The non-genomic effects of endocrine-disrupting chemicals on mammalian sperm

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Abstract

Exposure to toxicants present in the environment, especially the so-called endocrine-disrupting chemicals (EDCs), has been associated with decreased sperm quality and increased anomalies in male reproductive organs over the past decades. Both human and animal populations are continuously exposed to ubiquitous synthetic and natural-occurring EDCs through diet, dermal contact and/or inhalation, therefore potentially compromising male reproductive health. Although the effects of EDC are likely induced via multiple genomic-based pathways, their non-genomic effects may also be relevant. Furthermore, spermatozoa are transcriptionally inactive cells that can come in direct contact with EDCs in reproductive fluids and secretions and are therefore a good model to address non-genomic effects. This review thus focuses on the non-genomic effects of several important EDCs relevant to mammalian exposure. Notably, EDCs were found to interfere with pre-existing pathways inducing a panoply of deleterious effects to sperm function that included altered intracellular Ca^{2+} oscillations, induction of oxidative stress, mitochondrial dysfunction, increased DNA damage and decreased sperm motility and viability, among others, potentially jeopardizing male fertility. Although many studies have used non-environmentally relevant concentrations of only one compound for mechanistic studies, it is important to remember that mammals are not exposed to one, but rather to a multitude of environmental EDCs, and synergistic effects may occur. Furthermore, some effects have been detected with single compounds at environmentally relevant concentrations.

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Introduction

In 1992, a landmark report revealed a decrease of sperm concentration from an average of $113\text{--}66 \times 10^6$ of spermatozoa/ml of semen over a 50-year period in men with no history of infertility (Carlsen *et al.* 1992). Furthermore, a reduction in mean semen volume was also described, thereby indicating a decrease in total sperm count (Carlsen *et al.* 1992). Subsequently, other studies have reported diminished sperm quality determined by the reduction in concentration, motility and/or normal morphology (Auger *et al.* 1995, Toppari *et al.* 1996) accompanied by an increasing trend in testicular cancer and abnormalities in male reproductive organs, such as cryptorchidism and hypospadias (Toppari *et al.* 1996). Although this is a controversial subject and several factors have been suggested to affect male reproductive health and fertility worldwide, both in humans and wildlife, it has been extensively argued

that certain toxicants present in the environment, mainly substances that possess (anti)estrogenic- or antiandrogenic-like activities dubbed endocrine-disrupting chemicals (EDCs), may play an important role in such reproductive dysfunction.

Environmental EDCs can affect the action of endogenous hormones and deregulate hormonal balance through multiple mechanisms. Some of these substances can mimic reproductive hormones such as testosterone and estradiol (E_2) and interfere with their synthesis, binding and/or action, therefore potentially affecting male reproductive capacity (Phillips & Tanphaichitr 2008). Although structural similarities are often noted between EDC and endogenous hormones, this does not constitute a rule. In fact, EDCs are a highly heterogeneous group of compounds that include synthetic substances originated from industrial processes such as pesticides, polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated

biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), phthalates, and bisphenol A as well as natural compounds with hormonal activity, mainly phytoestrogens and mycotoxins. In general, EDCs are potentially serious hazards to the broad human and animal populations given their ubiquitous distribution and persistency in the environment. Even though many have been banned or restricted in most countries as agreed in the Stockholm Convention regarding persistent organic pollutants (POPs; e.g., PCDDs, PCBs, several pesticides, and PAHs), EDCs may be very resistant to degradation and be present via air, soil, water and food contamination (e.g. fish, meat, and milk; Hauser *et al.* 2002). Furthermore, it is important to mention that due to their lipophilic nature, POPs bioaccumulate in the food chain. In fact, as these substances can be stored preferentially in body fat for long time periods, top food chain species are the most susceptible, because in addition to being directly exposed, they feed on organisms already with accumulations of these compounds in their body tissues (Guillette 1994).

Besides being detected in urine (Jonsson *et al.* 2005, Xia *et al.* 2009, Jurewicz *et al.* 2013), breast milk (Chikuni *et al.* 1991, Mocarelli *et al.* 2011), and blood serum (Younglai *et al.* 2002, Mocarelli *et al.* 2008), important amounts of EDCs have also been found in secretions of the female reproductive tract, cervical mucus and follicular and seminal fluids (Tsutsumi *et al.* 1998, Younglai *et al.* 2002, Drbohlav *et al.* 2005). This is particularly relevant as spermatozoa steep in these reproductive fluids/secretions on their journey toward the oocyte and are therefore in direct contact with such toxicants, an important route of exposure that should not be neglected. Importantly, mounting evidence has shown that these man-made or naturally occurring compounds may act through multiple pathways. Besides modulating gene transcription, they may equally promote non-genomic actions that may ultimately compromise sperm fertilizing ability and jeopardize male fertility. To this extent, spermatozoa are excellent models for the analysis of such putative effects given their transcriptional inactivity, a consequence of the highly condensed nature of sperm chromatin due to histone replacement by protamines. With this in mind, the present review mainly focuses on the direct effects of EDCs (Table 1) and explores their mechanisms of action in mature mammalian spermatozoa.

Man-made chemicals: sources of exposure and effects on spermatozoa

Pesticides

With the purpose of preventing, abolishing or controlling any pest, pesticides represent an extensive and diverse group of substances that have been shown to possess antiandrogenic and/or estrogenic activity *in vivo* and

in vitro (Lemaire *et al.* 2004, Sengupta & Banerjee 2014). The use of synthetic pesticides has increased worldwide over the last decades, mostly as a way of obtaining higher crop yields, although their use in home formulations are also a reality (Sengupta & Banerjee 2014). With more than 140 000 tons used annually only in the European Union (Bjørling-Poulsen *et al.* 2008), it is safe to assume that their ubiquitous presence in the environment may pose a risk to human and animal health.

