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Effect of the herbicide atrazine and its metabolite DACT on bovine sperm quality

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ABSTRACT

Atrazine (ATZ), one of the most extensively used herbicides, is considered a ubiquitous environmental contaminant. ATZ is a known endocrine disruptor, and deleterious effects on reproductive function have been shown, even at low, ecologically relevant doses (0.1–3 μg/L). Once it enters the body, ATZ is metabolized to various metabolites, which are further detected in the urine, serum and tissues. In mammals, the major ATZ metabolite is diaminochlorotriazine (DACT). The current study focuses on direct effects of low doses of ATZ and DACT on bovine sperm isolated from ejaculates or epididymis compartments (head, body and tail). Sperm were incubated under capacitation conditions with or without 0.1–10 μM ATZ or 1–100 μM DACT. The integrity and functionality of sperm membranes (plasma, acrosomal and mitochondrial) were examined simultaneously by fluorescence staining at 0, 2 and 4 h of incubation. Acrosome reaction (AR) was induced by Ca^{++} ionophore, after capacitation. The findings indicated that both ATZ and DACT adversely affect sperm, expressed by damaged sperm membranes. ATZ had a prominent effect on epididymal-tail sperm, expressed as disruption of all examined membranes, mostly at low (0.1 or 1 μM) concentrations; pseudo-AR and that induced by Ca^{++} ionophore were both affected by exposure to 0.1 μM ATZ ($P < 0.05$ and $P < 0.00004$, respectively). A similar pattern was documented for sperm isolated from ejaculates ($P < 0.002$ and $P < 0.001$, respectively). ΔYm was affected by ATZ in sperm isolated from the epididymis tail (1 μM, $P < 0.0009$), but not in that isolated from ejaculates. DACT reduced sperm viability at all examined concentrations and in all fractions. DACT at 1 μM impaired ΔΨm in sperm isolated from the epididymis tail and ejaculate ($P < 0.005$). DACT at 100 μM did not induce pseudo-AR in sperm isolated from the ejaculate, but did in sperm isolated from the epididymis tail ($P < 0.05$). Induction of AR by Ca^{++} ionophore was impaired in sperm isolated from ejaculate and exposed to 10 or 100 μM DACT ($P < 0.05$) and in sperm isolated from the epididymis tail and exposed to 1, 10 or 100 μM DACT ($P < 0.0004$). These findings reveal the harmful effect of exposure to ATZ and DACT, mainly at low ecologically relevant doses, on sperm viability, AR and mitochondrial function. We conclude that sperm at advanced stages of spermatogenesis, through its passage and storage in the epididymis compartments as well as in the ejaculate, is sensitive to herbicide. The results suggest that ATZ- or DACT-induced disruptions of sperm membranes might impair sperm fertilization competence.

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1. Introduction

Atrazine (ATZ) is one of the best known chlorotriazine herbicides, extensively used to control growth of broadleaf and grassy weeds in agricultural crops [1]. ATZ is considered a ubiquitous environmental contaminant, as it is frequently detected in ground and surface water, as a result of its mobility in soil [2]. It is estimated that people who use ground water as their primary drinking-water source are exposed to at least 0.2 ppb (i.e. μg/L) of ATZ [3]. ATZ can even be found in regions where it is not used, in the ground water (21 ppb), surface water (42 ppb), and up to 40 ppb in rainfall agricultural areas [4]. ATZ use has been banned in Europe since 2004, but it is still utilized in about 70 countries, including the USA, Brazil, Argentina, Mexico, China and Israel [5]. The maximal level of ATZ allowed in the drinking water in the USA is 3 ppb and in Europe, 0.1 ppb.

Some trace amounts of ATZ can be transferred to the human circulation via the food chain [6,7]. For instance, it has been detected in

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human amniotic fluid (0.6 μg/L) and urine (0.1 μg/L) [8]. Wildlife and domestic animals can be exposed to this herbicide via consumption of contaminated food or water, inhalation of pesticide spray or absorption through the skin. The endocrine-disruptive effects of triazines and their metabolites have been shown in mammalian and aquatic species [9]. Several studies have suggested that as an endocrine disruptor, ATZ can alter reproductive function in different species, such as amphibians and rats, even at low, ecologically relevant doses (0.1–3 μg/L). ATZ has a demasculinization/feminization effect in amphibians [10] and has been shown to disrupt reproductive tract development and function in rodents by altering steroid levels [9,11]. Testicular lesions, associated with reduced germ cell numbers, were observed in fish, amphibians, reptiles and mammals exposed to ATZ [1]. Exposure primary cultures of Leydig cells as well as in BLTK1 murine Leydig cells to triazines altered the expression of genes associated with steroidogenesis [3,9]. Other studies have shown that ATZ elicits a depletion of the antioxidant defense system in mice and rat testis, indicating an oxidative stress [1,12–14]. ATZ inhibit mitochondrial function in human sperm by binding to F_1F_0–ATP synthase [15] and found to be toxic to the mitochondria in human liver carcinoma (HepG2) and rat skeletal muscle (L6) cell lines, via downregulation of TFAM and SIRT1 genes which involve in mitochondrial function [16]. In mice, ATZ exposure interfered with normal meiosis and thus affected spermatozoa production [17]. Moreover, ATZ was shown to reduce progressive motility in porcine sperm [18] and increase spontaneous AR in boar sperm [19].

Once it enters the body, ATZ is metabolized in the liver by P450 enzymes into various metabolites, detected in the urine and serum [2,20,21]. In mammals, the major and most frequently detected metabolite is diaminochlorotriazine (DACT), shown to induce oxidative stress and disrupt endocrine function [9,22]. DACT forms covalent adducts with various proteins, presumably as a chemical-induced toxicity step [23]. However, less is known about direct effects of DACT on sperm during spermatogenesis.

Spermatogenesis occurs throughout the adult male's life. The process requires approximately 1 month in mice, 2 months in humans and 61 days in bulls [24]. Spermatozoa that have completed morphogenesis are moved from the testicular tube to the rete testis and then transferred to the epididymis. In the epididymis compartments, caput (head), corpus (body) and cauda (tail), the sperm undergoes maturation, in a stage-dependent manner [25–27], acquiring motility and fertilization competence through a series of post-translational modifications [27,28]. Finally, the mature sperm are stored in the tail of the epididymis [29]. In the female reproductive tract, the ejaculated spermatozoa undergo further maturation, known as "capacitation", a crucial process through which spermatozoa acquire the ability to bind to the oocyte's zona pellucida (ZP) and subsequently undergo AR [27,30]. Both capacitation and AR are essential processes for fertilization [31,32]. Given the intensive changes occurring during the continuous and lengthy process of spermatogenesis, it is reasonable to assume that spermatozoa are sensitive to environmental stressors, such as ATZ, at various developmental stages. Gely-Pernot et al. [17] recently reported that ATZ affects the epigenetic process of meiosis in male mice. Iinterruption of meiosis, a key step in gametogenesis, may lead to the production of abnormal spermatozoa and the reduced sperm quality as found in ATZ-treated animals [33,34]. Nevertheless, the mechanisms underlying these effects are not clear. Moreover, less is known about the direct effects of ATZ and its major metabolite DACT at advanced stages of spermatogenesis i.e., on the epididymis-stored sperm or the ejaculate itself.

In the current study, we examined, in vitro, the effects of low doses of ATZ and DACT on both capacitation and AR in bovine sperm isolated from epididymis compartments (head, body and tail) and ejaculate. In-vitro capacitation designed to mimic sperm capacitation in the female oviduct was achieved by providing specific culture conditions [35]. Spontaneous or pseudo-AR (i.e., AR that is induced without a known, controlled stimulation) [36] was recorded. In-vitro induction of AR was performed after 4 h of in-vitro capacitation by Ca^{++} ionophore [37–39]. We therefore studied the degree of pseudo-AR vs. induced AR [19] and found it to be an adequate approach to examining stimulatory or inhibitory effects of environmental compounds.

2. Materials and methods

2.1. Reagents and materials

All reagents were purchased from Sigma (Rehovot, Israel), unless otherwise specified. Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine (ATZ; lot #421-55A, purity: 98.9%) and diaminochlorotriazine; 2-chloro-4,6-diamino-1,3,5-triazine (DACT; lot# 404-99A, purity: 96.7%) were purchased from Chem Service Inc. (West Chester, PA, USA). A 1000 mM stock solution of ATZ was prepared in absolute EtOH and a 10 mM stock solution of DACT was prepared in dimethyl sulfoxide (DMSO) according to the manufacturer's instructions (Sigma).

Sperm membrane integrity was evaluated using fluorimetric probes: (1) double stranded DNA by 4′,6-diamidino-2-phenylindole (DAPI); (2) plasma membrane integrity by propidium iodide (PI); (3) AR by fluorescein isothiocyanate-conjugated *Pisum sativum agglutinin* (FITC–PSA); mitochondrial membrane potential ($\Delta\Psi m$) by 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide fluorescent probe (JC-1; ENZOBiochem, New York, NY, USA).

2.2. Sperm preparation

Bovine sperm was supplied by "SION" Artificial Insemination Center (Hafetz-Haim, Israel). All of the experiments were performed in accordance with the 1994 Israeli guidelines for animal welfare. Ejaculated bull sperm was obtained with an artificial vagina, and the "swim up" technique was applied to obtain motile sperm. Sperm cells were washed three times by centrifugation (600*g* for 10 min at 25 °C) in NKM buffer (110 mM NaCl, 5 mM KCl, 20 mM MOPS [3-N-morphilino propanesulfonic acid, pH 7.4]) and allowed to swim up after the last wash. The washed cells were counted and maintained at 39 °C until use. Only semen that contained at least 80% motile sperm cells were used in the experiments.

2.3. Extraction of epididymal spermatozoa

Bovine testes were brought from the slaughterhouse in 4 °C saline solution. The epididymis was recovered from testes immediately upon arrival to the laboratory. Thereafter, the epididymis head, body and tail were dissected to facilitate sperm release from each individual compartment. The epididymis content was transferred into tubes and washed twice by centrifugation (600*g* for 10 min at 25 °C) in NKM buffer. The washed cells were counted and maintained at 39 °C until use.

