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Boar spermatozoa successfully predict mitochondrial modes of toxicity: Implications for drug toxicity testing and the 3R principles

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ABSTRACT

Replacement of animal testing by *in vitro* methods (3-R principles) requires validation of suitable cell models, preferably obtained non-invasively, defying traditional use of explants. Ejaculated spermatozoa are highly dependent on mitochondrial production and consumption of ATP for their metabolism, including motility display, thus becoming a suitable model for capturing multiple modes of action of drugs and other chemicals acting via mitochondrial disturbance. In this study, a hypothesis was tested that the boar spermatozoon is a suitable cell type for toxicity assessment, providing a protocol for 3R-replacement of animals for research and drug-testing. Boar sperm kinetics was challenged with a wide variety of known frank mito-toxic chemicals with previously shown mitochondrial effects, using a semi-automated motility analyser allied with real-time fluorescent probing of mitochondrial potential (MitoTracker & JC-1). Output of this sperm assay (obtained after 30 min) was compared to cell viability (ATP-content, data obtained after 24–48 h) of a hepatome-cell line (HepG2). Results of compound effects significantly correlated ($P < 0.01$) for all sperm variables and for most variables in (HepG2). Dose-dependent decreases of relative ATP content in HepG2 cells correlated to sperm speed ($r = 0.559$) and proportions of motile ($r = 0.55$) or progressively motile ($r = 0.53$) spermatozoa. The significance of the study relies on the objectivity of computerized testing of sperm motility inhibition which is comparable albeit of faster output than somatic cell culture models. Sperm suspensions, easily and painlessly obtained from breeding boars, are confirmed as suitable biosensors for preclinical toxicology screening and ranking of lead compounds in the drug development processes.

1. Introduction

Between 40 and 100 million animals are annually used for experimental research world-wide, figures exceeding 12 million animals within the European Union (EU) (COM, 2010, 511/final 2), 0.5 million only in Sweden, according to the European definition of research animal (EU Directive 86/609; EU Directive 2010/63). Most of these animals are euthanized to obtain tissues or explants for cell culture or biochemical analysis, either sequentially within the experimental layout, or terminally (post-mortem). Many are also used for investigative and regulatory toxicity testing, as well as disease modeling for pharmaceutical discovery. A significant proportion of these animals are used by the pharmaceutical industry to predict toxicity of drug-candidates before entering clinical testing (Nuffield Council on Bioethics, May 2005). International law within the EU and OECD (Organization for Economic Cooperation and Development) countries, as well as other national legal frameworks, is becoming increasingly restrictive regarding use of animals in research (EU Directive 2010/63). Legislation is currently applying the 3R-Principles for Reduction, Replacement and Refinement of experimentation with animals (Russell and Burch, 1959). Although the scientific community strives toward these principles, the continued use of large animal numbers strongly calls for the

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development and evaluation of new *in vitro* methods with the potential to *reduce* and *replace* them (or animal-derived tissues and cells) for toxicity testing, based on better mechanistic understanding of molecular initiating events triggering various adverse outcome pathways (AOPs), and the ability of *in vitro* methods to encompass these.

In agreement with current legislation, regulatory toxicity testing of drug candidates is to be conducted in two animal species (one rodent and one non-rodent), according to OECD guidelines (OECD, 2014). However, current practice is to "front-load" safety in the design phase of drug molecules using a variety of *in silico* and *in vitro* methods, the latter using cells and tissues *ex vivo* from animals (Gomez-Lechon et al., 2003). This practice is thus increasing the use of animals early in drug discovery, for safety testing, and provides strong possibilities for developing alternatives.

The induction of cardio- and hepatotoxicity are common causes of attrition of drug candidates, both in the preclinical phase and in clinical development. One of the most important and common modes of action in this is via disturbance of mitochondrial function and intactness via a number of discrete mechanisms (Dykens and Will, 2007). The prediction of disturbance to mitochondrial function has traditionally been studied in freshly isolated mitochondria from animals sacrificed on a daily basis (Porceddu et al., 2012). Several groups have focused on alternative sources of biological material for studying mitochondrial function, including the spermatozoon, which possesses exquisite energy dependence for membrane homeostasis and motility. This energy is supplied both by a subset of mitochondria (usually 75–100) located in the sperm midpiece, surrounding the base of the flagellum (Kamp et al., 2003; Zhang and Dai, 2012) and by cytoplasmic glycolysis (Silva and Gadella, 2006). Derived ATP is then used as an energy source required for motility and for plasma membrane functionality and integrity (Januskauskas and Rodriguez-Martinez, 1995; Peña et al., 2009). Phenotypes are easily monitored using computerized motility analysers (Tejerina et al., 2008, 2009) and suitable fluorophores (Johannisson et al., 2009; Pimenta Siqueira et al., 2011). Ejaculates from domestic animals shown high total sperm numbers, i.e. >100 billion spermatozoa in boar, which can be routinely collected without discomfort from this particular (and other) livestock species, thus warranting provision of numerous test cells without sacrificing experimental animals. Boar semen is routinely collected for commercial artificial insemination (AI) purposes breeding sires highly selected for sperm quality, with semen suspended in simple extenders and maintained at 16–20 °C for several days (Rodriguez-Martinez and Wallgren, 2010). Such a source warrants low viability variation, which is a pre-requisite for the repeatability of *in vitro* assays. Sperm viability and kinematics can be observed under the microscope and changes induced by a variety of compounds affecting mitochondria are thus amenable, both qualitatively and quantitatively. Despite this simple layout, few studies of cytotoxicity testing *in vitro* have systematically used spermatozoa (Andersson et al., 1998, 2004; Eskov et al., 2007, 2008; Hoornstra et al., 2003).

The present study evaluated an extended, yet focused battery of substances whose modes of action involve disturbances of mitochondrial function, via triggering of a variety of AOPs on boar spermatozoa and a human hepatoma cell line (HepG2,) often used by the pharmaceutical industry to assess drugs with known human hepatotoxicity profiles. The hypothesis tested was that the boar spermatozoon is a suitable cell type for toxicity assessment that also provides a protocol for 3R-replacement of animals for research and testing. The results clearly show that the sperm motility assay tested provides a good model for detection of chemically-induced mitochondrial dysfunction. The applicability of the sperm-base assay in a pharmaceutical screening paradigm, and the potential 3R wins are discussed in this work.