Based on their chemical structure, pesticides can be divided into several subclasses, with organochlorines, organophosphates and pirethroids being the most commonly known (Sengupta & Banerjee 2014). Although with some exceptions probably attributed to the dissimilar concentrations detected in body fluids, exposure to these compounds detected in urine, seminal or blood serum samples have been associated with poor standard sperm parameters (Sengupta & Banerjee 2014).

Sperm motility in particular is of paramount importance for a given spermatozoon to reach and penetrate the oocyte and without which it does not have any fertilizing potential. *In vitro* studies have clearly shown that, whether determined by a computer-assisted sperm analyzer (CASA) system or simple phase-contrast microscopy, the pirethroids deltamethrin, cypermethrin and fenvalerate, as well as the organophosphates dimethoate, malathion and diazinon and the herbicides alachlor, bendiocarb, fenoxaprop-ethyl, and atrazine, can significantly reduce such parameter in humans, rodents, rabbit and boar (Table 1; Betancourt *et al.* 2006, Grizard *et al.* 2007, Abdallah *et al.* 2010, 2012, Yuan *et al.* 2010, Krockova *et al.* 2012) followed by an increase in cell death (Betancourt *et al.* 2006, Grizard *et al.* 2007, Abdallah *et al.* 2010, 2012). In parallel, the functional status of mitochondria, which is well correlated with sperm fertilizing capability (Amaral *et al.* 2013) and widely characterized by the determination of the mitochondrial membrane potential (MMP), is also affected by EDCs (Table 1). In fact, the pesticide alachlor was found to depolarize human sperm mitochondria prior to related damage on sperm motility, leading the authors to suggest that the harmful effects may be correlated with mitochondrial dysfunction (Grizard *et al.* 2007). Additionally, a decrease in sperm antioxidant scavenging defenses and/or an increase in the production of reactive oxygen species (ROS) levels have been detected in human and rat spermatozoa after exposure to deltamethrin, dimethoate and alachlor (Grizard *et al.* 2007, Abdallah *et al.* 2010, 2012), suggesting that oxidative stress may play a key role in EDC-mediated effects. Such an imbalance between ROS generation and ROS scavenging may lead to downstream repercussions such as DNA fragmentation (critical for the full expression of the fertility potential), peroxidation of plasma membrane lipids and apoptosis, all of which have already been reported in human spermatozoa exposed *in vitro* to pirethroids,

Table 1 Experimental evidence showing the direct effects of EDCs on sperm parameters/features

Sperm parameter/ feature	EDC	Species	Concentrations tested (no. of samples, replicates or experiments)	Main outcome	References
Motility	Pesticides	Human	0.18, 0.37, 0.90, and 1.85 mM (10)	Decreased	Grizard <i>et al.</i> (2007) Pant <i>et al.</i> (2013)
	Alachlor		76.94, 384.7, and 769.4 µg/l (3)		
	β-HCH		39.04, 195.2, and 390.4 µg/l (3)		
	Lindane		57.49, 287.45, and 574.9 µg/l (3)		
	p,p'-DDD		53.89, 269.45, and 538.9 µg/l (3)		
	p,p'-DDE	Rat	10, 25, 50, and 100 µM (5)		Tavares <i>et al.</i> (2015) Abdallah <i>et al.</i> (2010) Abdallah <i>et al.</i> (2012) Yuan <i>et al.</i> (2010)
	Deltamethrin		10, 50, 100, and 200 µM (8)		
	Dimethoate		50, 100, and 200 µM (10)		
	Permethrin		1, 4, 16, and 64 µM (10)		
	Cypermethrin		1, 4, 16, and 64 µM (10)		
	Atrazine	Boar	50, 100, and 500 µM		Betancourt <i>et al.</i> (2006)
	Fenoxaprop-ethyl				
	Diazinon		50, 100, and 500 µM		
	Malathion	Rabbit	50, 100, and 500 µM		Krockova <i>et al.</i> (2012)
	Bendiocarb		0.054, 0.107, 0.161, 0.214, and 0.268 mg/ml (5)		
	PCBs				
	PCB54	Human	0.1–10 ng/ml		Roediger <i>et al.</i> (1989)
	Aroclor 1254	Rat	10⁻⁹, 10⁻⁸, and 10⁻⁷ M (6)		Aly (2013)
	PAHs	Human	0.001, 0.01, and 0.1, 1 µg PAHs/ml		Fredricsson <i>et al.</i> (1993)
	Diesel particle extract				
	Phthalates				
	DBP	Human	0.4, 4, and 40 mM		Pant <i>et al.</i> (2011)
	DEP		0.33, 330, and 3300 µM		
	DMP		9, 94, and 940 µM		
	DOP		64 and 640 µM		
	DEHP		0.8, 8, and 80 mM		
	DBP		13.47, 67.35, and 134.7 µg/ml		
	DEHP		5.73, 28.65, and 57.3 µg/ml		
	BPA				
	BPA	Mouse	0.0001, 0.01, 1, and 100 µM (4)		Rahman <i>et al.</i> (2015)
	Mycotoxins	Boar			Tsakmakidis <i>et al.</i> (2006)
	ZEA		125, 187.5, and 250 µM (6)		
	α-ZOL	Horse	1 pM– 100 µM (5)		Filannino <i>et al.</i> (2011)
Mitochondrial function	Pesticides	Human	0.18, 0.37, 0.90, and 1.85 mM (10)	Decreased	Grizard <i>et al.</i> (2007) Tavares <i>et al.</i> (2015)
	p,p'-DDE		10, 25, 50, and 100 µM (5)		
	Dioxins	Mouse	1 and 5 nM (3)		Fisher <i>et al.</i> (2005)
	TCDD				
	PCBs	Rat	10⁻⁹, 10⁻⁸, and 10⁻⁷ M (6)		Aly (2013)
Cellular ATP content	Pesticides	Human	25, 50, and 100 µM (4)	Decreased	Tavares <i>et al.</i> (2015)
	p,p'-DDE				
	PCBs	Rat	10⁻⁹, 10⁻⁸, and 10⁻⁷ M (6)		Aly (2013)
	Aroclor 1254				
Oxidative stress	BPA	Mouse	0.0001, 0.01, 1, and 100 µM (4)		Rahman <i>et al.</i> (2015)
	BPA				
	Pesticides	Human	0.37 and 1.85 mM (12)	Increased	Grizard <i>et al.</i> (2007) Abdallah <i>et al.</i> (2010) Abdallah <i>et al.</i> (2012)
	Alachlor				
Lipid peroxidation	Deltamethrin	Rat	10, 50, 100, and 200 µM (8)	Increased	Abdallah <i>et al.</i> (2010) Abdallah <i>et al.</i> (2012)
	Dimethoate		50, 100, and 200 µM (10)		
	PCBs	Rat	10⁻⁹, 10⁻⁸, and 10⁻⁷ M (6)		Aly (2013)
	Aroclor 1254				
	B[a]P	Human	500 µM (4)		Russo <i>et al.</i> (2006)
Chromatin/ DNA integrity	Pesticides	Human	0.37 and 1.85 mM (8)	Decreased	Grizard <i>et al.</i> (2007)
	Alachlor				