2.4. Sperm capacitation

In-vitro capacitation of bovine sperm was induced as described previously [40,41]. Briefly, sperm pellets were resuspended to a final concentration of 10^8 cell/mL in mTALP (modified Tyrode solution containing 100 mM NaCl, 3.1 mM KCl, 1.5 mM $MgCl_2$, 0.92 mM KH_2PO_4, 25 mM $NaHCO_3$, 20 mM HEPES [pH 7.4], 0.1 mM sodium pyruvate, 21.6 mM sodium lactate, 10 IU/mL penicillin, 1 mg/mL BSA, 20 μg/mL heparin, 2 mM $CaCl_2$). Cells were incubated in mTALP for 4 h at 39 °C with 5% CO_2. Sperm capacitation state was

confirmed by examining the ability of the sperm to undergo AR, induced by addition of 20 μM Ca^{++} ionophore A23187 for an additional 20 min of incubation.

Note that sperm from epididymis head and body do not have the ability to capacitate [30]. Thus, only sperm retrieved from the epididymis tail or ejaculate was subjected to in-vitro capacitation.

2.5. Treatments

The experiments were performed separately for ATZ and DACT. Sperm (10^8 cell/mL) was exposed to 0.1, 1 or 10 μM final concentration of ATZ, or 1, 10 or 100 μM final concentration of DACT. These concentrations were guided by data from well-known experimental model studies [9,42–46]. Following the manufacturer's instructions (Sigma), ATZ was dissolved in EtOH (0.4% maximal concentration) and DACT was dissolved in DMSO (0.01% maximal concentration). Neither of these vehicles was found to have a deleterious effect on sperm viability at the final concentrations used in the current study. Sperm incubation was performed in mTALP with or without ATZ or DACT at 39 °C under an atmosphere of 5% CO_2 in air. Analysis of sperm was performed after 0, 2 and 4 h of incubation, and an additional 20 min incubation with 20 μM Ca^{++} ionophore as a positive control for AR.

2.6. Simultaneous fluorimetric assessment of sperm membranes

Simultaneous fluorimetric assessment of sperm membranes (plasma, acrosomal and mitochondrial) was performed as described previously [47], with some modifications. Briefly, sperm pellets were resuspended to a final concentration of 25×10^6 cell/mL in mTALP. A 150-μL aliquot of semen diluted in mTALP medium was put into a warmed microcentrifuge tube. DAPI (17 μL of a 0.1 mg/mL solution) was added and the sample was incubated for 10 min at 37 °C. The sample was then centrifuged and 100 μL mTALP medium was added to the pellet. In addition, 3 μL of PI (0.5 mg/mL), 2 μL of JC-1 (153 μM) and 50 μL of FITC–PSA (1 mg/mL) were added. The sample was incubated for 10 min at 37 °C, then centrifuged and the pellet was resuspended in 40 μL mTALP medium. A 10-μL sample was put on a glass slide, coverslipped and immediately evaluated by epifluorescent microscopy (Nikon Eclipse, TE-2000-u, Tokyo, Japan) using Nis Elements software (Nikon, Tokyo, Japan) and equipped with a digital camera (DXM1200F; Nikon, Tokyo, Japan), with excitation at 450–490 nm and emission at 515–565 nm using a triple filter. At least 200 sperm cells were examined per slide and classified based on the fluorescence emitted from each probe. The cells were scored by a single skilled individual using ImageJ software (version 1.47 v, Wayne Rasband, National Institutes of Health, USA) and manual counting (Fig. 1).

2.7. Statistical analysis

Statistical analysis was performed separately for the ATZ and DACT experiments and did not include cross-comparison between the examined compounds or vehicles. All values for incidence of the various classes of spermatozoa in the fluorimetric assessment were converted to percentages and normalized to T0 (time 0, preincubation time point) before analysis. Data were analyzed by JMP-7 software (SAS Institute Inc., 2004, Cary, NC, USA) and an ANOVA model using concentration, time and their interaction as fixed effects, and bull as a random effect. Posthoc comparisons were performed by Contrast *t*-test (*LS-MEANS* Student's *t*-test). Data are expressed as mean ± SD of the percentages. For all analyses, $P < 0.05$ was considered significant; *P*-values between 0.05 and 0.1 were also reported as trends that might be real and worthy of note. For each set of experiments, sperm samples from at least three bulls were tested.

3. Results

As already noted, the maximal concentrations of the solvents used as vehicles (0.4% for EtOH and 0.01% for DMSO) did not have any significant effect on any of the examined parameters when tested as solvent-effect controls.

3.1. Effect of ATZ on bovine sperm

3.1.1. Ejaculated sperm

Exposure to ATZ (0.1, 1 or 10 μM) for 4 h increased the proportion of dead sperm relative to the control group (34.5 ± 2.4, 32.9 ± 4.4 and 33.7 ± 5.8 vs. 14.2 ± 0.9%, respectively, $P \leq 0.05$; Fig. 2A). All the three doses similarly increased the proportion of dead sperm. ATZ had no significant effect on ΔΨm of sperm isolated from ejaculate (Fig. 2B). Exposure to ATZ (0.1 or 1 μM) for 2 h increased the proportion of sperm that underwent a pseudo-AR relative to the control (40.3 ± 15.8 and 36.8 ± 13.0 vs. 17.4 ± 6.6%, respectively, $P \leq 0.002$; Fig. 2C). On the other hand, exposure to ATZ (0.1 or 1 μM) for 4 h subsequently decreased the proportion of sperm that responded to Ca^{++} ionophore, expressed as a reduced proportion of acrosome-reacted cells relative to the control (35.3 ± 8.0 and 30.6 ± 10.2 vs. 54.5 ± 4.9%, respectively, $P \leq 0.001$; Fig. 3A)

3.1.2. Effect of ATZ on sperm isolated from the epididymis

ATZ increased the proportion of sperm with damaged plasma membranes in a dose-responsive manner (Fig. 4). Nevertheless, it did not affect the integrity of the plasma membrane in sperm isolated from the head of the epididymis (Fig. 4A'). Sperm isolated from the body of the epididymis were affected only at the highest ATZ concentration (10 μM), expressed by a higher proportion of dead cells relative to the control group (14.1 ± 1.3 vs. 6.8 ± 2.6%, respectively, $P \leq 0.006$). The rate of dead sperm at 2 h did not differ from that recorded at 4 h of incubation (Fig. 4A″). The most prominent effect was recorded for sperm isolated from the tail of the epididymis and exposed to 0.1, 1 or 10 μM ATZ for 2 h, relative to the control (21.2 ± 6.9, 25.0 ± 11.3 and 28.1 ± 9.9 vs. 12.5 ± 3.0%, respectively, $P \leq 0.001$; Fig. 4A‴).

Exposure to 1 μM ATZ for 4 h significantly increased the ΔΨm of sperm isolated from the tail of the epididymis, relative to the control (2.8 ± 1.1 vs. 0.4 ± 0.2, $P \leq 0.0009$; Fig. 4B).

Exposing sperm to ATZ (0.1, 1 or 10 μM) significantly elevated the proportion of sperm that underwent pseudo-AR after 2 h of capacitation, relative to the control group (38.8 ± 5.2, 42.4 ± 5.5 and 44.8 ± 9.2 vs. 23.7 ± 4.9%, respectively, $P \leq 0.05$; Fig. 4C). The same pattern was recorded after 4 h of incubation with 0.1, 1 or 10 μM ATZ, relative to the control group (35.3 ± 23.9, 40.5 ± 5.0 and 40.2 ± 6.6 vs. 26.2 ± 1.7%, respectively, $P \leq 0.0003$; Fig. 4C). ATZ also decreased the proportion of sperm that responded to Ca^{++} ionophore, expressed by a reduced rate of induced AR relative to the control group (24.7 ± 6.1, 27.4 ± 1.8 and 23.6 ± 5.9 vs. 39.2 ± 1.4%, respectively, $P \leq 0.00004$; Fig. 3B Table 1).

3.2. Effect of DACT on bovine sperm

3.2.1. Effect of DACT on ejaculated sperm

Incubation of sperm with 10 or 100 μM DACT increased the proportion of dead sperm relative to the control group after 2 h incubation (32.3 ± 3.2 and 30.8 ± 3.2 vs. 8.8 ± 3.3%, respectively, $P \leq 0.05$), and after 4 h incubation (31.6 ± 1.6 and 46.7 ± 4.6 vs.16.4 ± 1.3%, respectively, $P \leq 0.05$; Fig. 5A).

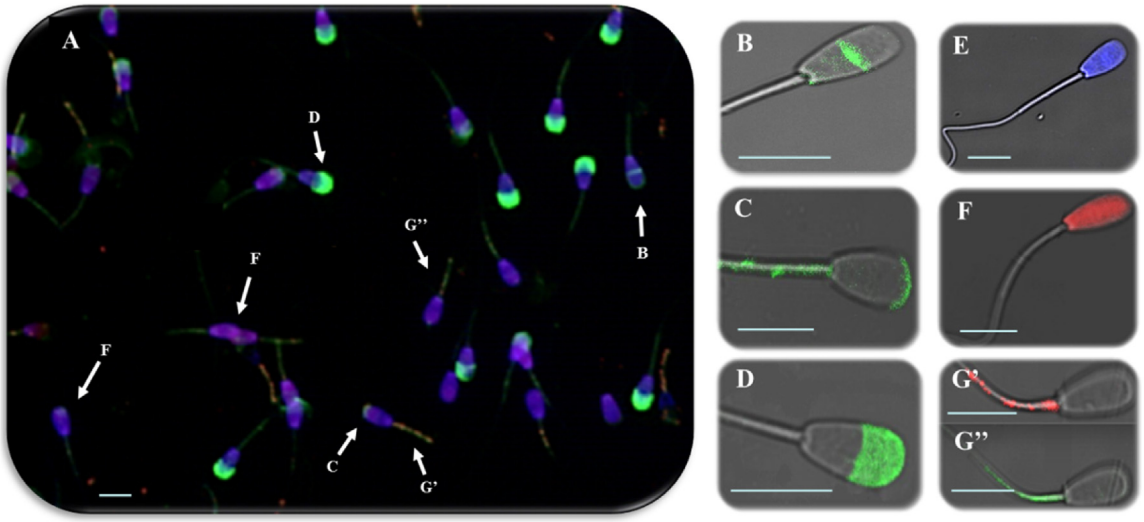

Fig. 1. Epifluorescence photomicrography of sperm cells stained with fluorescent probes. (A) Simultaneous staining with the four probes: propidium iodide (PI), 4′,6-diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC–PSA) and 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide fluorescent probe (JC-1). (B) Acrosome-reacted sperm cell with residual equatorial staining. (C) Acrosome-reacted sperm cell with residual upper staining. (D) Acrosome-damaged sperm cell with stained acrosome. (E) Sperm cell nucleus staining. (F) Dead sperm cell with damaged plasma membrane. (G′) Sperm cell with high $\Delta\Psi$m. (G″) Sperm cell with low $\Delta\Psi$m. Scale bars = 10 μM.

