1061 \pm 0.0021	0.1032 \pm 0.0016	0.1008 \pm 0.0036	0.1029 \pm 0.0016	0.109 \pm 0.0041	0.1038 \pm 0.0056	0.6545	
MC	0.0977 \pm 0.0031	0.1049 \pm 0.0023	0.1034 \pm 0.0024	0.0959 \pm 0.0017	0.1024 \pm 0.0045	0.1083 \pm 0.0012	/	0.0509	
Dif	0.0482 \pm 0.0019	0.0471 \pm 0.0035	0.044 \pm 0.0045	0.0499 \pm 0.0029	0.0523 \pm 0.0011	/	/	0.3971	
TT	0.4181 \pm 0.0351	0.3617 \pm 0.0109	0.3617 \pm 0.0109	0.3761 \pm 0.0156	0.4041 \pm 0.0157 ****	/	/	< 0.0001	
Cypro	0.3255 \pm 0.1247	0.4126 \pm 0.1318	0.4126 \pm 0.1318	0.3796 \pm 0.0188	0.3853 \pm 0.0696	0.5086 \pm 0.1062	/	0.6031	
CYP19A1	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM		P-values	
PT	0.7554 \pm 0.0342	0.774 \pm 0.0367	0.7379 \pm 0.0392	0.7512 \pm 0.003	0.776 \pm 0.0221	0.7936 \pm 0.0052	0.1945 \pm 0.0117 ****	< 0.0001	
MC	0.7899 \pm 0.009	0.8183 \pm 0.0214	0.8376 \pm 0.0177	0.8222 \pm 0.0269	0.7253 \pm 0.147	0.3634 \pm 0.08 **	/	0.0017	
Dif	0.3594 \pm 0.0418	0.4034 \pm 0.0287	0.3536 \pm 0.0208	0.3716 \pm 0.0421	0.4227 \pm 0.0152	/	/	0.6192	
TT	0.8257 \pm 0.0287	0.7402 \pm 0.0263	0.7402 \pm 0.0263	0.6722 \pm 0.0435	0.7695 \pm 0.0277 ****	/	/	< 0.0001	
Cypro	0.52 \pm 0.1456	0.5093 \pm 0.0671	0.5093 \pm 0.0671 *	0.6974 \pm 0.1579	0.6399 \pm 0.2211	0.7899 \pm 0.1415	/	0.3675	
CYP51	0 μM	0.01 μM	0.1 μM	1 μM	10 μM	100 μM		P-values	
PT	0.7805 \pm 0.0204	0.7927 \pm 0.0345	0.7981 \pm 0.0299	0.8068 \pm 0.0185	0.7933 \pm 0.0196	0.8036 \pm 0.0226	0.7996 \pm 0.0169	0.8500	
MC	0.8783 \pm 0.0061	0.8771 \pm 0.0242	0.8483 \pm 0.0263	0.8697 \pm 0.0056	0.8472 \pm 0.0165	0.8987 \pm 0.0098	/	0.2868	
Dif	0.3031 \pm 0.0181	0.3506 \pm 0.0188	0.3214 \pm 0.0109	0.3280 \pm 0.0033	0.3355 \pm 0.0035	/	/	0.1068	
TT	0.7581 \pm 0.0265	0.7661 \pm 0.0009	0.7524 \pm 0.0173	0.7433 \pm 0.0199	0.1630 \pm 0.0060 ****	/	/	< 0.0001	
Cypro	0.0229 \pm 0.0013	0.0205 \pm 0.0001	0.0234 \pm 0.0012	0.0217 \pm 0.0008	0.0220 \pm 0.0008	0.0229 \pm 0.0011	/	0.4082	

CYP11A1 and HSD3B at the highest non-cytotoxic concentration ($p < 0.05$) without variations in STAR and CYP19A1 levels. The western blot results are represented in [Supplemental Data 9](#).

3.7. Effect of triazole exposure on AHR protein concentration in hGCs

We examined the protein content of AHR in primary granulosa cells after exposure to triazoles for 48 h. Dif decreased AHR from 1 μM ($p < 0.05$); however, no significant variations were observed for PT, MC, TT, and Cypro. The results are summarised in [Table 5](#). Western blot

Table 3

Relative mRNA expression of the AHR (aryl hydrocarbon receptor) mRNA gene (A) and protein concentration (B) in the KGN cell line after exposure to triazoles (prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro)) for 48 h. For each triazole compound, each triazole concentration was compared to the control (without exposure) for mRNA, and for proteins, the values were normalized with the expression of vinculin (VCL) for each sample and compared to the control condition (without triazole) * 100. Purple values are at cytotoxic concentrations. Data are means \pm SEM of three independent experiments. Stars (*) correspond to ordinary one-way ANOVA significance followed by Dunnett's multiple comparison test or Kruskal-Wallis significance followed by Dunn's multiple comparison test corresponding to the comparison between control (without exposure) and the other triazole concentrations. * : $p < 0.05$; ** : $p < 0.01$; *** : $p < 0.001$; **** : $p < 0.0001$.

