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IN VITRO EFFECTS OF TYRE DEBRIS ORGANIC EXTRACT ON THE KINETIC AND MORPHOLOGIC TRAITS OF RABBIT SPERMATOZOA

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Abstract: The present study aims at evaluating the effects of the organic extract of tyre debris (TDOE) from tyre wear on the kinetic and morphologic features of rabbit spermatozoa. Rabbit sperm were incubated for 4 h with 0, 5, 10, 50 and 75 µg/mL of TDOE. Sperm motility was evaluated by computer-assisted semen analysis. Phosphatidilcholine (PS) externalization (apoptosis) and plasma membrane breakage (necrosis) were assessed using the annexinV/propidium iodide assay. The sperm ultrastructure was observed by scanning (SEM) and transmission electron microscopy (TEM). A relevant decrease in the motility rate, in PS externalization, and in plasma membrane breakage of spermatozoa was observed after incubation with TDOE at concentrations higher than 50 µg/mL. The most frequent ultrastructural anomalies detected in the analysed specimens were: plasma and/or acrosomal membrane breakage, swollen and disorganized mitochondria, and altered axonemal patterns. Taken together, these results suggest that the organic extract of tyre debris can be toxic to rabbit spermatozoa – affecting their movement and structural integrity – when present in seminal plasma at a concentrations higher than 50 µg/mL. Although rabbit sperm has been proven to be a suitable model for testing the *in vitro* effects of many chemical compounds, including TDOE, the obtained results must be considered preliminary and cannot be extrapolated yet to the *in vivo* outcomes because of scanty data. The results encourage, however, further research in this field.

Key Words: rabbit, semen quality, spermiotoxicity, tyre debris organic extract.

INTRODUCTION

Particulate matter (PM) is a complex mixture of dust, dirt, and soot or smoke, as well as liquid droplets suspended in the air. PM standards are based on total mass and size, which can range from a few nanometers to tens of micrometers in an aerodynamic diameter. Coarse particles of 2.5 µm to 10 µm diameter ($PM_{2.5}$– PM_{10}) are of particular health interest due to their capacity to penetrate into the lung and potentially cause health effects (Wegesser and Last, 2008). Exposure to breathable PM has been associated with an exacerbation of asthma and chronic obstructive pulmonary disease, as well as with increased morbidity due to respiratory and cardiovascular diseases (Alexis *et al*., 2006). The mechanisms underlying PM toxicity are not fully understood. However, inflammation, oxidative stress (Risom *et al*., 2005) and DNA damage seem to be involved (Sørensen *et al*., 2003).

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Tyre debris (TD) is a component of PM produced by motor vehicle tyre wear. Five to seven percent of the breathable fractions of the PM_{10} size range is tyre debris (Fauser, 1999; Tappe and Null, 2002). Thus, a certain quantity of such debris may be inhaled by inhabitants – and the toxicity of this component must be considered. TD toxicity has been linked to inorganic components such as zinc (Smolders and Degryse, 2002) and organic compounds (Camatini *et al*., 2003). The potential toxicity of zinc leached by tyre material on water biota has been extensively described, while the toxicity related to the organic part has received little attention.

The organic portion of TD is mainly characterized by the presence of isoprene polymers and other organic hydrocarbons, such as long chain alkanes (Beretta *et al*., 2007). It has been demonstrated to induce a dose-dependent increase in cell mortality, DNA damage, and a significant modification in cell morphology. These particulate compounds are not mutagenic per se, but become so only after metabolic activation (Gualtieri *et al*., 2005a, 2008).

Global declines in semen quality are frequently associated with enhanced exposure to environmental chemicals due to an increased use of pesticides, plastics, and other anthropogenic materials. A significant amount of toxicology data based upon laboratory and wildlife animals studies suggests that exposure to certain chemicals cause reproductive toxicity and impaired semen quality (reduced sperm concentration and motility, altered morphology) and thus reduced male fertility (Phillips and Tanphaichitr, 2008).

Rabbit semen can be easily collected by an artificial vagina and the fertility of sperm tested (Morton, 1988). In particular, seminal endpoints (sperm concentration, viability, motility and morphology) are very accessible and can be easily analyzed by well-established procedures (Foote and Carney, 2000; Castellini *et al*., 2009).