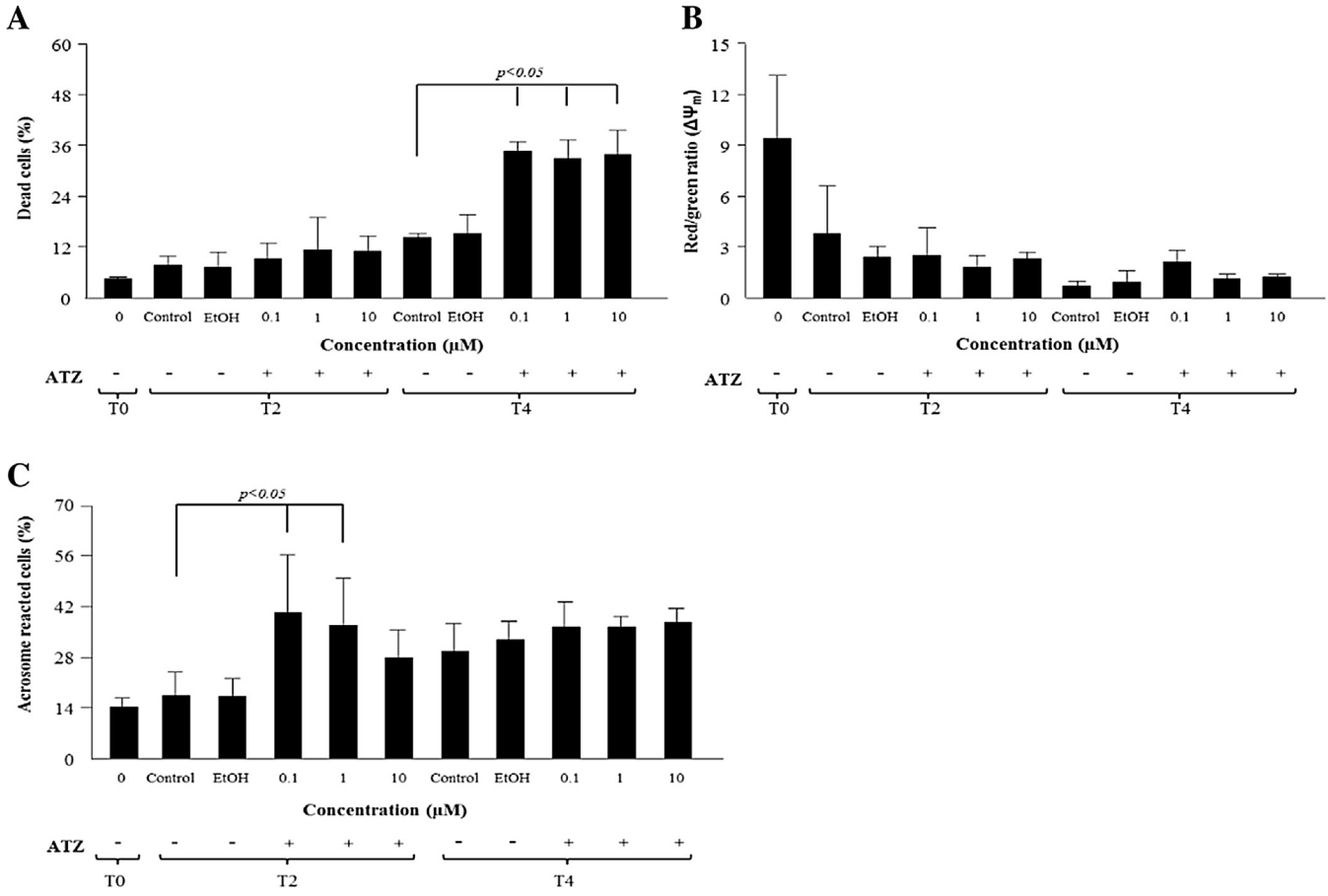

Fig. 2. Effect of ATZ on ejaculated sperm. Spermatozoa were incubated for 4 h with 0.1, 1 or 10 μM ATZ dissolved in EtOH. (A) Sperm viability was determined with PI fluorescent probe. (B) Mitochondrial membrane potential ($\Delta\Psi$m) was determined with JC-1 fluorescent probe and presented as the mean proportion of red (high potential)/green (low potential) stained sperm. (C) Acrosome reaction was determined with FITC–PSA fluorescent probe. Data are presented as the mean proportion ± SD of the examined cells, calculated for 3 replicates, with at least 200 sperm/group examined.

The effect of DACT on $\Delta\Psi$m was characterized by a bidirectional pattern. The proportion of sperm with high $\Delta\Psi$m decreased sharply in groups exposed to 1, 10 or 100 μM DACT for 2 h relative to the control group (1.7 ± 0.3, 2.3 ± 1.8 and 1.8 ± 1.3 vs. 6.3 ± 0.6,

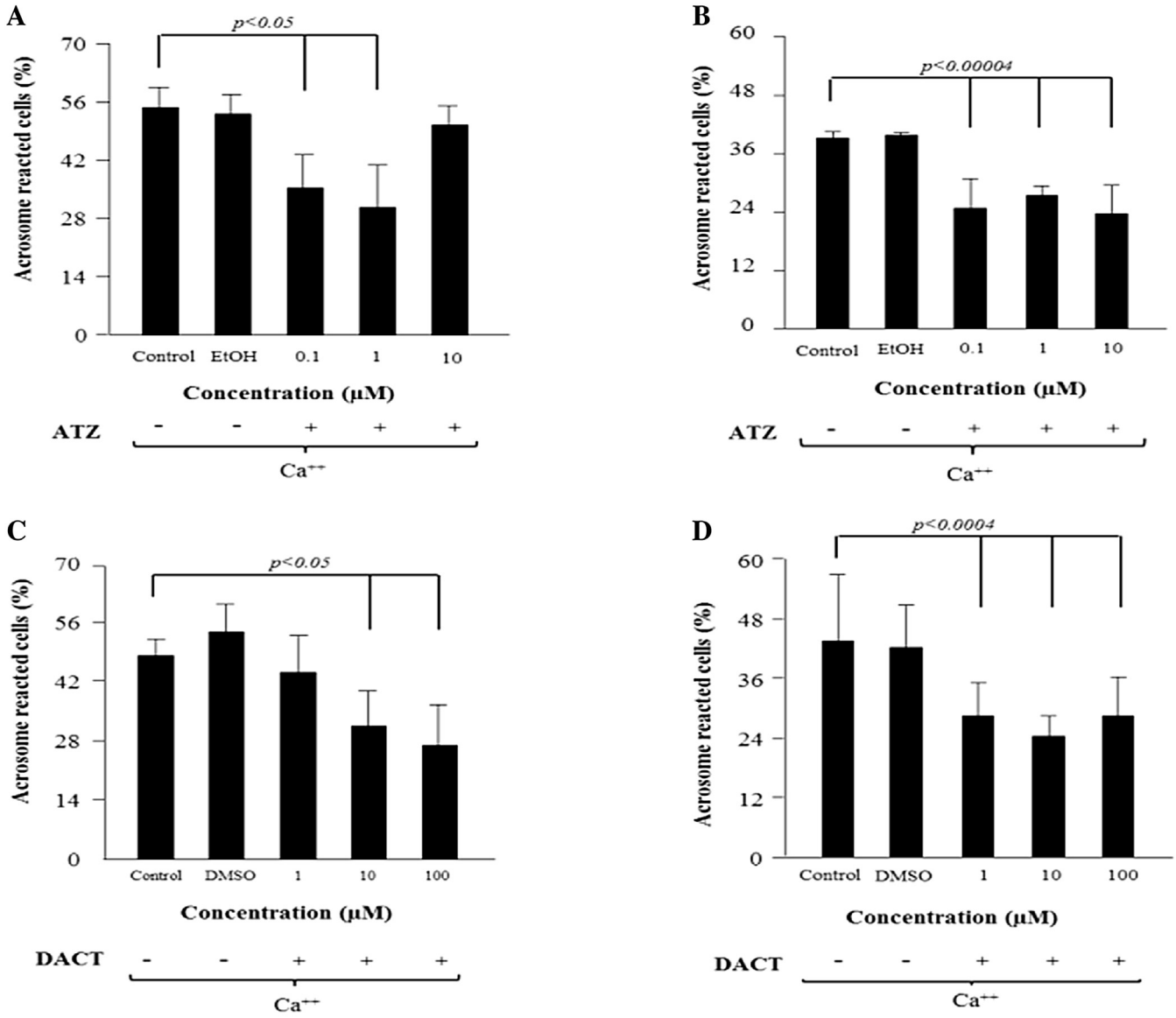

Fig. 3. Effect of ATZ and DACT on Ca^{++} ionophore-induced acrosome reaction (AR). Spermatozoa were isolated from ejaculates (A, C) or epididymis tail (B, D) and incubated for 4 h with 0.1, 1 or 10 μM ATZ dissolved in EtOH (A, B) or with 1, 10 or 100 μM DACT dissolved in DMSO (C, D). AR was induced by incubating the sperm for an additional 20 min with 20 μM Ca^{++} ionophore and was determined with FITC–PSA fluorescent probe. Data are presented as the mean proportion ± SD of the examined cells, calculated for 3 replicates, with at least 200 sperm/group examined.