Table 1 Continued.

Sperm parameter/ feature	EDC	Species	Concentrations tested (no. of samples, replicates or experiments)	Main outcome	References
Chromosomal anomalies	Parathion Paraoxon PCBs	Mouse	0.05–0.8 mM		Contreras <i>et al.</i> (1999)
	Aroclor 1254 PAHs	Rat	10^{-9} , 10^{-8} , and 10^{-7} M (6)		Aly (2013)
	B[a]P	Human	500 µM (4) 10 and 25 µM (4)		Russo <i>et al.</i> (2006) Sipinen <i>et al.</i> (2010)
	BPDE Mycotoxins ZEA	Boar	5, 20, and 50 µM (4–6) 10, 20, and 30 µg/ml (4)		Tsakmakidis <i>et al.</i> (2008)
	α-ZOL α-ZAL α-ZOL β-ZOL ZEA	Horse	25×10^{-6} –250 nM (3)		Minervini <i>et al.</i> (2010)
	PAHs B[a]P	Human	200 µg/ml	Increased structural aberrations	Watanabe & Kamiguchi (2001)
	Pesticides p,p'-DDE Atrazine	Human Boar	25, 50, and 100 µM (5) 8, 20, and 40 µM (5)	Decreased	Tavares <i>et al.</i> (2015) Maravilla-Galván <i>et al.</i> (2009)
	Fenoxaprop- ethyl BPA BPA PAHs B[a]P	Mouse Human	12, 30, and 60 µM (5) 0.0001, 0.01, 1, and 100 µM (3) 12.5, 25, 50, and 100 µg/ml	Increased Decreased Increased	Rahman <i>et al.</i> (2015) Mukhopadhyay <i>et al.</i> (2010)
	Phytoestrogens Genistein	Human Boar Mouse	1, 10, and 100 nM (4) 0.001, 0.01, 0.1, 1, 10, and 100 µM (4) 1, 10, 100, and 1000 nM (3–6)		Fraser <i>et al.</i> (2006) Mohamed el <i>et al.</i> (2011) Adeoya-Osiguwa <i>et al.</i> (2003)
	Pesticides Lindane	Human	0.1, 1, 5, 15, and 30 µM	Increased	Silvestroni & Palleschi (1999)
Acrosome integrity	p,p'-DDE Atrazine	Boar	1, 10, 25, and 50 µM (6) 8, 20, and 40 µM (5)	Decreased	Tavares <i>et al.</i> (2013) Maravilla-Galván <i>et al.</i> (2009)
	Fenoxaprop- ethyl Parathion Paraoxon PCBs	Mouse	12, 30, and 60 µM (5) 0.05–0.8 mM		Contreras <i>et al.</i> (1999)
	PCB54 Aroclor 1254 PAHs B[a]P	Human Rat Human	0.1–10 ng/ml 10^{-9} , 10^{-8} , and 10^{-7} M (6) 12.5, 25, 50, and 100 µg/ml	Increased Decreased	Roediger <i>et al.</i> (1989) Aly (2013) Mukhopadhyay <i>et al.</i> (2010)
	BPA BPA Phytoestrogens Genistein	Mouse Human Boar Mouse	0.0001, 0.01, 1, and 100 µM (3) 1, 10, and 100 nM (4) 0.001, 0.01, 0.1, 1, 10, and 100 µM (4) 1, 10, 100, and 1000 nM (3–6)		Rahman <i>et al.</i> (2015) Fraser <i>et al.</i> (2006) Mohamed el <i>et al.</i> (2011) Adeoya-Osiguwa <i>et al.</i> (2003)
	Mycotoxins ZEA	Boar	125, 187.5, and 250 µM (6)	Increased	Tsakmakidis <i>et al.</i> (2006)
	α-ZOL β-ZOL	Horse	125, 187.5, and 250 µM (6) 1 pM–100 µM (5)	Decreased	Filannino <i>et al.</i> (2011)

Table 1 Continued.

