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Research Article

Ex Vivo Assessment of Testicular Toxicity Induced by Carbendazim and Iprodione, Alone or in a Mixture

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Summary

To measure the testicular toxicity of two fungicides (carbendazim and iprodione), alone or in a mixture, we used a rat *ex vivo* model of seminiferous tubules, greatly reducing the number of rodents used, in accordance with the 3R rule (Replacement, Reduction, and Refinement). This model allows the representation of puberty, a critical life period with regard to endocrine disruptors. The cellular modifications were followed for three weeks through transcriptomic and proteomic profiling analysis. A quantitative and comparative method was developed to estimate how known pathways were disturbed by each substance. This pathway-driven analysis revealed a strong alteration of steroidogenesis and an impairment of meiosis in all cases, albeit the initial molecular events were different for both substances. The *ex vivo* cytogenetic analysis confirmed that both fungicides alter the course of the first meiotic prophase. In addition, the mixture of both substances triggered effects greater than the sum of their cumulative effects and compromised future sperm motility after a shorter time of exposure compared with the fungicides tested separately. The alliance of an *ex vivo* culture with omics strategies complemented with a physiological examination is a powerful combination of tools for testing substances, separately or in a mixture, for their testicular toxicity. In particular, proteomics allowed the identification of systematically differentially expressed proteins in the secretomes of exposed cultures, such as FUCO and PEBP1, two proteins linked with the motility and fertilizing ability of spermatozoa, respectively. These proteins may be potential biomarkers of testicular dysfunction and infertility.

Keywords: pesticides, toxicogenomics, spermatogenesis, endocrine disruption, biomarker

1 Introduction

The recent awareness of the residual presence in foods, wines and drinking water of many pesticides requires urgent investigation of their potential toxicities to human health and the environment. Global pesticide consumption has increased steadily since the 1940s and this widespread use of pesticides in a massive manner for many years is now suspected to represent one of the main environmental causative factors related to public

health problems, such as some cancers, allergies, neurological and reproductive disorders (Mehrpour et al., 2014; Mostafalou and Abdollahi, 2013). In particular, since the middle of the twentieth century in developed countries, studies have reported a significant decrease in human fertility associated with lowered sperm quality and an increase in reproductive diseases such as testicular and ovarian cancers (Jensen et al., 2002). Reduced semen quality seems to be so frequent that it may impair fertility rates (Jorgensen et al., 2012). According to the 2013 report

Abbreviations

EDC, endocrine disrupting chemical; CBZ, carbendazim; ER, endoplasmic reticulum; IPR, iprodione; CCK, cocktail or equimolar mixture of carbendazim and iprodione; BTB, blood-testis barrier; FC, fold change; SC, synaptonemal complex; PI, pachytene index; L/Z, leptotene/zygotene transition

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of the World Health Organization (WHO) and United Nations Environment Program (UNEP) entitled “Endocrine Disrupting Chemicals”, close to 800 molecules are known or suspected to be capable of disrupting the hormonal system and only a few of these have been studied to date (WHO, 2012). Among fungicides, benzimidazoles and dicarboximides are suspected to impair fertility. In particular, two substances, carbendazim (CBZ) and iprodione (IPR), respectively representative of these two families, are persistent in water and food (Nougadere et al., 2012). CBZ is not approved by the European Food Safety Authority (EFSA). EFSA classified this substance as R60/61 (toxic for reproduction) and R46 (Cat 2 mutagen), and proposed it as a Cat 2 EDC (probable endocrine-disrupting chemical), although it was not “classified” as such.

CBZ is a systemic benzimidazole fungicide, and is the main metabolite of benomyl, used to control a broad range of diseases on arable crops (cereals, oilseed rape), fruits and vegetables, and also in post-harvest food storage (Hicks, 1998). CBZ is known to disrupt microtubular structures in the testis and to cause testicular toxicity in rats. More specifically, CBZ was found to inhibit the binding of guanosine triphosphate (GTP) to tubulin, interfering with initial events of microtubule polymerization (Winder et al., 2001), an event that is essential for the segregation of chromosomes during cell division. Lim and coworkers showed that susceptibility to CBZ toxicity is age dependent, adult rats being more responsive than younger ones. In adult rats, CBZ led to a sloughing of the seminiferous epithelium, contrary to the situation in the prepubertal rat (Lim and Miller, 1997b).

In rats, other effects have been related to CBZ exposure *in vivo*, such as a decrease in spermatid number and sperm motility, morphological abnormalities, failure of spermiogenesis, and infertility (Rama et al., 2014).

Regarding IPR, the European Union (EU) approves this chemical as a fungicide for fruits, vegetables and vines. EFSA classifies IPR as R43 (irritant) and 3R40 (Carcinogenic Cat. 3 for limited evidence of a carcinogenic effect). IPR is approved by EFSA and to date is not considered an EDC. In addition, CBZ and IPR are sometimes used simultaneously or in a mixture. In the case of aerial application (referred to as crop dusting), this may contaminate populations living in agricultural zones.

Although the literature is less abundant for IPR, this substance is suspected to be antiandrogenic (Wolf et al., 1999). Blystone and colleagues (2007) recently observed that IPR (i) induces Leydig cell tumors in rat testis in long-term studies, (ii) delays male rat pubertal development, (iii) reduces serum testosterone levels, and (iv) decreases *ex vivo* testicular testosterone production. In addition, Blystone and colleagues (2009) demonstrated the cumulative and antagonistic effects of a mixture of the two antiandrogens, IPR and vinclozolin, in the pubertal male rat.

Reproductive toxicity status is still pending for hundreds of substances. However, standard reproductive toxicity tests require the use of a large number of animals. The application of REACH will require the sacrifice of millions of animals if toxicological tests are performed *in vivo*. At the same time, there is a strong social and ethical pressure to reduce the number of animals sacrificed for the achievement of toxicological studies and to apply the rule of the “3Rs” (Replacement, Reduction, and

Refinement) (Russell and Burch, 1959). Moreover, *in vivo* regulatory tests do not allow the study of the cellular or molecular mechanisms of toxic substances and are therefore of limited interest in terms of understanding the mode of action of the evaluated molecules. This is particularly true for endocrine disrupting chemicals (EDCs). It is therefore essential for economical, ethical and scientific reasons to use alternative methods to change regulatory toxicology (Hartung, 2009). This will be possible if more data are collected and compiled. This is the reason why we have used an *ex vivo* rat model (Hue et al., 1998; Staub et al., 2000) to investigate the potential reproductive toxicity of the two fungicides, CBZ and IPR, alone or in a mixture. In addition, this model allows the number of rats used for testing to be reduced by a factor of twenty, respects animal welfare, and enables the analysis of cellular responses induced by chronic exposure to low doses of toxic substances for as long as three weeks. In the rat model, this three-week period corresponds to the development of spermatogenesis and, in particular, mimics puberty, a critical life period with regard to endocrine disruptors.

Our goal was to analyze, *ex vivo*, the molecular and cellular effects of these two fungicides, tested separately or in a mixture. The advent of new genomic techniques has allowed us to consider that issues of mixture toxicology may now be solved. In this work we used transcriptomics and proteomics to observe the adverse molecular effects of a mixture compared with the responses to the individual components. Immunocytochemistry was used as a physiological endpoint to assess the cytological changes induced by these substances on spermatogenesis using staining of the synaptonemal complexes (SCs) with an anti-SCP3 antibody.

To achieve this aim we (i) designed omics methods to assess dose- and time-dependent responses, (ii) utilized a single bioinformatics tool, Ingenuity Pathways Analysis (IPA), to summarize the modulation of thousands of genes and hundreds of proteins into hypotheses about specific cellular pathways of toxicity (PoT) towards these substances, (iii) observed the cytological changes of these specific cells after exposure to IPR and CBZ by immunostaining of the SCs, and (iv) analyzed the omics data sets transversely to identify potential biomarkers of testicular toxicity. This *ex vivo* model permitted the number of rodents used to be greatly reduced and could be well adapted for screening large libraries of compounds. It could potentially be developed into a very useful preliminary screening assay in the prioritization of compounds for subsequent *in vivo* toxicological evaluation.

2 Animals, materials and methods

2.1 Animals and explant cultures

All procedures were approved by the Scientific Research Agency (approval number 69306) and conducted in accordance with the guidelines for care and use of laboratory animals. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (2010/63/EU) for the care and use of laboratory animals. The entire study was performed *ex vivo* using cultures of seminiferous tubules. For these cultures, healthy 23-day-old male

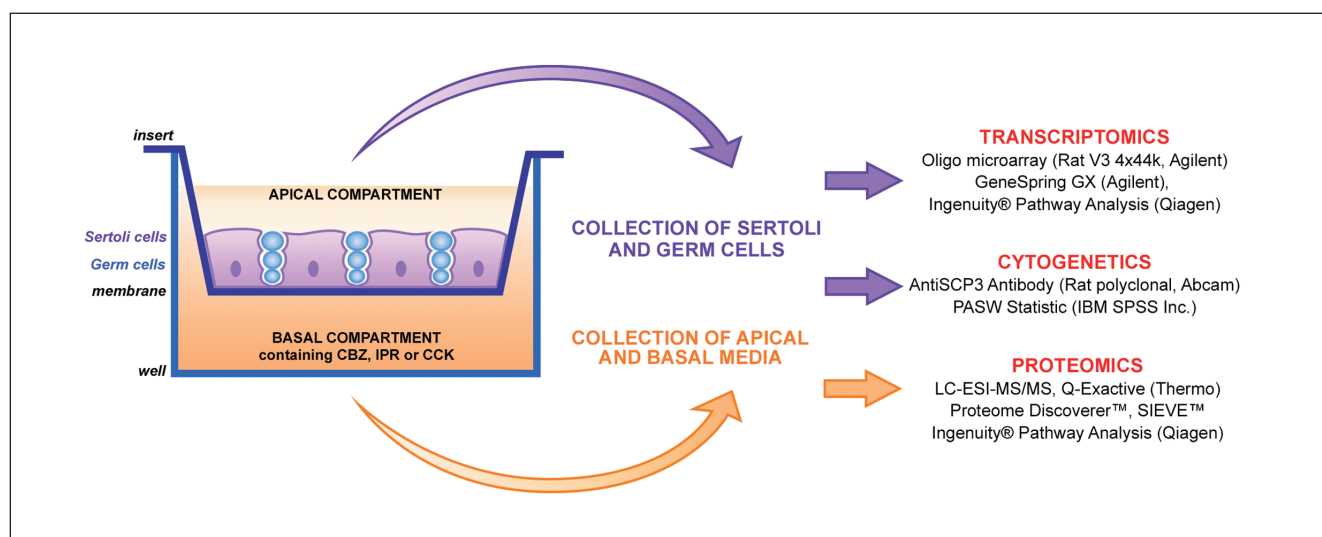


Fig. 1: Experimental procedure

Seminiferous tubules from 23-day-old Sprague-Dawley rats were cultured in a dual-compartment culture system. Cell samples were seeded (day 0) at about $7\text{--}8 \times 10^5$ cells/cm² on filter inserts. The same population was used to seed control and treated cultures. Toxicants were placed in the basal compartment in a “basal-to-apical” transport mode and the serum-free medium in the basal compartment was changed every two days. After 7, 14 and 21 days cells were collected for transcriptomics, and cytogenetics. Apical and basal media were collected for proteomics at the same time points.