A. Gene	Triazoles	0 μ M	0.01 μ M	0.1 μ M	1 μ M	10 μ M	100 μ M	1000 μ M	P-values
AHR	PT	0.7805 \pm 0.0204	0.7927 \pm 0.0345	0.7981 \pm 0.0299	0.8068 \pm 0.0185	0.7933 \pm 0.0196	0.8235 \pm 0.0381	2.321 \pm 0.0548 ****	< 0.0001
	MC	0.7911 \pm 0.0093	0.8507 \pm 0.0498	0.7919 \pm 0.0309	0.8235 \pm 0.0414	0.8386 \pm 0.0322	1.511 \pm 0.01 ****	/	< 0.0001
	Dif	0.182 \pm 0.008	0.1728 \pm 0.004	0.1746 \pm 0.0055	0.1903 \pm 0.0059	0.2081 \pm 0.0175	/	/	0.1764
	TT	3.3256 \pm 0.1481	2.8723 \pm 0.1992	3.3098 \pm 0.2112	3.4917 \pm 0.1277	5.5346 \pm 0.6421 **	/	/	< 0.0001
	Cypro	1.4471 \pm 0.2006	1.4659 \pm 0.302	1.5515 \pm 0.2121	1.6194 \pm 0.2737	2.4449 \pm 0.5166	1.8893 \pm 0.5204	/	0.4049
B. Protein	Triazoles	0 μ M	1 μ M	10 μ M	100 μ M	1000 μ M	5000 μ M	P-values	
AHR	PT	100 \pm 0	67.6 \pm 23.07	81.01 \pm 39.66	168.4 \pm 81.20	2638 \pm 1297	7103 \pm 6521	0.4130	
	MC	100 \pm 0	55.13 \pm 16.67	86.83 \pm 51.16	74.37 \pm 58.51	227.3 \pm 129.2	705.6 \pm 225.5 *	0.0096	
	Dif	100 \pm 0	61.57 \pm 41.63	138.0 \pm 45.32	262.1 \pm 0102.3	1126 \pm 606.6	1025 \pm 226.6	0.0417	
	TT	100 \pm 0	95.79 \pm 2.044	84.98 \pm 22.96	107.1 \pm 22.27	308.3 \pm 84.35 *	275.8 \pm 71.76	0.0136	
	Cypro	100 \pm 0	85.74 \pm 33.39	97.96 \pm 43.08	127.0 \pm 23.99	682.2 \pm 399.3	1303 \pm 1076	0.3937	

results are represented in [Supplemental Data 10](#).

All the results are summarized as a dose-response graphic in [Supplemental Data 7](#) for KGN cells and in [Supplemental Data 8](#) for hGCs. [Fig. 5](#) resumes the effects of prothioconazole, metconazole, difenoconazole, tetraconazole, and cyproconazole on the steroidogenic activity of KGN cells (A) and hGCs (B).

4. Discussion

In the present study, we demonstrated the presence of prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro) in the soil and water of the French Centre-Val de Loire region. We have shown for the first time that exposure of human primary granulosa cells (hGCs) to PT at below cytotoxic concentrations, specifically, a concentration 256 times higher than that found in underground waters (UGW), altered E2 secretion. At higher concentrations (1000 μ M, around 2.5×10^6 UGW), E2 production and the mRNA expression of STAR, CYP19A1, and AHR were also altered in KGN cells. Metconazole exposure in KGN cells inhibited STAR and CYP19A1 mRNA expression at 9000 and 90,000 times the UGW concentration. This was supported by an increase in AHR mRNA expression. However, in hGCs, only the protein level of HSD3B and Pg secretion were reduced. Regarding Dif, *in vitro* exposure did not impact steroidogenesis in doses below the cytotoxic threshold. In hGCs, Dif alters both Pg and E2 secretion through decreased protein levels of CYP11A1 and AHR. Perturbations happened at 2500 times the UGW concentration. Tetraconazole exposure reduced the secretion of both Pg and E2 in KGN cells and hGCs. This was associated with the inhibition of STAR, HSD3B, CYP19A1, and CYP51 and the upregulation of AHR mRNA expression in KGN cells at 33,000 times the UGW concentrations, and the decrease of STAR and P450 aromatase protein levels in hGCs. Cyproconazole exposure decreased E2 secretion in KGN cells related to an induction of STAR and CYP11A1 transcription at 3333 times the UGW concentration. However, in hGCs, both secretion of Pg and E2 were inhibited with the decrease in CYP11A1 and HSD3B protein levels.

[Fig. 5 A](#) and [5B](#) summarize the results of the present study in KGN cells and hGCs, respectively.