Sperm parameter/ feature	EDC	Species	Concentrations tested (no. of samples, replicates or experiments)	Main outcome	References
Intracellular Ca ²⁺ levels	Pesticides Lindane	Human	5, 10, 20, 30, 40, 50, and 60 µM (4)	Increased	Silvestroni & Palleschi (1999)
	p,p'-DDE		1 pM, 1 nM, 1, 10, 25, and 50 µM (5)		Tavares <i>et al.</i> (2013)
	p,p'-DDT		0.1, 1, and 10 µM (n=4–6)		Schiffer <i>et al.</i> (2014)
	Lindane		0.1, 1, and 10 µM (n=4–6)		
	Dieldrin		0.1, 1, and 10 µM (n=4–6)		
	Phthalates				
	DnBP				
	BBzP				
	DiBP		0.1, 1, and 10 µM (n=4–6)		
	DPP				
	Mycotoxins				
	α-ZOL		0.1, 1, and 10 µM (n=4–6)		
Viability	Pesticides	Human		Decreased	Grizard <i>et al.</i> (2007)
	Alachlor		0.18, 0.37, 0.90, and 1.85 mM (10)		Pant <i>et al.</i> (2013)
	β-HCH		76.94, 384.7, and 769.4 µg/l (3)		
	Lindane		39.04, 195.2, and 390.4 µg/l (3)		
	p,p'-DDD		57.49, 287.45, and 574.9 µg/l (3)		
	p,p'-DDE		53.89, 269.45, and 538.9 µg/l (3)		
			1, 10, 25, 50, and 100 µM (5)		Tavares <i>et al.</i> (2013, 2015)
	Deltamethrin	Rat	10, 50, 100, and 200 µM (8)		Abdallah <i>et al.</i> (2010)
	Dimethoate		50, 100, and 200 µM (10)		Abdallah <i>et al.</i> (2012)
	Diazinon	Boar	50, 100, and 500 µM (10)		Betancourt <i>et al.</i> (2006)
	Malathion				
	Atrazine				
	Fenoxaprop-ethyl		50, 100, and 500 µM (10)		
	Parathion	Mouse	0.05–0.8 mM		Contreras <i>et al.</i> (1999)
	Paraoxon				
	PCBs				
	Aroclor 1254	Rat	10⁻⁹, 10⁻⁸, and 10⁻⁷ M (6)		Aly (2013)
	Phthalates				
	DBP	Human	13.47, 67.35, and 134.7 µg/ml		Pant <i>et al.</i> (2011)
	DEHP		5.73, 28.65, and 57.3 µg/ml		
	Mycotoxins				
	ZEA	Boar	125, 187.5, and 250 µM (6)		Tsakmakidis <i>et al.</i> (2006)
	α-ZOL				
Sperm–oocyte interaction	PCBs				
	PCB77	Rat	0.01, 0.1, 1, and 10 µg/ml (3–5)	Decreased fertilization	Kholkute <i>et al.</i> (1994)
	BPA				
	BPA	Mouse	0.0001, 0.01, 1, and 100 µM (3)		Rahman <i>et al.</i> (2015)
	Phytoestrogens				
	Genistein	Mouse	100 nM (3)	Increased fertilization	Adeoya-Osiguwa <i>et al.</i> (2003)
		Bull	0.02, 0.2 , and 2 µg/ml	Decreased sperm–oocyte binding	Hinsch <i>et al.</i> (2000)

Concentrations in bold are the ones that induced the effects noted. BPA, bisphenol A; dioxins: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; micotoxins: ZEA, zearalenone; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol; α-ZAL, α-zearalanol; pesticides: β-HCH, β-hexachlorocyclohexane; PCB54, 2,2',6,6'-tetrachlorobiphenyl; PCB77, 3,3',4,4'-tetrachlorobiphenyl; phthalates: BBzP, benzyl butyl phthalate; DiBP, di-iso-butyl phthalate; DnBP, dibutyl phthalate; DPP, dipentyl phthalate.

organophosphates and alachlor (Table 1; Contreras *et al.* 1999, Grizard *et al.* 2007, Abdallah *et al.* 2010). Furthermore, co-incubation of spermatozoa with the potent antioxidants vitamin C and/or E has shown to prevent pesticide-induced effects (Abdallah *et al.* 2012).

Contrarily to organophosphates and pirethroids, organochlorine pesticides are extremely persistent

in the environment. Among them, the major and most stable dichlorodiphenyl trichloroethane (DDT) metabolite, p,p'-dichlorodiphenyl dichloroethylene (p,p'-DDE), has been the subject of multiple studies.

In vitro studies have shown that this compound, often used to control malaria-bearing mosquitoes in some countries (Aneck-Hahn *et al.* 2007), affects human sperm

function at concentrations found in reproductive fluids, potentially compromising male fertility (Tavares *et al.* 2013, 2015). Several parameters such as motility, MMP, cellular ATP levels and acrosomal integrity were found reduced upon exposure to p,p'-DDE whereas an elevation in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was attributed to the activation of the plasma membrane Ca^{2+} channel Catsper (Tavares *et al.* 2013, 2015). Overall, p,p'-DDE primarily promoted Ca^{2+} influx, which, due to its high and constant nature, may have impaired many cell parameters given that Ca^{2+} is heavily involved not only in acrosome reaction (which, if prematurely triggered, will render sperm non-functional) but also in several other signaling pathways (Jimenez-Gonzalez *et al.* 2006, Tavares *et al.* 2013, 2015). Accordingly, using p,p'-DDE doses found in human semen and concentrations that were five- and tenfold higher, Pant *et al.* (2013) have reported a significant concentration and time-dependent decline in motility after 24 h of p,p'-DDE exposure and decreased cell viability *in vitro*, although the latter was only observed at the higher doses tested. Furthermore, the same outcomes were found for the organochlorine pesticides β -hexachlorocyclohexane (β -HCH), lindane, and p,p'-dichlorodiphenyl dichloroethane (p,p'-DDD; Pant *et al.* 2013). Moreover, others have detected an increase in $[\text{Ca}^{2+}]_i$ due to Ca^{2+} influx from the surrounding media in human spermatozoa upon exposure to the organochlorine lindane. However, in this case, lindane was able to inhibit not only the acrosome reaction but also the sperm responsiveness to progesterone, a physiological agonist of the acrosome reaction (Silvestroni *et al.* 1997, Silvestroni & Palleschi 1999). Recently, the effects of almost 100 EDCs on human sperm were tested and not all were able to induce significant changes in intracellular Ca^{2+} levels. Moreover, those that did (e.g., pesticides, phthalates, mycotoxin, among others; Table 1) were not always acting via the Catsper channel (Schiffer *et al.* 2014). In accordance, others have found no effects on $[\text{Ca}^{2+}]_i$ upon bisphenol A and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure in human sperm (Luconi *et al.* 2001, Mota *et al.* 2012).