Table 1
Summary of ATZ effects on sperm features.

	Epididymis			Ejaculate
	Head	Body	Tail	
Viability	–	10 μM (T2)	0.1, 1, 10 μM (T2)	0.1, 1, 10 μM (T4)
PAR AR			0.1, 1, 10 μM (T2) 0.1, 1, 10 μM (T4)	0.1, 1 μM (T2)
ΔΨm			0.1, 1, 10 μM (Ca^{++}) 1 μM (T4)	0.1, 1 μM (Ca^{++}) –

Spermatozoa isolated from fresh ejaculates and epididymis compartments (head, body and tail) were incubated for 4 h with or without 0.1, 1 or 10 μM ATZ. Spermatozoa were collected after 0, 2 and 4 h of incubation (T0, T2, T4, respectively); viability, acrosome plasma damage (APD), induced (Ca^{++}) acrosome reaction (AR), pseudo-AR (PAR) and mitochondrial membrane potential ($\Delta\Psi_m$) were examined by simultaneous fluorimetric assessment. In the table are exposure times and ATZ doses that had significant effects and warranted further investigation. Shaded squares are parameters that were not examined.

respectively, $P \leq 0.005$). On the other hand, DACT increased the proportion of sperm with high ΔΨm in groups exposed to 10 or 100 μM for 4 h, relative the control group (8.4 ± 2.8 and 8.9 ± 2.2 vs. 4.4 ± 1.7, respectively, $P \leq 0.001$; Fig. 5B). The proportion of sperm with pseudo-AR was not affected by DACT in sperm isolated from the ejaculate (Fig. 5C). On the other hand, exposure to 10 or 100 μM DACT decreased the proportion of sperm that responded to Ca^{++} ionophore and underwent induced AR, relative to the control group (31.3 ± 8.4 and 26.7 ± 9.6 vs. 48.0 ± 3.9%, respectively, $P \leq 0.05$; Fig. 3C).

3.2.2. *Effect of DACT on sperm isolated from epididymis compartments*

DACT elevated the proportion of sperm with damaged plasma membrane in a dose-dependent manner. The effect was observed for all three examined epididymis compartments (Fig. 6). Sperm isolated from the epididymis head expressed a higher proportion of membrane damage than the control group after 2 h incubation

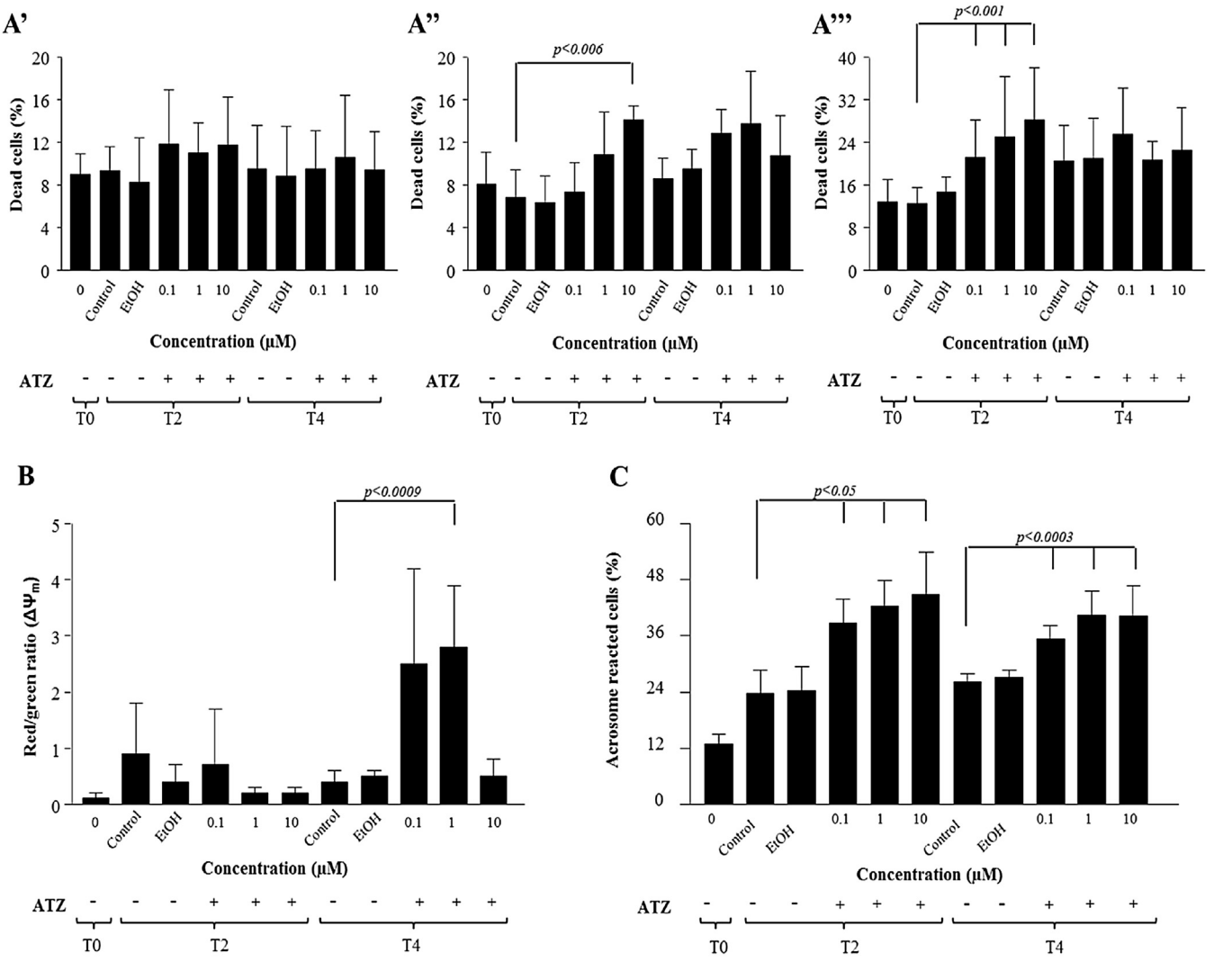

Fig. 4. Effect of ATZ on sperm isolated from the epididymis. Spermatozoa were isolated from epididymis compartments (head [A'], body [A″] and tail [A‴, B, C]) and incubated for 4 h with 0.1, 1 or 10 μM ATZ dissolved in EtOH. (A', A″, A‴) Sperm viability was determined with PI fluorescent probe. (B) Mitochondrial membrane potential (ΔΨm) was determined with JC-1 fluorescent probe and presented as the mean proportion of red (high potential)/green (low potential) stained sperm. (C) Acrosome reaction was determined with FITC–PSA fluorescent probe. Data are presented as the mean proportion ± SD of the examined cells, calculated for 3 replicates, with at least 200 sperm/group examined.

with 1, 10 or 100 μM DACT (29.5 ± 8.1, 36.2 ± 4.1 and 29.7 ± 5.4 vs. 19.6 ± 2.6%, respectively, $P \leq 0.0002$), and after 4 h incubation as well (30.1 ± 5.1, 29.5 ± 3.8 and 27.2 ± 5.0 *vs.* 14.9 ± 3.3%; $P \leq 0.0001$, respectively, Fig. 6A').

Sperm isolated from the body of the epididymis expressed a high proportion of cells with damaged plasma membrane relative to the control when exposed to 10 or 100 μM DACT for 2 h (20.0 ± 4.7 and 21.4 ± 2.3 vs. 10.2 ± 1.6%, respectively, $P \leq 0.003$) or 4 h (19.1 ± 1.4 and 18.0 ± 2.1 vs. 11.9 ± 1.1%, respectively, $P \leq 0.05$; Fig. 6A″). Sperm isolated from the epididymis tail expressed a higher proportion of damaged membranes than the control group after exposure to 1, 10 or 100 μM DACT for 2 h (23.2 ± 2.8, 26.3 ± 1.6 and 29.2 ± 1.1 vs. 15.2 ± 1.4%, $P \leq 0.0002$) or 4 h (25.2 ± 3.2, 29.1 ± 5.5 and 32.9 ± 8.6 vs. 15.6 ± 0.6%, respectively, $P \leq 0.00005$; Fig. 6A‴). DACT significantly increased the ΔΨm of sperm isolated from the tail of the epididymis when exposed to 1, 10 or 100 μM DACT for 2 h, relative to the control (0.6 ± 0.4, 1.1 ± 0.5 and 1.0 ± 0.4 vs. 0.2 ± 0.1, $P \leq 0.0005$), as well as for 4 h (1.4 ± 0.5, 1.2 ± 0.6 and 1.1 ± 0.3 vs. 0.5 ± 0.3, respectively, $P \leq 0.0005$; Fig. 6B). DACT significantly increased the proportion of sperm that underwent pseudo-AR when incubated for 4 h with the highest concentration (100 μM) relative to the control group (20.8 ± 4.4 vs. 9.3 ± 2.5%, respectively, $P \leq 0.05$; Fig. 6C). In addition, DACT (1, 10 or 100 μM) decreased the proportion of sperm that reacted to Ca^{++} ionophore and underwent induced AR, compared to the control group (28.5 ± 6.7, 24.2 ± 4.3 and 28.4 ± 7.9 vs. 43.5 ± 13.3%, respectively, $P \leq 0.0004$; Fig. 3D Table 2).