4.1. Triazoles can be cytotoxic for human granulosa cells

The most cytotoxic triazoles in this study were PT, Dif, and TT. PT was found in UGW of the French Centre-Val de Loire region at around 0.39 nM, the highest concentration among the pesticides detected. At 100 μ M, it decreased the viability of hGCs. However, this dose corresponds to 3×10^5 times¹⁶ the highest concentration found in the UGW. At less concentrated doses, PT also inhibited cell viability in extravillous trophoblast cells ([Dong et al., 2022](#)). Interestingly, in a study comparing the embryotoxic and pharmacologic potency of Dif and PT in rat whole embryo, PT was less potent than Dif ([Dimopoulou et al., 2017](#)). In our study, Dif was more cytotoxic for KGN cells than for hGCs. TT was toxic for KGN cells at 3×10^5 times the amount found in the UGW. It also showed cytotoxicity in bovine mammary epithelial cells, which was associated with an increase in apoptosis and autophagy ([Jeong et al., 2023](#)). The least cytotoxic triazole in human ovarian cells was MC. Indeed, no impact on cell viability was observed in primary granulosa cells, and KGN did not decrease cell viability before 1000 μ M. In an *in vivo* study performed on zebrafish, the LC50 of MC exposure was placed at 3.17 mg/L ([He et al., 2022](#)), a dose 5×10^5 times higher than those found in surface water in our study. Difenoconazole was also cytotoxic for granulosa cells from 10 μ M, corresponding to 25×10^3 times the highest concentration found in surface water. In the cytotoxicity evaluation of Dif on HepG2 cells, LC50 was placed at 24.72 μ M, which is relevant to our study ([Wang et al., 2022](#)). The results this study obtained on Cypro toxicity were supported by the literature, where low concentrations (between 10 and 100 μ M) did not exert cytotoxicity on the human placental cell line or rat Leydig cell line ([Rieke et al., 2014](#); [Roelofs et al., 2014](#)) and high concentrations (1000 μ M) decreased HepG2 cell viability ([Wang et al., 2022](#)). Consequently, the results of our study after the threshold of cytotoxicity are only illustrated in [Supplemental Data 7 and 8](#), and only results at non-cytotoxic concentrations were shown. To summarize, the greatest effect of triazole toxicity in KGN cells was Dif>TT> Cypro> PT=MC, and in hGCs, it was PT > Dif = TT > Cypro > MC.

¹⁶ $(0.1 \times 10^{-6} \text{ g/L} / 344.26 \text{ g/mol}) \times 10^6 = 0.00290 \mu\text{M}$; $100 \mu\text{M} / 0.000290 \mu\text{M} = 3 \times 10^5$