Sprague Dawley rats from Charles River France Inc. supplied with a health status certificate (Janvier, France), having undergone no treatment, were used. Animals were housed three per cage, at a temperature of $21 \pm 3^\circ\text{C}$, under a light cycle of 12-12 (6 p.m. - 6 a.m.), given a diet of SDS VRF1 (from Special Diets Services), 0.1 μm -filtered water in a bottle system, and autoclaved sawdust bedding. Rats were anesthetized with chloroform and then sacrificed by guillotine. The tunica albuginea of testes was removed and seminiferous tubules were dissociated by collagenase enzymatic digestions at 32°C and mechanical dissociation. Between each step of this process, seminiferous tubule fragments were washed with F12/DMEM supplemented with 100 U/ml penicillin, 50 mg/ml streptomycin, and 20 U/ml nystatin. The culture medium consisted of 15 mM HEPES-buffered F12 / DMEM supplemented with 20 $\mu\text{g}/\text{ml}$ gentallin, 20 U/ml nystatin, 1.2 g/l sodium bicarbonate, 10 $\mu\text{g}/\text{ml}$ insulin, 10 $\mu\text{g}/\text{ml}$ human transferrin, 10^{-4} M vitamin C, 10 $\mu\text{g}/\text{ml}$ vitamin E, 10^{-7} M testosterone, 3.3×10^{-7} M retinoic acid, 3.3×10^{-7} M retinol, 10^{-3} M pyruvate (all from Sigma), and 1 ng/ml of FSH obtained through NIDDK (Rockville, MD). Cell samples were seeded (Day 0 of the experiment) at about $7\text{--}8 \times 10^5$ cells/cm² on filter inserts (Falcon, France) in the culture medium supplemented as above. Incubation was carried out at 32°C , 5% CO₂. Thereafter only the medium of the basal compartment was changed every other day. At the age of 22-23 days, the most advanced germ cells are late spermatocytes (Clermont, 1972), allowing study of the whole meiotic phase under our culture conditions (Staub et al., 2000). In order to counterbalance inter-animal variation, testes from eight rats were pooled in each culture and used immediately, as previously described (Staub et al., 2000).

The same population was used to seed control and treated cultures. Toxicants were placed in the basal compartment in a “basal-to-apical” transport mode (Fig. 1). Analyses were performed at days 7 (D7), 14 (D14) and 21 (D21) of the cultures.

2.2 Chemicals

The pesticides tested in this study were carbendazim (CBZ) and iprodione (IPR), obtained from Sigma-Aldrich (St. Louis, MO), ref. 45368, lot SZBA347XV with 99.2% purity (HPLC assay, Sigma) for CBZ, and ref. 36132, lot SZE6222X with 99.0% purity (HPLC assay, Sigma) for IPR. For LC-MS/MS analysis, MilliQ water (Merck Millipore, Billerica, MA) was used; all other solvents were obtained from Carlo-Erba Reagents (Val de Reuil, France) and were of HPLC grade or higher. Chemicals for protein preparation were purchased from Sigma-Aldrich (St. Louis, MO) and were of reagent grade. LC-MS-grade formic acid (FA) was obtained from Fluka (Sigma-Aldrich, St. Louis, MO). For protein digestion, sequencing-grade modified trypsin (Promega, Madison, WI) was used.

2.3 Preparation and culture of seminiferous tubules

The technique of seminiferous tubule culture has been described previously (Hue et al., 1998; Staub et al., 2000). Briefly, cultures ($n = 3$) were performed with and without fungicides. Stock solutions of CBZ, IPR and the mixture/cocktail (CCK) were prepared in 1% dimethyl sulfoxide (DMSO), and then diluted in serum-free culture medium to obtain the final concentrations of 500 nM or 50 nM in the basal compartment. The final medium and controls contained 0.03% DMSO. Basal media (with or without fungicides) were renewed every two days. For



CCK, an equimolar solution of the two fungicides containing 0.03% DMSO (50 nM or 500 nM) was prepared.

2.4 Microarray experiments

Cell exposure, RNA extraction, RNA labeling and hybridization

Two different pools of seminiferous tubules were exposed to two concentrations of CBZ, IPR or their mixture (50 nM and 500 nM) or to the complete medium with vehicle (control cells) for 7, 14, or 21 days. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA preparations were quantified with the Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and their quality was assessed with the Agilent 2100 Bioanalyzer. RNA samples were amplified and labeled with the cyanine-3 fluorophore using a Low Input QuickAmp Labeling Kit (Agilent Technologies, Palo Alto, CA, USA). Hybridization was performed using Agilent Oligo Microarrays (Rat V3 4x44K). Fluorescence was scanned and signal data were extracted with Feature Extraction Software (Agilent Technologies, Palo Alto, CA, USA).

Design and statistical analysis of transcriptomic experiments

In this experimental design, and for each fungicide (50 or 500 nM), six independent analyses were conducted *versus* each specific control for each time point. For example, for CBZ we analyzed the 50 nM CBZ-exposed cells *versus* control for 7 days, 14 days, and 21 days and 500 nM CBZ-exposed cells *versus* control for 7 days, 14 days, and 21 days. For each experimental point (i.e., for a specific dose and time point), six raw fluorescence data files (three tests and three controls) were submitted to GeneSpring Software GX12 (Agilent Technologies, Palo Alto, CA, USA) using a widely used method for determining the significance change in gene expression (Fisichella et al., 2014; Ludwig et al., 2011; Wright et al., 2012). The fold change cutoff between control and exposed samples was set to 1.5. Genes that were significantly up- or down-regulated were determined by an unpaired t-test, with a p-value ≤ 0.05 and a Benjamini-Hochberg false discovery rate correction. We thus obtained probe sets that were significantly induced or repressed after exposure to fungicides alone or in a mixture.

2.5 Cytological methods

Spreading and immunocytological localization of the synaptonemal complex (SC)

Spreading and immunocytological localization of SC axial and lateral elements were performed according to Metzler-Guillemain and Guichaoua (2000). Briefly, after spreading by cyto centrifugation at 30 g, the slides were fixed in 2% paraformaldehyde (Merck Darmstadt, Germany). A rabbit polyclonal anti-SCP3 antibody (Abcam, Cambridge, UK Ab 15093) was used at a 1:100 dilution to reveal SC axial and lateral elements. Detection was performed with an FITC-conjugated anti-rabbit immunoglobulin G (Abcam, Cambridge, UK) at a dilution of 1:100. Slides were mounted in antifade medium (Vectashield, Vector Laboratories, Burlingame, USA).

Microscopic analysis

A Zeiss Axioplan 2 Fluorescence Photomicroscope (Carl Zeiss, Oberkochen, Germany) was used to observe the spermatocyte

nuclei. Primary spermatocytes, stained with the anti-SCP3 antibody, were selected to evaluate the respective percentages of leptotene, zygotene, pachytene and diplotene stages. One to two hundred nuclei were analyzed for each culture, including the control cultures, and for each time point (D7, D14, and D21) and fungicide dose (50 or 500 nM) in a blinded manner. We evaluated the percentages of the three pachytene substages (P1-3) corresponding in the rat model to early (P1), mid (P2) and late (P3) pachytene substages (Geoffroy-Siraudin et al., 2012). These substages were defined according to the condensation degree of the sex bivalent during the pachytene stage. Regarding the evaluation of the pachytene substage experiments, fifty nuclei were analyzed for each experimental condition at D7 and D14. The pachytene index (PI) was evaluated for each culture, time point and fungicide dose. We defined the PI by the ratio $P3/P1+P2+P3$ (Geoffroy-Siraudin et al., 2012). The percentages of nuclei showing SC abnormalities were quantified at each time point, in both exposed and control cultures. For each stage, and for each type of abnormality, variations over dose and time were analyzed.

Statistical analysis for immunocytochemistry (ICC)

Statistical analysis was performed using PASW Statistics Version 17.0.2 (IBM SPSS Inc., Chicago, IL, USA). Continuous variables are expressed as means \pm SD. Comparisons of means between two groups were performed using a Student's t-test. All tests were two-sided. The statistical significance was defined as $p \leq 0.05$. Three biological replicates were analyzed for each time and dose. Each experiment included controls (vehicle only) and tests (fungicide alone or in a mixture).

2.6 Mass spectrometry

Protein preparation

The proteins present in the apical media samples were precipitated with 80% cold acetone overnight, and the protein content of the pellets was estimated by a micro Bradford assay (Quickstart Bradford reagent from BioRad, Hercules, CA). Each sample (between 30 μ g and 200 μ g) was first reduced with dithiothreitol and alkylated with iodoacetamide and then digested by adding 2 μ g sequencing-grade modified trypsin overnight at 37°C. Reactions were stopped by acidification and an equivalent of 30 μ g digested proteins were loaded onto a Varian Omix C₁₈, 100 μ l tip (Agilent Technologies, Palo Alto, CA, USA), washed following the manufacturer's instructions and eluted in 50 μ l 60% acidified acetonitrile [ACN in 0.1% trifluoroacetic acid (TFA)]. Eluted peptides were dried using a speed-vacuum system (Labconco, Freezone 2.5plus, Kansas, US) and suspended in 4% ACN, 0.05% TFA (v/v) to a final concentration of 1 μ g/ μ l. Each sample (4 μ l) was analyzed twice by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

Proteomic analysis by LC-ESI-MS/MS

LC-ESI-MS/MS instrumentation, chromatographic columns and other components were obtained from Thermo Scientific (Bremen, Germany) unless otherwise noted. An Ultimate 3000 nanoflow high performance liquid chromatography system online coupled to an Q-Exactive Orbitrap was used with a 75 μ m

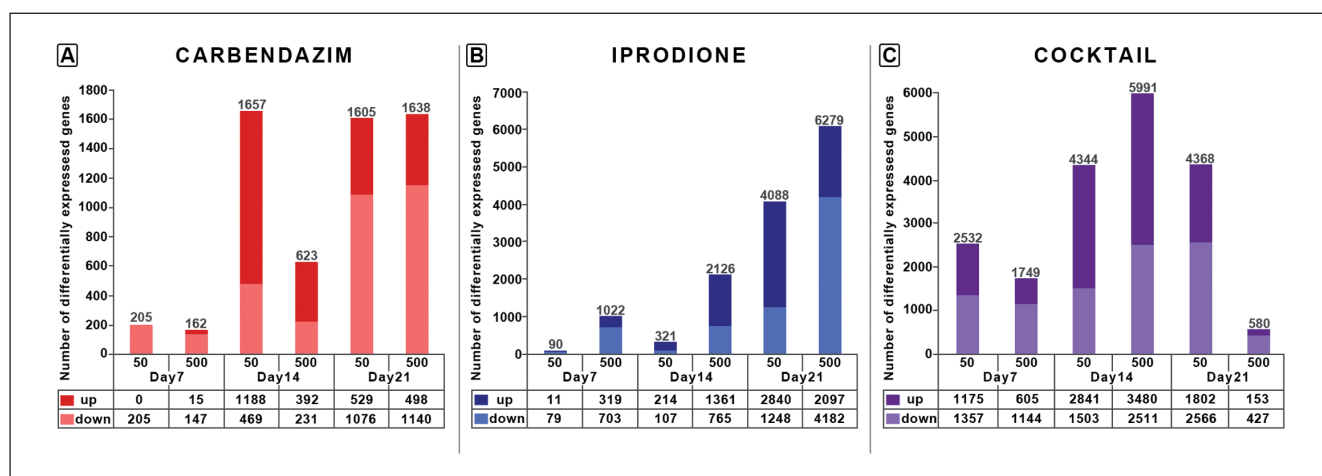


Fig. 2: Time- and dose effect on the number of significantly differentially expressed genes after exposure to carbendazim, iprodione and a mixture (named cocktail) of both fungicides

Ex vivo cell cultures of seminiferous microtubules from male Sprague-Dawley rats were exposed for 7, 14 and 21 days to two concentrations (50 nM and 500 nM) of A) CBZ, B) IPR and C) equimolar mixture of both substances (50 nM and 500 nM). Stacked bars represent the number of differentially expressed transcripts that were up- or downregulated after statistical analysis using Genespring GX12 software (Agilent) with p -value ≤ 0.05 and a cutoff value of 1.5.

x 250 mm Acclaim Pepmap 100, C_{18} , 3 μ m nanoviper column, maintained at 35°C. Peptides were loaded at 10 μ l/min for 6 min on a 300 μ m x 5 mm Pepmap 100, C_{18} , 5 μ m microcolumn and then resolved at 300 nl/min using a multistep gradient: from 4% to 25% ACN in 0.1% (v/v) formic acid in 88 min; then from 25% to 40% in 20 min, and finally from 40% to 90% in 4 min. Operating in positive polarity and data-dependent mode, the Q-Exactive Orbitrap acquired a full-range scan from 380 to 2000 Th (70,000 resolution, AGC target $3 \cdot 10^6$, maximum IT 200 ms) and then fragmented the top ten peptide ions in each cycle (17,500 resolution, AGC target 10^5 , maximum IT 100 ms, intensity threshold 2×10^4 , excluding charge-unassigned ions, NCE 27). Parent ions were then excluded from MS/MS for the next 30 s. The Orbitrap was continuously recalibrated using the lock-mass function on the ion m/z 445.12003. The software Xcalibur™ 2.2 and Chromeleon® Xpress were used to control the LC-ESI-MS/MS instrumentation.

