Transferability and inter-laboratory variability assessment of the in vitro bovine oocyte maturation (IVM) test within ReProTect

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A B S T R A C T

The new European chemicals policy for the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) will most probably impose a dramatic increase in the number of animals required for reproductive toxicity testing. For this purpose, the development and validation of alternative methods is urgently needed in order to reduce the use of laboratory animals. The present study describes the inter-laboratory variability and the transferability assessment of an in vitro test able to identify chemical effects during the process of oocyte maturation in a bovine model. The test was developed/optimised within ReProTect, an integrated research project funded by the European Union, joining together 35 partners with complementary expertise in reproductive toxicology. Eight chemicals with well-known toxic properties were tested (benzo[a]pyrene, busulfan, cadmium chloride, cycloheximide, diethylstilbestrol, ketoconazole, methylacetoacetate, mifepristone/RU-486 and DMSO as solvent) on the in vitro maturation (IVM) assay in two well-trained laboratories using the established Standard Operating Procedures. The statistical analysis demonstrated the concordance of results across the laboratories and the reproducibility of the test. We therefore conclude that the IVM test could advance toward the process of validation as alternative to the in vivo method that, in combination with additional in vitro tests, can become part of an integrated testing strategy in order to predict chemical hazards on mammalian fertility.

1. Introduction

Due to the complexity of the reproductive cycle and for the lack of validated alternative tests for most of the steps included in the cycle, testing in living animals is presently the only tool available for hazard assessment of reproductive toxicants.

The fifth statistical report showed that 10%, over the total amount of 1,026,286 of animals used in toxicity tests for toxicological and other safety evaluation in 25 European countries, were used in 2005 for reproductive/developmental toxicity testing as reported in the “Fifth Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union” [1]. Moreover, the future Community Policy for Chemicals will most probably increase the number of animals for reproductive/developmental toxicity testing, in order to ensure a high level of protection for human health and the environment [2–4]. Since the main goal of REACH is to use vertebrates only as last resort, the development and validation of alternative methods is urgently needed for safety testing of chemicals.

In order to provide reliable and relevant in vitro tests as building blocks for integrated testing strategies, a consortium of 33 partners (ReProTect, http://www.reprotect.eu) with complementary expertise in the area of reproductive toxicity was formed under the umbrella of the Sixth Framework Programme of the European Union [5]. Several in vitro and in vivo studies have demonstrated that a variety of processes occurring during maturation and subsequent fertilization can be blocked or impaired by chemicals or pharmacological compounds that can act with different modes of action resulting in infertile cycle or abnormal pregnancy outcome [6–12]. Recently, Lazzari and co-workers reported the successful development/optimization of in vitro maturation (IVM) and in vitro fertilization (IVF) of bovine oocytes for toxicity testing purpose [8] within ReProTect. In particular, the IVM...
test was proposed as alternative system to monitor the potential adverse effects on the maturation process after exposure of cumulus–oocytes complexes to testing substances, with special reference to the nuclear configuration changes within the oocyte as compared to control non-exposed oocytes. Successful achievement of the maturation stage (completion of meiosis up to the metaphase II) was selected as toxicological endpoint of this test. The development/optimisation of the IVM test was achieved by testing 15 chemicals under the developed Standard Operating Procedures (SOPs) in order to define the reproducibility within the developer laboratory (AVANTEA, Italy, partner 42 of the ReProTect project). In the prospective of validation process of the test, it was decided, within the ReProTect time frame, to assess the transferability and the inter-laboratory variability of the IVM assay. For this purpose, the toxicity effects of eight chemicals [benzo(a)pyrene, busulfan, cadmium chloride, cycloheximide, diethylstilbestrol, ketoconazole, methylacetoacetate, mifepristone and DMSO as solvent] were evaluated under the established SOPs in a second laboratory (Università degli Studi di Milano, UNIMI, partner number 38 of the ReProTect project). The selection of the mentioned substances was made by taking into account the mechanisms of action of the compounds, the available in vitro and in vitro data summarised in Table 1 of Lazzari et al. [8] and following a decision-making process that took place within the ReProTect project with advices of external reviewers. The selection was conducted adopting the ECVM Principles on Test Validation procedures [13], by the definition of chemical classes and/or ranges of molecular descriptors in order to cover the full range of toxic effects.