4. Discussion

The current study provides new evidence for direct effects of herbicides at low doses on sperm quality. In particular, we report that not only the herbicide, ATZ, but also its metabolite, DACT, deleteriously affect sperm viability and function. These alterations were mainly expressed on the sperm membranes: (1) damage to the plasma membrane, (2) impaired acrosome membrane integrity, and (3) alterations in ΔΨm. The most prominent effects were documented for sperm isolated from the epididymis-tail compartment, as it was affected by the lowest ATZ and DACT concentrations.

The plasma membrane is responsible for maintaining cellular osmotic equilibrium, by acting as a barrier between intracellular and extracellular mediums. Thus, maintenance of sperm viability

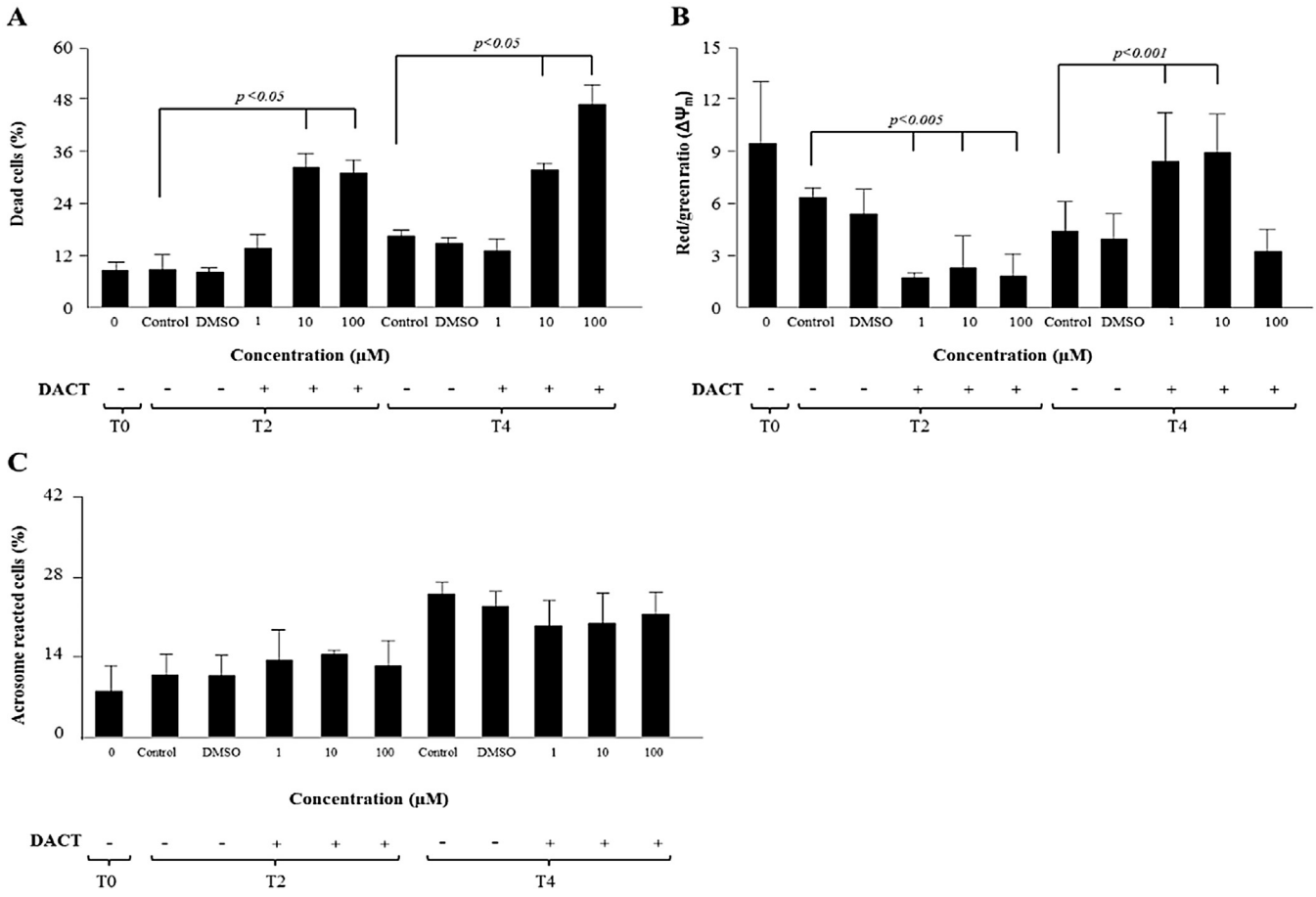

Fig. 5. Effect of DACT on ejaculated sperm. Spermatozoa were incubated for 4 h with 1, 10 or 100 μM DACT dissolved in DMSO. (A) Sperm viability was determined with PI fluorescent probe. (B) Mitochondrial membrane potential (ΔΨm) was determined with JC-1 fluorescent probe and presented as the mean proportion of red (high potential)/green (low potential) stained sperm. (C) Acrosome reaction was determined with FITC–PSA fluorescent probe. Data are presented as the mean proportion ± SD of the examined cells, calculated for 3 replicates, with at least 200 sperm/group examined.

Table 2
Summary of DACT effects on sperm features.

	Epididymis			Ejaculate
	Head	Body	Tail	
Viability	1,10, 100 μM (T2) 1,10, 100 μM (T4)	10, 100 μM (T2) 10, 100 μM (T4)	1, 10, 100 μM (T2) 1, 10, 100 μM (T4)	10, 100 μM (T2) 10, 100 μM (T4)
PAR AR			100 μM (T4) 1, 10, 100 μM (Ca^{++})	10, 100 μM (Ca^{++})
$\Delta\Psi_m$			1,10,100 μM (T2) 1,10,100 μM (T4)	1,10, 100 μM (T2) 1,10 μM (T4)

Spermatozoa isolated from fresh ejaculates and epididymis compartments (head, body and tail) were incubated for 4 h with or without 1, 10 or 100 μM DACT. Spermatozoa were collected after 0, 2 and 4 h (T0, T2, T4, respectively) of incubation; viability, induced (Ca^{++}) acrosome reaction (AR), pseudo AR (PAR) and mitochondrial membrane potential ($\Delta\Psi_m$) were examined by simultaneous fluorimetric assessment. In the table are exposure times and DACT doses that had significant effects and warranted further investigation. Shaded squares are parameters that were not examined.

and fertilization potential depends on the integrity and functionality of sperm membranes. Induced damages to plasma membrane integrity might result in impaired homeostasis, reduced viability and fertilizing ability and cell death. In humans, decreased sperm viability is associated with exposure to various endocrine-disrupting compounds, such as bisphenol A [48,49] and phthalates (DEHP) [50]. In porcine, in-vitro exposure to the herbicide fenoxaprop-ethyl decreased sperm viability [18]. The cryopreservation procedure is known to reduce sperm viability due to damage to the sperm plasma membrane, as a result of temperature changes and osmotic stress [51]. In-vitro exposure of boar sperm to relatively high ATZ concentrations (40, 100 and 500 μM) reduced sperm viability [18,19]. Here we report that exposing sperm to low concentrations of ATZ (0.1, 1 or 10 μM) or its metabolite, DACT (1 or 10 μM), reduces viability of sperm isolated from the epididymis or ejaculate, as determined by PI fluorescent probe that binds to the DNA of cells with a damaged plasma membrane. It should be noted, that all the examined ATZ-doses similarly increased the proportion of dead sperm. While the mechanism in not defined, the results indicate that exposure even to a low ATZ concentration may result in a similar harmful effect received from exposure to higher concentrations. Given that the maximal concentrations of EtOH and DMSO used in the current study did not affect any of the examined parameters when tested for solvent effects. Therefore, the observed alterations can be related to the examined compounds rather than the vehicle.