2. Material and methods

2.1. Toxic compounds and pharmaceutical drugs

A total of 130 toxic compounds and pharmaceutical drugs were used to challenge viability in HepG2 cells and boar spermatozoa. The compounds included were classified in nine groups according to their intracellular targets and/or mechanism of action (see Table 1, including their CAS numbering): (1) *COX I and II inhibitors*: Acetaminophen, Benoxaprofen, Bromfenac, Celecoxib, Diclofenac, Fenclozic acid, Glafenine, Ibufenac, Ibuprofen, Indomethacin, Mefenamic acid, Naproxen, Nimesulide, Salicylic Acid, Suprofen and Zomepirac; (2) *Ion Channel Inhibitors*: Aminopyrine, Amiodarone, Amlodipine, Chlorpropamide, Dantrolene, Furosemide, Glibenclamide, Nifedipine, Pinacidil and Verapamil; (3) *Antibiotics, antifungal, antiparasitics*: Amoxicillin, Cycloserine, Dapsone, Dicloxacillin, Flucloxacillin, Fumagillin, Fusidic Acid, Inavir, Isoniazid, Ketoconazole, Nifurtimox, Praziquantel and Terbinafine; (4) *Nuclear receptors and co-factor inhibitors*: Acitretin, Benzafibrate, Ciprofibrate, Clobetasol propionate, Dexamethasone, Estradiol, Flutamide, Gemfibrozil, Pioglitazone, Rosiglitazone, Tamoxifen and Troglitazone; (5) *Neuronal receptor agonists, antagonists and modulators*: Alpidem, Amitriptyline, Aricept, Buspirone, Caffeine, Carbamazepine, Carbidopa, Chlorpromazine, Clozapine, Dilevalol, Diphenylhydramine, Dopamine, Felbamate, Flumazenil, Imiloxan, Iproniazid, Isoproterenol, Ketotifen, Levetiracetam, Methapyrilene, Naltrexone, Nefazodone, Nomifensine, Olanzapine, Pargyline, Phensuximide, Physostigmine, Practolol, Ranitidine, Rimonabant, Sulpiride, Tacrine, Ticlopidine, Tolcapone, Valproic Acid and Zimeldine; (6) *Inhibitors of DNA and RNA synthesis*: Acyclovir, Azathioprine, Betaine, Carmustine, Cyclophosphamide, Dacarbazine, Doxorubicin, Fialuridine, Fluorouracil, Leflunomide, Levofloxacin, Mitomycin, Mitoxantrone, Nitrofurantoin, Rifampicin, Stavudine and Trovafloxacin; (7) *Other targets*: Amodiaquine, Bortezomib, Bosentan, Cinchophen, Cyclosporin A, Dihydralazine, Disulfiram, D-Penicillamine, Gliclazide, Mebendazole, Menadione, Methimazole, Methyldopa, Picotamide, Pyridoxine, Simvastatin, Tolmetin, Warfarin and Zileuton; (8) *Inhibitors of kinases*: Dasatinib, Imatinib Mesylate, Sorafenib Tosylate and Sunitinib Malate; and finally (9) *Inhibitors of membrane transporters*: Perhexilline, Probenecid and Tienilic acid.

Eight compounds whose primary mode of action involves mitochondrial disturbance were also tested: Antimycin A, Atractylocide (not tested in HepG2 cells), Arsenic Trioxide, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), 2,4 dinitrophenol (DNP), Oligomycin, Potassium cyanide (KCN) and Rotenone.

All chemicals and pharmaceutical drugs were obtained either from SIGMA–ALDRICH (St. Louis, MO, USA) or from AstraZeneca (AstraZeneca, Sweden).

2.2. Human hepatoma cells (HepG2)

The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC) (Rockville MD, USA). The HepG2 cells were seeded in collagen coated 96-well plates (Becton Dickinson, Stockholm, Sweden) at a density of 5000 cells/well in 95 µl of medium (DMEM with 4 mM glutamine (Gibco, Life Technologies, Stockholm, Sweden) with 10% FCS and 1 M HEPES), and cultured at 37 °C and 5% CO_2 for at least 18 h before addition of the test compound.

2.3. Boar spermatozoa

Breeding boar semen doses for commercial AI (Quality Genetics, Hållsta, Sweden), with a concentration of 20 million

Table 1
Calculated IC50:s (in μM) for the 130 pharmacological drugs and toxic compounds tested for each of the sperm variables and HepG2 cells.

