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Innovative non-animal testing strategies for reproductive toxicology: the contribution of Italian partners within the EU project ReProTect

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Summary. Reproductive toxicity, with its many targets and mechanisms, is a complex area of toxicology; thus, the screening and identification of reproductive toxicants is a main scientific challenge for the safety assessment of chemicals, including the European Regulation on Chemicals (REACH). Regulatory agencies recommend the implementation of the 3Rs principle (refinement, reduction, replacement) as well as of intelligent testing strategies, through the development of *in vitro* methods and the use of mechanistic information in the hazard identification and characterization steps of the risk assessment process. The EU Integrated Project ReProTect (6th Framework Programme) implemented an array of *in vitro* tests to study different building blocks of the mammalian reproductive cycle: methodological developments and results on male and female germ cells, prostate and placenta are presented.

Key words: alternative methods, male and female germ cells, prostate, placenta, REACH.

Riassunto *(Strategie innovative per i metodi alternativi in tossicologia riproduttiva: il contributo dei partners italiani nel progetto europeo ReProTect)*. La tossicità riproduttiva, con i suoi bersagli e meccanismi di azione, è una branca complessa della tossicologia: lo screening e l'identificazione di sostanze tossiche per la riproduzione è una delle maggiori sfide scientifiche per la valutazione della sicurezza delle sostanze chimiche, previsto anche dal Regolamento Europeo REACH. Le autorità regolatorie raccomandano l'applicazione del principio delle 3R e lo sviluppo di strategie sperimentali razionali per mettere a punto metodi *in vitro* e l'uso, nell'ambito del processo di valutazione, di un approccio meccanicistico nell'identificazione e caratterizzazione del rischio. Il progetto europeo ReProTect ha sviluppato un insieme di saggi *in vitro* per studiare le diverse fasi del ciclo riproduttivo dei mammiferi: sviluppi metodologici e risultati ottenuti su spermatozoi, ovociti, prostata e placenta sono presentati in questa rassegna.

Parole chiave: metodi alternativi, cellule germinali maschili e femminili, prostata, placenta, REACH.

INTRODUCTION

The ReProTect Project: conceptual framework and outcomes

The identification of reproductive toxicants and their mechanisms of action is a major scientific challenge during safety assessment of chemicals; indeed, reproductive toxicology is one of the most complicated fields of toxicology, due to multiple organs and tissues involved, potentially different modes of toxicant action and dependence on the endocrine system. In particular, endocrine disrupters (EDs) represent a big challenge for experimental toxicology, due to their complex effects on signal networks and programming [1]. Besides the scientific chal-

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lenge, the assessment of the impact of chemicals on the reproductive cycle is also a major ethical and social issue and needs to be considered since it deals with life stages that are potentially more susceptible and are pivotal for the protection of new generations (see also the new paradigm of "sustainable food safety") [2].

For the determination of reproductive toxicity, only *in vivo* studies are currently accepted by the regulatory authorities. Indeed, there is a major concern that reproductive toxicity testing will significantly increase due to the requirements of the new EU regulatory framework for the Registration, Evaluation and Authorization of Chemicals (REACH) [3]. Several thousands of existing and new chemicals are expected to require testing under new and more stringent safety provisions; in turn, this will require using a high number of experimental animals in time-consuming and expensive tests, leading to a slow assessment and management of many, potentially noxious chemicals. A number of useful and promising *in vitro* models are already available but they need to be converted into tests with a predictive power for toxicological safety testing [4]. Then, it is not just a matter of making available efficient test methods by developing or validating *in vitro* assays but it is necessary to develop integrated and/or intelligent testing strategies that are advocated within REACH [5] as well as in other risk assessment frameworks. For instance, the European Food Safety Authority (EFSA) points out reproductive toxicity studies as one area where the development of alternative methods is more difficult; whereas a number of *in vitro* methods are under development and some are undergoing validation, at present they cannot yet provide the information that can be derived from currently used *in vivo* methods. In recent years several bodies (*e.g.,* the Scientific Committee on Health and Environmental Risks, the European Food Safety Authority, the International Forum Towards Evidence-Based Toxicology) recommended that risk assessment should more and more rely on the capacity to integrate *in vitro* assays in a testing strategy covering a number of critical targets as well as exploiting the new molecular biology tools [6-8]. Therefore, besides bringing about a decrease in the number of animal tests, a new testing strategy for reproductive toxicology should provide more detailed information on mechanisms of toxicity in the different target tissues, leading to a new, more science-based risk assessment paradigm. Indeed, mechanistic toxicology evolves by relying, to a large extent, on methodologies that substitute or complement traditional animal tests. To date, the impressive developments of biotechnology and informatics of the last decades have been embraced by regulatory toxicology in a possibly too slow and cautious way; however, several agencies have initiated a debate about how to create a novel, evidence-based toxicological paradigm. Such paradigm would fully exploit information on identified toxicity pathways by making the best possible use of human cell cultures, system biology and *in silico* modelling [9].

The EU Integrated Project ReProTect ("Development of a novel approach in hazard and risk assessment of reproductive toxicity by combination and application of *in vitro*, tissue and sensor technologies", 6th Framework Programme) has represented the first and most relevant response of the European research system to the many questions posed by the new developments of reproductive toxicology. ReProTect was committed to provide an array of *in vitro* tests for the investigation of the different aspects and phases of the reproductive cycle as well as to contribute to the development of intelligent testing strategies for the compilation of reliable and valid safety information [10]. Thus, the short-term project achievement has been the development and optimization of a broad array of already available *in vitro* tests in order to make them amenable to subsequent formal validation studies. Indeed, within ReProTect all *in vitro* tests have been optimised following the procedure of the modular approach for test development [11] recommended by the European Centre for the Validation of Alternative Methods (ECVAM): this approach is based on a stepwise accomplishment starting with the definition of the test (mechanistic basis, standard operation procedure, identification of relevant toxicological endpoints). The performance of the assays is then assessed on a panel of recognized reproductive toxicants, in order to obtain dose-response curves of the selected endpoints and to demonstrate their repeatability. To date, the ensuing step (transferability of the test to another laboratory to determine the reproducibility of the results) has been completed for one test (see below "Effects of reproductive toxicants on the processes of oocyte *in vitro* maturation and fertilization in a bovine model") [12].

In addition, the ReProTect project has been much more ambitious, pivoting on two innovative concepts [10]:

a) to create a battery of test for reproductive toxicity targeting the building blocks of the reproductive cycle;
b) to integrate different approaches based on target cells/tissues and on mechanisms/pathways.

Overall, the complexity of organs and events involved in the reproductive cycle simply prevents to find "the" alternative test for reproductive toxicology [13]. However, the reproductive cycle can be broken up in building blocks (components and/or pathways), so to set up a comprehensive battery of tests, each of them addressing one component. This led to a project structure with three major research areas dealing with cell/tissue specific approaches and with the ambition of providing an array of test targeting the essential steps of reproductive cycle: fertility; implantation; prenatal development. Whereas the developmental toxicity tests were closer to optimization phase, the test batteries dealing with male and female fertility and implantation were more complex to develop en-

compassing a number of cells/tissues (sperm, Leydig cells, oocytes, ovary granulosa cells, trophoblasts, etc.) and of targeted parameters (DNA integrity, steroidogenesis, etc.).

Noticeably, during ReProTect some new targets were identified; one main example is prostate, a key functional gland for male fertility somehow overlooked in toxicity testing [14]. A fourth research area was represented by cross-cutting technologies, *i.e.,* those approaches focussed primarily on toxicant modes of action and potentially relevant to many or all components of the reproductive cycle; in this area, immediate success was obtained with assays on the transactivation of human estrogen and androgen receptors [15, 16] as well as with the use of structure-activity relationship modelling to improve predictivity of toxicology data sets [17].