In our previous study, the use of a computer-assisted semen analyzer (CASA) along with scanning (SEM) and transmission electron microscopy (TEM) proved to be useful for discriminating the effects of certain metal compounds on kinetic traits and the ultrastructure of rabbit spermatozoa. These parameters were proposed as valid quantifying spermiotoxicity endpoints (Castellini *et al*., 2009).

The present study was undertaken to investigate whether the *in vitro* incubation of semen with organic extract of tyre debris (TDOE) can produce adverse effects on the kinetic and morphological features of rabbit spermatozoa.

MATERIALS AND METHODS

Tyre debris particles and organic extract

Tyre debris particle samples were furnished by Prof. E. Bolzacchini (Department of Environmental Sciences, University of Milano-Bicocca) and obtained from Milan air samples that contained particles with an aerodynamic diameter of less than 100 μm. Some 5-7% of PM_{10} is composed of inhalable TD.

The TDOE was prepared according to the method proposed by Gualtieri *et al*. (2005a,b) using a Soxhlet extraction apparatus (SER 148, VELP® SCIENTIFICA, Milan, Italy). Briefly, tyre debris was Soxhlet-extracted for six hours with 150 mL of dichloromethane and then dried under a flow of nitrogen. Dry TDOE was then suspended in sterile dimetylsulfoxide (DMSO) at a final concentration of 10 mg/mL and stored at −20 °C. The final DMSO concentration never exceeded 0.5%, and at this level, no obvious toxicity to rabbit spermatozoa was observed.

Semen collection and study design

Semen collection from five mature New Zealand White rabbits was performed by means of an artificial vagina (containing water at 37 °C) once a week for five consecutive weeks (a total of 25 samples). To reduce the biological variability of semen samples, the experiments were performed on pooled specimens.

Sperm cell concentration was measured by means of a Thoma-Zeiss counting cell chamber and a light microscope (Olympus CH-2) set at 400× objective magnification.

To investigate the potential effects of the TDOE on semen quality, aliquots of pooled ejaculated rabbit spermatozoa prepared in Tyrode's albumin lactate pyruvate buffer (TALP) were incubated with TDOE at a concentration of 0, 5, 10, 50 and 75 μg/mL for 4 h at 37 °C in 5% CO_2. The range of concentrations was selected considering the data reported by Gualtieri *et al*. (2005b) while the time of exposure was determined by preliminary experiments (data not shown).

Evaluation of sperm movement by CASA

The kinetic characteristics of TDOE-treated and untreated spermatozoa were analyzed using a CASA (model ISAS® Valencia, Spain) computer-assisted sperm analyzer. This system consisted of a negative phase contrast optical system (Olympus CH-2) equipped with a Sony CCD camera. The set-up parameters of the CASA were those previously established by Castellini and Lattaioli (1999) and the acquisition rate was set at 100 Hz. For each sample, two drops and six microscopic fields were analysed. Recorded sperm motility parameters were motility (%) and curvilinear velocity (VCL, mm/sec). Motility is calculated as the number of motile spermatozoa within the analysis field divided by the sum of the motile plus immotile spermatozoa within the field. VCL is defined as the sum of the incremental distances moved in each frame along the sampled path divided by the time taken for spermatozoa to cover the track.

AnnexinV/propidium iodide assay

An annexinV/propidium iodide assay was carried out on TDOE-treated and untreated specimens to quantify the presence of necrosis and evaluate surface changes, such as phosphatidylserine (PS) translocation from the inner side of the plasma membrane to the outer layer, which is a critical stage of the apoptotic process. The annexin V protein preferentially binds to negatively charged phospholipids, such as PS in the presence of Ca^{2+}, whereas propidium iodide (PI) stains the cells with broken membranes, a primary symptom of necrosis.

Detection of PS externalization was performed by using the Vybrant apoptosis assay kit (Invitrogen Ltd, UK). Samples were washed with phosphate buffered saline (PBS), centrifuged, and suspended in annexin-binding buffer (ABB) to obtain a cell density of approx. 10×10^6. Following the manufacturer's instructions, 10 μL of conjugated-FITC annexin V and 1 μL of PI (100 mg/mL) working solution were added to 100 μL of cell suspension. The spermatozoa were incubated at room temperature for 15 min. After a careful wash with ABB, a drop of sperm cell suspension was smeared on each glass slide. Slides were mounted in glycerol containing 5% n-propylgallate. Observations were made and photographs taken with a Leitz Aristoplan (Leica, Wetzlar, Germany) light microscope equipped with fluorescence apparatus. A total of 100 spermatozoa from each rabbit sample were scored.