The present study describes the inter-laboratory and the transferability assessment of the IVM test between the developer laboratory and the second laboratory (UNIMI), in accordance with the requirements laid down in the ECVM Modular Approach [13]. This information will allow the evaluation of the practicability of the test and will provide an estimation of the amount of training that will be necessary to successfully transfer the test to an experienced laboratory, as well as to identify possible sources of between-laboratory variability.

2. Materials and methods

2.1. Standard operation procedures (SOPs) transfer

The transfer of the SOPs for bovine oocyte maturation was achieved through a series of meetings and e-mail contacts between the lead laboratory and the second laboratory within 6 weeks. No practical training was necessary for the SOPs transfer since the technique of in vitro maturation of bovine oocytes is routinely performed in the 2nd laboratory. However, although both partners are expert in the field of oocyte maturation, fertilization and embryo culture, a number of technical steps had to be discussed in detail to ensure that the same procedures were carried out in both laboratories.

The established SOPs of the IVM assay were transferred from the lead laboratory to the experienced 2nd laboratory. All materials/reagents/products were purchased as outlined in SOPs. Cycloheximide, which is acting as positive control in the assay, was tested 5 times in order to assess the robustness of the test.

No further material transfer agreement was necessary since both partners have signed the consortium agreement of ReProTect.

2.2. Chemicals and reagents

All chemicals and reagents were purchased from the same suppliers of the developer’s laboratory (Sigma Chemical Company, St. Louis, USA), unless otherwise stated in the text.

2.3. Oocyte collection

Bovine ovaries were recovered at a local abattoir (INALCA BS S.p.A., Ospeedaletto Lodigiano, LO, IT 22700 CE, Italy) from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/EEC and subsequent modifications. The ovaries were transported to the laboratory within 2 h, in sterile saline maintained at 26 °C. All the subsequent procedures, unless differently specified, were performed at 35–38 °C. Cumulus–oocytes complexes (CDCs) were isolated from follicles of 3 mm or above of diameter by aspiration with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane, QLD, Australia) with a vacuum pressure of −28 mmHg. Afterwards, the CDCs were cultivated in a humidified atmosphere of 5% CO2/95% air at 38.5 °C under 5% CO2 in humidified air for 24 h. IVM medium was TCM 199 supplemented with 10 ng/ml Epidermal Growth Factor (human recombinant EGF, Peprotech EC Ltd., London, UK), FSH/lH 0.1 IU each (Menogon, Ferring Spa, Milan, Italy), 0.10 mg/ml Glutamine and 0.11 mg/ml Sodium Pyruvate. An average of 15–20 oocytes per dish/replicate was matured in presence of selected chemicals at different concentrations reported in Table 1 of Lazzari et al. [8]. After the maturation time the oocytes were washed, freed of cumulus cells and fixed in 500 µl of 60% ethanol in Dulbecco’s phosphate buffered saline (PBS) for 30 min at 4 °C. The oocytes were stained with 0.5 mg/ml of 4',6-diamidino-2-phenylindole (DAPI) and 1 mg/ml Propidium Iodide to evaluate nuclear morphology by observation at 200–400 x magnification. The toxicological endpoint evaluated in this study, as defined in Lazzari et al. [8], is the completion of meiosis I up to the metaphase II stage, which is normally only reached after 24 h in vitro culture. Matured oocytes were characterized by the extrusion of the first polar body associated to the second metaphase plate. Oocytes arrested at the germinal vesicle (GV) stage or that had progressed beyond the GV stage but had not completed the maturation process were classified as not matured. Results are expressed as percentage of metaphase II oocytes calculated on the total number of oocytes cultured in maturation medium.

2.5. Transferability and assessment of the inter-laboratory variability test

Eight chemicals [benzo(a)pyrene, busulfan, cadmium chloride, cycloheximide, diethylstilbestrol, ketoconazole, methylacetoacetate and DMSO as solvent] were selected to assess the transferability and the inter-laboratory variability of the IVM test from a set of previously tested substances [8] and listed in Table 1. All the chemicals were dissolved in DMSO (Sigma-Aldrich) to 10 mM, with the exception of cycloheximide that was dissolved in water. The maximum dose of DMSO used was 15 µL. At this concentration DMSO did not show adverse effects on the selected toxicological endpoint. The stock solution for each chemical varied from 100 to 0.1 mM depending on the chemical and its solubility. Aliquots were stored at −20 °C and used within 3 months. Cycloheximide was included as positive control in all replicates at the EC50 dose (0.39 μM) established in preliminary experiments [8]. Every test run was accompanied by a replicate in which the maximum concentration of DMSO used as solvent was added in the culture medium in absence of the chemical. This replicate was the control solution.