Fenoxaprop-ethyl, atrazine, and organophosphates such as parathion and its active metabolite paraoxon were also found to induce the acrosome reaction *in vitro* (Contreras *et al.* 1999, Maravilla-Galván *et al.* 2009). However, before spermatozoa become able to undergo the acrosome reaction, they must undergo capacitation, a process without which they do not become functionally competent, and little is known about the potential effects of pesticides in this process. Apart from p,p'-DDE and atrazine, which were able to suppress capacitation markers in human and boar sperm (Maravilla-Galván *et al.* 2009, Tavares *et al.* 2015), and fenoxaprop-ethyl, which was found to increase capacitation (Maravilla-Galván *et al.* 2009), to our knowledge no other *in vitro* studies have been performed. Nevertheless, the

concentrations used to obtain such findings cannot be considered environmentally relevant.

Dioxins, PCBs and PAHs

Polychlorinated dibenzo-p-dioxins

PCDDs are a well-established family of POPs commonly referred to as dioxins. The representative compound TCDD has been originated from industrial sources as an unintended by-product in the production of certain chlorophenols or chlorophenoxy acid herbicides, waste incineration, metal production and fossil fuel and wood combustion (Harnly *et al.* 1995). Considered the most powerful biological agent ever made by man, it interacts primarily with the aryl hydrocarbon receptor (AhR; Buchanan *et al.* 2000), a cytosolic-ligand transcription factor that upon binding translocates to the nucleus where it heterodimerizes with the aryl hydrocarbon nuclear translocator and elicits (anti)estrogenic responses in many tissues (Buchanan *et al.* 2000, Boverhof *et al.* 2006). In fact, TCDD has been used as a prototype for a great class of halogenated aromatic hydrocarbons on the basis that other related congeners will show the same toxicity of TCDD but with altered potency, determined by their relative AhR agonism and pharmacokinetics (Van den Berg *et al.* 2006).

Despite the extensive data generated over the years pointing toward male reproductive system susceptibility to dioxin exposure, in particular TCDD, studies have mostly focused on possible genomic effects (Mably *et al.* 1992, Jin *et al.* 2008, Mocarelli *et al.* 2008, 2011). Notwithstanding, Fisher *et al.* (2005) have described a dose-dependent loss of MMP in epididymal sperm due to increased ROS production in mice exposed to TCDD, an effect that was not perceptible in spermatozoa from AhR knockout mice but that was detected in mouse epididymal spermatozoa after 45 min of direct exposure to 1 and 5 nM TCDD. Taken together, these findings showed not only the involvement of AhR in mediating TCDD-induced oxidative stress in the male reproductive system but also that AhR is capable of mediating non-genomic TCDD-induced effects even though many of AhR-mediated effects involve alterations at the gene expression level (Fisher *et al.* 2005). However, contrarily to the results obtained by these authors, a recent study showed no alteration in human sperm mitochondrial function following 24 h of *in vitro* exposure to similar and higher TCDD doses (Mota *et al.* 2012). We may therefore infer that either mouse sperm mitochondria diverge greatly from their human sperm counterparts or human sperm are more resistant to TCDD, which is in fact suggested by the features of the human AhR, more closely related to the allele existent in TCDD-resistant DBA/2J mice (Mota *et al.* 2012). Additionally, no direct effects were observed on human sperm viability, capacitation, acrosome reaction and motility even at

1 μM TCDD (Mota *et al.* 2012), a concentration far higher than background levels (Tsutsumi *et al.* 1998). Accordingly, Hanf *et al.* (1992) also reported the lack of *in vitro* effects of various dioxin congeners on human sperm motility over a period of 60 h. Altogether, both reports seem to suggest that several aspects of human sperm functionality would not be compromised when in contact with naturally occurring levels of dioxins in both seminal fluid and female genital tract secretions (Hanf *et al.* 1992, Mota *et al.* 2012). It seems that the reported effects of TCDD on human sperm parameters that may affect male fertility may result from changes during spermatogenesis and/or during the transit through the male reproductive tract rather than through a direct exposure (Mota *et al.* 2012).

Polychlorinated biphenyls

PCBs are a class of compounds that include a total of 209 different congeners with varying patterns of toxicity. A small group of 12 congeners known as dioxin-like or co-planar PCBs resemble the TCDD molecular conformation, binding to AhR and presenting the greatest toxicity of all PCBs (Safe 1994). The non-dioxin-like congeners, on the other hand, although not believed to primarily interact with this receptor, are still able to induce toxic effects on diverse systems as demonstrated by manifold *in vivo* studies, potentially acting via the estrogen receptor (Hansen 1999, Pflieger-Bruss *et al.* 2006a).

For a long time, PCBs were being generated through industrial processes and used in hydraulic fluids, capacitors, lubricants, plasticizers, and electrical insulators (Hauser *et al.* 2002). Although banned, they continue to enter the environment due to leaks in equipment-containing PCBs still in use or inadequate clearance practices and via *de novo* formation through high-temperature procedures such as incineration.