By staining cells with FITC-Annexin V (AnV, green fluorescence) and simultaneously with PI (red fluorescence) non-vital dye it was possible to identify four types of cell populations: intact cells (AnV negative, PI negative); early apoptotic cells (AnV positive, PI negative); damaged sperm with PS externalization (AnV positive, PI positive); damaged necrotic sperm (AnV negative, PI positive).

Electron microscopy

The ultrastructural sperm analysis was carried out in TDOE-treated and untreated pooled rabbit semen samples by transmission and scanning electron microscopy (TEM and SEM respectively). For TEM, sperm samples were fixed in a cold Karnovsky fixative and maintained at 4 °C for 2 h. Fixed semen were washed in 0.1 mol/L cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1 h at 4 °C, then dehydrated and embedded in Epon Araldite. Ultra-thin sections were cut with a Supernova ultramicrotome (Reickert Jung, Vienna, Austria), mounted on copper grids, stained with uranyl acetate

and lead citrate and then observed and photographed with a Philips CM10 TEM (Philips Scientific, Eindhoven, the Netherlands).

For SEM, sperm samples were fixed as described above and smeared on poly-lysine (1%) coated cover slides. After dehydration, specimens were dried by using the critical point technique and then coated in gold and examined with a Philips CM 515 SEM (Philips Scientific, Eindhoven, the Netherlands).

For each rabbit sample, one hundred spermatozoa were analyzed using both microscopic techniques.

Sperm cells were considered to have an altered ultrastructure when revealing alteration of the plasma membrane and/or acrosomal membrane, mitochondria, axonemal pattern and chromatin status.

Statistical evaluation

A linear model was used to verify the effect of TDOE dose (0, 5, 10, 50, 75) on sperm characteristics (Statacorp®, 2005). Differences were considered statistically significant when $P< 0.05$.

RESULTS AND DISCUSSION

It is known that various environmental pollutants have detrimental effects on reproductive function of human beings and animals (Duty *et al*., 2004). In this study, we evaluated the *in vitro* effects of the organic part of TD on rabbit spermatozoa. To our knowledge, this is the first study addressing this issue.

The concentrations used in these experiments were chosen following Gualtieri *et al*. (2005b), who tested the effects of TDOE at 10, 50, 60 and 75 μg/mL concentrations on the human lung A549 cell line for 24, 48, and 72 h. Concentrations were calculated by considering the amount of TD in the air in a polluted urban area and inhaled daily by humans.

The choice of rabbit spermatozoa was based on the ease with which semen can be collected with an artificial vagina, so avoiding the use of invasive methods (surgery) or expensive and difficult procedures (Castellini *et al*., 2006). In addition, there is no need to sacrifice the animal as occurs with the use of other experimental animals such as mice and rats. Unlike human beings, the diet and lifestyle of rabbits are standardized – so excluding potential interferences with analytical outcomes. Moreover, one of the

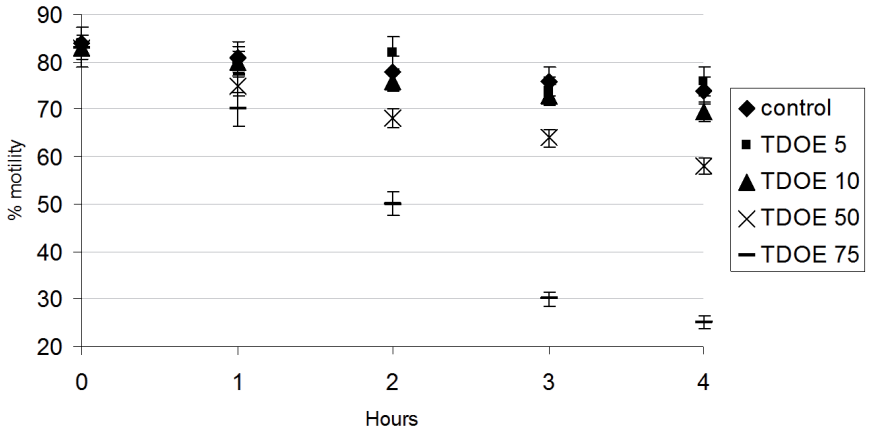

Figure 1: Effect of the organic extract of tyre debris (TDOE) concentrations on motility rate of rabbit spermatozoa during incubation for 4 h (95% upper and lower confidence interval).