When the tested compound was water-soluble, the solvent control was the simple control.

For the maturation test, immature oocytes were treated for 24 h with the selected chemicals at the concentration range reported in Table 2.

2.6. Assessment of general cytotoxicity on bovine cumulus–oocyte complex

Cytotoxicity was determined by trypan blue staining. For this purpose at the end of the period of chemical exposure, COCs were recovered from the incubation medium and washed in collection medium. Cumulus cells were partially removed by gently pipetting with a fire-polished glass pipette and only the cells surrounding the oocyte were left. These complexes were incubated with a solution of trypan blue (0.4% in PBS with 1 mg/ml PVA) diluted 1:1 in collection medium. After 15 min from the start of the staining the cells were recovered from the trypan blue solution and washed twice in collection medium and observed at the stereomicroscope. Blue cells were considered non-viable. The percentage of stained cumulus cells was calculated on the total number of oocytes cultured in maturation medium.

2.7. Statistical analysis

The analysis of chemical effects was carried out by evaluating 12–20 oocytes for each concentration/independent run. The data are expressed as percentage of matured oocytes. At least three independent experiments were carried out for each substance/dose.

Statistical analysis was carried out using the statistical software package R [15]. Laboratory effects on maturation in the controls were examined using a generalized linear model with binomial response, laboratory as fixed effect, and experiment as random effect (function glmnRQ in R-package MASS). Significance of the difference between labs was tested by conditional t-testing of the respective fixed effect at α = 0.05.
### Table 1
Training set of chemicals tested in the bovine oocyte in vitro maturation transferability test.

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS. No.</th>
<th>Classification (EU)</th>
<th>Product class</th>
<th>Mode of action</th>
<th>References providing in vivo data and in vitro data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo[a]pyrene</td>
<td>50-32-8</td>
<td>R45-R46-R60-R43-R50-53</td>
<td>Environmental pollutant and in tobacco smoke</td>
<td>Benzo[a]pyrene belongs to a group of environmental pollutants, polycyclic aromatic hydrocarbons that have the ability to bind covalently DNA, giving rise to adducts that constitutes a potential source of carcinogenic damage [34].</td>
<td>In vivo: decrease of ovarian volume (mouse: [35]); destruction of primordial follicles (mouse: [36]); increased gestation period (mouse: [37]); abnormal sperms (mouse: [38]); reduced progressive motility of stored sperm (rats: [39]); Detection of DNA-adducts in oocytes, luteal cells and stromal arteries of human ovaries post-mortem (human: [40]). In vitro: increased chromosomal aberrations in human spermatozoa following metabolic activation by rat liver S9 [41].</td>
</tr>
<tr>
<td>Busulfan</td>
<td>55-98-1</td>
<td>R53-R36/37/39-R45</td>
<td>Alkylating agents, antineoplastic and drug/therapeutic agent</td>
<td>Busulfan is an alkylating agent and induces chromosomal aberrations, DNA–DNA and DNA–protein cross linking [42].</td>
<td>In vivo: reduced number of germ cells, primordial, primary and preantral follicles (mouse: [43,44]; rat: [45,46]); destruction of stem cells and differentiating spermatogonia (mouse: [42]).</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>7790-78-5</td>
<td>R45-R46-R60-R26-R25-48/23/25-R50-R53</td>
<td>Pesticide and fungicide</td>
<td>Cadmium chloride is a heavy metal and binds to DNA inducing DNA strand breaks [47]. It is considered as a general toxicant.</td>
<td>In vivo: impaired gonadal development in mouse embryos [48]; sterility (rat: [49]); decreased number of oviductal eggs and implantation sites (rabbit: [50]); mutagenic effect on oocyte chromosomes (hamster: [47]). In vitro: gametes exposed to cadmium chloride give rise to embryos of low viability [51].</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>66-81-9</td>
<td>R61-R28-R51/53-R68</td>
<td>Agricultural chemical, antibiotics, antifungal, antiparasitic, drug/therapeutic agent, fungicide, bactericide, wood preservative and growth regulator/fertilizer</td>
<td>Cycloheximide causes a dose-dependent decrease of protein synthesis.</td>
<td>In vivo: Inhibition of ovulation (hamster: [10]); prevention or delayed follicular growth, resumption of meiosis, cumulus expansion, altered steroid profile (hamster: [52]). In vitro: inhibition of oocyte maturation (pig: [42]; pig and mouse: [53]).</td>
</tr>
<tr>
<td>DES (diethylstilbestrol)</td>
<td>56-53-1</td>
<td>R45-R61-R36/37/R51/53</td>
<td>Agricultural chemical, antineoplastic agents, contraceptives, postcoital, synthetic and drug/therapeutic agent</td>
<td>DES is a non-steroidal estrogen. It has a depolymerising effect on meiotic microtubules and interferes with meiosis delaying progression to metaphase I and formation of the metaphase spindle. DES binds to γ-tubulin disaggregating the mitotic spindle that breaks up in micro-spindles and dispersed chromosomes. In addition DES can act by inhibiting cyclin proteolysis and therefore by reducing the level of maturation promoting factor within the oocyte [13].</td>
<td>In vivo: reproductive tract defects and reduced fertility after prenatal exposure (mouse: [54]; mouse and rat: [55]); reduced fertility, increased rates of ectopic pregnancy, spontaneous abortion and preterm delivery after in utero exposure (human: [56]). In vitro data: delayed oocyte maturation and meiotic spindle disruption (mouse: [31]). In vivo: ovarian aberrations and increase in polycytoplastic follicles [57–59] and reduced endometrium thickness in mice [59].</td>
</tr>
</tbody>
</table>
**Table 1 (Continued)**