Sequest HT was run by Proteome Discoverer™ 1.4 to match the acquired MS/MS spectra, with a tolerance of 10 ppm/0.02 Da, to a *Rattus norvegicus* protein database, with the sequences for human transferrin and bovine insulin. Sieve™ 2.1 was used to derive the relative quantitation ratio between treated and control samples. Average ratios were calculated using variance weighting on unique peptides. Proteins with an average ratio ≥ 2 or ≤ 0.5 and a p -value ≤ 0.05 were selected for further computer analysis.

2.7 Biological analysis

Lists of genes and proteins significantly induced or repressed after exposure to fungicides were uploaded into QIAGEN's Ingenuity® Pathway Analysis software (IPA®, QIAGEN Redwood City, <http://www.qiagen.com/ingenuity>) for biological analysis by comparison with the Ingenuity Knowledge Data-

base. These lists of altered genes and proteins were then processed to investigate their functional distribution, as defined by Gene Ontology. For each substance and time point, datasets and known canonical pathway associations were measured by IPA by using a ratio of the number of genes or proteins from a dataset that match to a specific pathway divided by the total number of genes/proteins that map to this canonical pathway. This ratio is expressed as a percentage. A Fisher's exact test was used to determine a p -value representing the significance of these associations.

For each time point, protein datasets obtained for each substance were compared and five secreted proteins present in almost all tests at all time points were isolated as potential biomarkers.

3 Results

3.1 Transcriptomics

In a first step, gene expression changes depending on the tested substances, CBZ, IPR and CCK (a mixture – or cocktail – of IPR+CBZ), were evaluated in three biological replicates with commercial Agilent rat V3 4x44K microarrays. A specific cell control was run at each time point due to the differentiation of this organotypic cell culture during the 21 days of the experiment. The number of transcripts differentially expressed (up or down) by treated cultures compared with untreated cultures reflects the magnitude of the cellular disruption. Tab. S1 (<http://dx.doi.org/10.14573/altex.1601253s1>) lists all the genes up- and downregulated with each substance and at each time point.

Figure 2 shows the number of differentially expressed transcripts detected in response to 50 and 500 nM CBZ, IPR and CCK. Three durations (7, 14 and 21 days) of a chronic expo-

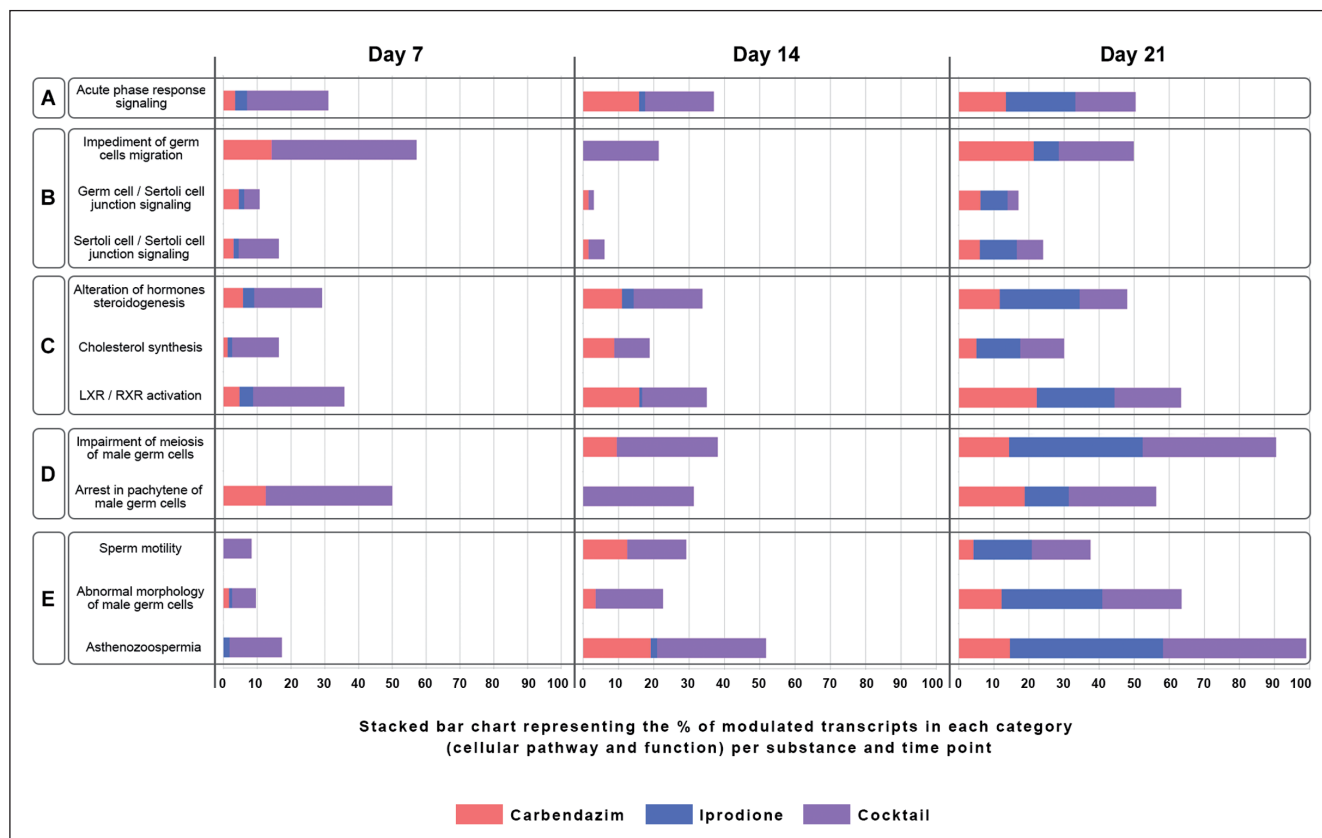


Fig. 3: Pathways of toxicity elicited by each substance tested individually or in an equimolar mixture

Ex vivo cell cultures of seminiferous microtubules from male Sprague-Dawley rats were exposed for 7, 14 and 21 days to 50 nM CBZ or IPR, or to CCK (a mixture of both substances, each at 50 nM). Whole gene expression experiments ($n = 3$) were performed with rat Agilent 4x44K microarrays. Probe sets were obtained that were significantly induced or repressed after exposure to the fungicides alone or in a mixture ($FC \geq 1.5$ and $p\text{-value} \leq 0.05$). Relevant canonical pathways disturbed after exposures were revealed with the Ingenuity® Pathway Analysis tool. Data sets obtained after cell exposure were compared quantitatively with the genes known to be part of the main canonical pathways of spermatogenesis. The contribution of each pathway, for each experimental condition, is represented by a percentage. We focused mainly on the altered biological processes that were specifically related to the testicular tissue, then gathered these into five groups, each including several characterized pathways.

- A. Acute phase response signaling (pathway related to nonspecific chemical toxicity);
- B. Alteration of germ cell migration across the seminiferous epithelium;
- C. Endocrine disruption by interaction with steroidogenesis;
- D. Impairment of meiosis;
- E. Compromising the future spermatozoa.

The stacked bars indicate quantitatively how a biological process is disturbed by a given substance according to the duration of exposure of the cell culture.

sure were investigated in order to cover a period of three weeks, which in the rat model corresponds to the development of spermatogenesis.

For CBZ, a time-dependent response was observed, ranging from 205 to 1,657 significantly differentially expressed transcripts at 50 nM and from 162 to 1,638 differentially expressed transcripts at 500 nM. At D7, few transcripts were found to be modulated relative to controls, not exceeding 205 transcripts at both doses. Interestingly, the strongest response to 50 nM CBZ was observed at D14, with approximately 1,000 more modulated transcripts than at 500 nM and the highest number of up-

regulated transcripts compared to downregulated ones. At D21, the same range (1,605 and 1,638 for 50 nM and 500 nM, respectively) was observed at both concentrations.

For IPR, a time- and dose-dependent response was observed, ranging from 90 to 4,088 significantly differentially expressed transcripts at 50 nM and from 1,022 to 6,279 differentially expressed transcripts at 500 nM. IPR induced a much greater number of modulated transcripts than CBZ. Seventy percent of the genes differentially expressed by exposure to 50 nM IPR were also altered with 500 nM IPR and this was true at D7 and D14. At D21, this rate was 50%. For the cocktail, a time-dependent

response to CCK was observed from D7 to D21, ranging from 2,532 to 4,368 and from 1,749 to 580 significantly differentially expressed transcripts at 50 nM and 500 nM, respectively. From the beginning of the culture, and whatever the concentration, the cocktail altered more genes than each fungicide used separately, and even more than the sum of the altered genes by each compound, except for D21. For example, at 50 nM D7, the cocktail altered 2,532 genes *versus* 295, which is the sum of 205 (for CBZ) plus 90 (for IPR); this corresponds to a ratio of 8.6 more altered genes. At 50 nM D14, the cocktail altered 4,344 genes *versus* 1,978 (sum of 1,657 plus 321); corresponding to a ratio of 2.2 more altered genes. This phenomenon was not observed at D21, whatever the concentration, where ratios of 0.77 and 0.07 were observed at 50 and 500 nM, respectively.

In a second step, and for each substance (CBZ, IPR and CCK) and time point (D7, D14 and D21), gene datasets were analyzed with Ingenuity Pathway Analysis for annotation, and the results are reported in Table S2 (<http://dx.doi.org/10.14573/altex.1601253s2>). All data sets were examined for their association with known canonical pathways, which are defined by a discrete number of genes. The quantification of this association was performed by calculation of multiple ratios, linking a specific dataset to several canonical pathways as described in the Methods section.

In a third step, we focused on the altered biological functions and pathways that were specifically related to testicular tissue. These altered processes after exposure to CBZ, IPR, or CCK (50 nM) were gathered into five groups, representing specific characteristic dysfunctions:

- A) Altered pathways related to chemical toxicity (*Acute phase signaling*);
- B) Alteration of germ cell migration across the seminiferous epithelium (*Impediment of germ cell migration, germ cell/Sertoli cell junction signaling, Sertoli cell/Sertoli cell junction signaling*);
- C) Endocrine disruption by interaction with steroidogenesis (*Alteration of hormone steroidogenesis, cholesterol synthesis, LXR/RXR activation*);
- D) Impairment of meiosis (*Impairment of meiosis of male germ cells, arrest in pachytene of male germ cells*);
- E) Compromising the future spermatozoa (*Sperm motility, abnormal morphology of male germ cells, asthenozoospermia*).

The results are combined in Figure 3 as stacked bars representing, for each substance, the percentages of modulated transcripts in each pathway. The results are presented per substance (CBZ, IPR and CCK) at the concentration of 50 nM and for the different time points (D7, D14 and D21). At both concentrations (50 nM and 500 nM), the same pathways are involved. Nevertheless, at 500 nM (data not shown), many other secondary pathways are also at work and are interconnected. Consequently, the conclusions are less pertinent than at the lower dose of 50 nM.

CBZ acts earlier than IPR on the *ex vivo* cultured cells, starting from D7. IPR exerts a more clear toxic effect at D21, corresponding to the last time point of the experiment. The cocktail acts from D7, as for CBZ alone, and produces a greater effect than each substance tested individually towards all the considered pathways.

We paid special attention to the transcriptional change of meiotic genes.