During the final year of the ReProTect project, a ring trial, named "Feasibility Study", was conducted, in which blinded chemicals with toxicologically well-documented profiles were tested by employing a battery of 14 *in vitro* assays developed within the project itself [18]. This comparative analysis together with a weight of evidence approach allowed a robust prediction of adverse effects on fertility and embryonic development caused by the 10 test chemicals *in vivo*: the vast majority of the predictions made on the basis of the *in vitro* results turned out to be correct when compared to the whole animal data [18]. The core feasibility study was flanked and supported by satellite studies, such as those conducted with the newly developed PSA secretion assay to screen substances targeting the prostate [14]. At the end of project, the ReProTect test battery holds promise for use as a screening approach for reproductive toxicity testing according to the EU REACH legislation.

Overall, ReProTect has involved 32 European partners from academia, industry and governmental institutes, with a major participation of Italian partners: ENEA, (Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Rome, Italy), Istituto Sperimentale Italiano Lazzaro Spallanzani (Rivolta d'Adda, Cremona, Italy), Istituto Superiore di Sanità (The Italian National Institute of Health, Rome, Italy, led by A. Mantovani, coordinator of the WP IV on cross-cutting technologies), Avantea (Cremona, led by G. Lazzari, coordinator of the WP I on fertility); University of Siena and University of Milano were involved contributing with innovative approaches covering main components of the reproductive system, namely the male and female gametes (WorkPackage/WP I, Fertility), the prostate (WP IV, Cross cutting technologies) and the placenta (WP II, Implantation). Functional as well as molecular parameters were exploited both as potential predictors of *in vivo* effects and as building blocks in a modular testing battery [10, 19]. The outcomes of specific activities and their relevance for a new testing strategy on reproductive toxicity are discussed in the ensuing paragraphs.

TESTS ON MATURE SPERMATOZOA

Methods used to evaluate reproductive toxicity, including male-mediated developmental toxicity, require a large number of animals and validated *in vitro* alternatives are not yet available [20]. The strategy pursued by the ReProTect project was to break down the mammalian reproductive cycle into several *in vitro* workable tiers. In this context, within the framework of the fertilization tier, the tests proposed and described below focus on two crucial features of the mammalian sperm, which are acquired extratesticularly during its transit through the epididymus. Actually, it is during the epididymal maturation that sperm gain the capacity for progressive motility and the newly reorganized sperm nuclear DNA around the protamine core reaches its zenith of compactation when the inter- and intra-protamine disulfide bridges between cysteine residues are formed to provide a highly rigid structure. It is speculated that the generation of a more protected genome (sperm has practically no DNA repair system) and of a more hydrodynamic sperm head can speed transit through the female reproductive tract. Therefore, both processes, namely nucleus remodeling and acquisition of motility, seem essential to fulfill the primary goals of the male gamete genome, that is a successful fecundation and a fully sustained pregnancy. Consequently, it is reasonable to assume that any impairment to these concurrent, apparently non correlated, differentiation pathways should hamper the proper expression of the sperm reproductive capability.

Direct assays on spermatozoa, which represent the functional end-product of the whole intra-testicular spermatogenesis process and its maturation during epididymal transit, have the main advantage of being able to detect effects on the terminally-differentiated male gametes ready to undergo fertilization. For participating in fertilization spermatozoa must be alive and motile, able to undergo acrosome reaction and deliver an intact male genome into the oocyte to create an embryo with a high chance of completing a full-term healthy pregnancy. Any damage to these properties (motility, plasma membrane integrity, genetic integrity) could hamper its reproductive capacities before or after the fertilization process. Notably, both motility and genetic integrity have been demonstrated to be predictors of human fertility in the general population [21, 22].

Ideally, an *in vitro* sperm assay should rely on a readily accessible source of sperm obtainable from animals without invasive procedures. Furthemore, in order to ensure the repeatability of the test and the possibility of easy inter-laboratory exchange an *in vitro* sperm assay should involve sperm which are homogeneous, constant over time and disposed to be stored for long period storage at low temperature without losing their functional/structural properties. The use of bovine sperm, easily collected in sufficiently large amount, matches many of these ideals [20] and has been selected in the ReProTect trials. The use of frozen semen represents an innovative approach of great interest for testing the toxic effect of chemicals on fertility, because

it does not involve invasive procedures which compromise animal welfare. Frozen bovine semen is readily accessible and represents a material that can be stored for long periods in liquid nitrogen, without losing its biological properties, in order to ensure the repeatability of the tests and to afford the possibility of exchange among different laboratories.

Mammalian sperm motility: a tool for in vitro *evaluation of chemical toxicity*

One of the most important laboratory tests to evaluate sperm quality has historically been the visual estimation of sperm motility. The introduction of a computerized measurement by image analysers improved the accuracy of the method with the advantage of eliminating the human factor and led to a more objective analysis. Within the ReProTect project sperm motility parameters measured on mature bovine sperm were used to determine the toxicity of chemical compounds [23]. Sperm motility has been chosen as endpoint, since this parameter is considered to be a sensitive marker for epididymal toxicants [21]. Straws of 500 µl containing about 15 x 10^6 spermatozoa were thawed and subsequently incubated for one hour with different test compounds. After incubation, Total Motility/TM (%), Progressive Motility/PM (%) and Average Path Velocity/VAP (µm/sec) were measured by a Computer Assisted Semen Analysis system (HTM-IVOS, Hamilton Thorne) [24].

This approach was tested on 36 compounds, some of which were recognized or suspected as reproductive toxicants with different mechanisms of action while others were known to have a toxic effect on cells. Each compound was dissolved in an appropriate vehicle depending on its physical and chemical properties. The concentration of vehicle was determined in a preliminary test (0.2% for Ethanol and to 1% for Acetone, Demi water and DMSO). The compounds were the following: Acetone (vehicle), Antimycin A, Benzalkonium chloride, Benzene, Butyl-4-hydroxybenzoate, Cadmium chloride, 3-Chloro-1,2,-propanediol, Colchicine, Cycloheximide, Demi water (vehicle), 1,2-Dibromo-3-chloropropane, 2,4-Dichlorophenol, 2,4-Dinitrophenol, Diethylstilbestrol, Diethyldithiocarbamate, DMSO (vehicle), Epichlorohydrin, Ethanol (vehicle), 5-Fluoracil, Hexachlorophene, Hexachlorocyclohexane, 4-Monochlorophenol, Methyl mercury (II) chloride, Mifepristone, Nitrobenzene, 4-Octylphenol, Ouabain, Pentachlorophenol, Salicylic acid, Sodium azide, Sodium cholate hydrate, Sodium fluoride, 2,3,4,5-Tetrachlorophenol, 2,4,5-Trichlorophenol, Triton X-100 (positive control) and Zinc chloride.

The testing of chemicals was developed in two sequential steps: the Dose-Range Finding study (DRF) and the main study. DRF studies were performed in duplicate for all the compounds at final chemical concentration of 10, 100 and 1000 µg/ml and an IC_{50} (concentration of chemical at which the motility of the sperm cells is 50% lower than the motility of the control sperm cells [= control with vehicle] after an incubation time of 60 minutes) was calculated for the endpoint TM (percentage of spermatozoa with VAP > 25 µm/sec). The Main study was performed only on 21 compounds, in which an IC_{50} value was reached in the DRF. A number of 7 concentrations were chosen in a narrowed range around the IC_{50} value with a factor of 1.5. The Main study was tested in triplicate and an IC50 was calculated for TM.