Pharmacological drugs and toxic compounds			HepG2 cells viability	Spermatozoa		
Group	Compound	CAS number		Motile	Progressive motile	Speed
Cox I and II inhibitors	Acetaminophen	103-90-2	>250	>1000	>1000	>1000
	Benoxaprofen	51234-28-7	182.5	910	780	623
	Bromfenac	91714-93-1	>250	>1000	>1000	>1000
	Celecoxib	169590-42-5	49.58	35	30	22
	Diclofenac	15307-86-5	>250	86	20	83
	Fenclozic acid	17969-20-9	>250	>1000	777	972
	Glafenine	3820-67-5	>250	>1000	>1000	>1000
	Ibufenac	1553-60-2	>250	502	496	523
	Ibuprofen	15687-27-1	117.9	>1000	>1000	>1000
	Indomethacin	53-86-1	>250	455	472	507
	Mefenamic acid	61-68-7	>250	109	79	102
	Naproxen	22204-53-1	>250	>1000	>1000	>1000
	Nimesulide	51803-78-2	>250	48	38	88
	Salicylic acid	69-72-7	>250	>1000	>1000	>1000
	Suprofen	40828-46-4	>250	>1000	>1000	>1000
	Zomepirac	33369-31-2	>250	>1000	>1000	>1000
Ion channels inhibitors	Aminopyrine	58-15-1	>250	>1000	>1000	>1000
	Amiodarone	1951-25-3	115.4	10.7	7.7	10
	Amlodipine	88150-42-9	22.35	259	226	360
	Chlorpropamide	94-20-2	>250	>1000	>1000	>1000
	Dantrolene	7261-97-4	>250	>1000	>1000	>1000
	Furosemide	54-31-9	>250	>1000	>1000	>1000
	Glibenclamide	10238-21-8	>250	603	477	416
	Nifedipine	21829-25-4	>250	478	320	152
	Pinacidil	85371-64-8	>250	620	336	234
	Verapamil	52-53-9	171.2	>1000	>1000	>1000
Antibiotics. antifungal. antiparasitics	Amoxicillin	26787-78-0	>250	>1000	>1000	>1000
	Cycloserine	68-41-7	>250	>1000	>1000	>1000
	Dapsone	80-08-0	>250	>1000	>1000	>1000
	Dicloxacillin	3116-76-5	>250	>1000	>1000	>1000
	Flucloxacillin	5250-39-5	>250	>1000	>1000	>1000
	Fumagillin	23110-15-8	>250	>1000	>1000	>1000
	Fusidic acid	6990-06-3	>250	244	221	205
	Inavir	203120-17-6	>250	>1000	>1000	>1000
	Isoniazid	54-85-3	>250	>1000	>1000	>1000
	Ketoconazole	65277-42-1;79156-75-5	217.8	133	91	94
	Nifurtimox	23256-30-6	100	>1000	>1000	>1000
	Praziquantel	55268-74-1	>250	>1000	>1000	>1000
	Terbinafine	91161-71-6	>250	12.2	10.6	22
Nuclear receptors and co-factor inhibitors	Acitretin	55079-83-9	>250	>1000	>1000	>1000
	Benzafibrate	41859-67-0	>250	>1000	>1000	>1000
	Ciprofibrate	52214-84-3	>250	>1000	>1000	>1000
	Clobetasol propionate	25122-46-7	>250	>1000	>1000	>1000
	Dexamethasone	50-02-2	>250	>1000	>1000	>1000
	Estradiol	50-28-2	>250	>1000	>1000	>1000
	Flutamide	13311-84-7	237.6	104	72.3	87
	Gemfibrozil	25812-30-0	>250	>1000	745	>1000
	Pioglitazone	111025-46-8;105355-27-9	>250	>1000	>1000	>1000
	Rosiglitazone	122320-73-4	>250	205	240	86
	Tamoxifen	10540-29-1	>250	28.7	16.3	19.3
	Troglitazone	97322-87-7	226.7	48	29.7	28.7
Neuronal receptor agonists. antagonists and modulators	Alpidem	82626-01-5	>250	>1000	>1000	>1000
	Amitriptyline HCl	50-48-6	70.61	729	612	462
	Aricept	120011-70-3;110119-84-1	>250	>1000	>1000	>1000
	Buspirone	36505-84-7	>250	>1000	>1000	>1000
	Caffeine	58-08-2	>250	>1000	>1000	>1000
	Carbamazepine	298-46-4	>250	>1000	>1000	>1000
	Carbidopa	28860-95-9	236.3	200	117	104
	Chlorpromazine	50-53-3	38.83	143	63.3	109
	Clozapine	5786-21-0	173.8	577	493	464
	Dilevalol	75659-07-3	>250	>1000	>1000	>1000
	Diphenylhydramine	147-24-0	>250	>1000	>1000	>1000
	Dopamine	51-61-6	>250	>1000	>1000	>1000
	Felbamate	25451-15-4	>250	>1000	>1000	>1000
	Flumazenil	78755-81-4	>250	>1000	>1000	>1000
	Imiloxan	81167-16-0	>250	>1000	>1000	>1000
	Iproniazid	54-92-2	>250	>1000	>1000	>1000
	Isoproterenol	7683-59-2	>250	>1000	>1000	>1000
	Ketotifen	34580-13-7	183.2	>1000	>1000	>1000

	Levetiracetam	102767-28-2;51052-62-1	>250	>1000	>1000	>1000
	Methapyrilene	91-80-5	>250	>1000	>1000	>1000
	Naltrexone	16590-41-3	>250	>1000	>1000	>1000
	Nefazodone	83366-66-9	142.2	>1000	338	237
	Nomifensine	24526-64-5	>250	>1000	>1000	>1000
	Olanzapine	132539-06-1	>250	>1000	>1000	>1000
	Pargyline	555-57-7	>250	>1000	>1000	>1000
	Phensuximide	86-34-0	>250	>1000	>1000	>1000
	Physostigmine	57-47-6	>250	>1000	>1000	>1000
	Practolol	6673-35-4	>250	>1000	>1000	>1000
	Ranitidine	66357-35-5	>250	>1000	>1000	>1000
	Rimonabant	168273-06-1	>250	130	32	30
	Sulpiride	15676-16-1	>250	>1000	>1000	>1000
	Tacrine	321-64-2	>250	>1000	>1000	>1000
	Ticlopidine	55142-85-3	248.1	190	183	306
	Tolcapone	134308-13-7	176.2	15.8	13	24.5
	Valproic Acid	99-66-1	>250	>1000	>1000	>1000
	Zimeldine	56775-88-3	178.5	>1000	>1000	>1000
Inhibitors of DNA and RNA synthesis	Acyclovir	59277-89-3	>250	>1000	>1000	>1000
	Azathioprine	446-86-6	>250	>1000	>1000	>1000
	Betaine	107-43-7	>250	>1000	>1000	>1000
	Carmustine	154-93-8	>250	>1000	>1000	>1000
	Cyclophosphamide	50-18-0	>250	>1000	>1000	>1000
	Dacarbazine	4342-03-4	>250	>1000	>1000	>1000
	Doxorubicin HCl	23214-92-8	63.74	113	92	41
	Fialuridine	69123-98-4	>250	>1000	>1000	>1000
	Fluorouracil	51-21-8	>250	>1000	>1000	>1000
	Leflunomide	75706-12-6	241.1	658	538	187
	Levofloxacin	82419-36-1;100986-85-4;83380-47-6	>250	>1000	>1000	>1000
	Mitomycin	50-07-7	101.6	>1000	>1000	>1000
	Mitoxantrone	65271-80-9	41.27	40	53	18
	Nitrofurantoin	67-20-9	245.6	651	382	163
	Rifampicin	13292-46-1	>250	>1000	564	316.5
	Stavudine	3056-17-5	>250	>1000	>1000	>1000
	Trovafloxacin	146836-84-2	>250	>1000	>1000	>1000
Other targets	Amodiaquine	86-42-0	>250	491	332	244
	Bortezomib	179324-69-7	>250	>1000	>1000	>1000
	Bosentan	147536-97-8	>250	295	205	225
	Cinchophen	132-60-5	>250	>1000	>1000	>1000
	Cyclosporin A	79217-60-0;59865-13-3	>250	>1000	>1000	>1000
	Dihydralazine	484-23-1	>250	>1000	>1000	>1000
	Disulfiram	97-77-8	119.6	1.3	0.5	1.66
	D-Penicillamine	52-67-5	>250	>1000	727	700
	Gliclazide	21187-98-4	>250	>1000	>1000	>1000
	Mebendazole	31431-39-7	100	>1000	>1000	>1000
	Menadione	58-27-5	21.57	118	67	25.5
	Methimazole	60-56-0	>250	>1000	>1000	>1000
	Methyldopa	555-30-6	>250	>1000	>1000	>1000
	Picotamide	32828-81-2	>250	>1000	>1000	>1000
	Pyridoxine	65-23-6;12001-77-3	>250	>1000	>1000	>1000
	Simvastatin	79902-63-9	26.1	33.5	16	45.5
	Tolmetin	26171-23-3	>250	>1000	>1000	>1000
	Warfarin	81-81-2	>250	>1000	>1000	>1000
	Zileuton	111406-87-2	>250	>1000	>1000	>1000
Inhibitors of kinases	Dasatinib	302962-49-8	167.1	147	125	168
	Imatinib mesylate	220127-57-1	>250	>1000	>1000	>1000
	Sorafenib tosylate	475207-59-1;4750207-59-1	166.3	82	38.5	18
	Sunitinib malate	341031-54-7	83.62	336	217	140
Inhibitors of membrane transporters	Perhexilline	6621-47-2	12.3	12	8.5	21.5
	Probenecid	57-66-9	>250	>1000	>1000	>1000
	Tienilic acid	40180-04-9	>250	>1000	>1000	>1000