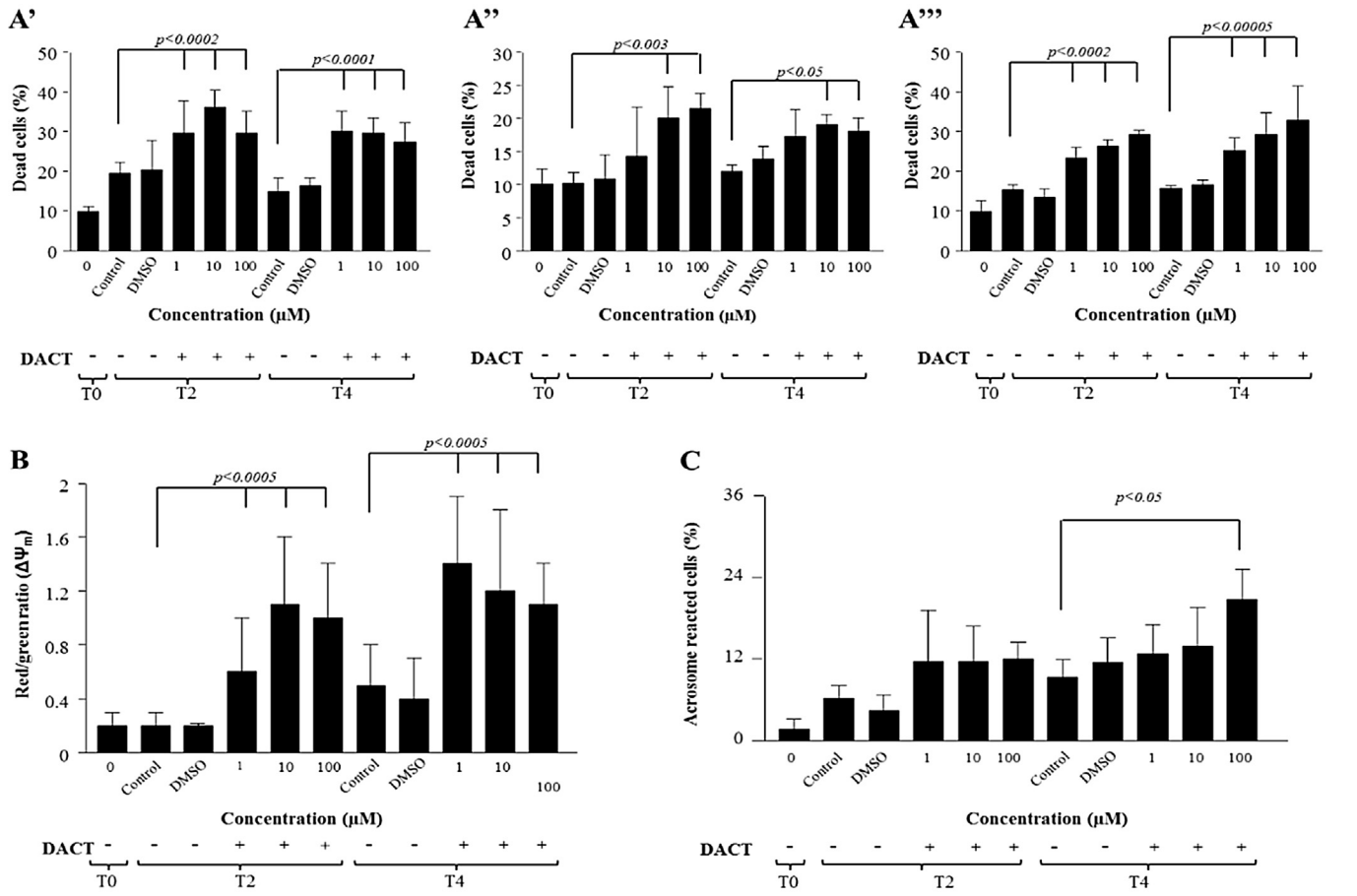

Fig. 6. Effect of DACT on sperm isolated from the epididymis. Spermatozoa were isolated from epididymis compartments (head [A'], body [A″] and tail [A‴, B, C]) and incubated for 4 h with 1, 10 or 100 μM DACT dissolved in DMSO. (A', A″, A‴) Sperm viability was determined with PI fluorescent probe. (B) Mitochondrial membrane potential (ΔΨm) was determined with JC-1 fluorescent probe and presented as the mean proportion of red (high potential)/green (low potential) stained sperm. (C) Acrosome reaction was determined with FITC–PSA fluorescent probe. Data are presented as the mean proportion ± SD of the examined cells, calculated for 3 replicates, with at least 200 sperm/group examined.

Our study explores the risk of exposure to low concentrations of a hazardous compound, which in some cases might be environmentally relevant. It is important to note that ATZ did not affect viability of sperm isolated from the head of the epididymis, and affected those isolated from the epididymis body only at a relatively high dose (10 μM). On the other hand, its metabolite DACT affected sperm isolated from all of the examined epididymal compartments, suggesting that DACT is more harmful than ATZ. Previous studies in which laboratory animals were orally administered ATZ reported an adverse indirect effect on testicular sperm number, motility, viability and morphology [13]. To the best of our knowledge, the current study provides first evidence that DACT has a direct effect on mature sperm. Given that ATZ is metabolized in the body into various metabolites [12–14], both its direct and indirect effects on sperm viability should be taken into account.

Disruption of acrosome membrane integrity, as found in the current study, is one of the potential mechanisms by which ATZ and DACT impair sperm viability and its competence to undergo AR. In mammals, AR involves fusion of the outer acrosome membrane and the overlying plasma membrane and progressive, resulting in the release of acrosomal enzymes. These are essential events for zona-pellucida penetration and further merging of the sperm with the oocyte [52]. Determining the spermatozoa's ability to undergo induced AR in vitro constitutes a useful parameter in evaluating sperm quality and male fertility [38,53,54]. Several fluorescent techniques are suitable for verification of acrosome integrity, including fluorescein isothiocyanate-conjugated Peanut agglutinin (FITC–PNA) or FITC–PSA, which was used in our study [47,53]. Thus, the reported acrosome membrane alterations due to ATZ and DACT exposure might explain the reduced proportion of sperm isolated from the epididymis tail and ejaculate that underwent AR induced by Ca^{++} ionophore (Fig. 6).

Interestingly, ATZ, but not DACT, increased the proportion of sperm that underwent pseudo-AR during capacitation, in particular those isolated from the epididymis tail. Pseudo-AR is defined as a non-controlled, spontaneously occurring reaction (i.e., due to an unknown factor) that is independent of the fertilization process. Sperm cells that undergo pseudo-AR are no longer capable of undergoing AR, and therefore cannot fertilize the oocyte [55]. Pesticides have been found in the mammalian reproductive tract [56]. For example, ATZ was detected in women's amniotic fluid and urine (at 0.6 and 0.1 μg/L, respectively). Diazinon was detected in umbilical cord plasma of pregnant women at delivery (0.6 pg/g) [8]. Given that sperm of ejaculate origin is sensitive to ATZ and DACT, good-quality semen might be damaged by exposure to ATZ or DACT in the female reproductive tract. This suggestion warrants further investigation.

Based on our findings, exposure to 0.1 μM ATZ, an environmentally relevant dose, during the first 2 h of capacitation increased the proportion of pseudo-AR in sperm originated from ejaculate and epididymis tail. Maravilla-Galván et al. [19] reported that ATZ at relatively high concentrations (8–40 μM) increases the occurrence of pseudo-AR in boar sperm. Taken together, the increased pseudo-AR reported for ATZ-treated sperm might explain, in part,

the decrease in the Ca^{++} ionophore-induced AR in that group. On the other hand, given that DACT did not increase pseudo-AR during capacitation, there might be another explanation. Inhibition of calcium signaling has been reported in LβT2 pituitary cells exposed to 300 μM DACT for 24 h [21]. As intracellular calcium influx is a mandatory event for AR occurrence [57], inhibition of calcium signaling by DACT may provide an explanation for AR inhibition. This point needs to be further explored.

Impairment of sperm mitochondria was also documented. Using JC-1 staining, we examined the effects of ATZ and DACT on sperm ΔΨm. We found a prominent effect of 1 μM ATZ on epididymis-tail, but not ejaculated sperm, expressed by hyperpolarization of the mitochondrial membrane. Sperm mitochondria are arranged in a helicoidal form in the midpiece and play a pivotal role in flagellar beat and sperm motility [28]. Generally, spermatozoa that exhibit high ΔΨm have intact acrosome function, normal motility and morphology and high fertilization capacity [58]. On the other hand, sperm cells with low ΔΨm are associated with reduced fertilization rate and an elevated level of reactive oxygen species [59]. In bovine, sperm in the epididymis head are not motile and are characterized by silent mitochondria [30]. On the other hand, sperm in the epididymis tail are motile and characterized by active mitochondria with high membrane potential, indicating that sperm maturation through the epididymis passage involves activation of sperm mitochondria [60]. While not entirely clear, the findings of the current study suggest that the effect of ATZ/DACT on ΔΨm is dependent on developmental stage, i.e., the sperm stored in the epididymis are more sensitive than those isolated from the ejaculate. Hase et al. [15] reported that 100 μM ATZ is required to inhibit sperm mitochondrial function. Here we report that ATZ at low, ecologically relevant doses (0.1 and 1 μM) is sufficient to disrupt ΔΨm in bull sperm. Moreover, a deleterious effect on the mitochondrial membrane was also observed when ejaculate was exposed to 1, 10 or 100 μM DACT. A previous study in somatic cells documented pesticide-induced damage to mitochondrial activity and cellular respiration [61]. A similar response was documented for cells of the freshwater microalga *Chlamydomonas reinhardtii* exposed to 0.25 μM ATZ for 3 h [62], and for human liver carcinoma HepG2 cells exposed to 0.1 μM bisphenol A after 72 h of treatment [63]. Other studies have reported a transient increase in ΔΨm induced by a variety of compounds [64]. For example, the pesticide alachlor was found to depolarize human sperm mitochondria [65]. Similar effects were reported in SH-SY5Y human neuroblastoma cells, induced by the organophosphorus compounds parathion, trio-tolyl-phosphine and triphenylphosphite [66]. Taken together, cellular stress caused by various compounds, including endocrine disruptors, may lead to a transient increase in ΔΨm, which in turn might cause transient hyperpolarization, increased production of free radicals and apoptosis [64,67]. As a decrease in ΔΨm is followed by release of cytochrome *c* [64], it is possible that ATZ- or DACT-induced impairments in ΔΨm might lead to apoptosis in sperm.

In summary, our results shed some light on the potential hazards associated with exposure to ATZ or its metabolite DACT. Exposure for only for a few hours to low doses of ATZ (0.1 μM) or DACT (1 μM) hyperpolarized the mitochondrial membrane, decreased sperm viability and interrupted AR. These findings are highly important because ATZ is frequently detected at these doses in ground and surface water in agricultural areas, and even in regions where it is not used. Drinking ATZ-contaminated water seems to be the main source of ATZ found in the circulation, amniotic fluid, testes and urine. Although ATZ has been shown to alter reproductive function in various species, the exposure risk to humans is not yet clear.