A negative (sperm and vehicle) and positive (sperm and 7.5% Triton X-100, the concentration inducing ~ 75% inhibition of motility) control was always included.

For 6 chemicals chosen on random basis a repetition of the test was performed in order to evaluate the repeatability of the assay and at the same time a viability test [25] was carried out to distinguish between deactivation of sperm motility and cell death due to unspecific toxicity. This test is based on a double-DNA staining with SYBR-14 (stains living sperm nuclei in green) and propidium iodide (stains dead cells nuclei in red) and a flow cytometric analysis. The percentage of live sperm cells (Intact Membrane, IM) was measured and the IC_{50} was calculated.

For data analysis, non-linear curve fitting and IC_{50} estimation were performed using the function multdrc R-package drc [26].

The data were fitted using the four-parametric log-logistic model:

$$f(x) = c + (d - c) \cdot [1 + \exp(b(\log(x) - e))]^{-1}$$

This model does not assume the response to approach 0 at high doses. The minimum response is estimated by non-linear regression. Parameters c and d estimate the lower and upper bounds of the response and are constrained to be ≥ 0. Parameter e estimates the log (IC_{50}).

An overview of all values for TM and IM measured in the main study is given in *Table 1*.

For assessment of intra-laboratory IC_{50} variability (repeatability) data from two experiments performed for 6 of the compounds were fitted with the four-parameter log-logistic model and the estimated IC_{50} ratio with 95% confidence interval was calculated using function SI of R-package drc. If this confidence interval did not include 1, there was evidence of poor IC_{50} repeatability.

All of the confidence intervals included 1 and the range between first and second measurement were considered acceptable for *in vitro* measurements, therefore none of the duplicated experiments indicated poor IC_{50} repeatability.

This study was focused on the usability and repeatability of bovine sperm as *in vitro* model for reproductive toxicity and the results obtained suggest that this system has the potential to serve as an useful *in vitro* screening test.

Total motility was a sensitive marker for toxic effect on sperm and a viability test should always be included to distinguish between deactivation of sperm motility (*e.g.* Ethanol) and cell death due to unspecific toxicity (*e.g.* 1,2-Dibromo-3-chloropropane).

Incubation of bovine sperm with toxic compounds and analysis for motility and viability appeared to be fast and simple. This assay met the criteria for an ide-

Table 1 | *Summary table for endpoint TM and IM. Main columns indicate availability of dose range finding and main study data, respectively. IC_{50} columns show the IC_{50} estimates for each experiment. IC_{50} ratio columns show the estimated ratio of IC_{50} values for a given comparison of experiments [23]. TM: total motility; IM: intact membrane.*

Compound	Main (TM)	IC_{50}	IC_{50}	IC_{50}	IC_{50} ratio	Main (IM)	IC_{50}	IC_{50}
		Exp1	Exp2					
1,2-Dibromo-3-chloropropane	X	2.274	2.291	mM	0.991	X	2.352	mM
2,3,4,5-Tetrachlorophenol	X	0.332		mM				
2,4,5-Trichlorophenol	X	0.742	0.857	mM	0.775	X	3.069	mM
2,4-Dichlorophenol	X	1.190		mM				
2,4-Dinitrophenol	X	0.571		mM				
4-Monochlorophenol	X	2.144	2.610	mM	0.822	X	2.984	mM
4-Octylphenol	X	0.668		mM				
Acetone								
3-Chloro-1,2,-propanediol								
Antimycin-a	X	451.991		µg/ml				
Benzalkonium Chloride	X	379.400		µg/ml				
Benzene								
Butyl-4-hydroxybenzoate	X	0.877		mM				
Cadmium Chloride								
Colchicine								
Demi Water								
Diethyldithiocarbamate								
Diethylstilbestrol	X	0.223		mM				
Dmso								
Epichlorohydrin								
Ethanol	X	221.269	278.471	mM	0.795	X	1205.85	mM
Hexachlorophene	X	0.287		mM				
Hexachlorocyclohexane	X	0.826		mM				
Methylmercury (Ii) Chloride	X	0.068		mM				
Mifepristone								
Nitrobenzene								
Ouabain								
Pentachlorophenol	X	0.085	0.065	mM	1.300	X	0.171	mM
Salicylic Acid	X	5.160		mM				
Sodium Azide	X	0.325		mM				
Sodium Cholate Hydrate	X	0.986		mM		X	0.863	mM
Sodium Fluoride	X	2.770		mM				
Triton X-100	X	0.649	0.471	mM	1.364	X	0.714	mM
Zinc Chloride								
5-Fluoracil								
Cycloheximide								

ally *in vitro* sperm assay: a) obtainable from animals without invasive procedures; b) involve sperm cells which are homogeneous and constant over time.

However, due to the lack of useful *in vivo* data for the effects on sperm motility, a prediction model could not be developed.

Mammalian sperm exposed in vitro *to mutagenic reproductive toxicants*

Sperm genetic integrity is pivotal for the full expression of individual fertility potential, to ensure a flawless transmission of father's heritable complement to the progeny, and to determine the sperm capacity to achieve and sustain a pregnancy. There is a wealth of epidemiological evidence linking paternal exposure to chemical agents with an increased risk of pregnancy loss, developmental and morphological defects, infant mortality, infertility, and genetic diseases in the offspring, including cancer [27]. Furthermore, exposure of rodents to germ cell mutagens can have markedly adverse consequences on fertility and pregnancy outcomes, presumably by altering chromosome number

or structure or through unrepaired DNA damage [28]. The final stages of gamete differentiation in male mammals are sensitive targets of DNA-reactive chemicals: once germ cells, deprived of the cytoplasm that houses protective enzymes, including those implicated in DNA repair, are released from germinal tissue they can no longer rely on the protection of Sertoli cells and are vulnerable to DNA damage by a variety of xenobiotics. In the mature male gamete, only the extremely condensed chromatin remains to represent a protective factor shielding the genetic material from exogenous assaults.

Several methods have been developed to detect genetically defective sperm. Assays such as Fluorescence In Situ Hybridization (FISH), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), Sperm Chromatin Structure Assay (SCSA) and Comet assay have been improved and applied in different fields such as diagnosis of male infertility, occupational, pharmacological, epidemiological, and toxicological studies [29]. Nevertheless, their application in *in vitro* detection of male germ cell mutagens is rather scanty and the studies addressing this issue and published so far are listed in *Table 2*. The majority of studies were conducted, prevalently applying the Comet assay, in a few laboratories and positive results were mostly imputed to oxidative DNA lesions. Comet assay is a genotoxicity testing method widely applied, both *in vivo* and *in vitro*, to cells from different organs and tissues. This method can be applied to non-proliferating cells and for this reason it is one of the few cytogenetic assays suitable to detect DNA damage in mature spermatozoa [30]. The test detects DNA single and/or double strand breaks by a differential electrophoretic migration of broken versus unbroken DNA of single cells [31]. Most chemicals affect DNA by covalent modifications, which do not straightforwardly lead to nick production. In somatic cells, DNA adducts can be uncovered by Comet assay through breaks formed during repair processes. Sperm, however, are essentially devoid of repair enzymes and, consequently, it is unlikely that the Comet assay (and the other DNA fragmentation assays as well) can detect DNA lesions induced in sperm by most chemicals. To overcome such a limitation, we proposed a modification of the Comet assay based on the addition of a crude protein extract from somatic cells [32, 33], under the rationale that repair enzymes contained in the extract could ignite the incision process and produce DNA-strand breaks. Purified repair enzymes can be included in the Comet assay and are used to detect specific DNA lesions such as oxidized or alkylated bases [31, 34]. However, the specificity for a particular kind of lesion makes these enzymes unfit for the screening of compounds with unknown mechanisms of action. The capacity of a crude cellular extract to recognize and cut chemically modified DNA of agarose-embedded nuclei had been previously demonstrated and applied to evaluate inter-individual differences in DNA repair efficiency [35]. Our approach represents an original attempt to supplement sperm repair enzymes deficiency in the assessment of chemically induced DNA lesions.

