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In vitro alachlor effects on reactive oxygen species generation, motility patterns and apoptosis markers in human spermatozoa

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Abstract

Due to its extensive production and application, the toxicity of chloracetanilide herbicide alachlor[2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide] should be evaluated to establish minimum effects. In this study, we have examined the *in vitro* effects of alachlor on human sperm motion using a compunter-assisted sperm analyser (CASA). An evaluation of both reactive oxygen species (ROS) and markers of apoptosis was also performed to investigate the mechanism by which alachlor modifies the sperm movement.

After exposure up to 2 h to alachlor (0, 0.18, 0.37, 0.90 and 1.85 mM), the percentage of viable, motile spermatozoa and sperm velocities were concentration and/or time dependently decreased. The most sensitive parameters were progressive motility, mean average path velocity and mean straight velocity. Alachlor (1.85 mM) induced an increase in ROS production. A decrease of mitochondrial membrane potential ($\Delta\Psi_m$), an increase of both phosphatidylserine (PS) externalization and DNA fragmentation, which were concentration and/or time dependent, were also observed.

It is possible that toxic effects of alachlor result in an oxidative stress which could act as a mediator of apoptosis. Alachlor could also contribute to some hypofertility cases.

Keywords: Alachlor; Apoptosis markers; CASA; Human spermatozoa; Reactive oxygen species

1. Introduction

Alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)-acetanilide] is an herbicide commonly used in the production of corn, soy beans, rice and peanuts. Due to its extensive production and application, everyone could be exposed [1] and the detrimental effect of alachlor on life should be evaluated to establish minimum effects. Some findings, from epidemiological studies on populations exposed to alachlor, suggest an increased incidence of cancer [2,3]. In rodents, alachlor exposure is also associated with tumor formation [4,5]. Moreover, in animals the interaction of alachlor with estrogen and progesterone receptors is suspected of having endocrine disrupting effects [6]. Alachlor metabolites measured in urine samples were found present above the limit of detection among men from agricultural mid-Missouri, an area where alachlor is largely used. In addition, an association between alachlor exposure evaluated by these metabolites and reduced sperm quality (decrease in sperm concentration, motility and morphologically normal sperm) was demonstrated [7,8]. However, to our knowledge, the levels of alachlor in blood, testis or accessory sex glands of men following environmental or occupational exposure to this herbicide have never been measured. In addition, no reproductive effects have been demonstrated either in human or animal studies. Several *in vitro* studies looking at DNA damage, in a numerous mammalian cells, demonstrate the DNA damaging potential of alachlor. Nevertheless, it is difficult to determine whether this DNA damage was a consequence of direct interaction of alachlor with DNA or a result of a secondary effect on cellular processes. Some findings suggest that the lipophilic alachlor interacts

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with lipid and protein components of the plasma membrane modifying its structure and functions [4]. It is also possible that the toxic effects of alachlor may be associated with an oxidative stress [9].

In vitro toxicity screening using animal sperm has been developed for the past decade. The large number of cells isolated from animals facilitates designing experiments to assess either the toxicity of compounds to which subjects could be exposed or the reproductive risk. Because sperm motion is important for sperm functional capacity [10] not only the percentage of motile spermatozoa but also sperm movement characteristics might give information regarding quantitative and qualitative evaluation of toxic effects of chemicals. The use of computer assisted sperm analysis (CASA) system allows analysis of a large number of sperms in a short time and provides multiple parameters of sperm motion useful for detecting toxicity [11–14]. In addition, spermatozoa may be a good model to help to better understand the molecular mechanism of the alachlor cytotoxicity. Sperms produce reactive oxygen species (ROS) mostly originated from normal metabolic activity but excessive ROS generation induced by a wide variety of stimuli have detrimental effects on sperm function [15–17]. Ejaculated spermatozoa have also been shown to demonstrate changes consistent with apoptosis in particular a decrease in mitochondrial membrane potential ($\Delta\Psi_m$), externalization of phosphatidylserine (PS) at the plasma membrane and a nuclear fragmentation [18]. Such phenomenon may be induced by intrinsic and extrinsic factors such as toxin exposure and oxidative stress [19].

The aim of the present study was to analyse the *in vitro* effects of alachlor on human sperm motility by investigating sperm motion parameters using the CASA system. In addition, to investigate the mechanism by which alachlor modifies the sperm movement, an evaluation of both ROS production and sperm features of cells undergoing apoptosis were performed.