The current study provides clear evidence that ATZ affects spermatogenesis, not only at earlier stages but also in sperm isolated from the epididymis compartments. Sperm from the epididymis tail, the main storage compartment in the testis for mature cells, was found to be highly sensitive to ATZ and its metabolite DACT. In light of these findings, we postulate reduced fertilization competence due to short exposure of sperm to low concentrations of ATZ or DACT at advanced stages of spermatogenesis. A further controlled fertility study is required to confirm this assumption in the animal model. The knowledge that stands to be gained will be relevant to ensuring public reproductive health.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

[1] G.D.C. Severi-Aguiar, E.C.M. Silva-Zacarin, P. De Pós-graduação, C. Biomédicas, C.U. Hermínio, Effects of herbicide atrazine in experimental animal models, Herbicides–Prop, Synth. Control Weeds (2011) 285–296.
[2] D.B. Barr, P. Panuwet, J.V. Nguyen, S. Udunka, L.L. Needham, Assessing exposure to atrazine and its metabolites using biomonitoring, Environ. Health Perspect. 115 (2007) 1474–1478, http://dx.doi.org/10.1289/ehp.10141.
[3] S.O. Abarikwu, A.B. Pant, E.O. Farombi, Effects of quercetin on mRNA expression of steroidogenesis genes in primary cultures of Leydig cells treated with atrazine, Toxicol. In Vitro 27 (2012) 700–707, http://dx.doi.org/10.1016/j.tiv.2012.11.005 (Elsevier Ltd).
[4] T.B. Hayes, A. Collins, M. Lee, M. Mendoza, N. Noriega, A.A. Stuart, et al., Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 5476–5480, http://dx.doi.org/10.1073/pnas.082121499.
[5] H.M. LeBaron, J.E. McFarland, O.C. Burnside, The triazine herbicides [Internet], in: The Triazine Herbicides, Elsevier, 2008, http://dx.doi.org/10.1016/B978-044451167-6.50004-0.
[6] E. Diamanti-Kandarakis, J.-P. Bourguignon, L.C. Giudice, R. Hauser, G.S. Prins, A.M. Soto, et al., Endocrine-disrupting chemicals: an Endocrine Society scientific statement, Endocr. Rev. 30 (2009) 293–342, http://dx.doi.org/10.1210/er.2009-0002.
[7] S. Peighambarzadeh, S. Safi, S. Shahtaheri, M. Javanbakht, A. Rahimi Forushani, Presence of atrazine in the biological samples of cattle and its consequence adversity in human health, Iran. J. Public Health 40 (2011) 112–121 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3481744&tool=pmcentrez&rendertype=Abstract.
[8] A. Bradman, D.B. Barr, B.G. Claus Henn, T. Drumheller, C. Curry, B. Eskenazi, Measurement of pesticides and other toxicants in amniotic fluid as a potential biomarker of prenatal exposure: a validation study, Environ. Health Perspect. 111 (2003) 1779–1782 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1241723&tool=pmcentrez&rendertype=Abstract.
[9] A.L. Forgacs, M.L. D'souza, I.T. Huhtaniemi, N.A. Rahman, T.R. Zacharewski, Triazine herbicides and their chlorometabolites alter steroidogenesis in BLTK1 murine leydig cells. [Internet], Toxicol. Sci. (2013), http://dx.doi.org/10.1093/toxsci/kft096.
[10] T.B. Hayes, Welcome to the revolution: integrative biology and assessing the impact of endocrine disruptors on environmental and public health, Integr. Comp. Biol. 45 (2005) 321–329, http://dx.doi.org/10.1093/icb/45.2.321.
[11] R.L. Cooper, Laws ĀSC, P.C. Das, M.G. Narotsky, J.M. Goldman, E.L. Tyrey, et al., Atrazine and reproductive function: mode and mechanism of action studies, Birth Defects Res. B Dev. Reprod. Toxicol. 80 (2007) 98–112, http://dx.doi.org/10.1002/bdrb.
[12] Y. Jin, X. Zhang, L. Shu, L. Chen, L. Sun, H. Qian, et al., Oxidative stress response and gene expression with atrazine exposure in adult female zebrafish (Danio rerio), Chemosphere 78 (2010) 846–852, http://dx.doi.org/10.1016/j.chemosphere.2009.11.044, Elsevier Ltd.
[13] S.O. Abarikwu, A.C. Adesiyan, T.O. Oyeloja, M.O. Oyeyemi, E.O. Farombi, Changes in sperm characteristics and induction of oxidative stress in the testis and epididymis of experimental rats by an herbicide atrazine, Arch. Environ. Contam. Toxicol. 58 (2010) 874–882, http://dx.doi.org/10.1007/s00244-009-9371-2.
[14] S.O. Abarikwu, O.F. Akiri, M.A. Durojaiye, A. Adenike, Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats, Toxicol. Mech. Methods 25 (2014) 70–80, http://dx.doi.org/10.3109/15376516.2014.989349.
[15] Y. Hase, M. Tatsuno, T. Nishi, K. Kataoka, Y. Kabe, Y. Yamaguchi, et al., Atrazine binds to F1F0-ATP synthase and inhibits mitochondrial function in sperm,