Much concern has been raised regarding the reproductive health consequences of exposure to the environmental levels of PCBs as they have been associated with lower sperm quality (Bush *et al.* 1986, Hauser *et al.* 2002, 2003) and reduced couple fecundity (Buck Louis *et al.* 2013). Furthermore, although this was not true for every case, a number of human studies have pointed to inverse relationships between blood serum or seminal plasma PCBs within the range of current levels for most European populations and standard sperm parameters, particularly with sperm motility, which was found to be more vulnerable to PCB exposure than sperm concentration or morphology (Bush *et al.* 1986, Hauser *et al.* 2003). Supporting these findings, non-genomic studies have shown a decrease in motility (Table 1) after exposure to low levels of 2,2',6,6' tetrachlorobiphenyl (PCB54; Roediger *et al.* 1989) and 2,4,4'-trichlorobiphenyl (PCB28), 2,4,6-trichlorobiphenyl (PCB30), and 2,3',4,4',5-pentachlorobiphenyl (PCB118; Yurdakok *et al.* 2015) in human and bull spermatozoa. Moreover,

porcine sperm treated with an organochlorine mixture containing several PCB congeners plus a panoply of other compounds, including pesticides, were also found to reduce both motility and viability *in vitro* (Campagna *et al.* 2002). In sharp contrast, exposure to reasonably high concentrations (2–20 $\mu\text{g}/\text{ml}$) of the non-dioxin-like 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) and dioxin-like PCB congeners 118, 126 (3,3',4,4',5-pentachlorobiphenyl) and 77 (3,3',4,4'-tetrachlorobiphenyl) failed to produce any effect on human sperm movement, viability and spontaneous acrosome reaction after 5 h of incubation (Pflieger-Bruss *et al.* 2006a,b). Nevertheless, using a validated *in vitro* 3-day approach (Tavares *et al.* 2013, 2015) that better mimics sperm exposure to EDCs in the female reproductive tract, we found that PCB77 was able to reduce both motility and mitochondrial function in human normozoospermic samples before promoting cell death after 48 h of exposure at the highest concentration, as shown in Fig. 1. The fact that a greater incubation period was used may explain the discrepancies between these studies and highlights the types of experimental approaches that can be undertaken with human samples.

Besides affecting conventional sperm parameters and, in some cases, acrosomal status (Roediger *et al.* 1989), PCBs may also interfere with nuclear DNA integrity (Table 1). A potential mechanism whereby PCBs may produce such damage is oxidative stress, which has been already reported to occur in rat sperm exposed to Aroclor 1254, a commercial mixture of PCBs (Aly 2013). In fact, in this case, direct exposure to low Aroclor 1254 concentrations (10^{-9} , 10^{-8} , or 10^{-7} M) for 3 h not only induced oxidative stress and increased sperm DNA damage but also increased lipid peroxidation and decreased sperm motility, ATP levels, acrosome reaction, MMP and viability. Furthermore, Aroclor 1254 promptly promoted cytochrome c release and caspase-3 activation, thus culminating in mitochondria-mediated apoptosis (Aly 2013). Interestingly, Kholkute *et al.* (1994) have also reported an inhibition of fertilization rates when capacitated sperm and oocytes obtained from non-exposed mice were cultured *in vitro* in a PCB77-containing medium.

Polycyclic aromatic hydrocarbons

PAHs comprise a wide range of organic compounds with multiple aromatic rings. In general, PAHs are present in fossil fuels (oil and coal), tar deposits and overcooked food and are generated when incomplete combustion of organic material occurs. Exposure to these compounds has been correlated with a higher risk of infertility as men with idiopathic infertility and abnormal sperm parameters were shown to have increased levels of urinary PAH metabolites when compared to controls (Xia *et al.* 2009). Furthermore, non-genomic studies (Table 1) have shown that although modest effects on human sperm motility were observed after immediate

exposure to diesel particulate extracts, a marked alteration in all CASA parameters reflecting sperm movement and hyperactivation (motility, curvilinear velocity, straight line velocity, linearity, and amplitude of lateral head displacement) was elicited upon 18 h of exposure (Fredricsson *et al.* 1993).

Owing to its reprotoxic and carcinogenic potential, benzo[a]pyrene (B[a]P) has received much attention,

being widely used as a surrogate for PAH exposure. Studies performed with B[a]P have, so far, mainly focused on the formation of DNA adducts, which may subsequently lead to DNA damage. B[a]P may be converted into reactive metabolites, such as benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE) that binds covalently to DNA (Pelkonen & Nebert 1982), or can be metabolized by the aldoketo reductase pathway, promoting oxidative stress and the establishment of catechol DNA adducts (Penning 2004). Either way, metabolites react with a multitude of molecules and thus exposure to B[a]P may disturb many cellular processes. Previous reports probably reflecting the genomic effects of B[a]P have described DNA lesions in mouse sperm after *in vivo* treatment (Olsen *et al.* 2010) and similar non-genomic BPDE-induced damage in human sperm following *in vitro* exposure (Sipinen *et al.* 2010). Surprisingly, B[a]P and BPDE-related DNA fragmentation were also observed in human sperm *in vitro* as measured by the alkaline comet assay. The finding that DNA damage may occur without DNA-adduct formation following direct B[a]P exposure in human sperm suggests a B[a]P metabolism independent of BPDE conversion (Sipinen *et al.* 2010). Additionally, B[a]P was also able to directly increase the number of human spermatozoa with structural chromosome aberrations, but only when metabolically activated by rat liver S9 (Watanabe & Kamiguchi 2001).

However, not many studies have evaluated the effects of B[a]P on other sperm parameters that indicate functional status and fertilizing potential. With that in mind, Mukhopadhyay *et al.* (2010) found that B[a]P directly affected sperm functional competence in human normozoospermic samples as evidenced by increased hyperactivation and premature acrosomal reaction *in vitro*. Furthermore, high exposure to B[a]P (500 μ M, 1 h) was also found to directly increase human sperm

Figure 1 Effect of continuous exposure to different PCB77 concentrations on sperm parameters as an example of experimental *in vitro* approaches using human samples. (A) Viability, (B) high mitochondrial membrane potential (MMP), and (C) total motility (progressive + *in situ* motility) were assessed by the LIVE/DEAD Sperm Viability Kit, mitochondrial JC-1 dye (Molecular Probes, Eugene, OR, USA) and phase contrast microscopy respectively (Tavares *et al.* 2015). Human normozoospermic samples were continuously exposed to PCB77 for 3 days at 37 °C and 5% CO₂ in a PBS-based medium supplemented with 0.9 mM CaCl₂, 0.5 mM MgCl₂, 5 mM D-glucose, 1.0 mM Na-pyruvate, 10.0 mM Na-lactate, 0.3% (w/v) BSA, and 1% (v/v) penicillin/streptomycin, pH 7.2–7.4, optimal to maintain spermatozoa for extended periods (Tavares *et al.* 2013, 2015). Solvent controls were performed by adding 0.5% (v/v) DMSO. Daily assessments were performed for each parameter in the same samples ($n=6$) and medium was changed every day following centrifugation (528 g, 10 min). Interestingly, sperm motility was affected prior to high MMP decline. Results represent mean percentage $\pm$ s.e.m. relative to the respective control (i.e. % in each PCB77 concentration/% in the vehicle DMSO $\times 100$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ symbolize significant differences when compared to the controls.