This novel approach, dubbed Repair Proficient Comet [ReProComet] assay, was tested with six chemical compounds known to have a genotoxic potential on male germ cells but with different mechanisms of action. The selected chemicals were: methyl methane sulfonate, a monofunctional direct alkylating agent known to induce dominant lethal mutation; melphalan, a mutagenic bifunctional direct alkylating agent, known to induce dominant lethal mutations and heritable translocations; mitomycin C, a mutagenic bi/trifunctional alkylating agent known to induce dominant lethal mutations; diethylsulfate, a mutagenic monofunctional direct alkylating agent known to be active in the late stages of spermatogenesis; bleomycin, a radiomimetic compound, inducing DNA breaks and abasic sites through the production of free radicals; and colchicine, an antimitotic compound, known to disrupt microtubules by binding to tubulin and preventing its polymerization. Due to its mechanism of action this compound was used as negative control. For all these compounds both the standard Comet and the ReProComet assay were applied and results were reported in two papers [32, 33].

Summarizing, results showed, as expected, that the mitotic poison colchicine did not result positive even by the modified assay. Dose-effect relationships were obtained with ReProComet after treatment with methyl methane sulfonate, diethylsulfate and melphalan, whereas no increase of the Comet assay parameters was observed using the standard Comet assay even at the highest concentrations tested. Results obtained with standard Comet assay for mitomycin C did not show significant increases of parameters at any of the tested doses. However, at the lowest doses, Comet assay parameters were statistically decreased with respect to matched controls. This observation is compatible with a mitomycin C-based mechanism of action including the formation of cross-links which could reduce electrophoretic DNA migration. By ReProComet assay no differences were detected between control and mitomycin C-treated samples. Bleomycin tested by standard Comet assay was always negative. After ReProComet, at all the concentrations but the highest, a significant, but not dose-dependent, increase of parameters was detected with respect to matched controls.

The results described represented a proof of concept that cellular extracts added to spermatozoa make ReProComet a more sensitive assay able to detect chemically induced sperm DNA modifications. However, the test is still in a preliminary step of development and further efforts are required in the validation process before its possible application as a pre-screening approach to identify germ cell genotoxins.

IN VITRO SCREENING OF REPRODUCTIVE TOXICANTS WITH A PROSTATE-MEDIATED EFFECT

Prostate, an overlooked target in *in vitro* alternative methods, is critical for male fertility. Within ReProTect,

Table 2 | *List of compounds tested in vitro on mammalian sperm and assessed by sperm DNA/chromatin damage tests*

Sperm species	Compounds tested	Assay		References
Human	3-amino-1-methyl-5H-pyrido (4,3-b)indole	Comet	+	[36, 39]
Human	2-amino-3-methylimidazo-4,5-f)quinoline	Comet	+	[36, 39]
Human	benzo[a]pyrene (with/without s9)	Comet	+	[49]
Human	benzo[a]pyrene-7,8-diol 9,10-epoxide	Comet	+	[49]
Human	bisphenol A	Comet, TUNEL	-	[43]
Human	chlorpyrifos	SCSA	+	[48]
Human	chlorpyrifos-oxon	SCSA	+	[48]
Human	daidzein	Comet	+	[37, 38, 40]
Human	diazinon	SCSA	+	[48]
Human	diazoxon	SCSA	+	[48]
Human	dibromochloropropane	Comet	+	[37, 38]
Human	1,2,3,4-diepoxybutane	Comet	+	[37]
Human	Diethylstilbestrol	Comet Comet, TUNEL	+-	[37, 40, 43]
Bovine	DNAse I	Comet, SCSA, TUNEL	+	[50]
Human	DNAse I	Comet, SCSA, TUNEL	+	[50]
Murine	DNAse I	Comet, SCSA, TUNEL	+	[50]
Human	1,2-epoxybutene	Comet	+	[37, 38]
Human	Equol	Comet	+	[40]
Human	β-estradiol	Comet Comet, TUNEL	+-	[37, 38, 40, 43]
Human	ethylene glycol monoethyl ether	Comet	+	[37]
Human	genestein	Comet Comet, TUNEL	+-	[37, 40, 43]
Human	Hydrogen peroxide	Comet	+	[40, 42, 45, 50]
Human	Hydrogen peroxide	SCSA	+	[51]
Murine	Hydrogen peroxide	Comet	+	[42]
Tammar Wallaby	Hydrogen peroxide	Comet	+	[42]
Human	2-hydroxyestradiol	TUNEL	+	[43]
Human	4-hydroxyestradiol	TUNEL	-	[43]
Human	kaempferol	Comet	+	[36, 39]
Human	lead acetate	Comet	+	[37]
Human	lead nitrate	Comet	+	[37]
Human	lead sulfate	Comet	+	[37]
Bovine	Mercury chloride	Comet	+	[41]
Human	methyl-parathion	SCSA	+	[48]
Human	methyl-paraoxon	SCSA	+	[48]
Human	myricetin	Comet	+	[36, 39]
Human	Nonylphenyl	Comet Comet, TUNEL	+-	[37, 40, 43]
Human	peritoneal fluid from healthy women vs.from women with endometriosis	TUNEL	-+	[47]
Human	Quercetin	Comet	+-	[36, 39, 43]
Human	Silymarin	Comet	+	[36, 39]
Murine	Ionizing radiation	Comet	+	[46]
Human	Ionizing radiation	Comet	+	[46]
Bovine	Ionizing radiation	TUNEL	+	[44]

a cellular model system was used to screen chemicals affecting prostate by a tiered approach integrating two toxicological endpoints: cell viability and PSA secretion. In particular, in WP(IV), dedicated also to toxicogenomics, a cell-based assay was employed to provide a phenotypic anchoring to gene expression profiling data [52]: since human prostate cell lines have been used as cellular model to investigate androgen receptor (AR)-dependent signaling, the selected cell-based, cell-specific, clinically used assay has been the prostate-spe-

cific antigen (PSA) secretion assay. Besides to be a supportive tool for the toxicogenomic approach *(Table 3)*, the PSA secretion assay has been implemented as an independent tool to investigate prostate-mediated effects on male reproduction [53].