A list of 305 meiotic genes given by IPA was taken as the reference. We compared this list with each of our datasets established at 50 nM. As a result, 27, 79 and 69 meiotic genes were differentially expressed at all time points with CBZ, IPR and CCK, respectively. In particular, for CBZ, seven genes were involved in meiotic recombination, and one coded for a synaptonemal complex protein. For IPR, 18 genes were involved in recombination and seven coded for synaptonemal complex proteins (most of the SC proteins). For CCK, 45% (31/69) of the genes involved in meiosis were also present in the IPR transcriptomic analysis, whereas only 17% (12/69) were also present in the CBZ transcriptomic analysis. The rest of the altered genes (26/69) were specific to CCK. In particular, numerous premeiotic and meiotic genes (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>) were modulated, such as *Papalb* (DNA replication), *Rec8* (a gene required during meiosis for the separation of sister chromatids and homologous chromosomes), *Cyp26b1*, *Syce1*, *Sycp1*, *Sycp2*, *Stag3* (genes encoding synaptonemal complex proteins), *Cyp26b1*, *HspA2* (functions in pairing), *Btrc* (checkpoint/meiotic control), *Dmcl* (encoding a protein essential for meiotic homologous recombination, pachytene checkpoint), *Bcl2*, *Bcl2A1*, *Casp3*, 4, 6 and 7, *Ccnal* (apoptosis), *Spol*, *Mae1*, *Exo1*, *Msh4*, *Brcal*, *Tex12*, *Tex15* (genetic recombination) and *Daz2* (expression restricted to premeiotic germ cells, particularly in spermatogonia) that encodes an RNA-binding protein important for spermatogenesis, *Ccne2* (essential for the control of the cell cycle at the late G1 and early S phase) and *Stmn1* (gene belonging to the stathmin gene family, involved in regulation of the microtubule filament system). *Piwil2* was found in common data sets obtained with IPR and CCK at D21. Genes known to be associated with postmeiotic germ cells, including protamine 1 (*Prm1*) and transition protein 1 and 2 (*Tnp1* and *Tnp2*) were downregulated with CCK.

3.2 Cytological results

The cytological observations were carried out with three biological replicates. A specific cell control was run at each time point due to the differentiation of this organotypic cell culture (D7 and D14).

Timing of meiosis

In control cultures, the percentages listed in Table S3 (<http://dx.doi.org/10.14573/altex.1601253s3>) of the four stages of the meiotic prophase (leptotene, zygotene, pachytene and diplotene) are of the same order of magnitude as those obtained previously (Ali et al., 2014; Geoffroy-Siraudin et al., 2010, 2012). These percentages were evaluated from 100 nuclei for control, pesticide-treated and cocktail-treated cultures. In this study, we showed that exposure of seminiferous tubules to CBZ, IPR and CCK during a time course experiment had an effect on the timing of meiotic prophase. The percentages of leptotene and pachytene stages (Tab. S3, <http://dx.doi.org/10.14573/altex.1601253s3>) in treated cultures (with 50 nM CBZ, IPR and CCK) were similar to control cultures at D7 and D14. A

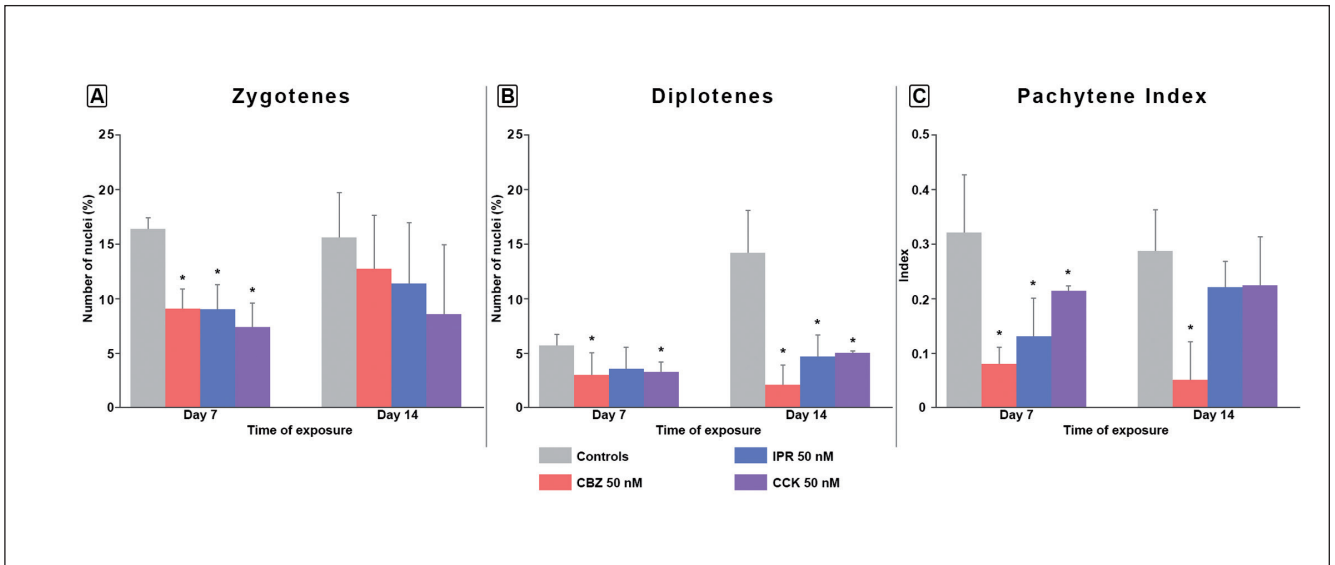


Fig. 4: Effects of CBZ, IPR and CCK on the chronology of meiosis

Primary spermatocytes stained with an anti-SCP3 antibody were selected to evaluate the respective percentages of leptotene, zygotene, pachytene and diplotene stages of the meiotic prophase. One to two hundred nuclei were analyzed for control cultures and each culture treated with 50 nM fungicide at D7 and D14. CBZ, IPR and CCK modified the percentages of nuclei at the zygotene (A) and diplotene (B) stages. The zygotene stage significantly decreased with CBZ, IPR and CCK for both time points. The diplotene percentage was slightly reduced at D7 for CBZ, IPR and CCK compared with control cultures. Unlike the control cultures, there was no increase in the diplotene stage in all treated cultures at D7 and D14. The pachytene index (C) measures the relative modulation of the pachytene substages (early, mid and late) and indicates the degree of condensation of the sex bivalent. The statistically significant differences are indicated by an asterisk ($p \leq 0.05$).

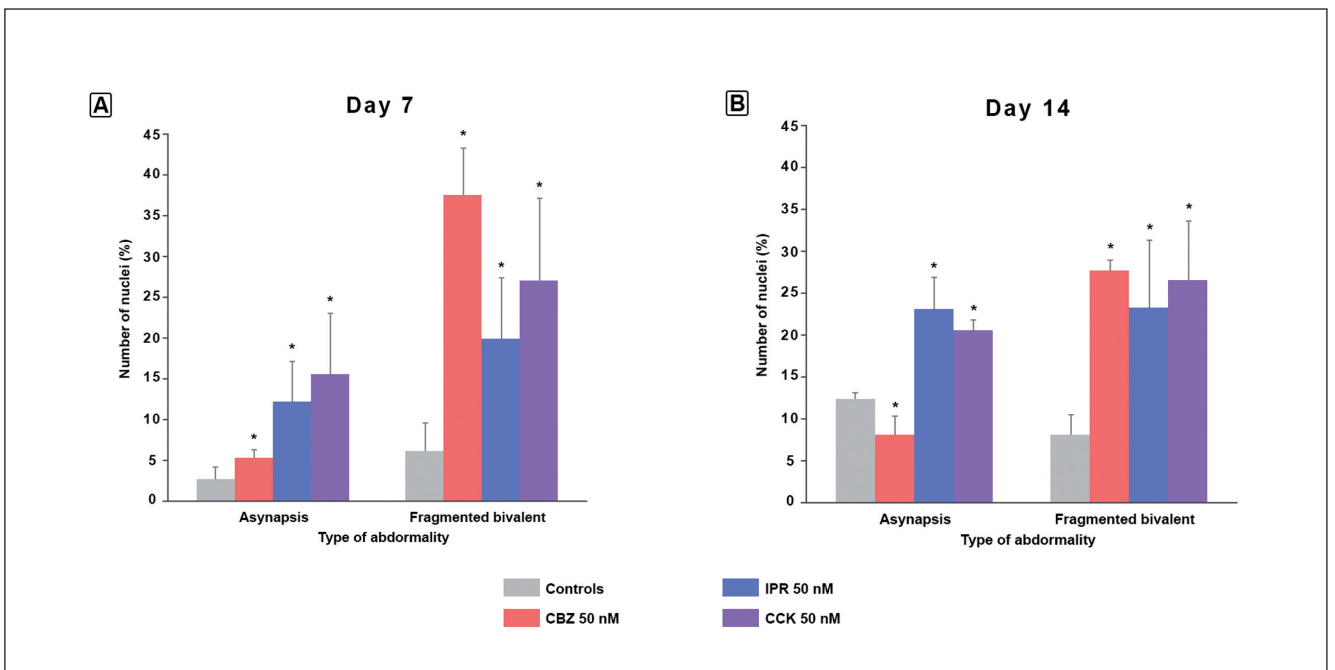


Fig. 5: Spermatocyte abnormalities

Primary rat spermatocytes stained with an anti-SCP3 antibody were selected to quantify synaptonemal complex abnormalities by fluorescence photomicroscopy. One to two hundred nuclei were analyzed in control cultures and in each culture treated with 50 nM fungicide. The analysis of SC abnormalities at D7 (A) and D14 (B) showed that asynapsis was more frequent with IPR and CCK, whereas fragmented SC was the predominant abnormality induced by CBZ. All differences were statistically significant (*, $p \leq 0.05$).

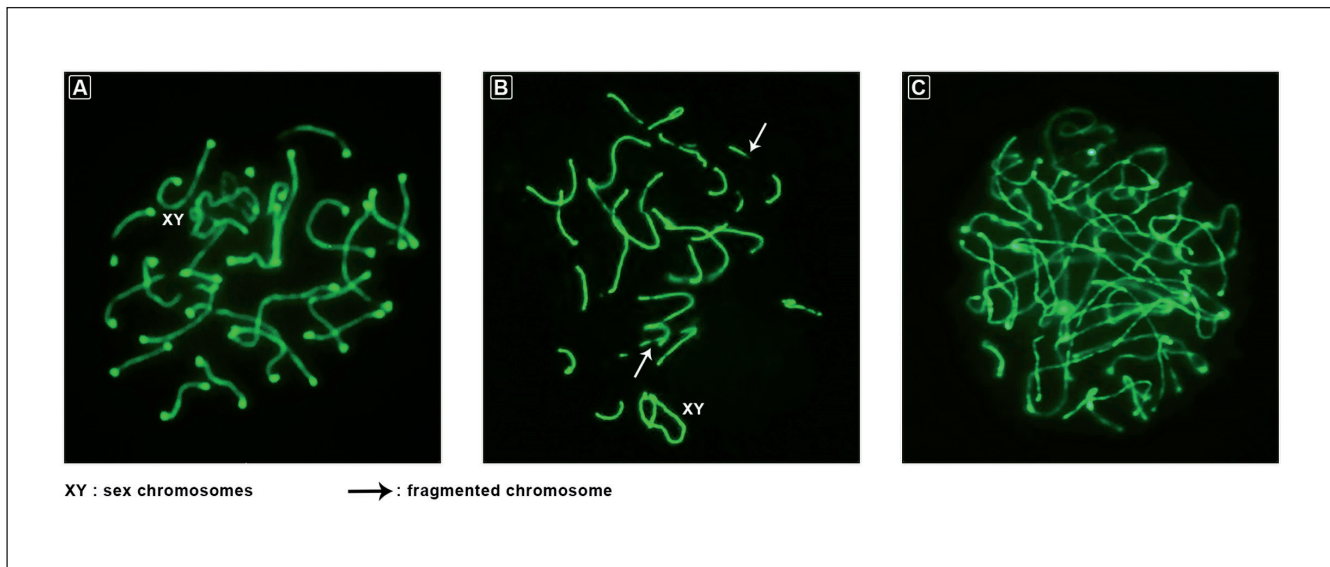


Fig. 6: Synaptonemal complex abnormalities observed with the two fungicides

Primary rat spermatocytes stained with an anti-SCP3 antibody were selected to observe the spermatocyte nuclei showing synaptonemal complex abnormalities by fluorescence photomicroscopy. A) Control nucleus (pachytene stage). B) Fragmented SC is the most commonly observed abnormality in the presence of CBZ (50 nM for 7 days). C) Asynapsis occurs in the presence of IPR (50 nM for 7 days).

significant decrease was observed for the zygotene stage with CBZ, IPR and CCK at D7, while at D14 the difference was not statistically significant (Fig. 4A). The diplotene percentage was slightly reduced at D7 for CBZ, IPR and CCK compared with control cultures. Unlike the control cultures, there was no increase in the diplotene stage with the culture time between D7 and D14 in all treated cultures (Fig. 4B).