intracellular ROS and induce nuclear DNA damage, effects that were significantly counteracted with the addition of a Chilean propolis extract described to possess antioxidant properties (Russo *et al.* 2006). Yet, membrane lipid peroxidation was unaffected upon B[a]P treatment (Russo *et al.* 2006).

Phthalates and bisphenol A

Other compounds considered of interest in male reproduction are the diesters of 1,2-benzenedicarboxylic acid (phthalic acid), commonly identified as phthalates. They are usually used as plasticizers in a wide range of polyvinyl chloride products, including raincoats and footwear, toys, food packing and medical devices, and as solvents in personal care goods, coatings, and polishes (Fredricsson *et al.* 1993, Pant *et al.* 2011). Owing to their widespread use and non-covalent binding to products, phthalates can be constantly released and easily dispersed in the environment, being detected in individual populations worldwide (Duty *et al.* 2003, Pant *et al.* 2011, Jurewicz *et al.* 2013). Although these compounds have short half-lives (hours) and do not accumulate in the body, chronic exposure to low levels obtained through diet and commonly used products can provide continuous steady state concentrations in the living organisms (Duty *et al.* 2003).

In *in vivo* animal studies, exposure to phthalates has shown to adversely affect male reproduction, but data in humans have been inconsistent. Although a number of studies have associated urinary and seminal phthalate levels to a deterioration of important sperm parameters in environmentally exposed men, others failed to detect any alterations (Duty *et al.* 2003, Jonsson *et al.* 2005, Pant *et al.* 2011, Jurewicz *et al.* 2013). As significant temporal within-person variation has been described in metabolite concentrations determined in spot, first morning and 24-h urine samples, this discrepancy may be attributed, at least in part, to both variable phthalate levels and their short half-lives (Preau *et al.* 2010). To this extent, *in vitro* studies are an excellent option to study the effects of phthalate exposure and are also crucial in verifying their potential non-genomic actions in spermatozoa. As such, Pant *et al.* (2011) have tested the effects of two specific phthalates (di(2-ethyl-hexyl) phthalate (DEHP) and di-*n*-butyl phthalate (DBP)) on human sperm motility and viability using the concentrations detected in seminal samples from epidemiological findings (5.73 and 13.47 µg/ml for DEHP and DBP respectively) and concentrations that were five- or tenfold higher. They found that both EDCs induced a concentration- and time-dependent decrease in sperm viability, but cytotoxicity was only obvious at the maximum concentrations tested and following 96 h of exposure. However, when motility was monitored, a reduction at all DEHP doses was observed only after 12 h of *in vitro* exposure, but for DBP the same effect was only detected after an

18-h incubation period. Consistently, a more than 50% inhibition of motility was detected after 24 and 48 h of exposure to the environmental DEHP and DBP concentrations, respectively, confirming the higher toxicological potential of DEHP (Pant *et al.* 2011). Fredricsson *et al.* (1993) have also studied the effects of various phthalates on human sperm motility using a CASA system and observed that the various movement variables analyzed (curvilinear velocity, straight line velocity, and linearity) were differently influenced by the different phthalates. Additionally, they concluded that human normozoospermic samples were more susceptible to the action of DEHP and DBP *in vitro*, with the former being the most potent. In contrast, spermatozoa were found to better resist dimethyl phthalate (DMP) exposure than di-ethyl phthalate (DEP) and di-*n*-octyl phthalate (DOP).

Importantly, recent data have also indicated the presence of important levels of phthalates such as DEHP and its hydrolyzed metabolite mono(2-ethylhexyl) phthalate not only in media used for sperm washing and isolation but also in media used to perform IVF and in added protein sources (human serum albumin or serum substitute; Takatori *et al.* 2012). Such evidence reinforces the need of assessing the effects of phthalates *in vitro* on several sperm functional parameters other than motility, viability and, recently, Ca²⁺ levels (Table 1), which to our knowledge were the only parameters evaluated so far.

Another compound with potential estrogenic activity is bisphenol A, which is used in epoxy resins and polycarbonate plastics needed to produce baby bottles and plastic containers, among other products. Similarly to phthalates, BPA is heavily produced annually and can constantly leach into the environment.

In vitro studies have shown no effect of BPA in inducing DNA damage (Bennetts *et al.* 2008), altering [Ca²⁺]_i and affecting spontaneous or progesterone-induced acrosome reaction in human sperm directly (Luconi *et al.* 2001). Nevertheless, in a very recent study Rahman *et al.* (2015) have shown that high BPA exposure (100 µM) for a relative short period of time directly decreased motility and motion kinematics by reducing ATP levels in mice sperm *in vitro*. Furthermore, a decrease in capacitation and premature acrosome reaction were found at 100 µM BPA, ultimately resulting in decreased fertilization and blastocyst formation rates (Table 1).

Natural compounds: sources of exposure and effects on spermatozoa

Phytoestrogens and mycotoxins

Among the natural-occurring EDCs, phytoestrogens and mycotoxins have been the most studied. Phytoestrogens are known to be produced by plants and are mainly

consumed through diet because they are present in legumes, fruits, cereal, nuts, and oilseeds (Mazur & Adlercreutz 2000). These compounds have raised a certain level of concern due to the fact that they are able to bind to estrogen receptors, acting as agonists or as antagonists (Mohamed el et al. 2011). Because of the high prevalence and risk of exposure to such compounds, several studies have been performed to assess their possible effects on male fertility.