Human primary granulosa cells

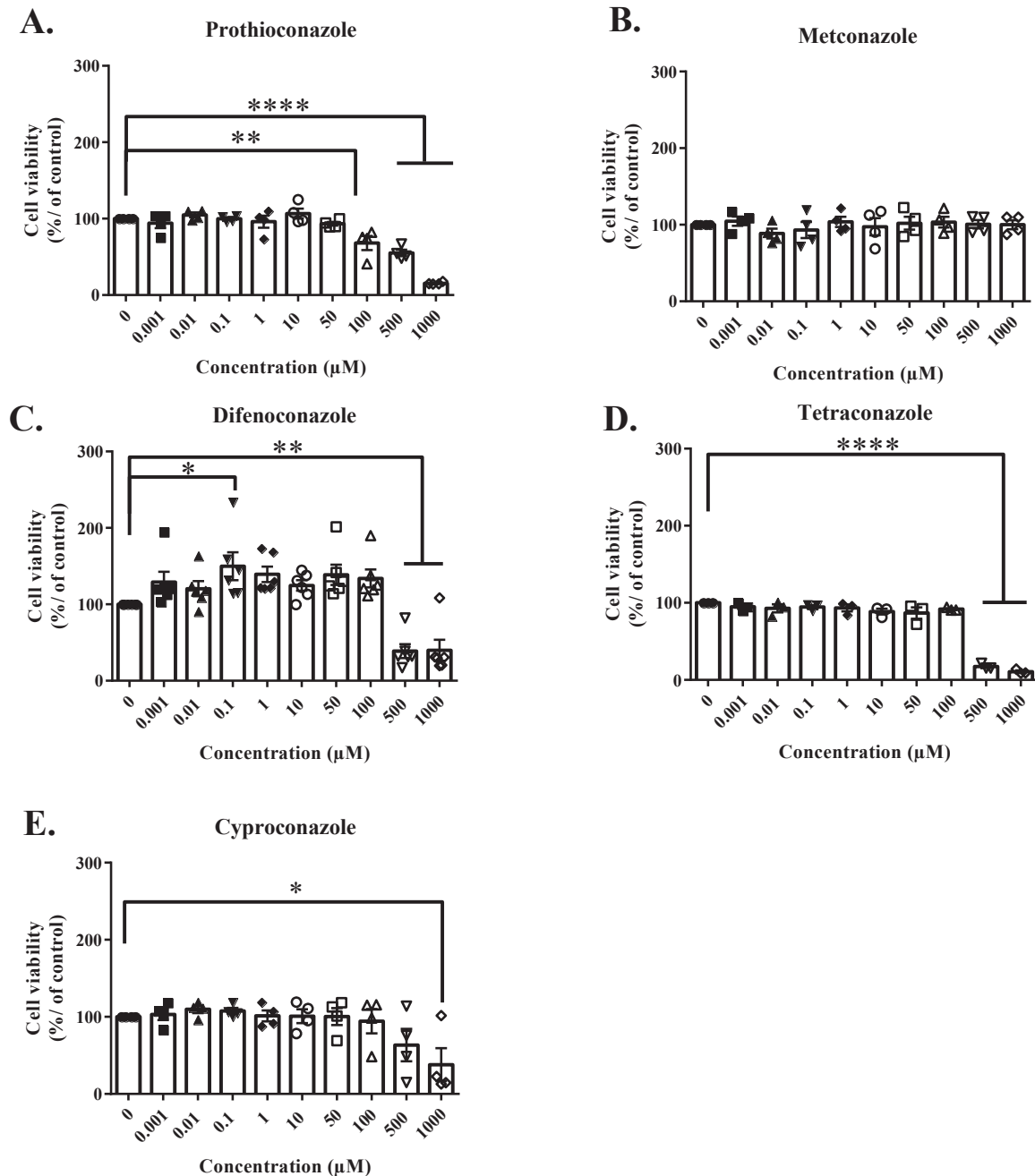


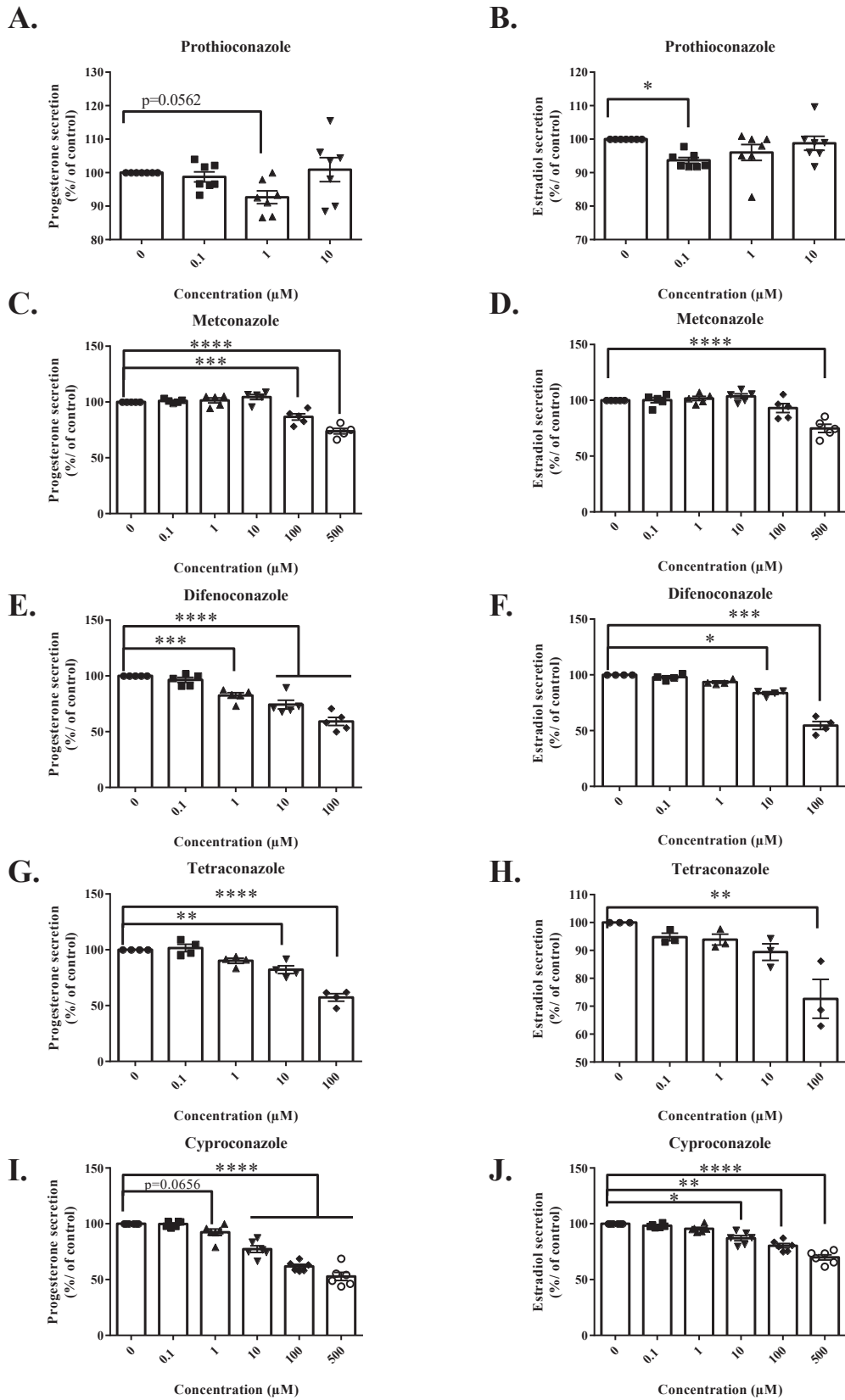
Fig. 3. Effect of triazoles exposure on human primary granulosa cell (hGCs) viability Dose effect (0.001–1000 μM) of five triazoles (Prothioconazole (A), Metconazole (B), Difenoconazole (C), Tetraconazole (D) and Cyproconazole (E) for 24 h on cell viability of hGCs. Data are means \pm SEM of 3, 4 or 6 independent experiments. For one experiment of hGCs, a pool of cells from 3 patients was used for each triazole. The values were normalized with the control (without triazole exposure). Stars (*) correspond to the one-way ANOVA significance followed by the Dunnett's multiple comparison test or the Kruskal Wallis significance followed by the Dunn's multiple comparison corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