Regarding male reproductive functions, spermatogenesis, semen quality of the ejaculate and function of specific testicular cell types (*i.e.* Leydig and Sertoli cells) represent the main toxicological endpoints [21, 54]. However, the main male accessory sex gland, prostate, has received so far limited attention as a target in reproductive toxicity assays: indeed, it is essential for male fertility since it secretes the prostatic fluid (constituting ~ 30% of the whole ejaculate). Sperm functional competence is depending on prostatic fluid that provides proteins (*e.g.* PSA), trace elements (*e.g.* zinc) and other molecules (*e.g.* citrate) essential to sperm cell activation and capacitation [55-57]. PSA has a central role in semen liquefaction and an increased PSA secretion is an established prostate cancer/PCa biomarker [58, 59]. Noteworthy, from a toxicological point of view human-derived cell lines may be considered more representative for hazard assessment since rodent prostate is not physiologically overlapping to humans due to differences in the ejaculation process as evidenced by the lack of KLK2 and PSA/KLK3, regulating human liquefaction of the clot in the prostatic fluid [60]. Androgens strongly regulate PSA production and secretion within the prostatic fluid [61]: under pathological conditions (*i.e.* PCa, benign prostatic hyperplasia/BPH), androgens loose their ability to regulate the AR-mediated signaling pathway leading to a constitutive activation of AR-signaling target genes (*e.g.* PSA), by other proliferative signals [58, 62]; PSA secretion becomes also partially mistargeted and reaches the blood flow rather than being fully addressed to the prostatic ducts [56, 58]. Most PSA in human serum is complexed to proteins, although a significant fraction is free: the role of free PSA is not known but its concentration is apparently higher in BPH than in PCa [63]. Thus, ratio of free to total PSA might be detecting factors that predispose or promote malignant transformation in prostate cells. PSA secretion is maintained by a few established human cell lines, among them LNCaP that, although of tumor origin, has features of normal prostate epithelial cells, such as AR expression, androgen sensitivity and PSA-secretory capability [64, 65 and refs therein].

EDs with (anti)androgenic activity may exert their regulatory action on the AR by direct binding to AR or acting on its regulated signaling pathway. Thus, a novel *in vitro* approach was implemented to discriminate ED-like chemicals: the proposed *in vitro* alternative method takes advantage from the availability of commercial human prostate cell lines where both a general toxicity assay (cell viability and indirect proliferation assay) and a cell-specific functional effect (PSA secretion assay) can be used as toxicological endpoints [52, 53].

Since PSA blood levels is a recognized PCa biomarker [57, 59], we implemented it as an *in vitro* toxicological biomarker in human prostate cell lines to test and compare the role of both a well-known (anti)androgen set of EDs (ReProTect training set) and a ReProTect feasibilty set of blinded chemicals whose androgen-like activity had to be established [53]. The rationale for selection criteria have been described elsewhere: briefly, the ReProTect training set of chemicals has been selected on the basis of already known effects in each selected model system (in the case of prostate-mediated effects, see [53]), whereas the ReProTect feasibility set has been selected by an independent expert group [18] following as main selection criteria the availability of *"high quality information on the reproductive toxicity of the test agents in the laboratory animal…, as well as on their mode of action… Due to serious time constraints it was decided that the majority of the agents should*

Table 3 | *Overview of the toxicogenomic approach showing the dual application of the same cell-based bioassay (PSA secretion assay): from phenotypic anchoring to a novel supportive alternative* in vitro *method*

Steps of the toxicogenomic approach	Steps of the toxicogenomic approach	AIMS
Cell manipulation and treatment	Manipulation of prostate cell lines (plating, starvation and treatments) for: - cytotoxicity (MTS) assay; - microarray experiments	For each tested compound, selection of NOAEC and/or LOAEC to be used in microarray experiments
Toxicogenomics core	Microarray experiments and i*n silico* data analysis: - RNA extraction and purification from harvested treated-cells; - Preparation of dye-labeled samples and chip hybridization; - Data collection and analysis; - Validation of microarray results by real time RT-PCR (qPCR)	Comparison of gene expression profiling; Identification of genes differentially modulated by AR-(anti)agonists and/or AR status
Cell-based bioassay	PSA secretion assay	Phenotypic anchoring in toxicogenomics;novel supportive tool as alternative *in vitro* method in reproductive toxicology

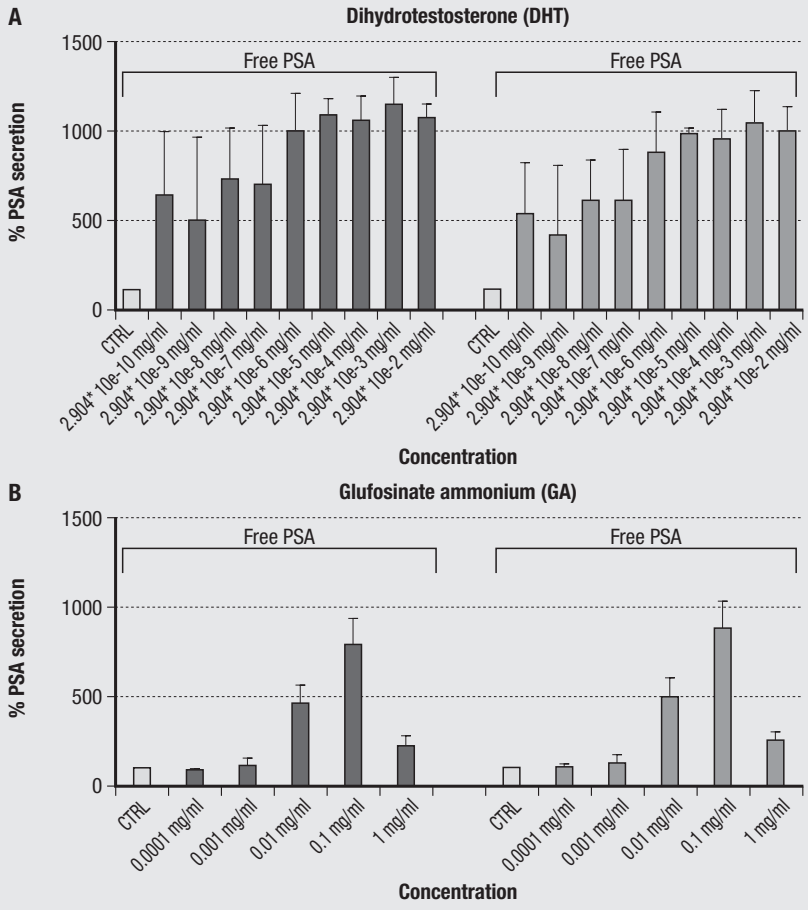

Fig. 1 | *Examples of dose-response curves of free and total PSA secretion obtained with known (A, dihydrotestosteone/DHT) or unknown (B, glufosinate ammonium/GA) androgen-like chemicals [48].*

be selected from the panel of ~ 150 ReProTect agents, for which a detailed toxicological database was already existing at that time". Specifically, the cell line LNCaP was used as a model system of prostate epithelial cell line, in which the analysis of cell viability and indirect proliferation (MTS assay) and the changes in free and total PSA secretion (DELFIA assay) were used to evaluate, respectively, general and cell-specific toxicity of EAS [53 and refs therein]. Indeed, LNCaP expresses a point mutated AR (ART^{877A}) that alters the ligand binding specificity [66]. Despite this, LNCaP represent the best known commercial cell line secreting high levels of PSA.