Proportion of motile spermatozoa (speed higher than 0 μm/s), proportion of progressive spermatozoa (speed higher than 5 μm/s), average velocity of the spermatozoa (μm/s).

spermatozoa/mL, were obtained on a weekly basis. Each AI-dose contained a sperm pool of three Hampshire boars, extended in Beltsville-Thawing Solution (BTS, IMV-Technologies, L'Aigle, France) (Pursel and Johnson, 1975) and prepared at 2.5×10^9 total spermatozoa per dose (80 mL), chilled to 16–20 °C and transported overnight. The extended sperm suspension was kept in a Styrofoam box at 18–20 °C for a maximum of 4 days, during which the experiments were run. Routine controls of motility and morphology were performed before starting each experiment and only samples with a motility >60% were considered acceptable for testing. Sperm morphology was periodically assessed for each boar by the company delivering the semen. Testing was initially performed at AstraZeneca R&D Södertälje (Sweden) and then at Linköping University (Department of Clinical and Experimental Medicine, IKE, Linköping, Sweden).

2.4. Preparation of the testing compounds

The chemical compounds were diluted in DMSO (SIGMA–ALDRICH, St. Louis, MO, USA) to prepare stock dilutions.

The maximum test concentration for each drug was dependent on the solubility of the compound, and empirically determined for the spermatozoa and fixed as a maximum of 250 μM for the HepG2 cells. Following dilution, aliquots were stored at −20 °C, until thawing immediately before analysis.

For testing on HepG2 cells, the thawed compounds were serially diluted in 100% DMSO to give 200X the desired test concentration (10 concentrations in a half-log series). The DMSO stocks were then diluted 20X in Glucose free DMEM (with 4 mM glutamine (Gibco, Life Technologies, Stockholm, Sweden) with 10% FCS and 1 M Hepes).

For sperm testing, the thawed compounds were serially diluted in XCELL (IMV-Technologies, L'Aigle, France) to the desired concentration (8 concentrations in a half-log dilution series). The DMSO concentration was adjusted to be equal in all boar spermatozoa incubations within the same experiment (in general 0.1–1% DMSO) as a dose–response effect of DMSO was observed in boar spermatozoa when the concentration of DMSO was ⩾2%.

2.5. Cytotoxicity in human hepatoma cells (HepG2)

Ten half-log dilutions of each studied compound (prepared as indicated above) were added to a 96 well-plate. Five hundred (500) μL of each dilution of every compound were added to each well with 5000 cells and incubated at 37 °C and 5% CO_2 for 24 h. Within all experiments the final DMSO concentration was 0.5%, as a decrease in ATP production was observed in HepG2 cells when the concentration of DMSO was ⩾1%. Controls consisted in 0.5% of DMSO in 100 μL of DMEM with 4 mM glutamine (Gibco, Life Technologies, Stockholm, Sweden) with 10% FCS and 1 M HEPES. Cell viability was determined by the relative concentration of ATP in exposed versus control cells with cell viability in controls set to 100% (while incubated in medium with 0.5% DMSO). ATP-concentration was determined using the Cell Titer Glo Luminescent Cell viability assay (Promega Biotech AB, Sweden) according to the manufacturer's instructions.

2.6. Cytotoxicity in boar spermatozoa

Eight half-log dilutions of each studied compound (prepared as indicated above) were added to a 96 well-plate. Fifty (50) μL of the sperm suspension concentrated to 40 million spermatozoa per mL were added to 50 μL of each dilution and carefully mixed. Two replicates were performed per experiment with four controls used in each plate. Controls consisted in 50 μL dilution of DMSO diluted in XCELL at the same concentration that the highest dilution of the tested compound with 50 μL of the sperm suspension carefully mixed. Once the 96 well-plate was prepared, it was incubated at 37 °C for 30 min on a shaking plate.

Following incubation, one aliquot of 2.5 μL of the sperm suspension was taken from each well, loaded into a Leja Standard Count 4 Chamber Slide (SC 20-01-04-B, Leja Products B V, Nieuw-Vennep, The Netherlands) and examined at 10× using an IX71 Olympus light inverted microscope equipped with a thermal plate (38 °C), positive phase contrast optics (10× objective, Olympus AB, Stockholm, Sweden), a Charge Coupled Device (CCD) camera (UI-1540LE-M-HQ, IDS Imaging Development Systems, Germany), and the ToxiSperm™ software (Biophos SA, Lausanne, Switzerland).

For each aliquot, in a microscope field per chamber and four chambers per slide, the following sperm variables were analyzed: average velocity (μm/s), numbers (million/mL), total motility (%, speed > 0 μm/s), progressive motility (%, speed > 5 μm/s), immotile cells, and total sperm number per microscope field.

2.7. Assessment of sperm mitochondrial integrity

To confirm that the cause of decrease in sperm motility in the cytotoxicity assay was due to an inactivation of their mitochondria, the organelle status was evaluated loading 94 nM of MitoTracker Red CMXRos (Molecular Probes, Life Technologies, Eugene, OR, USA, 9.4 μM diluted in XCELL) and/or 1 mg/mL of JC-1 (Molecular Probes, Life Technologies, Eugene, OR, USA, 9.4 μM diluted in XCELL) on each half-log dilution tested well, loading on a Leja chamber and examined as described above, using the uEye software (IDS Imaging Development Systems, Obersulm, Germany) to retrieve a snap-shot of the fluorescence per cytotoxicity level.