The pachytene index (for calculation see the Methods section) decreased significantly at D7 for CBZ, IPR and CCK. This decrease was statistically significant only for CBZ at D14 (Fig. 4C).

To summarize, the cell subtype percentages of zygotene and diplotene for CBZ-, IPR- and CCK-treated cell cultures followed the same decreasing trend compared with controls.

Synaptonemal complex abnormalities

Two types of abnormalities were frequently observed with the two fungicides and the cocktail: asynapsis and fragmented synaptonemal complexes (fragmented bivalent) (Fig. 5A and 5B at D7 and D14, respectively). Total abnormalities increased from 32% to 47% after culture treatments, compared to 8% and 20% for the control cultures at D7 and D14, respectively (Tab. S3, <http://dx.doi.org/10.14573/altex.1601253s3>). Compared with control nuclei (Fig. 6A), the predominant abnormality for CBZ was fragmented SC (Fig. 6B), whereas only IPR induced asynapsis (Fig. 6C).

3.3 Proteomics

Three cultures were treated with each fungicide (CBZ and IPR) or with their equimolar mixture (CCK) and compared with controls at each time point (D7, D14 and D21). The exoproteomic analysis of the culture media revealed that the apical chamber medium was richer in proteins than the basal medium

in all cases (data not shown). As a result, this current analysis will focus on the results obtained with the analyses of the apical medium.

An average of 273 ± 114 (106 min to 559 max) different proteins were identified in the apical chamber content of each culture treated with CBZ or IPR, or their untreated control.

The comparison performed by the Sieve™ software on the LC-MS/MS data files highlighted many proteins differentially expressed between a treated culture sample and the corresponding control sample at the same time, using a cut-off ≥ 2 and a Sieve™ p-value ≤ 0.05 . Table S4 (<http://dx.doi.org/10.14573/altex.1601253s4>) provides the complete list of these differentially expressed proteins, sample per sample. Table S5 (<http://dx.doi.org/10.14573/altex.1601253s5>) indicates which proteins were common to the cultures exposed to CBZ, IPR and to an equimolar mixture (50 nM each) of these two fungicides (CCK). Figure 7 shows the number of differentially expressed proteins (average ratio ≥ 2 or ≤ 0.5 and a p-value ≤ 0.05) according to the treatment and time of exposure. From 104 (treatment with IPR) to 268 (treatment with CBZ) modulated proteins were found at D7. At D14 and D21, the protein numbers decreased noticeably: 59 for IPR to 78 for CBZ at D14 and from 7 for CCK to 151 for CBZ at D21. Between D14 and D21 the numbers of modulated proteins increased in the single-compound treated cultures with CBZ (78 versus 151) and IPR (59 versus 91). The Venn diagrams in Figure 8 show how some of these proteins were found to be modulated by two or three substances at the same time point.

Five of the modulated proteins (APOE, CLUS, FUCO, LG3BP and PEBP1) were found in almost all samples at almost all times, as shown on Table 1. We used SecretomeP 2.0 to confirm the secretory feature of these proteins (all including

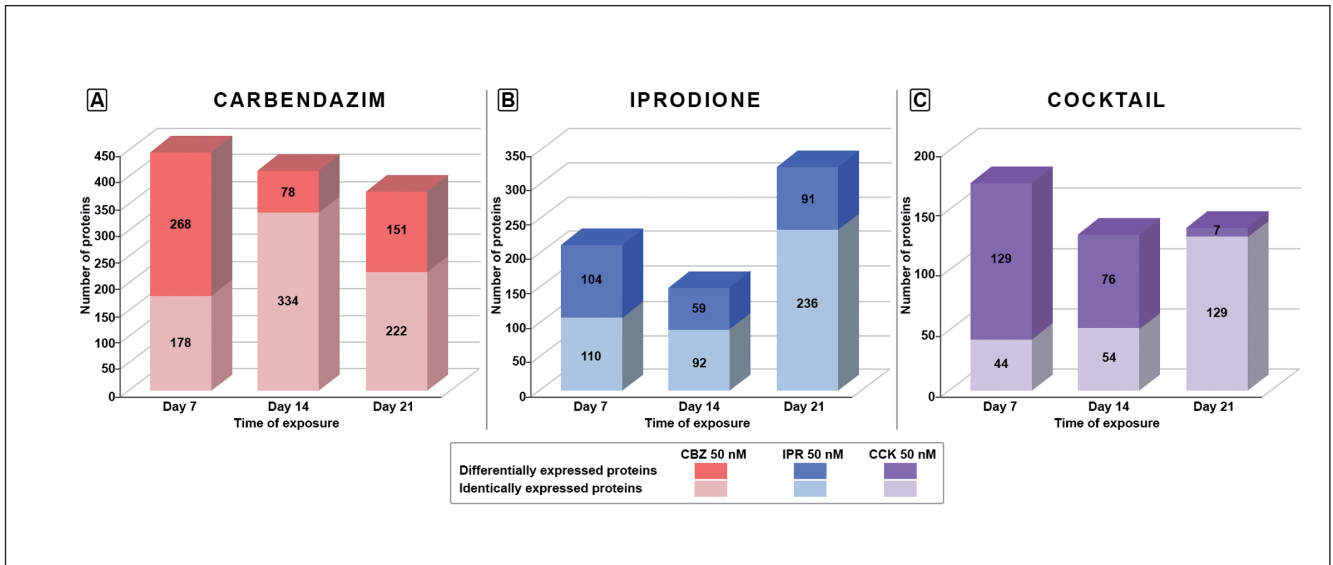


Fig. 7: Evolution of the number of differentially expressed proteins compared to control cultures secreted during culture and identified by mass spectrometry

Ex vivo cell cultures of seminiferous microtubules from male Sprague-Dawley rats were exposed for 7, 14 and 21 days to 50 nM CBZ or IPR, or to an equimolar cocktail of both substances (50 nM each). The protein content of the apical medium was extracted, digested with trypsin, identified by nanoLC-MS/MS (analysis in duplicate) and compared using the SIEVE™ software (label-free semiquantification). Secreted proteins were found to be differentially expressed in the samples exposed to the toxicants compared with control samples cultured for the same number of days (ratio $\geq \pm 2$, SIEVE™ p-value ≤ 0.05). The charts represent the number of proteins identified by mass spectrometry in cultures treated with A) CBZ, B) IPR, and C) CCK. Stacked histograms represent proteins that are statistically differentially expressed (dark color) and proteins that are expressed at the same level (light color) when compared with untreated control cultures, respectively.

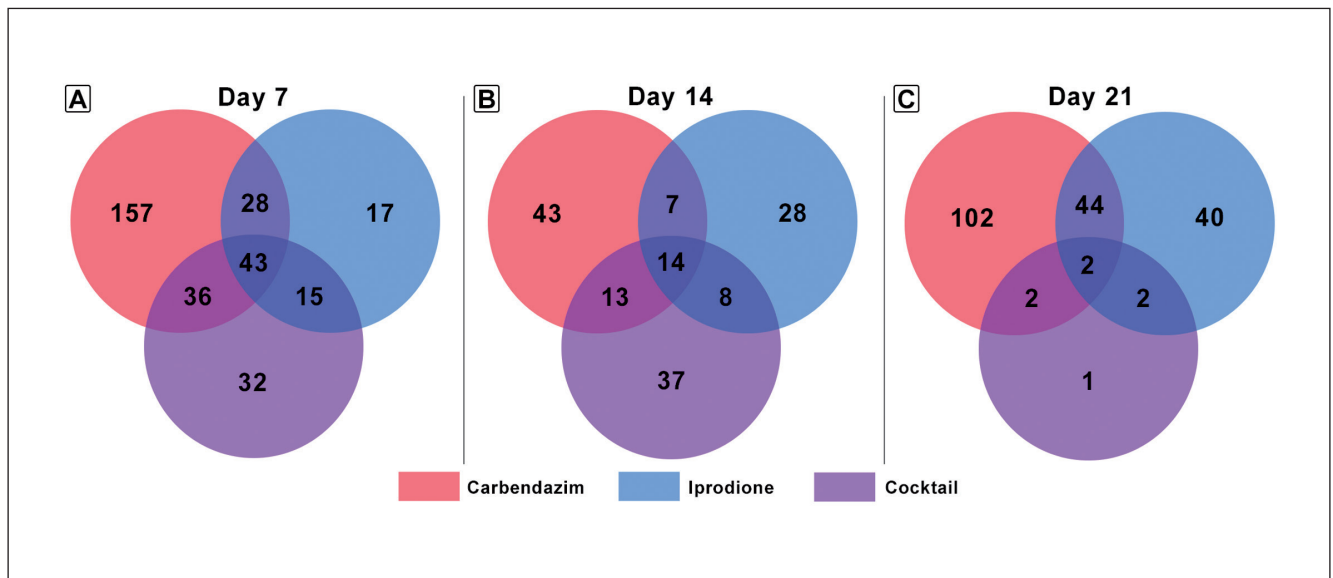


Fig. 8: Comparison of differentially expressed proteins identified in supernatants of cells treated with each substance

Ex vivo cell cultures of seminiferous microtubules from male Sprague-Dawley rats were exposed for A) 7 days, B) 14 days and C) 21 days to 50 nM CBZ or IPR, or to an equimolar cocktail of both substances (50 nM each). For each time point, protein datasets were compared and common proteins were identified. These proteins are indicated in Tab. S5 (<http://dx.doi.org/10.14573/altex.1601253s5>) with their expression fold change *versus* untreated cells.

Tab. 1: Key proteins differentially expressed after exposure

Ex vivo cell cultures of seminiferous microtubules from male Sprague-Dawley rats were exposed for 7, 14 and 21 days to 50 nM carbendazim (CBZ) or iprodione (IPR) or to an equimolar cocktail of both substances (50 nM each).

We isolated five secreted proteins present in almost all tests, at all time points. The expression fold changes of these proteins are indicated, *versus* untreated cells at the same time point, as calculated by comparison of the LC-MS peak intensities of the tryptic peptides of these proteins by Sieve software; Sieve p-value ≤ 0.001 except for (*) p-value ≤ 0.05 . Standard deviations (SD) are reported next to each ratio. ND, undetected proteins.

Carbendazim (50 nM)	Uniprot accession number	Day 7	Day 14	Day 21
APOE	P02650	3.36 ± 0.44	-2.92 ± 0.36	2.43 ± 0.06
CLUS	P05371	5.35 ± 0.16	-5.96 ± 0.55*	-1.08 ± 0.04
FUCO	P17164	6.16 ± 0.93	-1.29 ± 0.13*	ND
LG3BP	O70513	5.07 ± 0.51	-2.07 ± 0.17	6.82 ± 0.08
PEBP1	P31044	2.61 ± 0.28	ND	3.80 ± 0.49

Iprodione (50 nM)	Uniprot accession number	Day 7	Day 14	Day 21
APOE	P02650	ND	-6.20 ± 0.33	-1.20 ± 0.09
CLUS	P05371	-2.02 ± 0.11	-3.80 ± 0.11	-1.21 ± 0.05
FUCO	P17164	ND	ND	2.64 ± 0.30*
LG3BP	O70513	ND	-2.73 ± 0.23	3.66 ± 0.17
PEBP1	P31044	-2.38 ± 0.23	-2.14 ± 0.09	1.95 ± 0.13*

Cocktail (50 nM each)	Uniprot accession number	Day 7	Day 14	Day 21
APOE	P02650	-3.56 ± 0.36*	-3.46 ± 0.06	-6.19 ± 0.66
CLUS	P05371	-1.21 ± 0.05	-4.22 ± 0.33	-2.41 ± 0.18
FUCO	P17164	-2.22 ± 0.15	-8.70 ± 0.79	-2.38 ± 0.42*
LG3BP	O70513	-3.38 ± 0.11*	-6.64 ± 0.41	ND
PEBP1	P31044	-5.49 ± 0.29	-80.22 ± 10.67	-2.76 ± 0.42*

a signal peptide, except PEBP1, and all having a score ≥ 0.5). We will comment mainly on the biology of these proteins in the next section.