<table>
<thead>
<tr>
<th>Name</th>
<th>CAS. No.</th>
<th>Classification (EU)</th>
<th>Product class</th>
<th>Mode of action</th>
<th>References providing in vivo data and in vitro data</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ketoconazole</em></td>
<td>65277-42-1</td>
<td>R25</td>
<td>Antifungal and drug/therapeutic agent</td>
<td><em>Ketoconazole acts mainly by inhibiting the enzyme cytochrome P450 14-alpha-demethylase. This enzyme is in the sterol biosynthesis pathway that leads from lanosterol to ergosterol and it is involved in steroidogenesis. Moreover it inhibits a key enzyme for the synthesis of meiosis activating sterol that acts to induce meiosis resumption in mouse oocytes [29].</em></td>
<td><em>In vivo</em>: prologation of estrous cycle, increase of estradiol, LH and FSH, suppressed ovulation, reduced epididymis and accessory sex organ weights (rat: [30,60]).&lt;br&gt;<strong>In vitro</strong>: inhibition of oocyte maturation (mouse: [29]; pig: [28]). Decrease sperm motility (rat: [61]). Disruption of steroidogenesis in mouse follicles [62].&lt;br&gt;<em>Mifepristone/RU-486</em>&lt;br&gt;Not reported Pharmaceutical RU-486 is an antiprogestogens with high affinity not only for the glucocorticoid receptor but also for the progesterone receptor. It prevents progesterone (and glucocorticoids) from binding to hormone receptors.</td>
</tr>
<tr>
<td><em>Methyl acetoacetate</em></td>
<td>105-45-3</td>
<td>R36</td>
<td>Solvent for cellulose ethers, component of solvent mixtures for cellulose esters, chemical intermediate for organic compounds. It also use as herbicides and pesticides</td>
<td>Not available.</td>
<td><em>In vivo</em>: no effects on fertility [63].</td>
</tr>
</tbody>
</table>

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*a* The Europe (EU) classification is based on the data collected from the European Chemicals Bureau (ECB) within ESIS (European Chemical Substances Information System). Web site: http://ecb.jrc.it. The data shown represents the risk phrases.<br>*b* The product class data were obtained from ChemIDPlus Lite on the web site: http://chem.sis.nlm.nih.gov/chemidplus/.<br>*c* The mode of action was determined by a literature research and the data publicly available.
Table 2