4.2. Triazoles alter steroidogenesis in human granulosa cells

Difenoconazole exposure for 48 h decreased the secretion of Pg and E2 in granulosa cells through the inhibition of CYP11A1, known to convert cholesterol to pregnenolone, and a tendency for inhibition of HSD3B, which converts pregnenolone into Pg. This was at 2500 times the UGW concentration; however, it corresponds to 4.8 times the concentration found in the blood of birds living in a vineyard in France (Angelier et al., 2023). This result is in good agreement with Zhai et al.'s

study (2023), in which it was found that Dif inhibited 3 β -HSD1 enzymatic activity in human placenta microsomal extracts. Moreover, it has also been shown in the literature that Dif (10 μM) increased CYP11A1 and HSD3B expression on HepG2, associated with a decrease in E2 secretion (Wang et al., 2022). This endocrine-disrupting effect was also observed with zebrafish in the literature (Chen et al., 2022; Teng et al., 2018). Furthermore, in our study on hGCs, Dif reduced the expression of AHR protein at the same concentration with which it caused a decrease in E2. It has been shown that Dif can activate AHR in human hepatic

Human primary granulosa cells



(caption on next page)

Fig. 4. Effect of triazoles exposure on steroid production by human primary granulosa cells (hGCs) Dose effect (0.001–1000 μM) of five triazoles (Prothioconazole (A-B), Metconazole (C-D), Difenoconazole (E-F), Tetraconazole (G-H) and Cyproconazole (I-J) for 48 h on progesterone (A-C-E-G-I) and estradiol (B-D-F-H-J) production of hGCs. Data are means \pm SEM of *, 4 or 6 independent experiments. For one experiment of hGCs, a pool of cells from 3 patients was used for each triazole. The values were normalized with the control (without triazole exposure). Stars (*) correspond to the one-way ANOVA significance followed by the Dunnett's multiple comparison test or the Kruskal Wallis significance followed by the Dunn's multiple comparison corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Table 4

Protein expression of STAR, CYP11A1, HSD3B, and CYP19A1 aromatase proteins after triazole exposure in human primary granulosa cells.

Triazoles		0 μM	0.1 μM	1 μM	10 μM	100 μM	500 μM	P-values
STAR	PT (n = 5)	100 \pm 0	143.44 \pm 13.47	104.27 \pm 36.29	76.98 \pm 18.19	/	/	0.091
	MC (n = 4)	100 \pm 0	111.72 \pm 40.34	201.27 \pm 53.94	72.17 \pm 3.16	150.74 \pm 74.71	237.33 \pm 170.81	0.6057
	Dif (n = 6)	100 \pm 0	81.37 \pm 35	47.73 \pm 26.37	72.6 \pm 28.74	67.08 \pm 33.27	/	0.3062
	TT (n = 4)	100 \pm 0	59.99 \pm 17.11	65.32 \pm 40.55	31.59 \pm 19.94	13.45 \pm 9.95 *	/	0.0321 *
	Cypro (n = 6)	100 \pm 0	87.71 \pm 17.49	102.02 \pm 24.08	109.58 \pm 59.85	87.68 \pm 31.56	94.03 \pm 35.09	0.8435
	CYP11A1	0 μM	0.1 μM	1 μM	10 μM	100 μM	500 μM	P-values
CYP11A1	PT (n = 5)	100 \pm 0	156.54 \pm 108	205.13 \pm 124.59	204.96 \pm 82.66	/	/	0.9935
	MC (n = 5)	100 \pm 0	134.72 \pm 44.39	227.81 \pm 69.94	245.19 \pm 83.74	99.25 \pm 33.24	202.22 \pm 104.06	0.4648
	Dif (n = 4)	100 \pm 0	107.19 \pm 37.1	74.27 \pm 28.36	72.85 \pm 34.27	19.84 \pm 5.41 *	/	0.0786
	TT (n = 7)	100 \pm 0	294.54 \pm 118.57	95.44 \pm 26.6	110.41 \pm 37.97	281.12 \pm 145.26	/	0.6088
	Cypro (n = 6)	100 \pm 0	55.34 \pm 11.67	57.65 \pm 14.81	75.71 \pm 37.38	97.05 \pm 48.49	18.79 \pm 14.34 **	0.0736
	HSD3B	0 μM	0.1 μM	1 μM	10 μM	100 μM	500 μM	P-values
HSD3B	PT (n = 5)	100 \pm 0	116.64 \pm 20.72	78.56 \pm 31.91	103.48 \pm 42.6	/	/	0.5347
	MC (n = 5)	100 \pm 0	219.95 \pm 96.35	308.88 \pm 181.4	636.9 \pm 405.1	21.78 \pm 3.87 *	43.04 \pm 17.79	0.0221 *
	Dif (n = 4)	100 \pm 0	86.82 \pm 9.48	57.62 \pm 6.39	70.19 \pm 12.78	72.6 \pm 13.77	/	0.1105
	TT (n = 5)	100 \pm 0	94.27 \pm 32.97	95.76 \pm 20.65	85.34 \pm 17.39	105.36 \pm 30.39	/	0.9846
	Cypro (n = 6)	100 \pm 0	91.21 \pm 30.41	103.91 \pm 39.58	103.21 \pm 42.7	79.19 \pm 33.89	13.77 \pm 7.56 *	0.0488 *
	CYP19A1	0 μM	0.1 μM	1 μM	10 μM	100 μM	500 μM	P-values
CYP19A1	PT (n = 4)	100 \pm 0	146.26 \pm 32.61	128.16 \pm 42.22	70.42 \pm 26.35	/	/	0.3302
	MC (n = 7)	100 \pm 0	121.29 \pm 17.57	283.53 \pm 81.08	207.27 \pm 45.08	232.28 \pm 71.69	335.23 \pm 139.75	0.4807
	Dif (n = 6)	100 \pm 0	116.9 \pm 47.29	93.01 \pm 43.07	85.68 \pm 33.4	53.47 \pm 7.59	/	0.396
	TT (n = 3)	100 \pm 0	34.6 \pm 4.53	59.3 \pm 11.95	69.14 \pm 18.46	32.78 \pm 10.91 *	/	0.0579
	Cypro (n = 3)	100 \pm 0	62.32 \pm 30.41	178.13 \pm 116.29	457.84 \pm 300.54	166.09 \pm 87.19	6.57 \pm 3.44	0.4456