4 Discussion

Carbendazim (CBZ) is well documented to elicit reproductive and developmental toxicity in animals while iprodione (IPR) is much less well documented in this regard, even if it is suspected to be deleterious to reproduction. The purpose of this study was to identify the cellular and molecular mechanisms that could explain their toxicities, in a demonstrative way for CBZ and a predictive one for IPR. Furthermore, studying the mixture (CCK) of these two fungicides, which are often used simultaneously in agriculture, should allow us to assess, using the same methods, how their molecular effects combine.

The concentrations of fungicides were selected after analysis of the literature and considering toxicokinetics and plasma concentrations when available. For CBZ (Ermler et al., 2013; Lim

and Miller, 1997a; Moffit et al., 2007; Rajeswary et al., 2007; Yu et al., 2009), the concentrations tested (50 and 500 nM) were below the lowest peak plasma concentrations found in the literature, i.e., 13 μ M after 24 h in nude mice according to Jia and colleagues (2003). For IPR, the concentrations tested in this report (50 nM and 500 nM) were consistent with the lowest concentrations tested in previous studies (Blystone et al., 2009, 2007; Perez-Carreón et al., 2009; Washington and Tchounwou, 2004). Toxicokinetic data could not be found for IPR.

We used a rat seminiferous tubule culture model mimicking the *in vivo* situation in the pubertal rat (Staub et al., 2000). Seminiferous tubules from 23-day-old Sprague Dawley rats were cultured in a dual-compartment culture system (Fig. 1). At this age there are no round spermatids in the rat testes. Thus, the round spermatids originate from the meiotic divisions occurring *ex vivo* (Staub et al., 2000). The basal compartment contained the chemicals, thus this was regarded as the blood side of the system bringing the toxic substances that have to cross the blood–testis barrier (BTB) to reach the germ cells. The medium containing the chemical(s) was renewed every two days

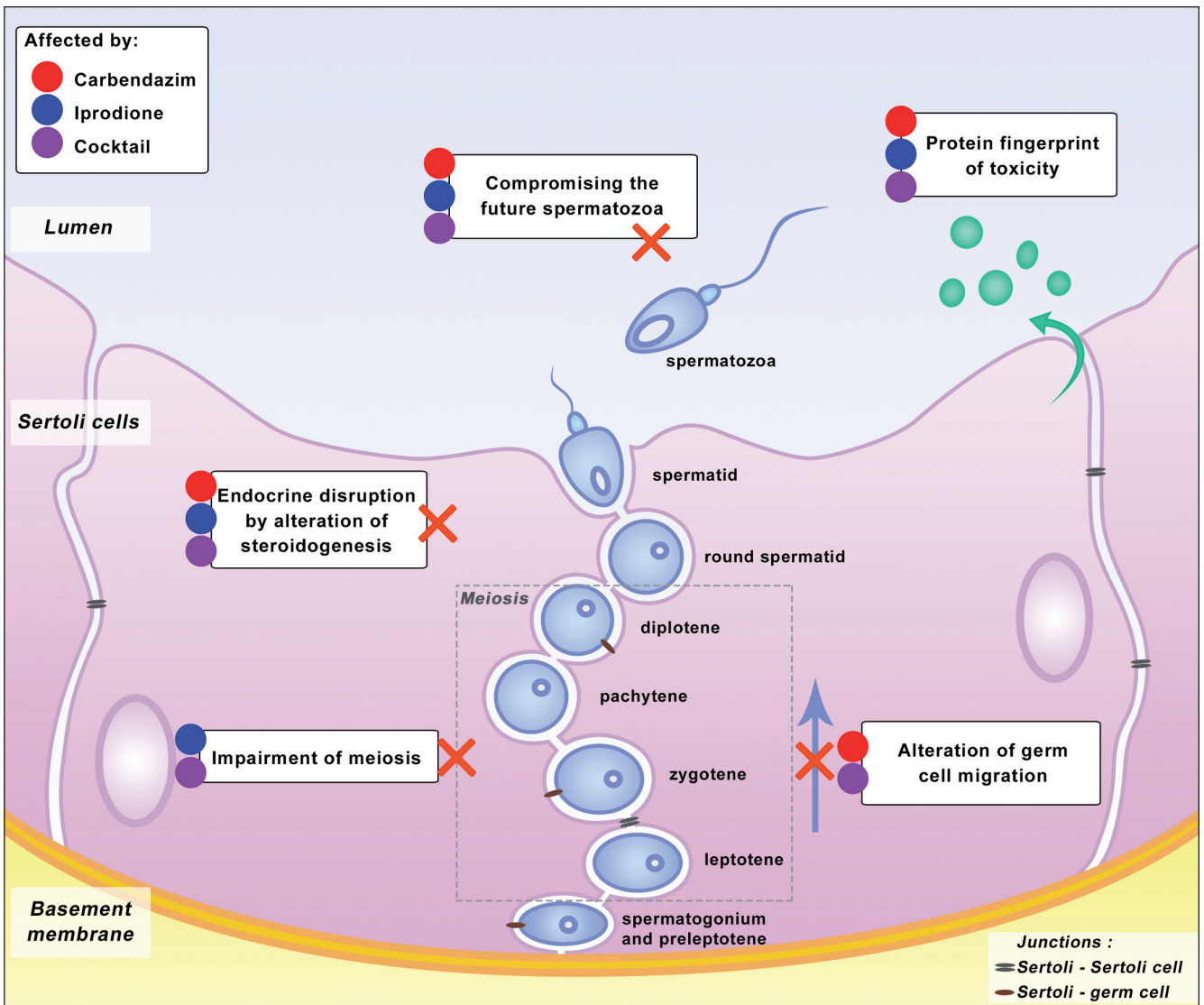


Fig. 9: General cellular scheme

Diagram of the seminiferous epithelium representing migrating germ cells (blue) surrounded by Sertoli cells (purple). As the spermatocytes cross the BTB, the Sertoli-Sertoli cell junctions open and close like a zipper. The germ cells then migrate and differentiate up to the final production of sperm in the lumen. CBZ, IPR and their mixture (or cocktail) impair this process via different initial molecular events, indicating several pathways of toxicity, as shown in the inserts, but leading to a similar mode of action, namely an impairment of spermatogenesis.

in this compartment. The apical compartment represented the luminal side, where proteins are secreted. This model allows the analysis of cellular responses induced by exposure to toxic substances for three weeks. In rats, this period of time corresponds to the development of spermatogenesis and mimics puberty, a critical life period, particularly with regard to endocrine disruptors (Geoffroy-Siraudin et al., 2010, 2012).

We applied microarray-based gene expression analysis, as well as proteomics of the extracellular compartment, to examine the toxicity of the two fungicides, CBZ and IPR, alone or as a cocktail (CCK), on this model. In addition, we examined cytogenetic modifications by counting the different populations

of germ cells and abnormalities provoked by these substances compared with control cells. To account for the evolution of cultures due to cell differentiation over time, specific control cells were used for each experimental time point. Such a physiotoxicogenomic approach has already been used for the toxicological assessment of bisphenol A (Ali et al., 2014) in the same cell culture type, combining the necessary expertise of physiologists and toxicologists.

We performed a gene expression profiling study to identify the modulated transcripts after chronic exposure to CBZ, IPR and CCK for three weeks. The number of transcripts differentially expressed by treated cultures compared with untreated

cultures reflects the magnitude of the cellular disruption. This parameter was time dependent for CBZ and CCK, and dose- and time dependent for IPR. From the beginning of the culture, and whatever the concentration, the cocktail altered more genes than each fungicide tested separately, and even more than the sum of genes altered by each compound. The drastic decrease with CCK at D21 is likely to be due to the loss of germ cells with high RNA content, as described by Carette and coworkers (2015).

We then revealed the canonical pathways most affected by each substance. To achieve this, we developed an original method (Pisani et al., 2015) to quantify the association of our data for each experimental point with known canonical pathways. We compared our data sets (genes/proteins) obtained after cell exposure with the genes/proteins known to be part of the main canonical pathways of spermatogenesis quantitatively. The result is, for each experimental condition, that the contribution of each pathway is represented by a percentage. This calculation mode, along with biological expertise, allowed us to categorize the main pathways of toxicity connected with testicular dysfunction and to compare the different cell treatments with respect to these pathways. We then gathered these disturbed pathways into five categories (Fig. 3), each representing a characterized dysfunction: nonspecific chemical toxicity or acute phase response signaling (A), alteration of germ cell migration (B), endocrine disruption (C), impairment of meiosis (D), and compromising the future spermatozoa (E). Figure 3 shows how these pathways were disturbed by each substance as a function of time within the differentiation of testicular culture. The metabolic and signaling pathways help to understand, in a rapid and comprehensive manner, the interactions between the modulated genes and the cellular mechanisms to which they belong. Thus, looking at the overall scheme rather than at isolated genes allows for a better understanding of the cellular mechanisms involved in the response to a toxic substance. From this broad overview, wanting to analyze the induction or repression of each modulated molecule would seem to be illusive in complex eukaryotic organisms and with the current state of our knowledge except in a case where the trend is largely confirmed with multiple doses and time points. This represents the ideal configuration for highlighting an indicator of effect. That is why we searched for the secreted proteins common to most substances and time points, whatever the direction of their modulation.

For a better overview of the issue, Figure 9 represents a schematic section of the seminiferous epithelium and summarizes the main conclusions of the study. Germ cells migrate between Sertoli cells using the principle of a zipper: in the process of the differentiation and growth of germ cells towards their fate (spermatozoa), the junctions between Sertoli cells open to allow germ cells to migrate, then close again after their passage (Mruk and Cheng, 2004).

Below we present our observations by distinguishing the adaptive cell responses (altered pathways related to nonspecific chemical toxicity) from the main cellular dysfunctions specifically related to the testicular tissue. To monitor the changes specifically related to spermatogenesis induced by

the tested fungicides, we used a quantification method associating the main canonical pathways of spermatogenesis with each experimental condition. In the subsequent discussion we have exemplified the results with some genes and proteins that are representative of a given pathway because they are well documented, but beyond these, more genes and proteins demonstrate the existence of a specific pathway. The strength of the evidence is based precisely on the multiplicity of genes of a data set belonging to a canonical pathway and extracted via IPA (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>). Briefly, when comparing several experimental conditions, the canonical pathway may be conserved whereas individual components may vary.

4.1 Altered pathways related to nonspecific chemical toxicity

After seven days, exposure to 50 nM CBZ, IPR or CCK triggered cellular effects including NRF₂-mediated oxidative stress, acute phase signaling, xenobiotic response signaling, NFκB signaling, TREM-1 signaling, and immune response. Many chemicals are known to trigger these pathways and most of the time they are an early adaptive cell response (Meng et al., 2009; Nel et al., 2013; Pisani et al., 2015). For the calculation (IPA results), see the Methods section.

At 50 nM, these canonical pathways were altered at a higher ratio by CCK than by either of the two substances alone. At the highest concentration tested (500 nM), the same pathways were involved (data not shown). Nevertheless, at 500 nM many other secondary pathways, which are interconnected with each other, were also at work. Therefore, the conclusions are more obvious and pertinent at 50 nM, and we will mainly focus the discussion on this dose. The analysis of the protein content of the supernatants also revealed many proteins (ALDHA, HSP70 and 90, GSTM, PRDX1 and 2, TAU) related to the classical acute phase response to toxic chemicals. These proteins were secreted in the presence of each substance and their mixture (Tab. S4, <http://dx.doi.org/10.14573/altex.1601253s4>). The pathways of toxicity associated with an acute phase response are elicited by almost all chemicals and are not tissue specific. Consequently, we focused on the following pathways of toxicity, which are testis specific.

4.2 Alteration of germ cell migration across the seminiferous epithelium

The study of the transcriptome (at both doses) showed that CBZ alters all types of junctions: tight junctions (Sertoli cell-Sertoli cell junctions) and gap junctions (Sertoli cell-germinal cell junctions) from D7. Key genes affected by CBZ, IPR and CCK were *Tnfa* and *Tgfb*, genes encoding cytoskeletal proteins such as actins, tubulin and nectin (*Pvr1*) and also the claudins family (*Cldn*), E-cadherin, occludin, ZO1 (*Tjp1*), ZO2 (*Tjp2*), and epsin 3, in coherence with most of the recent literature concerning blood-testis barrier (BTB) dynamics (Jiang et al., 2014; Kopera et al., 2009).