The proposed tiered approach integrating the two above mentioned endpoints allows to investigate chemicals affecting prostate epithelium functionality by the contemporary measurement of both toxicological endpoints in the same cell cultures distinguishing changes due to a direct effect on PSA secretion or due to a secondary effect subsequent to cytotoxicity. Since androgens tightly regulate PSA secretion, it may be used in reproductive toxicology to detect EDs with a regulatory role on androgen-regulated pathways, which in turn affect PSA secretion itself. To evaluate the feasibility and applicability of the novel approach, as well to obtain a set of reference profiles of both free and total PSA secretion [53], both assays were firstly applied on the ReProTect training set (5α-dihydro testosterone/DHT, 17α-methyl testosterone/MT, 2-hydroxy-flutamide/2-OH-FTA, linuron/LIN and di-n-butyl phthalate/DBP) and afterwords used to screen the ReProTect feasibility set (methylacetoacetate, glufosinate ammonium/GA, methoxyacetic acid/MAA, sodium chloride, cadmium chloride hemipentahydrate, carbendazim, nitrofen, nitrobenzene, vinclozolin/VIN, bisphenol A/BPA) [53]. As shown in *Figure 1*, adapted from [53], the AR-agonist DHT *(Figure 1A)* increased both free and total PSA secretion, whereas – within the ReProTect feasibility (double-blinded) set – glufosinate ammonium/GA *(Figure 1B)* [67] unexpectedly resulted to induce both free and total PSA secretion at 0.1 mg/ml, suggesting a weak androgen-like properties of GA by comparison to DHT [48]: indeed, at the higher concentration (1.0 mg/ml) tested GA shown a clear toxic effect *(Figure 1B)*. The levels of PSA secretion induced by GA at 0.01 and 0.1 mg/ml is exactly overlapping the levels of PSA secretion induced by physiologically relevant concentration (from 2.9 x 10e-10 e 2.9 x 10e-7 mg/ml) of the endogenous androgen DHT: hence, a differential affinity of the GA and DHT ligands toward the mutated ART^{877A} could be clearly envisaged. The effect on

PSA secretion after GA treatment in LNCaP was clearly detectable also after normalization for cell viability/indirect proliferation confirming its target cell-specific effect. Interestingly, the PSA secretion profiles of recognized anti-androgens, as the environmental contaminants LIN and DBP [53] – a herbicide and a plasticizer, respectively – overlapped with a blinded chemical that, upon un-blinding [18] resulted to be another environmental contaminant, the fungicide VIN, evidencing the feasibility of the PSA secretion assay in correctly determining the AR-mediated role of the tested chemicals (data not shown) [53]. Furthermore, another chemical within the *ReProTect feasibility set* was recognized as a specific modulator of PSA secretion: the plasticizer BPA, an estrogen-like compound, showed a significant decrease in PSA secretion although cell viability increased [53]. The PSA secretion profile of BPA also overlapped to those ones of the known anti-androgens LIN and DBP, although it was previously shown that BPA cannot recognize the wild-type form of the AR [66]. Hence, we hypothesized that BPA shows its anti-androgenic properties only in presence of a mutated AR. On the contrary, 7 out of 10 chemicals within the ReProTect feasibility set [53] behaved similarly to one of them, MAA: their free and total PSA secretion profiles changed on the basis of cell viability and indirect proliferation changes, evidencing that their role in decreasing PSA secretion was directly proportional to the number of viable cells (data not shown) [53]. Thus, the selected cell-specific bioassay, PSA secretion, already established in the clinical field as a biomarker, has been also shown to be applicable in *in vitro* toxicology and – without detecting any false positive or negative – may represent a valuable toxicological biomarker for the screening of EDs that may interfere with prostate-mediated male fertility.

Overall, the proposed tiered approach – used as a means to screen androgen like-chemicals with a prostate-mediated effect – constitutes a reliable and feasible integrated *in vitro* toxicological assessment to detect chemicals affecting the male reproductive system at a commonly overlooked target. In particular, our integrated approach [53] to the prostate-mediated male reproductive toxicity allowed to: i) individuate a putative endocrine disrupter (GA), whose role as AR-interfering chemical has yet to be characterized; and ii) by comparison to LIN's and DBP's PSA secretion profiles, detect both a blinded anti-androgen as VIN and a blinded estrogen-mimicking chemical as BPA. Finally, to implement the described *in vitro* approach on prostate-mediated toxicity, molecular assays have to be included: a) to assess if the ED of interest is an AR ligand, an AR binding assay [16] has to be performed to define the mechanism of action as receptor-mediated or not; and b) to completely characterize the mechanism of action, the modulation of gene expression of AR and of its direct molecular target PSA has to be proven as well.

EFFECTS OF REPRODUCTIVE TOXICANTS ON THE PROCESSES OF OOCYTE *IN VITRO* MATURATION AND FERTILIZATION IN A BOVINE MODEL

The reproductive cycle is an integrated physiological process that encompasses a number of complex steps involving the development of female and male gametes, with their supportive somatic cells, and the long process of embryo development bringing to the establishment of pregnancy and ending up with the birth of the offspring. In this context, the oocyte represents the repository of transcripts and energy substrates that allow the initiation of embryo development with the formation of female and male pronuclei after fertilization, followed by the first embryonic cleavages. It is known that a variety of processes occurring during oocyte maturation and fertilization can be blocked or impaired by chemicals/drugs that can act with different mechanisms of action resulting in infertile cycle or gestation losses. Among them substances as Nocodazole or Carbendazim interfer with spindle dynamics causing aneuploidy [68, 72], others, as Cycloheximide and Genistein affect protein synthesis [73, 74] or phosphorylation [69], or as Ketoconazole inhibit steroidogenesis and oocyte maturation [71, 75]. General toxicants such as cadmium chloride also have very detrimental effects on the oocyte causing DNA and oxidative damage [76].

Therefore *in vitro* tests capable of revealing specific adverse effects on the oocytes are an important component of an alternative testing strategy for reproductive toxicity. For this reason within the ReProTect project two tests have been developed: the *in vitro* bovine oocyte maturation test (IVM test) and the *in vitro* bovine oocyte fertilization test (IVF test) [12, 18, 70].

The IVM test detects adverse effects on the oocytes following exposure to testing substances during the maturation process. Successful achievement of the maturation stage (completion of meiosis up to metaphase II) is the toxicological endpoint of this test. The IVF test reveals adverse effects following chemical exposure during oocyte fertilization. Sperm penetration into the oocytes and formation of the two pronuclei, female and male, are the toxicological endpoints.

The choice of the bovine model has been suggested by the fact that oocyte *in vitro* maturation and fertilization procedures are routinely applied for assisted reproduction purposes in animal breeding with high degree of success. Several thousands calves are born each year worldwide following the uterine transfer of *in vitro* produced embryos (see the Proceedings of the European Embryo Transfer Association at (www.aete.eu) for the statistics of bovine embryo transfers in Europe). These results indicate that bovine oocyte *in vitro* maturation and fertilization closely mimic the *in vivo* processes giving rise to viable embryos and offspring. Moreover, these tests represent promising methods to avoid additional terminations of animals

since bovine oocytes can be easily collected from abattoir ovaries of animals destined to enter the food chain.

Both tests have been optimised following the procedure of the modular approach for test development described above [11]; the IVM test is currently the one test that has accomplished module 3 (inter-laboratory reproducibility) [12].