Biochem. Biophys. Res. Commun. 366 (2008) 66–72, http://dx.doi.org/10.1016/j.bbrc.2007.11.107.
[16] S. Sagarkar, D. Gandhi, S.S. Devi, A. Sakharkar, A. Kapley, Atrazine exposure causes mitochondrial toxicity in liver and muscle cell lines, Indian J. Pharmacol. 48 (2016) 200, http://dx.doi.org/10.4103/0253-7613.178842.
[17] A. Gely-Pernot, C. Hao, E. Becker, I. Stuparevic, C. Kervarrec, F. Chalmel, et al., The epigenetic processes of meiosis in male mice are broadly affected by the widely used herbicide atrazine, BMC Genomics 16 (2015) 885, http://dx.doi.org/10.1186/s12864-015-2095-y.
[18] M. Betancourt, A. Reséndiz, E.C.Y.R. Fierro, Effect of two insecticides and two herbicides on the porcine sperm motility patterns using computer-assisted semen analysis (CASA) in vitro, Reprod. Toxicol. 22 (2006) 508–512, http://dx.doi.org/10.1016/j.reprotox.2006.03.001.
[19] R. Maravilla-Galván, R. Fierro, H. González-Márquez, S. Gómez-Arroyo, I. Jiménez, M. Betancourt, Effects of atrazine and fenoxaprop-ethyl on capacitation and the acrosomal reaction in boar sperm, Int. J. Toxicol. 28 (2009) 24–32, http://dx.doi.org/10.1177/1091581809333138.
[20] M.K. Ross, T.L. Jones, N.M. Filipov, Disposition of the herbicide 2-Chloro-4-(ethylamino) –6- (isopropylamino) – s –triazine (Atrazine) and its major metabolites in mice: a liquid Chromatography/Mass spectrometry analysis of urine, plasma and Tissue Levels, Drug Metab. Dispos. 37 (2009) 776–786, http://dx.doi.org/10.1124/dmd.108.024927 (annually).
[21] G.P. Dooley, R.B. Tjalkens, W.H. Hanneman, The atrazine metabolite diaminochlorotriazine suppresses LH release from murine LβT2 cells by suppressing GnRH-induced intracellular calcium transients, Toxicol. Res. (Camb) 2 (2013) 180–186, http://dx.doi.org/10.1039/C3TX20088D.
[22] Y. Jin, L. Wang, G. Chen, X. Lin, W. Miao, Z. Fu, Exposure of mice to atrazine and its metabolite diaminochlorotriazine elicits oxidative stress and endocrine disruption, Environ. Toxicol. Pharmacol. 37 (2014) 782–790, http://dx.doi.org/10.1016/j.etap.2014.02.014.
[23] G.P. Dooley, K.F. Reardon, J.E. Prenni, R.B. Tjalkens, M.E. Legare, C.D. Foradori, et al., Proteomic analysis of diaminochlorotriazine adducts in wister rat pituitary glands and l T2 rat pituitary cells, Chem. Res. Toxicol. (2008) 844–851.
[24] P.L. Senger, Pathways to pregnancy and parturition. Current Conceptions, Inc., 1615 NE Eastgate Blvd.; 1997; Available: http://www.cabdirect.org/abstracts/19982206441.html?freeview = true.
[25] L.R. França, G.F. Avelar, F.F.L. Almeida, Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs, Theriogenology 63 (2005) 300–318, http://dx.doi.org/10.1016/j.theriogenology.2004.09.014.
[26] P.M. Aponte, M.P.A. van Bragt, D.G. de Rooij, A.M.M. van Pelt, Spermatogonial stem cells: characteristics and experimental possibilities, APMIS 113 (2005) 727–742, http://dx.doi.org/10.1111/j.1600–0463.2005.apm_302.x.
[27] G.A. Cornwall, Role of posttranslational protein modifications in epididymal sperm maturation and extracellular quality control, Adv. Exp. Med. Biol. 759 (2014) 159–180, http://dx.doi.org/10.1007/978-1-4939-0817-2_8.
[28] E.M. Eddy, A. O'Brien, The spermatozoon [Internet], in: E. Knobil, J.D. Neill (Eds.), Knobil and Neill's Physiology of Reproduction, vol. 1, 2nd ed., Raven Press, New York, USA, 1994 https://books.google.com/books?id=6j-mOAcNnPUC&pgis=1.
[29] C. Reyes-Moreno, Characterization and identification of epididymal factors that protect ejaculated bovine sperm during In vitro storage, Biol. Reprod. 66 (2002) 159–166, http://dx.doi.org/10.1095/biolreprod66.1.159.
[30] R.J. Aitken, B. Nixon, M. Lin, A.J. Koppers, Y.H. Lee, M.A. Baker, Proteomic changes in mammalian spermatozoa during epididymal maturation, Asian J. Androl. 9 (2007) 554–564, http://dx.doi.org/10.1111/j.1745-7262.2007.00280.x.
[31] H. Breitbart, Signaling pathways in sperm capacitation and acrosome reaction, Cell Mol. Biol. (Noisy-le-grand). 49 (2003) 321–327 http://www.ncbi.nlm.nih.gov/pubmed/12887084.
[32] C. Patrat, C. Serres, P. Jouannet, The acrosome reaction in human spermatozoa, Biol. Cell. 92 (2000) 255–266 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1712232&tool=pmcentrez&rendertype=Abstract.
[33] S.O. Abarikwu, Q.C. Duru, O.V. Chinonso, R.-C. Njoku, Antioxidant enzymes activity, lipid peroxidation, oxidative damage in the testis and epididymis, and steroidogenesis in rats after co-exposure to atrazine and ethanol, Andrologia 48 (June 5) (2015) 548–557, http://dx.doi.org/10.1111/and.12478.
[34] S. Feyzi-Dehkhargani, R. Shahrooz, H. Malekinejad, R.-A. Sadrkhanloo, Atrazine in sub-acute exposure results in sperm DNA disintegrity and nuclear immaturity in rats, Vet Res forum an Int Q J. 3 (2012) 19–26 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4312814&tool=pmcentrez&rendertype=Abstract.
[35] J.J. Parrish, Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin, Theriogenology 81 (2014) 67–73, http://dx.doi.org/10.1016/j.theriogenology.2013.08.005.
[36] A. Abou-haila, D.R.P. Tulsiani, Signal transduction pathways that regulate sperm capacitation and the acrosome reaction, Arch. Biochem. Biophys. 485 (2009) 72–81, http://dx.doi.org/10.1016/j.abb.2009.02.003.
[37] J.J. Parrish, J. Susko-Parrish, M.A. Winer, N.L. First, Capacitation of bovine sperm by heparin, Biol. Reprod. 38 (1988) 1171–1180 http://www.ncbi.nlm.nih.gov/pubmed/3408784.
[38] J. Jankovicová, M.J. Simon Antalíková, L. Horovská, Acrosomal and viability status of bovine spermatozoa evaluated by two staining methods, Acta Vet. Hung. 56 (2008) 133–138, http://dx.doi.org/10.1556/AVet.56.2008.1.14.
[39] H.M. Florman, M.K. Jungnickel, K.A. Sutton, Regulating the acrosome reaction, Int. J. Dev. Biol. 52 (2008) 503–510, http://dx.doi.org/10.1387/ijdb.082696hf.
[40] J.J. Parrish, J. Susko-Parrish, M.A. Winer, N.L. First, Capacitation of bovine sperm by heparin, Biol. Reprod. 38 (1988) 1171–1180, http://dx.doi.org/10.1095/biolreprod38.5.1171.
[41] J.J. Parrish, J.L. Susko-Parrish, J.K. Graham, In vitro capacitation of bovine spermatozoa: role of intracellular calcium, Theriogenology 51 (1999) 461–472, http://dx.doi.org/10.1016/S0093-691X(98)00240-4.
[42] P.C. Das, W.K. McElroy, R.L. Cooper, Alteration of catecholamines in pheochromocytoma (PC12) cells in vitro by the metabolites of chlorotriazine herbicide, Toxicol. Sci. 59 (2001) 127–137 http://www.ncbi.nlm.nih.gov/pubmed/11134552.
[43] J.E. Graves, M.E. Richardson, R.S. Bernard, N.D. Camper, W.C. Bridges, Atrazine effects on in vitro maturation and in vitro fertilization in the bovine oocyte, J. Environ. Sci. Health B 37 (2002) 103–112, http://dx.doi.org/10.1081/PFC-120002982.
[44] S. Mu, X. Kang, Y. Wu, G. Li, J. Tong, Z. Zhang, Effects of atrazine on the acrosome reaction of spermatozoa in eriocheir sinensis, 5th Int Conf Bioinforma Biomed Eng. Ieee 2011 (2011) 1–3, http://dx.doi.org/10.1109/icbbe.2011.5780805.
[45] Z. Lin, J.W. Fisher, R. Wang, M.K. Ross, N.M. Filipov, Estimation of placental and lactational transfer and tissue distribution of atrazine and its main metabolites in rodent dams, fetuses, and neonates with physiologically based pharmacokinetic modeling, Toxicol. Appl. Pharmacol. Elsevier B.V. (2013), http://dx.doi.org/10.1016/j.taap.2013.08.010.
[46] Z. Liu, Y. Wang, Z. Zhu, E. Yang, X. Feng, Z. Fu, et al., Atrazine and its main metabolites alter the locomotor activity of larval zebrafish (Danio rerio), Chemosphere 148 (2016) 163–170, http://dx.doi.org/10.1016/j.chemosphere.2016.01.007.
[47] E.C.C. Celeghini, R.P. de Arruda, A.F.C. de Andrade, J. Nascimento, C.F. Raphael, Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes, Reprod. Domest. Anim. 42 (2006) 479–488.
[48] D.-K. Li, Z. Zhou, M. Miao, Y. He, J. Wang, J. Ferber, et al., Urine bisphenol-A (BPA) level in relation to semen quality, Fertil. Steril. 95 (2011) 625–630, http://dx.doi.org/10.1016/j.fertnstert.2010.09.026 (e1-4).
[49] J. Knez, R. Kranvogl, B.P. Breznik, E. Vončina, V. Vlaisavljević, Are urinary bisphenol A levels in men related to semen quality and embryo development after medically assisted reproduction? Fertil. Steril. 101 (2014) 215–221, http://dx.doi.org/10.1016/j.fertnstert.2013.09.030 (e5).
[50] N. Pant, A. Pant, M. Shukla, N. Mathur, Y. Gupta, D. Saxena, Environmental and experimental exposure of phthalate esters: the toxicological consequence on human sperm, Hum. Exp. Toxicol. 30 (2011) 507–514, http://dx.doi.org/10.1177/0960327110374205.
[51] H. Gürler, E. Malama, M. Heppelmann, O. Calisici, C. Leiding, J.P. Kastelic, et al., Effects of cryopreservation on sperm viability, synthesis of reactive oxygen species, and DNA damage of bovine sperm, Theriogenology 86 (2016) 562–571, http://dx.doi.org/10.1016/j.theriogenology.2016.02.007.
[52] E. Almadaly, I. El-Kon, B. Heleil, E.-S. Fattouh, K. Mukoujima, T. Ueda, et al., Methodological factors affecting the results of staining frozen-thawed fertile and subfertile Japanese Black bull spermatozoa for acrosomal status, Anim. Reprod. Sci. 136 (2012) 23–32, http://dx.doi.org/10.1016/j.anireprosci.2012.10.016.
[53] P. Lybaert, A. Danguy, F. Leleux, S. Meuris, P. Lebrun, Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa, Histol. Histopathol. 24 (2009) 999–1007 http://www.ncbi.nlm.nih.gov/pubmed/19554507.
[54] C.H. Whitfield, T.J. Parkinson, Relationship between fertility of bovine semen and in vitro induction of acrosome reactions by heparin, Theriogenology 38 (1992) 11–20 http://www.ncbi.nlm.nih.gov/pubmed/16727114.
[55] A. Wiser, S. Sachar, Y. Ghetler, A. Shulman, H. Breitbart, Assessment of sperm hyperactivated motility and acrosome reaction can discriminate the use of spermatozoa for conventional in vitro fertilisation or intracytoplasmic sperm injection: preliminary results, Andrologia 46 (2014) 313–315, http://dx.doi.org/10.1111/and.12068.
[56] R.S. Tavares, S. Escada-Rebelo, M. Correia, P.C. Mota, J. Ramalho-Santos, The non-genomic effects of endocrine-disrupting chemicals on mammalian sperm, Reproduction 151 (2016) R1–R13, http://dx.doi.org/10.1530/REP-15-0355.
[57] H. Breitbart, Intracellular calcium regulation in sperm capacitation and acrosomal reaction, Mol. Cell. Endocrinol. 187 (2002) 139–144, http://dx.doi.org/10.1016/S0303-7207(01)00704-3.
[58] F. Gallon, C. Marchetti, N. Jouy, P. Marchetti, The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability, Fertil. Steril. 86 (2006) 1526–1530, http://dx.doi.org/10.1016/j.fertnstert.2006.03.055.
[59] J.A. Espinoza, U. Paasch, J.V. Villegas, Mitochondrial membrane potential disruption pattern in human sperm, Hum. Reprod. 24 (2009) 2079–2085, http://dx.doi.org/10.1093/humrep/dep120.
[60] J. Ramalho-Santos, S. Varum, S. Amaral, P.C. Mota, A.P. Sousa, A. Amaral, Mitochondrial functionality in reproduction: from gonads and gametes to embryos and embryonic stem cells, Hum. Reprod. Update 15 (2009) 553–572, http://dx.doi.org/10.1093/humupd/dmp016.
[61] B.J. Thornton, T.E. Elthon, R.L. Cerny, B.D. Siegfried, Proteomic analysis of atrazine exposure in Drosophila melanogaster (Diptera: drosophilidae), Chemosphere 81 (2010) 235–241, http://dx.doi.org/10.1016/j.chemosphere.2010.06.032 (Elsevier Ltd).

[62] M. Esperanza, M. Seoane, C. Rioboo, C. Herrero, Á. Cid, Chlamydomonas reinhardtii cells adjust the metabolism to maintain viability in response to atrazine stress, Aquat. Toxicol. 165 (2015) 64–72, http://dx.doi.org/10.1016/j.aquatox.2015.05.012.
[63] L. Huc, A. Lemarié, F. Guéraud, C. Héliès-Toussaint, Low concentrations of bisphenol A induce lipid accumulation mediated by the production of reactive oxygen species in the mitochondria of HepG2 cells, Toxicol. In Vitro 26 (2012) 709–717, http://dx.doi.org/10.1016/j.tiv.2012.03.017.
[64] B. Kadenbach, S. Arnold, I. Lee, M. Hüttemann, The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases, Biochim. Biophys. Acta 1655 (2004) 400–408, http://dx.doi.org/10.1016/j.bbabio.2003.06.005.
[65] G. Grizard, L. Ouchchane, H. Roddier, C. Artonne, B. Sion, M.-P. Vasson, et al., In vitro alachlor effects on reactive oxygen species generation, motility patterns and apoptosis markers in human spermatozoa, Reprod. Toxicol. 23 (2007) 55–62, http://dx.doi.org/10.1016/j.reprotox.2006.08.007.
[66] K. Carlson, M. Ehrich, Organophosphorus compound-induced modification of SH-SY5Y human neuroblastoma mitochondrial transmembrane potential, Toxicol. Appl. Pharmacol. 160 (1999) 33–42, http://dx.doi.org/10.1006/taap.1999.8741.
[67] M. Hüttemann, I. Lee, A. Pecinova, P. Pecina, K. Przyklenk, J.W. Doan, Regulation of oxidative phosphorylation, the mitochondrial membrane potential, and their role in human disease, J. Bioenerg. Biomembr. 40 (2008) 445–456, http://dx.doi.org/10.1007/s10863-008-9169-3.