For IPR, only *Tnfa* and *Tgfb* were altered from D7, the other key genes were altered only at D21. Lui and Cheng (2007) have unraveled the crucial role of *TNFα* in the regulation of



junction dynamics in the testis. It is interesting to note that CBZ and CCK altered the same key genes from D7, but with much larger fold changes for CCK (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>). More specifically, intercellular adhesion molecule-1 (*Icam-1*), a regulator of BTB function (Xiao et al., 2012), was systematically repressed by CBZ and CCK at all doses and time points, but not by IPR. Many genes related to cell junctions (*Tnfa*, *Tgfb*, *Actin*, *Tubulin*, *E-adherin*, *Tjp1*, *Tjp2*, *Tnfr*, *Integrin*, *Epsin*, *Pvrl*, *Rho Gtpase*, *Cadherin*, *Cldn3*, *Ras* and *A2m*) diversely altered depending on pesticide doses and exposure times, are listed in Table S2 (<http://dx.doi.org/10.14573/altex.1601253s2>).

In addition, lipid metabolism was dysregulated by CBZ (pathway LXR/RXR in Fig. 2C). Lipid metabolism in sperm cells is important both for energy production and for cell structure. Like macrophages, Sertoli cells are highly phagocytic and need to efflux excess lipids from the cytoplasmic portions of elongated spermatids that are further cut during extrusion of differentiated sperm into the lumen of the seminiferous tubule. Additionally, any spermatogenic cells that undergo apoptotic death before complete maturation into spermatozoa are also phagocytized by Sertoli cells (Selva et al., 2004). RXR-regulated genes are required for the removal of lipids from Sertoli cells, such as the *Abca* multigene family, coding for the ATP-binding cassette transporter A. In transcriptomic datasets obtained with CBZ and CCK, we observed variations of the expression of *Abca* genes (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>). These transporters, highly expressed in the testis, mediate lipid efflux from Sertoli cells. The selective elimination of ABCA1 in *ABCA1*^{-/-} knockout mice results in Sertoli cell lipid accumulation, decreased spermatogenesis, and reduced fertility (Selva et al., 2004).

Testis germ cells are endowed with enzymatic scavenger systems to prevent lipoperoxidative damage. In particular, superoxide dismutase is present in the different developmental stages to defend the membranes against lipoperoxidation (Lenzi et al., 2002). In the current study, the expression of superoxide dismutase (SOD1) was modified in the secretome of cultures exposed to each individual substance or to their equimolar mixture. Superoxide dismutases are well known enzymes of antioxidant defense systems in the male reproductive organs (Fujii et al., 2003). The equilibrium between reactive oxygen species (ROS) and antioxidant enzymes is crucial for cell health and survival and SOD1 catalyzes the dismutation of superoxide radicals into hydrogen peroxides and oxygen (Luo et al., 2006). In fungi, IPR interfaces with cytochrome C reductase and a number of other flavin enzymes, leading to accumulation of toxic peroxy radicals and superoxide anions. These reactive radicals then cause the formation of lipid peroxides within the cell membrane, which is the ultimate cause of cell death in dicarboximide-treated fungi (Christopher and Nai, 1993).

The LXR/RXR pathway also concerns cholesterol homeostasis. Cholesterol plays a central role in many biochemical processes in addition to the synthesis of vitamin D and steroid hormones, such as the synthesis and maintenance of cell membranes (Robertson et al., 2005). Specifically, cholesterol is involved in

the plasticity of Sertoli cell membranes (Papadopoulos, 2005) and acts in particular as a membrane strengthener. LXR/RXR pathway disturbance was more marked with CBZ than with IPR and was also observed with CCK. The alteration of many genes involved in junctions between Sertoli cell-Sertoli cell and germ cell-Sertoli cell indicates a trend, namely membrane stiffening. A decrease of the plasticity of the junctions, which is essential for germ cell migration within the epithelium, may halt the germ cells at an intermediate stage of development. Interestingly, our proteomic datasets indicated that the protein, APOE, was either up- or downregulated with all treatments (CBZ, IPR and CCK), whatever the dose (50 or 500 nM) or exposure time point (D7, 14 and 21). APOE is known to facilitate the transfer of cholesterol/lipids between cells (Olson et al., 1994) and we observed here that its homeostasis was compromised in the presence of the tested compounds.

The possible reduction of sperm transport has already been evoked as a cause of the swelling of the testis and the increase of cytoplasmic content within the epididymis observed *in vivo* with CBZ (Nakai et al., 1992). Our results provide a molecular support of this observation. The movement of preleptotene/leptotene spermatocytes across the blood-testis barrier (BTB), also known as the Sertoli cell barrier, during the seminiferous epithelial cycle is one of the most important cellular events taking place in the mammalian testis. Without the passage of spermatocytes, spermatogenesis would be halted, resulting in transient or permanent sterility (Mruk and Cheng, 2012).

The metalloproteinase (MMP) transcripts were strongly repressed by CBZ, IPR, and CCK. In connection with this pathway, *Timp1*, coding for a tissular inhibitor of MMPs, was also downregulated with CCK (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>).

During testicular development and maturation in rats, extracellular matrix remodeling is a fundamental process that requires the presence of several proteases and protease inhibitors. Among the proteases, a pivotal role has been proposed for MMPs. These proteins are involved in the degradation and turnover of the extracellular matrix, with collagen as primary substrate. Collagen is abundant in the basement membrane of the seminiferous tubule in early spermatogenesis. The cleavage of collagen produces active fragments that are also involved in junction restructuring. Unlike its gene, the metalloproteinase MMP2 (gelatinase) was strongly induced (fold change, 13.4 at D7 and 4.7 at D21) in the supernatants of cultures exposed exclusively to 50 nM CBZ, and to a lesser extent to 50 nM IPR (fold change, 1.45 at D14 and 3.26 at D21).

With both fungicides, alone or in combination, many cytokine transcripts from the *Cxc* family (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>) were strongly downregulated. These molecules are often induced during inflammation, but it is interesting to note that we observed the opposite in this study. In particular *Tgfb* and *Il-1β* were downregulated in the presence of CBZ (up to 63 times). These molecules also play an important role in spermatogenesis, particularly in the junction plasticity and remodeling necessary for germ cell migration within the BTB (Li et al., 2009). By comparing the altered cytokine transcripts with the dysregulated proteins observed

by analysis of the secretomes by proteomics, only the cytokines TGF β 1 and TGF β 3 were observed as overexpressed following exposure to 50 nM IPR at D14 (FC 2.3) and 50 nM CBZ at D7 (FC 8.67). Recently, using the same *ex vivo* culture, Carette and colleagues suggested that increased levels of claudin-11 induced with CBZ might reinforce the interaction between Sertoli cells at the level of the BTB and thus impair the dynamic process of opening and closing this barrier for the normal progression of spermatogenesis (Carette et al., 2015). Proteomics after CBZ treatment also revealed the alteration of A2M (FC up to 3.44 at D7) and vinculin (FC up to 13.58 at D21), proteins involved in junctions.

4.3 Endocrine disruption by interaction with steroidogenesis

According to transcriptomic analysis, both CBZ and IPR and their mixture, exhibit the characteristics of endocrine disruptors. In this sense we do not necessarily mean that they bind to the androgen (AR) or estrogen (ER α , ER β) receptors but that they participate in the disruption of endocrine function by altering the expression of key enzymes of testosterone and estradiol-17 β biosynthesis. In particular, these fungicides inhibited key enzymes necessary for the synthesis of testosterone, which may result in a decrease of testosterone level, in our experiments.

CBZ, at 50 nM D14 and D21, inhibited NADP-dependent testosterone-17- β -oxidoreductase (*Akr1c3*) and 17- β -hydroxysteroid dehydrogenase (*Hsd17b3*) transcripts necessary for testosterone and estradiol biosynthesis. This is consistent with recent reports describing a decreased activity of HSD17B3 after treatment of rats with a single dose of CBZ (Adedara et al., 2013). Spermatogenesis requires a fine balance between androgen and estrogen levels (Carreau et al., 2012). It is likely that this balance was modified by CBZ and that this disturbed spermatogenesis.

IPR, at 50 nM from D7 to D21, also acted on the synthesis of androgens and estrogens but modulated many more enzymatic transcripts of this pathway. IPR inhibited the following transcripts: *Akr1c3*, *Hsd3b* and *Cyp17a1*, and also the aromatase *Cyp19a* from D14, which should cause a decrease in testosterone. Testosterone is converted to estradiol-17 β by the aromatase complex in Sertoli cells in immature rats (not in Leydig cells as in adults) (Cheng and Mruk, 2012). According to Blystone and colleagues (2007), IPR reduces the production of testosterone and progesterone *ex vivo*, and affects steroidogenesis in the testis, supposedly through the inhibition of enzymes involved in the biosynthesis pathways. Our data corroborate this hypothesis. IPR at 500 nM also repressed the *Esr1* and *Esr2* genes, but only at D21. These two receptors have been localized in the Sertoli cells of immature and adults rats (Lucas et al., 2011). Sertoli cells, through their paracrine regulation, are also involved in the synthesis of steroids, along with Leydig cells. IPR can, therefore, be considered as an endocrine disruptor for the time window of puberty.

CCK also acted on the biosynthesis of androgens and estrogens. The combination of CBZ and IPR cumulated the effects of both substances, suppressing key enzymes necessary for

the synthesis of testosterone and estradiol. With CCK, these effects occurred for shorter exposures (from D7 instead of D14), which renders CCK more toxic if dynamics is considered. With the 50 nM cocktail at D7, proteomic analyses also revealed a decrease in the protein 17- β -hydroxysteroid dehydrogenase (DHB4, product of the gene, *Hsd17b4*), which is involved in the pathway of fatty acid beta-oxidation, a part of cholesterol metabolism. This decrease was not observed when the two substances were tested alone.

These results are in line with a very recent memorandum on IPR published by United States Environmental Protection Agency (US EPA). This report, based on the weight of evidence, concludes that: "There is a potential for IPR to alter steroidogenesis, which may affect the estrogen and androgen pathways in mammals and wildlife." (Akerman and Blankinship, 2015).

4.4 Impairment of meiosis

Immunocytological studies of meiosis in the presence of CBZ and IPR showed that these pesticides impaired meiosis qualitatively and quantitatively. The evolution of cell subtype percentages was similar for the two fungicides and their mixture, and significantly different from controls. Partial arrest appeared at two stages of the first meiotic prophase. A first meiotic arrest probably occurred at the leptotene/zygotene transition, as suggested by the reduced percentage of zygotene nuclei. The leptotene/zygotene (L/Z) transition is a very transient stage contemporaneous with the onset of SC formation. During this stage, chromosome telomeres are spatially associated, and then telomere dispersal occurs rapidly. At the molecular level, this stage corresponds to strand exchange and formation of stable Holliday junctions in a homology search. According to Zickler and Kleckner (1998), the L/Z transition appears to be critical. Several mechanisms of action could be involved in the effect of the pesticides on this meiotic stage. The meiotic arrest could result from a transcript alteration of genes involved in recombination and pairing. Corroborating this hypothesis, transcriptomics showed that essential, known meiotic genes were up- or downregulated with 50 nM IPR and CCK, but to a lesser extent with CBZ, as detailed in the Results section. In fact, the CCK transcriptome analysis revealed that 69 meiotic genes were altered (Fig. 3). Of these, 31 (45%) of the genes identified as altered were also modulated in the IPR transcriptome, *versus* only 12 genes (17%) in the CBZ transcriptome, suggesting that IPR might be more active than CBZ towards meiosis. In fact, we know that the number of altered genes is directly related to the magnitude of the cellular response. Previous studies have shown that the number of modulated genes may be a good indicator of the level of cellular disturbance induced by xenobiotics such as drugs, chemicals, metals and nanoparticles (Lobenhofer et al., 2004; Pisani et al., 2015; Zarbl et al., 2010).

In the case of CBZ, we also saw above that the differential expression of genes involved in Sertoli cell-Sertoli cell junctions and germ cell-Sertoli cell junctions could impair germ cell migration within the epithelium and cause an arrest of the germ cells at an intermediate stage of development. It is



probable that the critical L/Z transition could be particularly susceptible to the alteration of the BTB functionality and the alteration of the relation between the Sertoli cells and the germ cells.