2.8. Statistical analysis

The HepG2 cell viability was calculated as a function of relative ATP content in cells with the dilutions of the testing compounds versus control cells. Plotting of the data, curve fit and calculation of the EC_{50} values were performed in the custom HTDR Origin Software (OriginLab Corporation, Northampton, MA, USA). Sperm kinematic data were processed using GraphPad Prism 5 (Graph Pad Software, La Jolla, CA, USA). For each experiment the mean (±SD) of the two duplicates were used to calculate dose–response curves considering the proportion of motile spermatozoa, proportion of progressive spermatozoa and mean sperm speed, setting as top value the average of the four controls and as low value 0 (understood as the absence of motility or speed value 0 μm/s) for each dose–response curve of each variable. An IC50 (defined as the concentration that reduces the values to half of the control value) of each compound for each variable measured was obtained. One-way ANOVA was performed using IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA) to test whether the different values observed in each dose–response curve were different and the *post hoc* test of Bonferroni was applied to determine when the differences observed became significant ($p < 0.05$). Spearman Rank correlations were run between IC50 values in HepG2 cells and sperm kinematics, using IBM SPSS Statistics 20 (IBM Corporation, Armonk, NY, USA).

3. Results

The IC50-values of the 130 test compounds tested in HepG2 cells (loss of cellular ATP content), and in boar spermatozoa (diminished average sperm velocity (μm/s), % of motile spermatozoa and % of progressive spermatozoa), are summarized in Table 1. Table 2 displays data obtained with eight compounds with established molecular initiating events and AOPs within mitochondria (mito-toxic compounds). In Table 1, compounds with an IC50 > 250 μM in HepG2 and/or an IC50 > 1000 μM in either one, two or all three sperm variables were compounds defined as non-toxic or without effect either in HepG2 cells and or spermatozoa.

The most potent compounds (lowest IC50s) of each group were: Celecoxib, Amlodipine, Ketoconazole, Troglitazone, Tolcapone, Mitoxantrone, Disulfiram, Sorafenib Tosylate and Perhexilline (see Table 1) while among mito-toxic compounds, the most potent was Antimycin A, with clearly lower calculated IC50:s for the spermatozoa than for the explored HepG2 (Table 2).

In order to assist cross-comparison of results obtained in the two cell systems, the test compound results were sub-divided into two groups based on their respective IC50s in HepG2 cells (<250 μM<) or spermatozoa (<300 μM<). Table 3 shows that 20 out of 32 compounds (62.5%) with an IC50 < 250 μM in HepG2 had a low IC50 (<300 μM) in the sperm assay. Moreover, from the 99 compounds with an IC50 > 250 μM in the HepG2 assay, 86

Table 2
Calculated IC50:s (nM, μM or mM) of different mito-toxic compounds tested in boar spermatozoa for each of the sperm variables assessed using the ToxiSperm™ software and in HepG2 cells.

Mito-toxic compounds	CAS number	Spermatozoa			HepG2 cells viability
		Speed	Motile	Progressive	
Antimycin A	1397-94-0	22 nM	60 nM	15 nM	>250 μM
Atractylocide	102130-43-8	>10 μM	>10 μM	>10 μM	Not tested
Arsenic trioxide	1327-53-3	94.7 μM	181 μM	83.8 μM	>250 μM
FCCP[a]	370-86-5	266 nM	270 nM	204 nM	>250 μM
2,4 Dinitrophenol (DNP)	51-28-5	39.5 μM	55.7 μM	40.3 μM	>250 μM
Oligomycin	1404-19-9	0.51 μM	4.9 μM	3.4 μM	>250 μM
KCN	151-50-8	576 μM	1.314 mM	491 μM	>250 μM
Rotenone	83-79-4	0.36 μM	2.0 μM	0.36 μM	48.6 μM

[a] Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone. Proportion of motile spermatozoa (speed higher than 0 μm/s), proportion of progressive spermatozoa (speed higher than 5 μm/s), average velocity of the spermatozoa (μm/s).

Table 3
Number of compounds fitting, with their IC50 levels, testing on either HepG2 cells or boar spermatozoa (μM segments).

		IC50 in HepG2 (μM)	
		<250	>250
IC50 in spermatozoa (μM)	<300	20	13
	>300	12	86

(87%) had an IC50 > 300 in the sperm assay, suggesting that the outcome of either assay was comparable, despite the divergence found for 25 compounds (20%) (see Table 1). In addition, the Spearman Rank correlation test showed significant correlations ($p < 0.01$) between methods ($r = .53–.559$) and sperm variables considered ($r = .933–.948$) (Table 4). Fig. 1 depicts dose–response curves obtained in boar spermatozoa for representative compounds of each sub-group of substances tested (see Section 2). Dose–response curves and associated IC50 data built on average sperm velocity, showed clear points of departure from control values in all cases, with sigmoidal responses thereafter until their respective maximal response, proving the disruption of the sperm energy production machinery and, thus, justifying the kinematic-based model (see also Fig. 2).

The calculated dose–response curves showed that when challenging spermatozoa with eight half-log dilutions of each tested compound, the studied sperm variables responded (decrease) in a dose–response manner to different toxic compounds ($p < 0.05$ in all curves). The *post hoc* test of Bonferroni showed that the first point being statistically ($p < 0.05$) different within the curve, was close to the IC50 for each dose–response curve.

Fig. 2(A–E) shows a typical decrease in mitochondrial activity alongside sperm motility after challenging boar spermatozoa with the mito-toxic compound Arsenic Trioxide. A decrease in the number of spermatozoa with fluorescent (live) mitochondria can be observed between the lower (panel A and C) and the higher (panel B and D) tested concentrations (higher magnifications in inserts), which follows the sperm velocity upon which the kinematic results are based on. In E, the decrease in mitochondrial activity is expressed by percentage variations in proportions of motile spermatozoa and their fluorescence intensity measured using the ImageJ (Public domain) alongside the eight half-log dilutions (compared to controls) after MitoTracker and JC-1 loading.