The procedures adopted for setting up the bovine oocytes IVM and IVF tests have been previously described [12, 18, 70]. The chemicals tested were Benzo[a]pyrene (binds to DNA forming adducts), Busulfan (alkylating agent), Butylparaben (weakly estrogenic), Cadmium Chloride (general toxicant), Carbendazim (spindle poison), Cycloheximide (protein synthesis inhibitor), Diethylstilbestrol (non steroid estrogen), Genistein (phytoestrogen, protein phosphorylation inhibitor), Ionomycin (calcium ionophore), Ketoconazole (steroidogenesis inhibitor), Lindane (inhibition of gap junction communication), Methylacetoacetate (solvent), Mifepristone (antiprogestogen), Nocodazole (spindle poison) and DMSO as solvent. The chemicals and the corresponding EC_{50} values obtained in both tests are listed in *Table 4*. The range of EC_{50} values covers several orders of magnitude and allows to discriminate highly effective/toxic chemicals from lesser effective/toxic ones. For example the chemicals Busulfan and Methylacetoacetate which, according to the literature, should not affect oocyte maturation and fertilization, were negative in both tests up to the highest tested dose of 1 mM. In contrast, other chemicals, which are known to affect both IVM and IVF (Ionomycin, Nocodazole, Carbendazim and Mifepristone/RU-486) have an EC_{50}, in the 10 μM order or lower. Cycloheximide, Genistein and Lindane inhibit oocyte maturation, with an EC_{50} between 0.4 and 40 μM, by different mechanisms that all interfere with meiosis resumption. The same chemicals/mechanisms are not relevant for oocyte fertilization and, in fact, no detrimental effects have been observed in the IVF test. This comparison of the results of IVM and IVF tests with the current knowledge about the tested chemicals, underlines their potential of becoming a valuable tool in the *in vitro* assessment of chemical effects on these reproductive processes. Moreover, the successful transferability of the IVM test, shown by the high inter-laboratory reproducibility, demonstrates the robustness of this test and its suitability to enter the official process of test validation by ECVAM. Further endorsement for both the IVM and IVF test comes from a recently published study in which chemicals were tested blindly and the data obtained were in full agreement with expected results [18].

Finally, since it is not possible to model the whole of the reproductive cycle *in vitro* with one approach, each segment of the system needs to be studied individually and then integrated into a testing strategy. This will allow the development and the validation of a battery of alternative tests that can cover the various steps of the reproductive cycle. Therefore, the bovine maturation and fertilization tests, in combination with additional *in vitro* tests, could become part of an integrated testing strategy in order to predict chemical hazards on mammalian fertility.

Table 4 | In vitro *bovine oocyte maturation (IVM) and fertilization (IVF) tests: results of the 14 chemicals tested by the developer laboratory (Avantea) in both the IVM and IVF test and results of the 8 chemicals selected for the IVM transferability study in the second laboratory (UNIMI)*

	***In vitro* maturation test**							***In vitro* fertilization test**	
	Concentration tested (mM)		**EC_{50} (mM)**			**Cytotoxicity (both test)**		**Concentration tested (mM)**	**EC_{50} (mM)**
Chemicals	**UNIMI**	**AVANTEA**	**UNIMI**	**AVANTEA**	**Both Labs**	**UNIMI**	**AVANTEA**	**UNIMI**	**AVANTEA**
Butylparaben	nd	50-150	nd	91.86 ± 4.74	nd	nd	+	10-500	364.50 ± 14.68
Carbendazim	nd	4-70	nd	13.99 ± 5.78	nd	nd	-	1-85	15.33 ± 5.94
Genistein	nd	10-80	nd	40.27 ± 5.05	nd	nd	-	80-250	> 250[a]
Ionomycin	nd	0.5-5	nd	1.84 ± 0.14	nd	nd	-	0.04-0.25	0.097±0.05
Lindane	nd	10-35	nd	33.94 ± 1.85	nd	nd	-	1-110	> 110[a]
Nocodazole	nd	0.01-0.1	nd	0.03 ± 0.01	nd	nd	-	0.01-30	0.17 ± 0.19
Benzo[a]pirene*	1-20	1-20	> 20[a]	> 20[a]	> 20[a]	-	-	0.1-10	>10[3]
Busulfan*	10-1000	40-1000	>1000	> 1000	>1000	-	-	40-1000	>1000
Cadmium Chloride*	1-5	1-5	3.07 ± 0.24	2.55 ± 0.18	2.84 ± 0.34	+	+	10-500	103.97 ± 17.97
Cycloheximide*	0.22-0.89	0.22-0.89	0.38 ± 0.05	0.40 ± 0.02	0.39 ± 0.04	-	-	0.1-1000	> 1000
DES*	2-20	2-20	5.04 ± 0.86	4.43 ± 0.64	4.69 ± 0.75	-	-	10-105	74.14 ± 6.55
Ketoconazole*	5-45	10-50	19.6 ± 9.12	26.4 ± 2.1	23 ± 7.01	-	-	20-50	29.33 ± 2.44
Methylacetoacetate*	10-1000	10-1000	>1000	>1000	> 1000	-	-	10-1000	> 1000
Mifepristone/RU-486*	1-50	10-90	16.4 ± 4.74	16.2 ± 6.46	16.3 ± 5.07	-	-	1-25	7.29 ± 0.08

[a] Solubility limit reached. Citotoxicity + or - : presence or absence of cytotoxic effect on oocyte and cumulus cells. Chemicals tested in both laboratories for demonstrating the transferability of the IVM test.*

REPRODUCTIVE TOXICANTS AND *IN VITRO* PLACENTAL EFFECTS

Placenta development, though largely studied in animals, presents many differences in humans from all the other species. For obvious ethical reasons, studies in human placenta can be performed only in tissues obtained after natural or elective termination of pregnancy. Therefore, *in vitro* models need to be developed to screen chemicals affecting placenta formation and development.

For this reason, within the ReProTect project, two *in vitro* models have been developed: the trophoblast-derived choriocarcinoma cell line BeWo and the primary cultures of chorionic villous explants from human placenta. These two models are described here focusing on para-Nonylphenol (*p*-NP) as a proof-of-concept study.

p-NP is a metabolite of alkylphenol ethoxylates used as surfactants in the manufacturing industry which accumulates in the environment where it acts with estrogen-like activity [77]. By mimicking or antagonizing the action of natural hormones, *p*-NP may disrupt endocrine function, promoting reproductive failure and carcinogenesis in estrogen-sensitive tissues [78, 79]. Studies in animals have shown that *p*-NP can pass the placenta and induce uterine and developmental toxicity [80]. However, little evidence is available on the effect of *p*-NP on the placenta itself. Human placenta is a potential target to the action of estrogens or estrogen-like chemicals, since it expresses estrogen receptor (ER)α and ERβ, mainly in the trophoblast [81, 82]. Trophoblast cells are the most involved in the formation and development of placenta. These cells are in direct contact with maternal blood and tissues and therefore greatly exposed to environmental chemicals.

On these bases, *in vitro* studies on *p*-NP in human placenta have been performed using the models described above. The two models were used in two sequential steps: first, the trophoblast-like BeWo cells, to evaluate chemical toxicity and secondly, the human chorionic villous explants, to examine the potential of non-toxic concentrations on placenta functions.

BeWo cells are representative of the differentiative pathway of the villous cyto-trophoblast in the syncytio-trophoblast [82]. This cell model was selected for the toxicological study because, due to its tumorigenic nature, it can be easily propagated allowing testing of numerous concentrations and replicates. For this study, BeWo cell cultures were exposed to *p*-NP at a wide range of concentrations, from 0.1 pM to 1 mM. After 24 hrs at 37 °C in 95% air – 5% CO_2, cultures were assayed for cytotoxicity, by the the decrease of cell viability, and endocrine toxicity, by the decrease of β-hCG secretion, as toxicological endpoints. Control cultures were exposed to medium plus the vehicle (ethanol 0.1%), the solution in which *p*-NP was dissolved, This study in BeWo cells was intended to select non-toxic chemical concentrations to be used in primary cultures of chorionic villous explants.