2. Materials and methods

2.1. Reagents and material

The 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] was purchased from Interchim (Montluçon, France). The TUNEL kit and DNase were obtained from Roche Diagnostics GmbH (Manheim, Germany). Alachlor (99.9% pure) and all other reagents were provided by Sigma–Aldrich (St. Quentin Fallavier, France).

All flow cytometry experiments were performed on an EPICS XL cytofluorometer (Beckman Coulter, Villepinte, France). The excitation wavelength was 488 nm supplied by an argon laser. Green (FITC-derived fluorescence) and red fluorescence (propidium iodide, PI) were detected with FL1 and FL3 detectors using a 525 and 675 nm filter. FL1 and FL3 fluorescence signals were recorded after logarithmic amplification.

2.2. Sperm preparation

Semen samples were obtained from men referred to the Biology of Reproduction Laboratory (CHU, Clermont-Ferrand, France) for routine semen analysis before *in vitro* fertilization or for semen analysis screening. All subjects were partners of women who had failed to conceive after 2 years of unprotected intercourse.

The ejaculates were collected by masturbation after a recommended sexual abstinence of 2–3 days. Immediately after liquefaction (about 30 min at 37 °C) sperm parameters were determined in accordance with guidelines of the WHO [20] and surplus semen confirmed to be in the normality limits set down by the WHO were used for experimental analysis.

To isolate sperm, an aliquot of semen was purified using a two-step discontinuous Percoll Gradient (47.5–95%) diluted in modified Earle medium (MEM; NaCl: 94.7 mM; KCl: 4.78 mM; $MgSO_4$: 0.81 mM; NaH_2PO_4: 1 mM; $NaHCO_3$: 3.9 mM; Na lactate: 21.3 mM; Na pyruvate: 0.33 mM; glucose: 5.56 mM; $CaCl_2$: 1.78 mM; HEPES: 105 mM; BSA: 0.3%; pH: 7.4).

After centrifugation at $500 \times g$ for 20 min, purified population of motile sperm (from the 95% layer) was recovered, washed in MEM and re-suspended in the same medium at a sperm concentration of 25×10^6 ml^{-1}.

2.3. Incubation procedure

Alachlor was dissolved in dimethylsulfoxide (DMSO). Spermatozoa (25×10^6 ml^{-1}) were exposed to 0.18, 0.37, 0.9 and 1.85 mM final concentrations of alachlor. These concentrations were guided by data of literature (1–400 μM according to the experimental model studied) [4,9,21] and characteristic of our experimental model (short duration of incubation). Samples with DMSO but without alachlor were used as controls. The final concentration of DMSO was kept at 0.5% (v/v) in all samples. The incubation was performed in 100 μl of MEM at 37 °C under an atmosphere of 5% CO_2 in air. Analysis of spermatozoa was performed after 5, 30, 60 and 120 min of incubation.

2.4. Sperm motion analysis

After incubation, the sperm suspensions were analyzed with the CASA system, Hamilton-Thorn Sperm Analyser (HTM Ceros model 12, Hamilton-Thorn Biosciences, Beverly, MA, USA).

A 3 μl sample was loaded into a pre-warmed disposable standard count chamber of 20 μm depth (Leja, Niew-Vennep, The Netherlands). The chamber was placed on the stage of the HTM which temperature was stabilized at 37 °C. The software settings of HTM are listed in Table 1 and at least 400 sperm trajectories were analyzed. For the attribute of motility, all spermatozoa travelling with a velocity >25 μm/s were considered as spermatozoa with a rapid progressive motility (Prog Mot, %). In addition, the following motion parameters were recorded: mean average path velocity (VAP, velocity of the smoothed average cell path, μm/s), mean curvilinear velocity (VCL, velocity of total distance between each sperm tracks, μm/s), mean straight line velocity (VSL, velocity between the beginning and the end of the track, μm/s), amplitude of lateral head displacement (ALH, mean width of the head oscillation, μm) and mean percentage linearity (LIN = VSL/VCL, departure of the cell track from a straight line, %) of all motile spermatozoa.

Table 1
CASA parameter settings used in the sperm motion analysis

Parameter	Setting
Frame/s (Hz)	60
Frames acquired (number)	30
Minimum contrast	80
Minimum cell size (pixel)	3
VAP progressive cell (μm/s)	25
VAP cut-off (μm/s)	5
Medium VAP cut-off (μm/s)	11
Head-size, non-motile (pixel)	6
Head intensity, non-motile	120
Static head size (pixel)	0.57–2.90
Static head intensity	0.4–1.40
Static elongation	0–80
Illumination intensity	2300
Magnification	1.84