4. Discussion

The present study investigated how basic phenotypic variables of boar spermatozoa (sperm speed and derived proportions of motile spermatozoa), assessed with an automated motility analyser capable of measuring huge sperm numbers within seconds (frame rate of 25/s and a frame time of 40 ms), respond to the *in vitro* exposure to eight chemicals with well-known effects on mitochondria (mito-toxic compounds) and to 130 toxic compounds and/or pharmaceutical drugs with varying targets. To validate the results, sperm motility data was compared to traditional toxicity testing outcome using HepG2 cells and the mitochondrial target was confirmed by exploring the organelle status after each dose step. The hypothesis tested was confirmed as valid. Ejaculated boar spermatozoa are suitable cells for quick, easy, cheap and repeatable respiration toxicity assays owing to their ability to display a phenotypically easily examinable kinematic behavior within seconds. The results obtained with boar spermatozoa were comparable to those obtained using other animal somatic cells that, however, need to be harvested via invasive methods, cultured and incubated for longer periods.

Our findings are in agreement with available literature regarding the high sensitivity displayed by boar spermatozoa to detect toxins disruptive of cell ion homeostasis, energy generation and mitochondrial function (Andersson et al., 2006; Apetroaie-Constantin et al., 2009; Hoornstra et al., 2003). As well, spermatozoa were equally sensitive to somatic cell lines used to indicate cytotoxicity by other mechanisms, besides genotoxicity (Severin et al., 2005; Jääskeläinen et al., 2003).

Table 4
Spearman Correlations (r) and significances (p-values) among evaluated endpoints in assays both cytotoxicity (boar spermatozoa kinetics and ATP content in HepG2 cells).

	HepG2 cells viability	% Motile spermatozoa	% Progressive motile spermatozoa	Sperm speed
Sperm speed	$r = 0.559$ $p < 0.01$	$r = 0.948$ $p < 0.01$	$r = 0.933$ $p < 0.01$	$r = 1$ $p < 0.001$
% Progressive motile spermatozoa	$r = 0.53$ $p < 0.01$	$r = 0.939$ $p < 0.01$	$r = 1$ $p < 0.001$	
% Motile spermatozoa	$r = 0.55$ $p < 0.01$	$r = 1$ $p < 0.001$		
HepG2 cells viability	$r = 1$ $p < 0.001$			