Table 1: *In vitro* effects of the organic extract of tyre debris (TDOE) at different concentrations on kinetic traits (motile cells, %; curvilinear velocity, VCL, μm/sec), apoptosis and necrosis (%), and transmission electron microscopy (TEM) evaluated morphology of rabbit spermatozoa after a 4 h exposure.

TDOE treatment	Motile cells (%)	VCL (μm/sec)	AnV−/ PI− %	AnV+/ PI– %	AnV−/ PI+ %	AnV+/ PI+ %	Altered sperm cells (TEM) %
0 μg/mL	76.30^{c}	183.65^{b}	81.50^{b}	6.25^{a}	5.75	6.50^{a}	7.75^{b}
10 μg/mL	76.64^{c}	186.17^{b}	83.02^{b}	8.35^{a}	7.05	6.52^{a}	8.00^{b}
50 μg/mL	59.93^{b}	168.34^{ab}	79.96^{b}	12.15^{ab}	4.25	7.54^{a}	9.21^{b}
75 μg/mL	28.23^{a}	142.13^{a}	36.67^{a}	17.67^{b}	8.67	37.0^{b}	46.67^{a}
Pooled SE	6.45	3.18	1.77	9.11	5.68	4.50	6.81

AnV−/ PI−: intact sperm, AnV+/ PI–: PS externalization (apoptosis), AnV−/ PI +: broken plasma membrane (necrosis), AnV+/ PI +: PS externalization and broken plasma membrane (apoptosis and necrosis).
^{abc} Data with different letters in the same column are statistically different ($P< 0.05$).

major problem for evaluating direct damage caused by chemical compounds in human samples is related to the well known wide variability of semen parameters such as concentration, morphology and motility detectable either in infertile or fertile individuals. On the contrary semen of genetically selected rabbits, such as those used in this study, is characterized by a low variability of kinetic parameters. This is an important factor for assuring a high repeatability of outcomes (Theau-Clèment *et al*., 2003).

The changes in motility rate of spermatozoa after incubation with TDOE at different concentration levels (0, 5, 10, 50 and 75 μg/mL) and different times of exposure (0, 1, 2, 3 and 4 h) are shown in Figure 1. A significant reduction of this kinetic variable was observed after treatment with TDOE at levels higher than 50 μg/mL (Table 1), and this variation was time-dependent. After a 4 h exposure, the percentage of motile spermatozoa was reduced by 21% ($P<0.05$) at 50 μg/mL concentration.

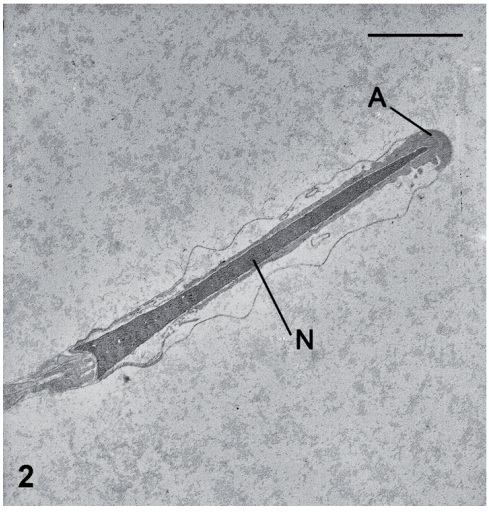

Figure 2: Transmission electron microscopy micrograph of a longitudinal section of a normal head of a rabbit spermatozoon after incubation for 4 h with the organic extract of tyre debris at a concentration of 10 μg/mL. Bar 1μm.

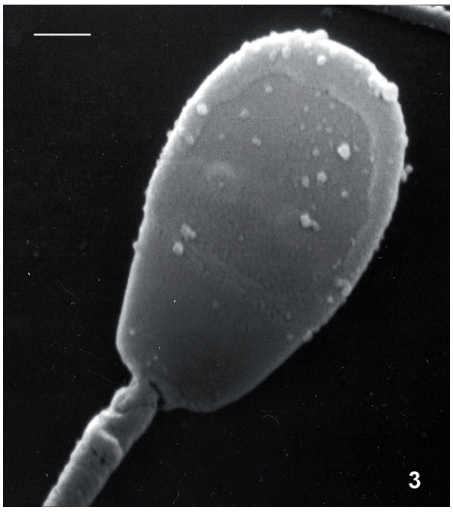

Figure 3: Scanning electron microscopy micrograph of rabbit sperm head after 4 h incubation with he organic extract of tyre debris at a concentration of 10 μg/mL. The plasma membrane appears intact. Bar 1 μm.