The effect of five triazoles (prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro)) on the protein levels of the cholesterol carrier STAR, CYP11A1, HSD3B, and CYP19A1 steroidogenic enzymes in primary human granulosa cells exposed *in vitro* for 48 h. Data are means \pm SEM of three to seven independent experiments. For one experiment with primary granulosa cells, a pool of cells from three patients was used for each triazole. The values were normalized with the expression of vinculin (VCL) for each sample and compared to the control condition (without triazole)* 100. Stars (*) correspond to the Kruskal-Wallis significance test, followed by Dunn's multiple comparison test corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. P-values represent statistical significance for the Kruskal-Wallis test.

HepaRG cells (Lasch et al., 2021) and that different results can be observed according to the cell type (Heise et al., 2015; Rieke et al., 2014). The literature has also shown that the inactivation of AHR leads to a decrease in E2 in ovaries (Baba et al., 2005). One hypothesis is that AHR, which is the natural ligand of AHR, could be modulated by Dif and inhibit AHR. AHR, known to modulate the expression of ER (Ohtake et al., 2009), once deactivated by Dif, binds the element response on the promoter of ER and consequently alters the secretion of E2.

Concerning PT exposure, our study shows that the secretion of E2 was disturbed at less than cytotoxic doses in human granulosa cells. This was associated with STAR, CYP19A1, and AHR mRNA expression variations in KGN but not with protein levels in hGCs. PT has been shown to

increase AHR protein concentration in HepG2 at toxic concentrations and bind AHR specifically at the tyrosine-137, Glycine-150, and serine-140 locations (Tian et al., 2022). In this same study, PT increased the number of atretic follicles in mice exposed *in vivo* by oral gavage for 30 days. AHR mRNA was also increased in the ovaries of mice by the main metabolite of PT, prothioconazole-desthio, which is also found in the soil of the French Centre-Val de Loire region. As has been demonstrated in human placental cells, PT can weakly induce aromatase (Kjeldsen et al., 2013), but in our study on granulosa cells, inhibition of the expression of CYP19A1 mRNA at the highest concentration was observed.

Metconazole decreased the secretion of Pg in hGCs at a non-cytotoxic

Table 5

Protein concentrations of AHR (Aryl Hydrocarbon Receptor) in human primary granulosa cells (hGCs) after exposure to the triazoles prothioconazole (PT), metconazole (MC), difenoconazole (Dif), tetraconazole (TT), and cyproconazole (Cypro) for 48 h. Data are means \pm SEM of two to six independent experiments. For one experiment with primary granulosa cells, a pool of cells from three patients was used for each triazole. The values were normalized with the expression of vinculin (VCL) for each sample and compared to the control condition (without triazole) \times 100. Stars (*) correspond to the Kruskal-Wallis significance test, followed by Dunn's multiple comparison test corresponding to the comparison between control (without exposure) and the other triazole concentrations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. P-values represent statistical significance for the Kruskal-Wallis test.