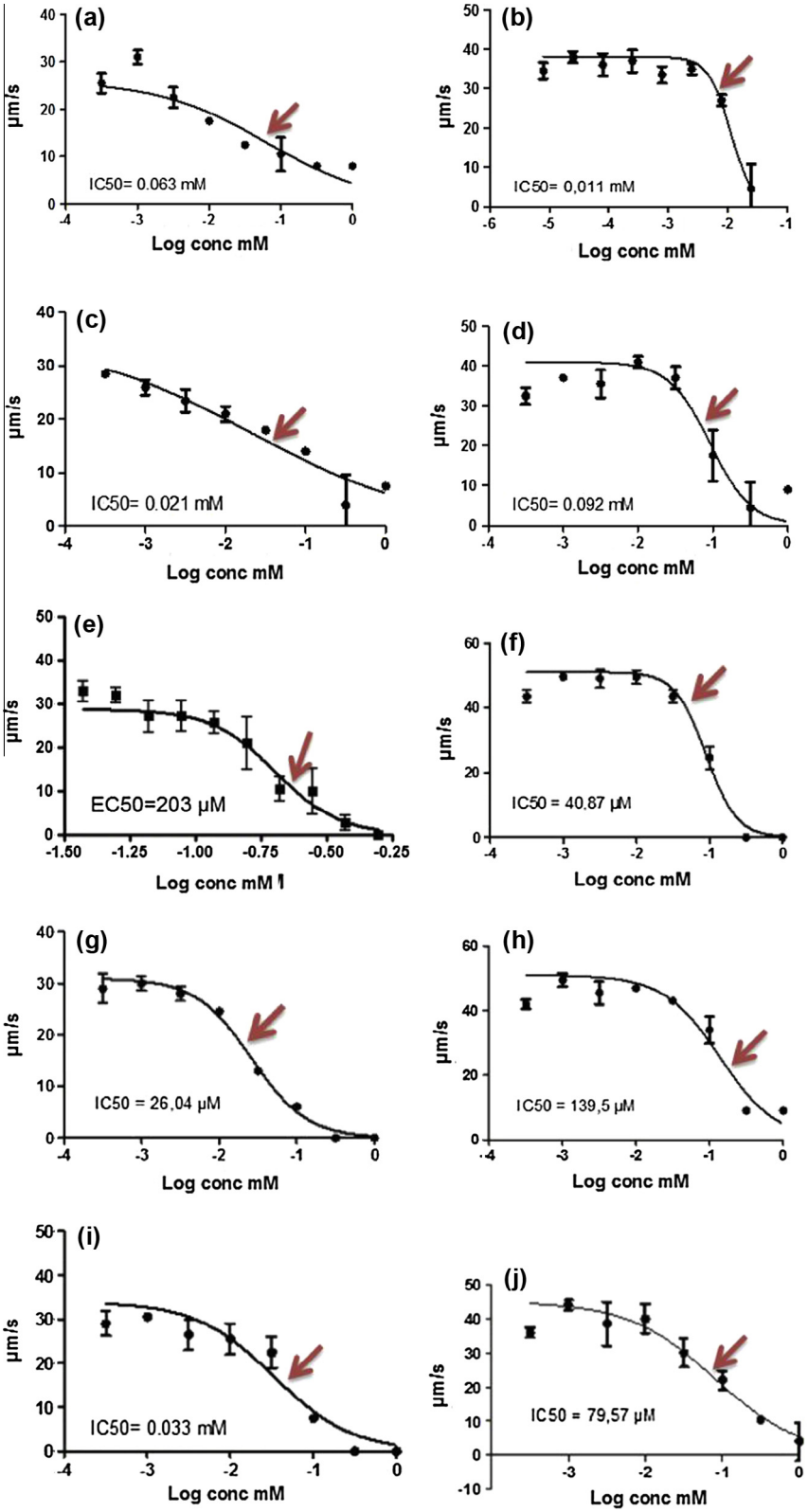

Fig. 1. Dose–response curves for average sperm velocity (µm/s) following exposure to representative compounds (Log conc mM): the IC50 is depicted numerically and marked in the curve (arrow). (a) COX I and II inhibitors: Diclofenac (COX I and 2); (b) Ion Channels Inhibitors: Amiodarone (Na, K-activated adenosine triphosphatase inhibitor); (c) Antibiotics, antifungal, antiparasitics: Ketoconazole (inhibition of 14-α demethylase, a cytochrome P-450 enzyme); (d) Nuclear receptors and co-factors inhibitors: Flutamide (andogen receptor ligand and antagonist); (e) Neuronal receptor agonists, antagonists and modulators: Chlorpromazine (antagonist (blocking agent) on different postsysnaptic receptors -on dopaminergic-receptors (subtypes D1, D2, D3 and D4) 5-HT1 and 5-HT2, H1-receptors, alpha1/alpha2-receptors muscarinic (cholinergic) M1/M2-receptors); (f) Inhibitors of DNA and RNA synthesis: Doxorubicin HCl (DNA and topoisomerase II); (g) Other targets: Menadione (Vitamin K dependent proteins); (h) Inhibitors of kinases: Subtinib Malate (multi-targeted receptor tyrosine kinase (RTK) inhibitor); (i) Inhibitors of transporters: Perhexilline (CPT-1 and 2); (j) Arsenic (III) Oxide (Mito-toxic compound. Pyruvate dehydrogenase inhibitor). The bars in the dose–response curves indicate the Standard Deviation.

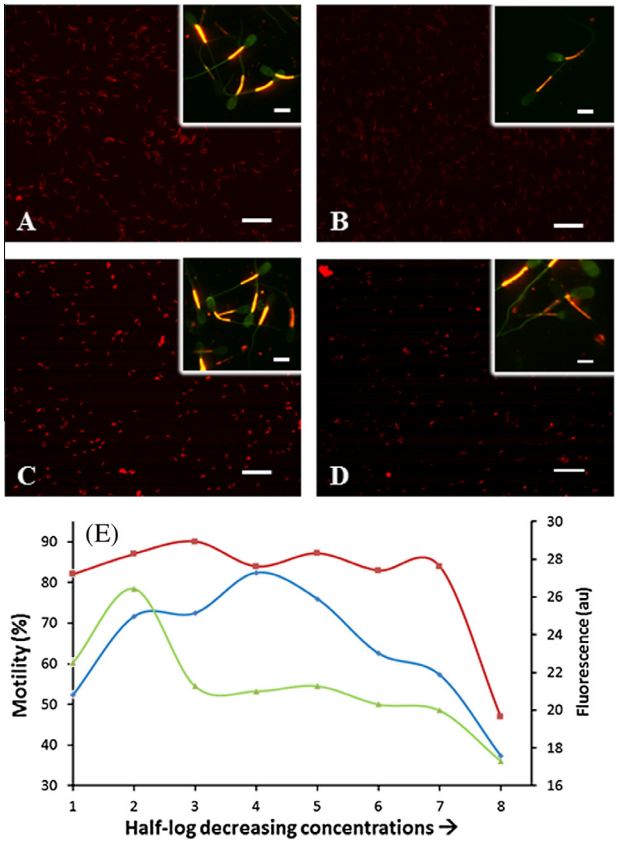

Fig. 2. (A–E) Intact mitochondria detected with MitoTracker (A–B, bar = 50 μm) and JC-1 (C–D, bar = 50 μm) after exposure to arsenic trioxide. A–C: 0.316228 μM (minimum tested concentration); B–D: 1 mM (maximum tested concentration) (Inserts, bar = 10 μm). E: fluctuations in fluorescence intensity to MitoTracker (blue line) and JC-1 (green line) (au: arbitrary units) and proportions of motile spermatozoa (red line) along the tested half-log decreasing concentrations of arsenic trioxide (In the *X*-AXIS: 1: 0.0003 mM; 2: 0.001 mM; 3: 0.003 mM; 4: 0.01 mM; 5: 0.03 mM; 6: 0.1 mM; 7: 0.3 mM; 8: 1 mM). Fluorescence filter U-MWIBA3 (Olympus AB, Stockholm, Sweden) with excitation filter BP460-495 and emission filter BA 510-550. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Toxicity testing has traditionally been performed in animals and/or explanted-cultured somatic cells with many different end-points, varying from the death of the cell to the display of different signs of distress or of death in animals. Although legislated for many countries (EU Directive 2010/63), a further reduction of the number of experimental animals, alongside their replacement by alternative, innovative methods following the 3R-principle (Russell and Burch, 1959) is a global need.

An important mechanism in drug-induced toxicity is the inactivation of mitochondria. The main function of the organelle is the production of ATP by oxidative phosphorylation (Dykens and Will, 2007; Ramalho-Santos and Amaral, 2013). When mitochondria are inactivated, cells are not able to produce/consume ATP which affects cellular membrane stability, disturbing cell homeostasis, eventually leading to cell death. Disruption of mitochondrial functions has been reported to be one of the major mechanisms of Drug-Induced Liver Injury (DILI, Chen et al., 2011) one of the most important pathologies to avoid when manufacturing commercial drugs (Temple and Himmel, 2002). Mitochondrial integrity is therefore a basic condition that must not be modified by any commercially available drug. Most toxicity testing is performed using primary cultured animal hepatocytes, as they have a large number of mitochondria (varying from 500 to 4000, with a predicted average of 2000 mitochondria per cell; Degli Esposti et al., 2012). To refine targeting mitochondria, toxicity assays have also been performed in freshly isolated mitochondria obtained from sacrificed experimental animals (Will et al., 2006; Porceddu et al., 2012). However, both explanted cell-cultured and isolated organelles require use of animals, a substantial difference compared with our proposed sperm-model that most closely follows the 3R-principles.

The number of available mitochondria is relevant to obtain reliable results. Hepatocytes in culture have 500–4000 mitochondria per cell, while the number of mitochondria that can be maximally isolated is 1–1500 per hepatocyte. Using spermatozoa for the same assays, as described in this study, the average sperm number per microscope field was ~1000, leading to a total of potentially 200,000 mitochondria per tested concentration and a total of 1.6×10^6 mitochondria testable per assay, as we had 2 duplicates of 8 half-log dilutions. Spermatozoa are very specialized cells and the number of mitochondria varies from one species to another, yet having around 100 organelles per spermatozoon (Peña et al., 2009). Considering the sperm numbers per ejaculate (in boar, around 120 billion in a total ejaculate volume of 200–250 mL; Rodriguez-Martinez et al., 2005) availability is far higher, decreasing costs, increasing mitochondria numbers per assay and avoiding euthanasia of experimental animals. However, it could be argued that there is an inherent genotypic difference between somatic parenchyma cells (as hepatocytes) and male gametes, the latter being haploid and genotypically heterogeneous, owing to the meiosis they underwent. On the other hand, their cell phenotype is highly homogenous, particularly in breeding boars, which are highly selected for sperm normality (motility and morphology). Assessment of sperm motility yielded IC50 values for several compounds, within 30 min, compared to 24 h when using HepG2 cells.