One of the most studied phytoestrogens is genistein, an isoflavone found in soy and legumes (Mazur & Adlercreutz 2000). Genistein exposure in human adult populations worldwide may vary according to individual eating habits, but in a rich-consuming soy diet, levels have been determined to range between 1.5 and 4.1 mg/day per person (Fukutake et al. 1996).

It has been shown that low doses of genistein *in vitro* are able to accelerate sperm capacitation and acrosome reaction *in vitro* (Table 1), not only in humans (Fraser et al. 2006) but also in mice (Adeoya-Osiguwa et al. 2003) and boar (Mohamed el et al. 2011). Because prematurely acrosome-reacted sperm cannot fertilize an oocyte, this compound could have an undesirable effect on fertility. On the other hand, Hinsch et al. (2000) have also shown that genistein may inhibit progesterone-induced acrosome reaction and binding to the oocyte zona pellucida, as determined by the hemizona assay, however, without compromising motility and viability in cryopreserved bull sperm. Nevertheless, IVF experiments using mouse spermatozoa that were pre-incubated with 0.1 μ M genistein have also been shown to increase the percentage of fertilized oocytes determined by the resumption of the second meiotic division and the presence of decondensed sperm heads, therefore suggesting the stimulation of sperm fertilizing ability (Adeoya-Osiguwa et al. 2003).

Mycotoxins, on the other hand, are secondary metabolites produced by some fungi, known as molds (Richard 2007), and, as is the case for phytoestrogens, animal and human populations are exposed to such compounds via dietary intake (Massart & Saggese 2010). Some of the main mycotoxins are aflatoxins, deoxynivalenol, fumonisins, zearalenone (ZEA), T-2 toxin, ochratoxin, and certain ergot alkaloids (Richard 2007). The most studied mycotoxin, ZEA, is a nonsteroidal estrogenic mycotoxin produced by *Fusarium* fungi (Zinedine et al. 2007) that contaminates corn, maize, barley, oats, wheat, sorghum, sesame, millet, and rice (Massart & Saggese 2010). On oral intake, ZEA is rapidly absorbed and metabolized in the liver and intestine into different metabolites such as α - and/or β -zearalenol (α - and β -ZOL), α - and/or β -zearalanol, and zearalanone (ZAN), which can be conjugated with glucuronic acid (Massart & Saggese 2010). Like phytoestrogens, ZEA has a similar structure to E_2 , acting as an estrogen receptor agonist (Sambuu et al. 2011). The effect of this mycotoxin has been studied in species that are the

most affected by ZEA such as porcine (Tsakmakidis et al. 2006, Sambuu et al. 2011) and equine (Minervini et al. 2010, Filannino et al. 2011). The *in vitro* studies performed in boar have reported the toxic effects of ZEA and its derivatives on sperm quality and function (Table 1), impairing its fertilizing capacity. Tsakmakidis et al. (2006) showed that exposure to ZEA led to a reduction in sperm viability, progressive motility and the percentage of sperm capable to undergo acrosome reaction. Moreover, in 2008, the same group revealed that ZEA also affected sperm chromatin integrity (Tsakmakidis et al. 2008). Contrarily to boar, horse sperm was thought to be less susceptible to the effects of ZEA. However, it has been found that the ZEA derivate, α -ZOL, decreased the percentage of motile sperm, changed the motility characteristic of the remaining motile sperm and induced the acrosome reaction (Filannino et al. 2011). Furthermore, a different study on chromatin structure stability showed that ZEA, α -ZOL, and β -ZOL are more toxic than the other derivatives on equine sperm chromatin structure (Minervini et al. 2010).

Conclusions and future perspectives

Many EDCs have been shown to interact with pre-existing signaling pathways in spermatozoa from diverse mammalian species and produce undesirable effects, contributing to a decrease in male fertility. Indeed, EDCs may affect sperm parameters such as motility, mitochondrial function, acrosomal status and/or DNA integrity, among others, via different modes of action, and this will inevitably have repercussions in terms of fertilization success and proper delivery of intact genetic information to the progeny.

Although little is known about EDC levels in reproductive fluids and secretions in heavily exposed populations (for instance, due to extremely high contamination, e.g., the Seveso disaster, or occupational exposure), they are expected to be higher than in populations exposed to environmental levels. Regardless, even though some authors may have used fairly higher concentrations in their *in vitro* experimental settings, one should always keep in mind that human and animal populations (and therefore spermatozoa) are not directly exposed to one but rather to a cocktail of environmental EDCs (Tsutsumi et al. 1998, Younglai et al. 2002, Drbohlav et al. 2005) and limited evidence exists about the possible synergistic effects of such toxicants on sperm functional parameters, an issue that should be seriously taken into account in further studies, although the unveiling of specific mechanistic effects of each individual EDC may be dampened in such experimental settings. Thus, when acting together, more environmentally relevant concentrations of several EDC may eventually hamper human sperm function and thus compromise male fertility.

Finally, studies performed in rodents have shown a new mode of EDC action with worrisome conclusions: EDCs may have the ability to reprogram the male germline during fetal gonadal sex determination and promote a transgenerational effect in which initial exposure effects may be transmitted to future generations in the absence of further exposure (Anway *et al.* 2006). Although there may be strain-specific effects, it has been postulated that EDCs may promote a permanent alteration in the germline epigenome that escapes epigenetic reprogramming at fertilization and early development. This germline epigenetic inheritance could alter the embryonic epigenome so individuals will be susceptible to develop adult onset diseases across generations, such as testis pathologies and a reduced fertilizing capability phenotype (Anway *et al.* 2006), meaning that aspects of male infertility may be perpetuated across multiple generations. This represents a totally new and promising research field that remains largely unexplored. Whether this occurs in humans is something that still needs to be verified.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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