The eight chemicals selected for the transferability test, their concentration range tested and EC_{50} values from partner 38 (UNIMI) and partner 42 (AVANTEA) laboratories.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration tested (µM)</th>
<th>UNIMI</th>
<th>AVANTEA</th>
<th>Both Labs</th>
<th>UNIMI</th>
<th>AVANTEA</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo[a]pyrene</td>
<td>1–20</td>
<td>1–20</td>
<td>&gt;20^+</td>
<td>&gt;20^+</td>
<td>&gt;20^+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Busulfan</td>
<td>10–1000</td>
<td>40–1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cadmium chloride</td>
<td>1–5</td>
<td>1–5</td>
<td>3.07 ± 0.238</td>
<td>2.55 ± 0.176</td>
<td>2.84 ± 0.341</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>0.22–0.89</td>
<td>0.22–0.89</td>
<td>0.38 ± 0.052</td>
<td>0.40 ± 0.020</td>
<td>0.39 ± 0.037</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DES (diethylstilbestrol)</td>
<td>2–20</td>
<td>2–20</td>
<td>5.04 ± 0.861</td>
<td>4.43 ± 0.644</td>
<td>4.69 ± 0.750</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>5–45</td>
<td>10–50</td>
<td>19.6 ± 9.12</td>
<td>26.4 ± 2.1</td>
<td>23 ± 7.01</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methylacetocetate</td>
<td>10–1000</td>
<td>10–1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mifepristone/RU-486</td>
<td>1–50</td>
<td>10–90</td>
<td>16.4 ± 4.74</td>
<td>16.2 ± 6.46</td>
<td>16.3 ± 5.07</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

^+ or −: presence or absence of cytotoxic effect on cumulus cells, respectively.

This chemical was toxic to the cumulus–oocyte complexes at all concentrations used (Table 2).

3.4. Assessment of inter-laboratory variability

Table 1 presents the data obtained in the two laboratories with eight reference chemicals. As shown in Table 1 and Fig. 1, for 3 of 8 test compounds laboratories UNIMI and AVANTEA did not find any consistent evidence of concentration effects (benzo[a]pyrene [max 20 µM], busulfan [max 1000 µM], and methylacetocetate [max 1000 µM]). For the rest of the compounds modelling concentration–response relationships and EC_{50} estimation was possible in both labs in all except one experiment. The only exception is a single experiment for ketoconazole at the lead laboratory, in which the concentration–response relationship shows a clear trend, but modelling is hampered by outlying observations.

Intra-laboratory variability of EC_{50} estimates for test compounds cadmium chloride, cycloheximide and DES is small with a coefficient of variation between 0.050 and 0.145 at AVANTEA and 0.078 and 0.171 at UNIMI. EC_{50} 95% confidence intervals are small.
for these compounds, the largest ones having a ratio of upper and lower limit of 2.66 at AVANTEA (cadmium chloride) and 2.96 at UNIMI (DES). At UNIMI, one of the EC_{50} estimates for ketoconazole is much smaller than the other two estimates resulting in a coefficient of variation of 0.47. Data set and fitted model underlying this estimate did not show any obvious problems. For Mifepristone intra-laboratory variability of EC_{50} estimates is moderate in both laboratories with coefficients of variation of 0.40 at AVANTEA and 0.29 at UNIMI. At the same time EC_{50} 95% confidence intervals are larger than for the other compounds, the largest ones having ratios of upper and lower limits of 8.83 at AVANTEA and 6.77 at UNIMI.

Comparison of mean EC_{50} estimates between the two labs (Fig. 2) shows good agreement, the relative ranking of compounds is identical. The largest observed ratio of means is 1.35 for compound ketoconazole.

Visual inspection of proportions of maturation in the solvent controls across experiments does not indicate a systematic difference between labs (Fig. 3). The respective statistical test of laboratory effects in a generalized linear mixed model was not significant (p = 0.5). Minor differences in the calculation of the EC50 estimates in comparison with those previously published by partner 42 [8] are due to the different statistical analysis applied in this study.

4. Discussion

The transferability of a test method from the developer laboratory to a second laboratory is a crucial step for demonstrating the robustness of the established SOPs. This step is necessary to evaluate the practicability of the test and to identify possible sources of intra- and inter-laboratory variability. Moreover it provides also an estimation of the amount of training that will be necessary to successfully transfer the test to an inexperienced laboratory.