The second blockade of the first meiotic prophase appeared before the diplotene stage, as the frequency of these nuclei did not increase at D14 in treated samples, unlike the nuclei in the control experiment. In fact, the arrest of meiotic progression occurred at the pachytene checkpoint as indicated by the decrease of the PI (see Fig. 4C) in the presence of the two fungicides and mixture. *Piwil2* belongs to the male germ cell-specific *ARGONAUTE* family, mainly expressed in testis (Sasaki et al., 2003). Friemel and colleagues recently showed that *Piwil2* was associated with male infertility by allele-specific DNA methylation (Friemel et al., 2014).

The study of SC abnormalities at the pachytene stage revealed some differences between the effects of CBZ and IPR (see Fig. 5). The predominant abnormality induced by CBZ was fragmented SC, whereas IPR and CCK induced both asynapsis and fragmented SC. With CCK, asynapsis came preferentially from the IPR.

Thus, although meiosis was partially arrested at the same stage with CBZ and IPR, with an equivalent low percentage of pulverized nuclei assimilated into apoptosis (Tab. S3, <http://dx.doi.org/10.14573/altex.1601253s3>), it is likely that the initial molecular event leading to the impairment of meiosis was different for the two fungicides.

4.5 Compromising the future spermatozoa

Differentiated spermatozoa are incapable of transcriptional or translational activity; this activity instead occurs during the transit along the male reproductive tract where the sperm surface undergoes major modifications in macromolecule composition (Sullivan et al., 2005). In particular, the machinery for sperm tail structure and motility is initiated during spermatocyte differentiation, well before the appearance of mature spermatozoa, during meiosis rather than after the completion of meiotic divisions (Shima et al., 2004). In the present study, CCK altered the sperm motility mechanism from D7, whereas CBZ and IPR tested separately altered this mechanism only from D14 and D21, respectively (see Fig. 3). The genes for which expression was affected include the *CatSper* gene family. These genes are involved in the mobility of the future sperm. Indeed, *CatSper1*^{-/-} and *CatSper2*^{-/-} male mice are infertile, and sperm cells from *CatSper1*^{-/-} and *CatSper2*^{-/-} mice are incapable of hyperactivation (Qi et al., 2007). The protein phospholipase A2, PLA2G2A, belongs to the PLA2 superfamily that catalyzes the hydrolysis of glycerophospholipids to yield fatty acids and lysophospholipids. To date, more than 30 enzymes that possess PLA2 or related activities have been identified in mammals. In our findings, the transcriptional downregulation of *Pla2g2a* is particularly striking (ranging from 6- to 38-fold, depending on the dose and duration of exposure) (Tab. S2, <http://dx.doi.org/10.14573/altex.1601253s2>). A recent study associated differential PLA2G2 protein expression with the sperm nuclear DNA fragmentation rate in men (Intasqui et al., 2013), suggest-

ing its use as a potential seminal plasma biomarker of sperm DNA integrity. Nevertheless, this protein was not detected in our proteomic datasets, unlike LGALS3BP, which was also described in the same study (Intasqui et al., 2013) as playing a similar role.

The gene, *Pdilt* (protein disulfide isomerase-like, testis expressed), was modulated by CBZ and CCK at all doses and time points, but only at D21 with IPR (FC 11). PDILT is a testis-specific ER chaperone protein (along with calnexin and calreticulin3) and is widely conserved in mammals, including humans. Sperm-fertilizing proteins (ADAM family proteins) require the action of PDILT as a unique quality control system for their maturation. Testis-specific quality control for nascent proteins in the ER is crucial in equipping spermatozoa with their fertilizing ability in humans (Tokuhiro et al., 2012). Consequently *Pdilt* plays a role in male infertility, and its downregulation in our data suggests that the tested substances as well as their mixture might impair sperm function. CBZ alone, or in a mixture with IPR, threatens the future of sperm motility.

There could be several reasons why CCK is more deleterious than IPR and CBZ tested separately. One chemical can affect the uptake rate of the other by, for example, competition at biological ligands or competitive inhibition of intracellular proteins. A chemical can either increase or decrease the rate of metabolism of another chemical, and the majority of synergistic interactions can be explained by interactions on metabolism (Cedergreen, 2014). From a proteomic perspective, the protein α -L-fucosidase (FUCO) encoded by the gene *Fuca1*, which is systematically downregulated in response to CCK treatment, is likely to play an important role in human reproduction (Khunsook et al., 2003). The analysis of secretomes also revealed the differential expression of phosphatidyl ethanolamine binding protein 1 (PEBP1), which belongs to the biological process of sperm development. PEBP1 is a receptor for decapacitation factors (DFs). Capacitation is a pivotal event for mammalian spermatozoa, involving the loss of surface proteins known as DFs and consequent acquisition of fertilizing ability. PEBP1 plays a fundamental role in capacitation by causing alterations in the sperm plasma membrane in both the head and flagellum, with functional consequences for membrane-associated proteins (Gibbons et al., 2005).

4.6 Secreted proteins as fingerprint of fungicide's toxicity

The analysis showed that the apical exoproteome environment was richer in proteins than the basal medium in all cases and that D14 seems to be a turning point in cellular development, as a critical time window for toxicant action. The basal compartment contained the chemicals, thus is regarded as the blood side of the system bringing the toxic substances. The medium in this compartment was renewed every two days with the studied chemicals. The proteins were thus very dilute in this medium contrary to the apical side where the medium was not changed. The apical compartment thus represented the lumen side where proteins were secreted.

Following CBZ, IPR and CCK treatments, the proteins identified as differentially expressed show that the disturbed metabolic pathways involve ubiquitination proteins, glycolysis, gluconeogenesis and an oxidative stress response pathway (NRF2-mediated). Correlating the results obtained by transcriptomic analysis, the pathway of lipid metabolism (LXR/RXR), with modulated expression of APOE, and matrix metalloproteinase expression (especially MMP2) also appear to be disturbed by the addition of all of the substances tested. With CBZ, several proteins that are strongly involved in membrane remodeling were differentially expressed, such as alpha-2-macroglobulin junction (A2MG), vinculin, cofilin-1, and tubulin beta-4B.

A comparison of the data sets obtained with CBZ, IPR and CCK at all time point reveals a series of proteins excreted in the apical environment that were systematically differentially expressed in most of the experimental conditions. These proteins are APOE, clusterin (CLUS), α -L-fucosidase (FUCO), galectin-3 binding protein (LG3BP), and phosphatidylethanolamine binding protein 1 (PEBP1). Interestingly, CLUS has already been reported by Plotton and colleagues (2006) to be linked to spermatogenic failure, with mRNA levels significantly lowered in testicular biopsies of azoospermic patients classified according to their testicular histology (Plotton et al., 2006). LG3BP, which was also systematically modulated in the current study, has already been associated, in humans, with abnormal semen, in particular with a strong DNA fragmentation (Intasqui et al., 2013). Very recently, Giacomini and colleagues demonstrated that LG3BP was underexpressed more than threefold in the seminal plasma proteome of men with idiopathic oligoasthenozoospermia (Giacomini et al., 2015). Galectin-3 has been identified as a proteolytic substrate for prostate-specific antigen (PSA) in prostasomes from human semen (Kovak et al., 2013). In addition, LG3BP and CLUS are both ligands for galectin-3 and components of human seminal plasma (Kovak et al., 2014).

Concerning DNA fragmentation, CBZ gave positive responses with the micronucleus assay *in vitro* and *in vivo* in mice (Bowen et al., 2011; Van Hummelen et al., 1995). CBZ is aneugenic and increases spermatozoon aneuploidy. Regarding IPR, at concentrations comparable to those used in practice, it can increase micronucleus formation and can be regarded as a potential aneugen in plants (Gadeva and Dimitrov, 2008). FUCO was downregulated with CCK at all time points. A deficiency of this protein has been associated with impaired sperm membrane modification, leading to a disturbance of events associated with epididymal sperm maturation, namely the acquisition of motility (Veeramachaneni et al., 1998). This last point is consistent with the transcriptomic analysis. As seen above, PEBP1 was also downregulated systematically with IPR and CCK (at both doses and at the three time points). Molecular modification of this decapacitation receptor could render spermatozoa irreversibly nonfertilizing.

Recently, White et al. (2014) demonstrated the capability of mass spectrometry to detect and quantify tens of dysregulated proteins occurring in renal carcinoma. Amongst these,

secreted proteins have been successfully analyzed in a multiplexing mode. The proteins targeted in the current study could be explored in a single analysis by liquid chromatography coupled to high-resolution mass spectrometry (LC-ESI MS/MS). Consequently, these secreted proteins should be the subject of further testing as potential biomarkers of reproductive dysfunction and infertility associated with reproductive toxicants.

4.7 Concluding remarks

In the current study, omics tools enabled the comparative analysis of multiple doses and times of exposure, identifying the molecular mechanisms of action of substances while pointing to potential biomarkers. Our data suggest that the two pesticides, carbendazim and iprodione are deleterious to spermatogenesis in the rat *ex vivo* model used. A pathway-driven analysis showed that these pesticides alter germ cell migration across the seminiferous epithelium, interfere with androgen and estrogen synthesis, and impair meiosis. Both substances appear to be endocrine disruptors, although with different molecular actions. CBZ acts more specifically on the stiffening of blood-testis barrier junctions, impacting on germ cell migration, whereas IPR is more deleterious to meiosis. These findings may have important implications in risk assessment for pesticide residues in food and water. *In vivo* assays remain the gold standard in toxicology. Nevertheless, the combination of this *ex vivo* culture model with omics technology is well suited for testing compounds, alone or in a mixture, and clearly contributes to the 3R rule to reduce, replace and refine animal testing. In particular, this *ex vivo* model permits the number of rodents used to be greatly reduced. Beyond this fundamental study to highlight genes and proteins involved in testicular dysfunction, we can infer what could be a preferred assay format arising directly from our current results. These five proteins could be tested *per se* in the apical medium under the form of enzyme-linked immunosorbent assays (ELISA) on fully automated laboratory equipment. Alternatively, multiplex analysis to screen and quantify several targeted markers could also be performed in a single analysis by LC-ESI MS/MS, without further assay design or the use of specific antibodies, contrary to ELISA. This would allow a first high-throughput screening of large libraries of chemicals, thus providing a tool for the prioritization of substances that may be toxic for reproduction prior to toxicological evaluation *in vivo*.

Concerning the effects of the mixture containing CBZ and IPR, i.e., CCK, it is perhaps illusory to speak of an overall combined effect, because this would require a greater number of doses tested in order to be able to trace the dose-response curves and chemical combination analysis with isobolograms. This was not the intention of this study. Nevertheless, it is clear that this mixture alters many more genes and proteins than each fungicide tested separately, and this occurs from the beginning of the exposure. For certain toxicity pathways (e.g., estradiol and testosterone synthesis) an additive effect was observed, whereas for other cellular pathways the mixture of the two substances triggered effects greater than the sum of their cumulative effects (e.g., impairment of meiosis). The dynamic



aspect of the response was also informative in the sense that an equimolar mixture of CBZ and IPR jeopardized certain cellular pathways (e.g., sperm motility) earlier than either fungicide tested separately at the same concentration (from D7, for the mixture, instead of D14 for CBZ and D21 for IPR).

The *ex vivo* cytogenetic analysis revealed that both fungicides, separately and as a mixture, altered the course of the meiotic cycle over the first two weeks of exposure. In addition, the transcriptomic analysis showed that the initial molecular events in the alteration of spermatogenesis were not necessarily the same for each fungicide, namely the alteration of germ cell migration for CBZ and the impairment of meiosis for IPR. Finally, the proteomic studies of secretome profiling highlighted several proteins (APOE, LG3BP, CLUS, FUCO, and PEBP1) linked to testicular dysfunction. LG3BP and CLUS, which are already linked to human oligoasthenozoospermia, are known to be ligands for galectin-3 and components of human prostasomes, and are consequently easily detectable in human semen. FUCO and PEBP1, which are related to motility and fertilizing ability, respectively, were differentially expressed in the secretomes at most test doses and time points using both fungicides and their mixture, suggesting them as potential new human biomarkers of infertility.

Availability of supporting data section

The data sets supporting the results of this article are available as supplemental files (five links are indicated in the text).

The transcriptomic raw data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO) repository and are accessible via GEO Series accession number (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75446>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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