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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.

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