In the present study, a set of eight chemicals, selected by taking into account the known mechanisms of action (Table 1), the in vivo and in vitro data available and in particular the results previously reported in Lazzari et al., 2008, were tested in order to assess the inter-laboratory variability and the transferability of the IVM test.

In the present work, both partners are well-trained laboratories, expert in the field of oocyte maturation, fertilisation and embryo culture as documented by several papers concerning oocyte meiotic and developmental capability in vitro and molecular mechanisms involved in the female gamete physiology [16–23]. Nevertheless, inexperienced laboratories will certainly need specific training in order to acquire the necessary skills to appropriately carry out all the procedures described in the established SOPs. In particular, proficiency in oocyte selection after follicle isolation, and citological evaluation at the end of the exposure period must be achieved [24].

The statistical analysis of the data obtained in the two laboratories demonstrated that there was good concordance of results across the laboratories and a high between-laboratory reproducibility of the test.

Amongst the tested chemicals we observed that Ketoconazole showed a higher intra- and inter-laboratory variability. A possible explanation can be referred to its role in sterol biosynthesis. Ketoconazole acts as inhibitor of lanosterol 14-demethylase (P45014DM) that is a key enzyme on formation of meiosis activating sterols (MAS) [25]. Byskov et al. [26] showed that MAS can induce in vitro meiotic maturation in mouse in a dose-dependent manner, and Faerge et al. [27] have reported the distribution of MAS binding sites in the oocytes of marmosets, cows and mice. However, while ketoconazole decreases the rate of GVBD in porcine oocytes [28] and mouse [29], it lacks to affect meiotic resumption in rat [30]. All together these findings suggest that the variability observed following ketoconazole exposure could depend upon the heterogeneous distribution of MAS receptors and the erratic activity of the sterol biosynthetic pathway within the oocyte population as well as species specificity.

In vitro maturation and fertilisation procedures are routinely applied for assisted reproduction purposes in animal breeding and their high success rate indicates that these procedures can closely mimic the in vivo processes of oocyte meiotic division and singamy giving rise to the formation of viable embryos and offspring [20]. Moreover, this test represents a promising method to avoid additional terminations of animals since bovine oocytes can be easily collected from ovaries of slaughtered animals.
Our findings indicate that the process of maturation is affected by low concentrations of chemicals as previously reported [8], suggesting that the oocyte can represent a relevant model for the development of a reliable test for reproductive toxicity. This high sensitivity is most likely a consequence of the complexity of cellular and molecular events that take place during meiosis resumption. This is particularly relevant since according to the current EC ECVAM validation process, the between-laboratory variability of a test can be assessed also with a limited number of test substances, providing that the set of test substances evaluated covers a wide range of toxic effects and relevant chemical classes [13]. The eight chemicals selected for the transferability test from the set of previously tested substances [8] include spindle poisons (DES), protein synthesis inhibitor (cycloheximide), and chemicals with steroidal-genic or anti-steroidal-genicity (DES, meflipristone), general toxicants (cadmium chloride, benzaldehyde) and drugs (ketconazole).

As previously observed, it is interesting to note that chemicals acting on mechanism such as protein synthesis (cycloheximide) in the present study has an active range 100 times lower for the maturation test as compared to the dose normally used to block protein synthesis in somatic cells in vitro (10 μM; [31]) and similar to the dose range reported for the inhibition of maturation of porcine oocytes [32]. These findings demonstrate that the meiotic process can reveal toxic effects at much lower concentration of active chemicals as compared to the mitotic process in somatic cells.

Furthermore, chemicals acting on microtubules such as DES interfere with microtubule dynamics that drive the oocyte chromatin in the different meiotic stages up to the metaphase II. As previously reported [33], altered chromatin configurations, with abnormal or multipolar spindles at metaphase/anaphase/telophase stages were observed.

At the same time, the chemicals busulfan and methylichlotoacetate did not affect oocyte maturation up to the highest tested dose of 1 mM in agreement with the lead laboratory.

The analysis of the data obtained by the transferability of the test demonstrated that there was good concordance of results across the laboratories and a high inter-laboratory reproducibility of the test. We therefore conclude that the IVM test could advance toward a building block for a testing strategy aiming to assess reproductive toxicity replacing or reducing the use of living animals. We therefore conclude that the IVM test could advance toward a testing strategy aiming to assess reproductive toxicity replacing or reducing the use of living animals.

Conflict of interest
No conflict of interest.

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