However, an IC50 does not fully define whether a cell is suitable to be used as a model for toxicological testing. The basic aspect to clarify is whether a certain cell detects toxic compounds while discarding non-toxic compounds. The results of kinematics in spermatozoa were comparable to the levels of cytotoxicity in HepG2 cells. Sperm testing, however, have several advantages. Lower cost due to cheaper cells, shorter incubation times, speed of analyses, etc is not to be forgotten. Moreover, spermatozoa can be obtained non-invasively from breeding boars, without incurring in animal welfare issues and, finally, without needing enormous animal numbers. HepG2 is a cell-line that has been used in research and toxicity testing for a long time (Havekes et al., 1983; Marroquin et al., 2007). When the challenge testing outcome was compared between HepG2 cells and spermatozoa, divergence of IC50s was seen in only 25/130 compounds, with a high degree of agreement for the majority of the tested compounds (Table 3). The significant agreement between the assays indicate that the sperm motility assay is as sensitive as indicator of cell toxicity as the HepG2 testing, but that 30 min sperm incubation produced information equivalent to 24 h incubation using the HepG2 cell line and a more cumbersome testing. However, not all compounds with low IC50 in spermatozoa had low IC50 in HepG2 (even that the correlation was significant). This discrepancy could be explained by a higher sensitivity of spermatozoa to display phenotypic motility inhibition following exposure to the toxic compounds than the explored ATP-changes recorded in HepG2 cells. While there was a good correlation between the toxicities for the two test cell systems, spermatozoa were much more sensitive in detecting mitochondrial toxicity, while escaping detection in the HEpG2 cell test. The most likely explanation is that HEpG2 cells up-regulate glycolysis (Crabtree-effect) in response to mitochondrial toxic compounds, while the so-called Crabtree-effect is absent in boar spermatozoa.

The explored end-points are also a major difference between the above cell-types. The more endpoints to be considered, the more difficult the interpretation of the testing becomes.

Hepatocytes suffer a series of changes after mitochondria have been damaged by respiration toxicity, from blebs in the plasma membrane to signs of necrosis, ending up in nuclear piknosis and shrinkage/disintegration of the cells. Spermatozoa reveal effects by decreasing speed and ultimately becoming immotile, a phenotypic response readily quantifiable. The boar spermatozoa used in this study were derived from commercial breeding AI-doses. The AI-doses were built by mixing the ejaculates of 3 boars, so that individual differences were minimized. The boars were selected for breeding owing to their high sperm quality fertility, and capacity to produce large litters (prolificacy). Boars were of similar age and continuously monitored for health status and semen characteristics. The spermatozoa were extended in rather uncomplicated, chemically-defined commercial extenders to sustain viability and potential fertilizing capacity for 3–5 days, stored at 16–20 °C, at concentrations of ≈30 million spermatozoa/mL with a similar sperm speed per batch. Taken together, this availability of a well-defined cell type is commendable, making assays repeatable. Sperm speed (mean velocity between 55 and 144 μm/s; Freking et al., 2012), decreases from just after ejaculation (~80% highly motile spermatozoa) to about half after overnight storage in BTS-extender (Rodriguez-Martinez et al., 2005). Sperm speed was, however, kept at ~40 μm/s thereafter, including steps of pre-warming during incubation alongside the present study (yet depicting about 70% of sperm motility). Such characteristics were kept for the 72 h experimental assay periods per week, and enabled proper testing.

It could be argued that the spermatozoa from other animal species (including human) could be used for the assay. However, boar spermatozoa have a medium-sperm-speed if we compare it with bull or ram spermatozoa (mean velocity of 68–162 μm/s; Katz and Dott, 1975) or to human spermatozoa (with 20–100 μm/s; Su et al., 2012). On the other hand, bull sperm is difficult to use. Liquid semen has a short survival and thus similar studies as the one presented here have been done using frozen-thawed semen (Eskov et al., 2007, 2008), even though it has been proved before to be 100 times less sensitive than boar spermatozoa to detect mitochondrial toxic compounds (Jääskeläinen et al., 2003). Human semen characteristics largely vary among men, and presents -compared to other animal species- a comparatively low sperm quality (including low sperm numbers). These arguments justify the use of boar spermatozoa as it has a velocity somewhere in the middle depicted by other species, have a large volume and concentration per ejaculate and, owing to an important male selection for sperm quality and fertility issued over decades, a clear homogeneity of repeated batches. In addition, boar spermatozoa have been proved as good sensors for detecting different levels of mito-toxic compounds causing foodborne illness and environmental diseases (Andersson et al., 2010; Mikkola et al., 1999; Peltola et al., 2004; Severin et al., 2005), a fact confirmed here (Fig. 2).

The semi-automated assay provided reliable dose–response kinematic changes comparable to those using isolated mitochondria, or one-cell type explants, albeit using different measuring end-points. The hereby described motility assay is based on the Qualisperm™ motility analyser, which has been validated for using spermatozoa from several animal species (Tejerina et al., 2008, 2009). Biomedical and toxicological research may need animals, as the multi-organic interactions and systemic responses vary from a single cell response or an explanted-cell culture to a whole animal organism. However, single cell models as ejaculated spermatozoa can be used to study and quantify early toxicity scrutiny of chemicals and drug candidates as shown in this article, reducing the number of animals in the total process of drug testing and toxicological assays and replacing them in the first steps. Also, our boar spermatozoa cell-model could be used to rank lead compounds in the drug development processes. Nevertheless, further research to complete our knowledge about this proposed boar ejaculated spermatozoa drug toxicity testing cell-model must be performed. This research should cover the intracellular pathways that explain the changes observed in the studied variables as well as the presence of plasmalemmal receptors that interiorize the challenged compounds and activates the intracellular signaling mechanisms.

In conclusion, conventional HepG2 testing (relative ATP concentration) and testing of boar sperm motility using the ToxiSperm™ software (Biophos SA, Switzerland), showed a high degree of agreement. However, the quicker output for the latter assay (within 30 min versus 24–48 h for HepG2), suggests the boar sperm motility assay model is more user- and animal-friendly (thus benefitting the 3R-principles) while detecting drugs that disturb mitochondrial function.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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