In the second phase of the study, villi explant cultures were exposed to chemicals at non-toxic concentrations. At different times of incubation, 24-72 hrs, depending on the analyses, cultures were assayed for several biological parameters characterizing processes occurring during placentation. Unlike isolated cell cultures, this model has, in fact, the advantage of preserving the topology of intact villi thus representing a physiological model of placenta establishment and development [83]. This is particularly reliable when, as in the case of our studies, placenta samples are collected from first trimester human pregnancy. Placental tissues were obtained after elective termination of pregnancy at weeks 7-12 of gestation with the consent of patients and approval of the hospital Ethics Committee (Siena, May 2004). Villous explants were dissected as described by Caniggia *et al.,* [84]. Briefly, small fragments of villous tips (15-20 mg wet weight) were placed on culture dish inserts, previously coated with matrigel and inserted in 24-well plates. Tissue explants were cultured in serum-free medium and incubated overnight at 37 °C in 95% air-5% CO_2 for attachment to the matrigel. The next day the culture medium was replaced with medium supplemented with *p*-NP dissolved in 0.1% ethanol, or with medium plus ethanol (control). *p*-NP concentrations (0.1 pM - 1 nM) were selected on the basis of the toxicological study on BeWo cells.

At the end of incubation, culture medium was collected and assayed for β-hCG release, villous explants were removed from matrigel and assayed for caspase-3 cleavage. The two parameters tested, β-hCG and caspase-3, are respectively indicative of the syncytialization of trophoblast and of its apoptotic shedding [83]. The release of cytokines by *p*-NP treated or vehicle exposed explant cultures was also examined. Cytokines are immunoregulatory molecules and key players of the biological and immunological mechanisms allowing placenta establishment in the maternal uterus [85]. As these molecules operate in a complex network in which a correct balance between them is crucial for successful pregnancy [86, 87], 10 cytokines were simultaneous assayed in the culture supernatants using the Human Ultrasensitive Cytokine 10-Plex Multiplex Bead Immunoassay (Invitrogen, Carlsbad, CA). The cytokines tested *e.g.* GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α, were selected on the basis of literature [86, 87].

Data obtained from the toxicological and the functional studies are summarised in *Table 5*.

Analysis on BeWo cells revealed toxicity of *p*-NP from μM to mM concentrations with a significant decrease in cell viability at exposure of 15 μM *p*-NP ($p < 0.05$) that became less than 50% at ≥ 50 μM. A significant decline in β-hCG secretion was seen at 15 μM *p*-NP exposure ($p < 0.05$) and it was dramatically dropped from 60 to 125 μM *p*-NP. Data were referred to vehicle-treated control cultures.

Functional studies in the chorionic villous explants

Table 5 | *Toxicological and biological effects of* p-*nonylphenol*/p-*NP on* in vitro *models of human placenta*

***In vitro* models**	**[*p*-NP]**	**Effects**
BeWo cell line	15 µM-1 mM 15-125 µM	Cytotoxicity endocrine toxicity
Chorionic villous explants from human placenta	1 nM10 pM	Increasing hormone (β-hCG) releaseand trophoblast apoptosisIncreasing cytokine (GM – CSF, IL-10) release

revealed that *p*-NP concentrations, from nM to pM, interfere with important physiological processes in human placenta. A significant increase of β-hCG secretion ($p < 0.05$) and caspase-3 fragmentation were both seen at 1 nM *p*-NP [81]. An increasing release of cytokines, including IL-1, IL-4, IL-10, IFN-γ and GM-CSF, was seen at even lower concentrations, statistically significant for GM-CSF and IL-10 at 10 pM *p*-NP [88].

From the results obtained, we can state that *p*-NP may exhibit toxicity on trophoblast cells and that even at non-toxic concentrations may interfere with physiological processes in placentation.

To our knowledge, no biological effects have been reported for *p*-NP at concentrations below µM, except for a dose-dependent inhibition of aromatase activity in human JEG-3 placenta cells in the range of nM-µM [89]. Effective concentrations of *p*-NP in placenta are also far below the levels found in human samples [90, 91]. The lowest effective concentration (10 pM) used in our study is about 2.2 pg/mL while the levels of *p*-NP detected in human samples vary from 0.3 to 221.7 ng/mL [90, 91].

Showing high sensitivity of human placenta to *p*-NP exposure, the data reported here on one single chemical support validity of the *in vitro* models used. The findings on *p*-NP are indeed consistent with those reported on Bisphenol-A, a chemical sharing with p-NP the usefulness in the manufacturing industry and the estrogen-like activity [92]. This chemical was also largely transferred by the placenta [92].

Such results raise concern about possible correlations with pregnancy disorders and/or fetal growth and development due to maternal exposure to chemicals with estrogenic activity.

FINAL REMARKS

So far, to assess reproductive toxicity, only *in vivo* studies are accepted by the regulatory authorities; this assumption finds its ground in the need to study complex effects on signaling networks, such as those induced by EDs, at organism level. However, an intelligent testing strategy on reproductive toxicology can be envisaged, integrating: a) an array of tests on major building blocks of the mammalian reproductive cycle (including still overlooked targets such prostate and placenta), and b) information on modes of action. An intelligent strategy affording a more cost-effective testing and a high level of consumer protection is urgently needed, indeed, the new criteria enforced by REACH aim at a high level of chemical safety in Europe, but these require an impressive number of animals to fit the current tests for hazard assessment of hundreds or thousands of substances [2-8]. In the meanwhile, the European Union has recently recommended new rules and principles to reduce the use of animal for scientific experimentation with the recently approved Directive 2010/63/UE [93]. ReProTect has provided a battery of tests that can be used to launch a modular *in vitro* testing strategy, in order to screen and prioritize reproductive toxicants as well as provide valuable information for hazard characterization [5, 6]. In the framework of ReProTect, the Italian partnership implemented the following *in vitro* assays: i) the mammalian sperm motility/CASA (WP I), ii) the dubbed Repair Proficient Comet/ReProComet (WP I), iii) the PSA secretion (WP IV), iv) the *in vitro* bovine oocyte maturation/IVM (WP I); v) the *in vitro* bovine oocyte fertilization/IVF (WP I), vi) the trophoblast-derived choriocarcinoma cell line BeWo (WP II) and vii) the primary cultures of chorionic villous explants from human placenta (WP II). The assays on male and females gametes are primarily focussed on direct effects on the target cells, including DNA integrity, whereas the assays on prostate and placenta are more relevant to EDs, in particular to alterations of nuclear receptor pathways. In several cases (*e.g.,* the ReProComet and the PSA secretion assays), the studies were mainly successful proof-of-concepts requiring further development, also because the limited *in vivo* data for innovative endpoints did not allow to develop a good prediction model, as for the CASA. Nevertheless, setting experimental protocols and testing a number of substances for relevant and innovative tests and parameters represents a remarkable success and a significant contribution to the overall outcome of ReProTect. Importantly, all assays have been set up following the procedure of the ECVAM modular approach to test validation [11] and based on such rules each assay was defined in terms of the underlying mechanistic basis and of the identification of relevant toxicological endpoints (module 1). Furthermore, Standard Operation Procedures/SOPs were compiled for each assay and, finally, a panel of known reproductive toxicants were applied to test assay performances in order to assess dose-response curves of the chemical effects on each specific toxicological endpoint (module 1) and to establish the within-laboratory variability (module 2). The above mentioned assays were performed until this stage [33, 53, 70, 92] and one of them (IVM) was further performed to accomplish the requirements of

module 3, the transferability of the assay to another laboratory [12].

Furthermore, within the framework of the "feasibility study" [18], some of the described tests were shown to correctly predict the already known *in vivo* toxicological profile, thus showing the applicability of a set of *in vitro* alternative methods as a screening tool in hazard assessment. Finally, the ReProTect project has provided a successful example of development and integration of *in vitro* tests that is becoming an internationally recognised model for advancing alternative testing research.

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Conflict of interest statement

There are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

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