Proteins	Triazoles	0 μ M	0.1 μ M	1 μ M	10 μ M	100 μ M	500 μ M	P-values
AHR	PT (n = 5)	100 \pm 0.00	103.2 \pm 33.68	83.10 \pm 15.4	132.00 \pm 40.72	/	/	0.9551
	MC	100 \pm 0.00	153.3 \pm 75.03	175.7 \pm 85.52	216.00 \pm 86.47	135.3 \pm 69.37		0.6845
	Dif (n = 5)	100 \pm 0.00	57.10 \pm 20.76	28.80 \pm 16.55 *	26.95 \pm 8.98 *	21.77 \pm 9.76 *	/	0.0253 *
	TT (n = 6)	100 \pm 0.00	261.2 \pm 135.5	114.1 \pm 25.28	71.82 \pm 0.1277	203.4 \pm 114.00	/	0.7593
	Cypro (n = 2)	100 \pm 0.00	16.46 \pm 8.47	22.98 \pm 15.47	37.52 \pm 29.44	19.53 \pm 5.26	10.24 \pm 0.08	0.4715

dose; this decrease was associated with a decrease in HSD3B protein levels. This effect could be cell type dependent because, in Zhai et al.'s study, no perturbation at the same concentration was observed in the HSD3B protein level in human placenta cells (Zhai et al., 2023). In our study, no alteration of steroid hormone secretion was noticed in KGN cells, but inhibition of STAR and CYP19A1 mRNA expression was observed to be associated with an increase of AHR mRNA expression but not with protein levels. This effect happened at 9000 times the UGW concentration and 333 times the concentration found in human urine in Brazil (Machado et al., 2019). Only a limited number of studies have investigated the connection between AHR and MC. In a previous study, we found that *in vitro* exposure of chicken testis to MC induced the transcription of AHR mRNA and was associated with steroidogenesis disruption (Serra et al., 2023). Zhuang et al. (2018) have shown that MC can bind specifically to cytochrome P450 CYP3A4, which plays a role in estrogen synthesis, metabolizing E2 into 2-hydroxyestradiol (Craig et al., 2011). Moreover, CYP3A4, a target of pregnane X receptor (PXR) present in ovarian cells (Gupta et al., 2008) with a potential steroidogenesis regulation (Yue et al., 2010), can regulate AHR (Gerbal-Chaloin et al., 2006). Consequently, two hypotheses concerning KGN cells can be made: i) MC binds to AHR, which will regulate, in the nucleus, the transcription of STAR and CYP19A1 mRNA, and ii) MC binds CYP3A4, which will change its conformation and is recognized by PXR which will modulate its transcription to regulate AHR and consequently alter the transcription of STAR and CYP19A1 mRNA.

Exposure of KGN cells and hGCs to TT led to a reduction of Pg and E2 secretion. This was associated in KGN cells with the inhibition of STAR, HSD3B, and CYP19A1 and the upregulation of AHR mRNA expression and in hGCs, with the reduction of STAR and CYP19A1 protein levels. AHR protein levels were also increased in hGCs, but at cytotoxic concentrations. The first effect was observed at 333 times the UGW concentration, 100 times the concentration found in human hair in Italy after the application season (Polledri et al., 2021) but below the concentration found in bird's blood in France (around 22 μ M) (Angelier et al., 2023). To our knowledge, this is the first time TT's endocrine disruption has been analyzed in cells of human origin. Very few studies have analyzed TT's effects on mammalian cells, and none focus on steroidogenesis. In a previous study by our lab, a negative impact on testosterone production related to HSD3B and CYP19A1 mRNA inhibition and an increase in AHR mRNA expression was observed in an explant of chicken testis (Serra et al., 2023). These effects occurred at concentrations higher than those observed in this study. Therefore, TT seems to exert an endocrine-disrupting effect on human granulosa cells, probably due to i) cholesterol synthesis alterations, ii) steroidogenic enzyme dysregulation, and iii) AHR activation.

Exposure of hGCs to cyproconazole (10 μ M; approximately 3333 times the concentration found in UGW) had an impact on the production of Pg and E2. Pg secretion was disturbed at 250 times the concentration

found in human urine in Brazil (Machado et al., 2019). This was associated with increased STAR and CYP11A1 mRNA expression levels in KGN cells and decreased CYP11A1 and HSD3B protein levels in hGCs. No induction of AHR mRNA before the cytotoxic threshold was observed. Perturbation of steroid production was also noticed in other cell types, such as JEG3 cells (Rieke et al., 2014), MA-10 cells (Roelofs et al., 2014), and HepG2 cells (Wang et al., 2022), but this is the first time it has been demonstrated in human granulosa cells.

To summarize the results, Dif, TT, and Cypro seem to have the greatest impact on the steroidogenic functions of human granulosa cells at concentrations from 256 to 9000 times those found in the soil and water of the Centre-Val de Loire region of France, but close to the human urine or bird blood concentrations measured by other researchers (Angelier et al., 2023; Machado et al., 2019). The next step would be the measurement of those triazoles in the follicular fluid (FF). Indeed, most chemicals are present in FF at ranges from ng/L to μ g/L (Craig et al., 2011; Petro et al., 2012; Petro et al., 2014). If triazoles are present in the FF, it could be dangerous for the development of oocytes (Petro et al., 2012) and increase the risk of infertility.