The VCL also reduced ($P<0.05$) after exposing spermatozoa to TDOE, but at the highest concentration level (75 µg/mL), VCL proved more resistant than motility.

These results lead us to hypothesize about a potential interaction of TDOE with crucial enzymes involved in oxidative phosphorylation coupling, or with the structural proteins in the axonemal microtubules that are involved in the flagellar movement of these cells − as proposed for organic metals and other toxicants (Castellini *et al*., 2009).

A 4 h incubation of rabbit spermatozoa with TDOE at a concentration level lower than 10 µg/mL did not produce any type of morphologic damage (Figures 2, 3). However, the percentage of intact spermatozoa decreased by half ($P<0.05$) after treatment with TDOE at the highest concentration level (Table 1). At the same time, the number of apoptotic (AnV+/ PI−) and apoptotic + necrotic (AnV+/ PI+) spermatozoa significantly increased ($P<0.05$) at that level of TDOE concentration. Nevertheless, necrotic cells did not significantly vary with increased TDOE concentration.

Besides the occurrence of PS externalization at the plasma membrane level, TDOE significantly affected the morphology of the sperm head as shown by TEM and SEM analysis. A concentration of 75 µg/mL of TDOE produced a considerable vesiculation and numerous holes in the plasma membrane of rabbit spermatozoa as shown by SEM analyses (Figure 4). Ultrastructural abnormalities were also confirmed by TEM (Figure 5). Spermatozoa showed broken plasma membranes and disrupted chromatin. TDOE-treated spermatozoa (50 µg/mL) often revealed broken plasma membranes and/or acrosomal membranes, swollen and disorganized mitochondria, and an altered axonemal pattern (absence of the usual 9+2 assembly). On the contrary, the chromatin was rarely disrupted (Figure 6). Changes in the number of morphological altered spermatozoa (as evaluated by TEM) after treatment with TDOE at increasing concentration levels

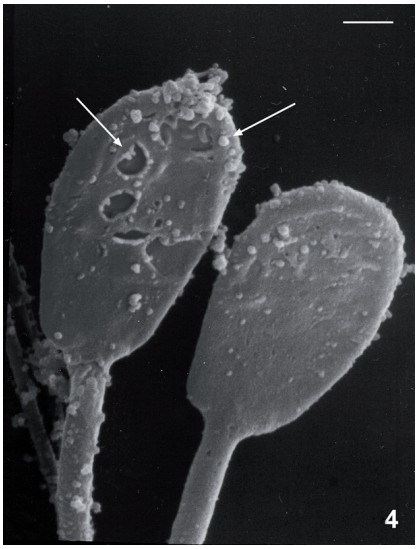

Figure 4: Scanning electron microscopy micrograph of rabbit sperm heads after 4 h incubation with the organic extract of tyre debris at a concentration of 75 µg/mL. The acrosomes are altered, highlighting the presence of holes and vesicles (arrows). Bar 0.5 µm.

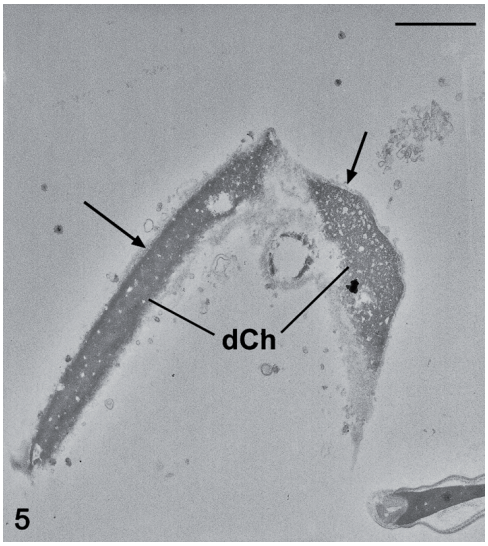

Figure 5: Transmission electron microscopy micrograph of cross section of rabbit spermatozoa after 4 h incubation with the organic extract of tyre debris at a concentration of 75 µg/mL characterized by nuclei with disrupted chromatin (dCh). The acrosomes are absent and the plasma membrane is broken (arrows). Bar 1 µm.

are reported in Table 1. A sperm cell was considered altered if at least one of the listed defects was present. In particular, the observed altered axoneme assembly and/or swollen and disorganized mitochondria of TDOE-challenged spermatozoa further support the detrimental effect of this toxicant on the sperm motion system. Further studies are needed to verify this hypothesis and explore the effects of single compounds of TDOE on this function.