5. Conclusions

This study demonstrated the cytotoxic and potential steroidogenesis-disrupting effects of five triazoles on human granulosa cells. We showed that for Dif and TT, these effects were observed at concentrations close to those found in the environment. However, we did not take into account the bioaccumulation in the food chain or "cocktail" effect of triazoles in ovarian functions, and the triazole content in human biological fluid, such as follicular fluid, also needs to be determined. Moreover, the molecular mechanisms of these triazoles on the negative effects of steroid production in human granulosa cells remain unclear. Further studies need to be carried out to understand the links between AHR and genes involved in the steroidogenesis process. Utilizing an *in vivo* approach involving mice with a genetically invalidated AHR gene could be a potential model for investigating the effects of triazoles on mammalian reproduction.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

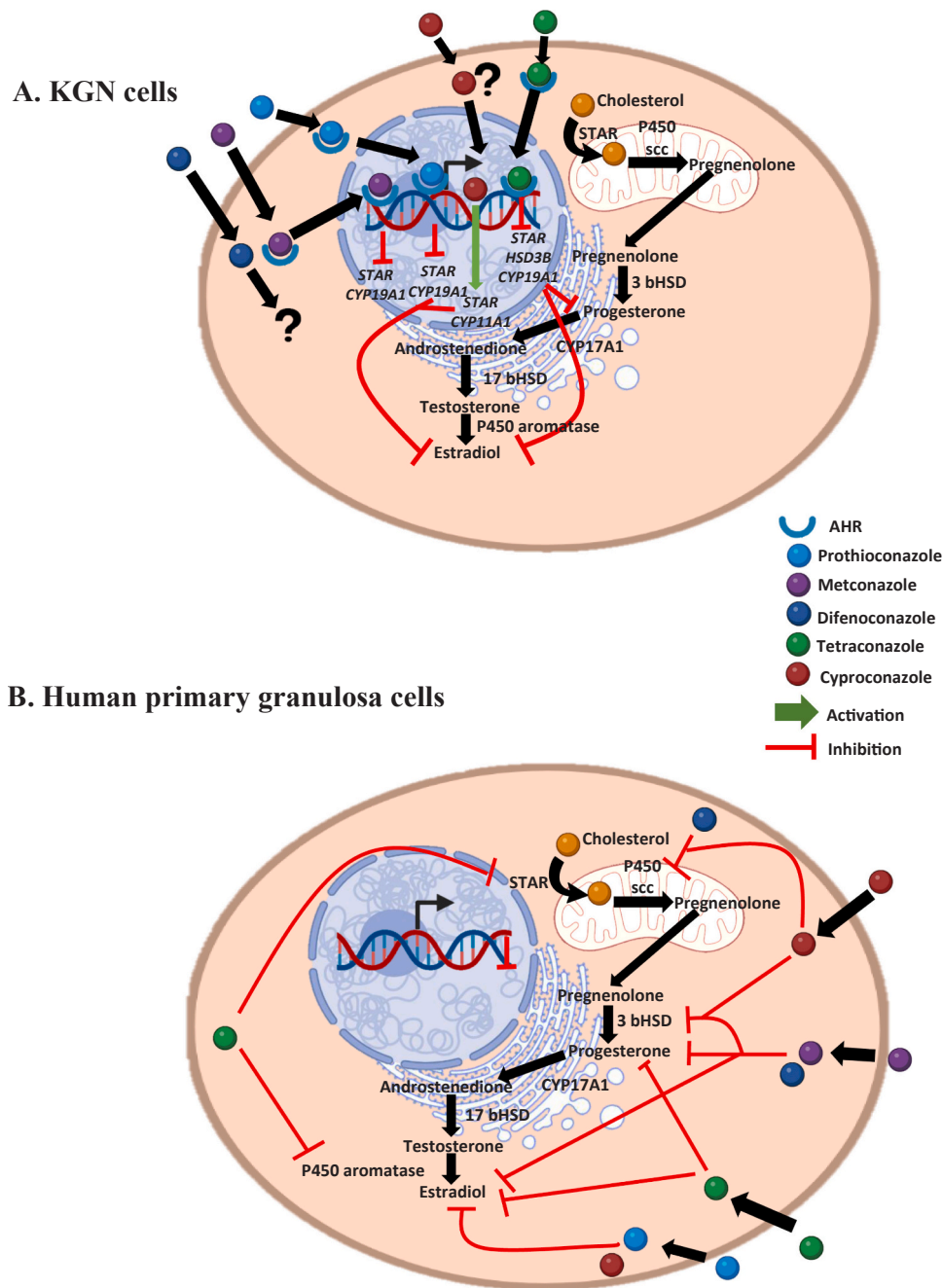


Fig. 5. Schematic representation of the effects of Prothioconazole, Metconazole, Difenconazole, Tetraconazole and Cyproconazole on KGN cells (A) and human primary granulosa cells (B).

the work reported in this paper.

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.etap.2023.104295](https://doi.org/10.1016/j.etap.2023.104295).

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