Besides the tail, the integrity of the spermatozoa head was seriously affected after incubation with TDOE at the highest concentration level. The most sensitive part of the sperm head to TDOE treatment was the plasma membrane, and particularly the part covering the acrosomal cap, as shown by SEM, TEM and PI staining. Damage occurring at plasma membrane level has also been reported in human lung epithelial cells after TDOE treatment (Beretta *et al*., 2007). Various membrane alterations were, moreover, observed by Castellini *et al*. (2009) after *in vitro* treatment of rabbit spermatozoa with different organic and inorganic metal compounds. The higher vulnerability of the plasma membrane coating the acrosome cap is due to its physiological structure, characterized by a certain instability to ensure a natural break down after interaction with the oocyte's zona pellucida. Nevertheless, molecular studies will be performed to clarify this aspect.

In addition to plasma membrane breakage, another important structural change in spermatozoa occurred after the TDOE challenge: the translocation of PS from the inner to the outer side of plasma membrane, as confirmed by the annexin V staining. This structural change has been considered as a precocious sign of apoptosis, even though it has been also detected during the spermatozoa capacitation and acrosome reaction (Avalos-Rodrigez *et al*., 2004). Taking into account these considerations, it can be hypothesized that TDOE may induce a precocious acrosome reaction and make sperm unable to naturally fertilize an egg.

A question that may naturally arise is how these compounds may *in vivo* reach the reproductive organs causing possible damage. The blood-testicular barrier is very selective and protects this tract from most of the toxicant agents accidentally introduced in the organism. A hypothesis is that TD, consisting in organic and inorganic compounds, may accumulate in the white adipose tissue, a known reservoir of lipophylic environmental pollutants (Mullerová and Kopecký, 2007). As for other hydrophobic environmental contaminants, TD may alter the metabolism and function of the adipose tissue and be chronically released in the whole organism. Furthermore, conditions affecting the integrity of the blood-testicular barrier (inflammation, infections) may render the access of TD to the reproductive organs feasible. Whether TD can accumulate in the adipose tissue and pass through the blood-testicular barrier has to be established by *in vivo* studies.

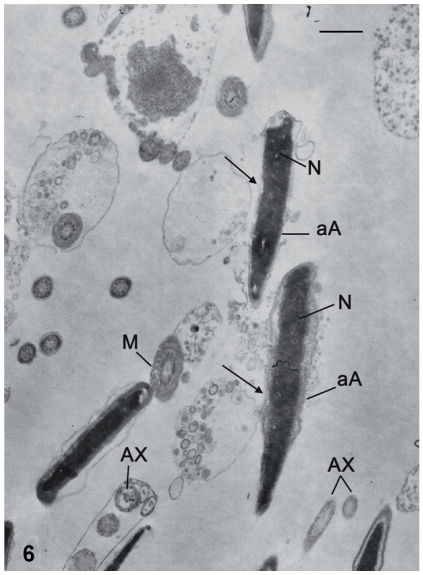

Figure 6: Transmission electron microscopy micrograph of longitudinal and cross sections of rabbit spermatozoa after 4 h incubation with the organic extract of tyre debris at a concentration of 50 µg/mL. Acrosomes are reacted or absent (aA), plasma membranes are broken (arrows). Mitochondria (M) are swollen and disassembled and axonemal pattern (AX) altered, nucleus (N). Bar 1 µm.

In conclusion, our results, although preliminary, support the evidence that the organic part of tyre debris is potentially harmful to spermatozoa over a threshold level (50-75 µg/mL), inhibiting motility and affecting head morphological integrity. Nevertheless, the results cannot yet be extrapolated to an *in vivo* outcome, because studies have not yet been performed. Finally, kinetic and morphological parameters could be useful quantifying endpoints for TD-related spermiotoxicity.

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