Effect of PM10 on Human Spermatozoa: An *In Vitro* Study

M. Geminiani[1], F. Moretti[1,3], R. Cardinali[4], P. Lattaioli[4], F. Cetta[2], C. Castellini[4] and G. Collodel*[,1,3]

[1]*Department of Biomedical Sciences, Applied Biology Section, University of Siena, Siena, Italy;* [2]*Department of Surgery, University of Siena, Siena, Italy;* [3]*Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena;* [4]*Department of Applied Biology, Animal Science Section, University of Perugia*

Abstract: This study was aimed at evaluating the *in vitro* effects of particulate matter (ranging from 2.5-10 μm in an aerodynamic diameter, PM10) from an urban area on the motility of human spermatozoa.

Samples of swim up selected human spermatozoa from three healthy men with normal semen parameters, according to WHO guidelines, were incubated for 4 hours with 10, 50 and 75 μg/ml of PM10. Kinetic parameters were evaluated with Computer-Assisted Sperm Analysis (CASA) and the presence of apoptosis and necrosis was assessed by AnnexinV/Propidium iodide assay.

Data was statistically elaborated.

Incubation of sperm with 10 μg/ml PM10 for 4 hours did not affect sperm motility. On the contrary, a decrease in progressive motility was evident at 50 μg/ml and 75 μg/ml PM10. Among the kinetic parameters assessed with CASA system, the linearity variable was significantly lower in sperm treated with 50 μg/ml and 75 μg/ml PM10 vs controls ($P<0.0001$) and the amplitude of the lateral head displacement variable was significantly higher in sperm treated with 50 μg/ml vs controls ($P=0.0211$). A substantial increase in necrosis, in up to 30% of sperm, was evident only in samples treated with 75 μg/ml PM10.

In conclusion, this preliminary study could indicate that PM10 is not potentially harmful to spermatozoa *in vitro*, except above a very high threshold (50-75 μg/ml) that is difficult to reach *in vivo* and that motility and viability are only partially affected.

Keywords: CASA, human sperm motility, sperm necrosis, PM10.

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INTRODUCTION

Particulate matter (PM) is a complex mixture of dust, dirt, soot, smoke and 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 (2.5-10 μm) [1].

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 [2, 3]. The mechanisms underlying PM toxicity are not fully known: inflammation [4], oxidative stress [5] and DNA damages [6] seem to be involved.

Recently, the effects of air pollution on reproductive and birth outcomes have attracted increased interest [7-9]. However, a limited amount of *in vivo* research has been conducted to examine the association between air pollution and male reproductive outcomes. A decline in semen quality, a phenomenon that occurs in developed countries, has been suggested to be associated with enhanced exposure to environmental chemicals that act as endocrine disrupters as a result of an increased use of pesticides, plastics, and other anthropogenic materials. A significant amount of toxicology data based upon laboratory and wildlife animal studies has suggested that exposure to certain chemicals is associated with reproductive toxicity involving reduced semen quality parameters (concentration, motility and morphology) and impaired fertility [10]. After analyzing the *in vivo* effects of air pollution on sperm quality, two different research groups reported a modest action of PM2.5 on sperm motility [11] and sperm head morphology [12].

Semen could be considered the most accessible reproductive endpoint, which can be easily analyzed by well established procedures [13].

The aim of this study was to investigate the *in vitro* effect of PM with a diameter of ≤10μM (PM10) on a fraction of swim up selected human spermatozoa, studying kinetic parameters with Computer-Assisted Sperm Analysis (CASA) and analysing the presence of apoptosis and necrosis by Annexin/Propidium iodide assay.

*Address correspondence to this author at the Department of Biomedical Sciences, Applied Biology Section, University of Siena, Policlinico Le Scotte, Viale Bracci, 14, 53100 Siena, Italy; Tel: 39-0577-233539; Fax: 39-0577-233527; E-mail: collodel@unisi.it

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MATERIALS AND METHODOLOGY

Particulate Matter (PM)

PM10 was collected at Torre Sarca, a site in the Milan area, considered representative of background urban atmospheric pollution concentration. The method used to sample PM10 was reported by Gualtieri *et al*. [14].

PM10 was stored in sterile Phosphate Buffer Saline (PBS) at a concentration of 2 μg/μL and then sonicated for 30 minutes at 37 °C.

Semen Analysis

Semen samples were collected from three healthy men (aged 30-35) with normal semen parameters referred to the Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena. The criteria for participation in the study included the presence of a normal karyotype and the absence of anatomical problems, such as varicocele, cryptorchidism, or genito-urinary infections. Patients were informed of and gave written consent for the procedures related to the study.

Semen samples were collected by masturbation after 4 days of sexual abstinence and examined after liquefaction for 30 min at 37 °C. Volume, pH, concentration and motility were evaluated according to World Health Organization guidelines [13].

Sperm Selection and PM10 Treatment

Motile sperm fractions were selected by swim up technique. A direct swim up of sperm from semen was performed: an aliquote of 1 ml of semen sample was placed in a sterile conical centrifuge tube and gently layered with 1.2 ml of Quinn's ® Sperm Washing Medium (Sage, *In vitro* Fertilization, Inc., Trumbull, CT, USA). The tubes, inclined at a 45° angle, were incubated for 45 min at 37 °C with 5% CO_2. The top 1ml of medium was then recovered, which contained highly motile sperm cells.

Selected sperm cells were incubated with PM10 in Biggers, Whitten, Whittingham (BWW) buffer at a concentration of 10, 50 and 75 μg/ml for 4 hours at 37 °C with 5% CO_2. The range of concentrations was selected considering the data reported by Gualtieri *et al*. [15] while the time of exposure was determined by Moretti *et al.* [16]. Aliquots of samples not incubated with PM10 were used as controls. An experiment was performed for each semen sample obtained, therefore the experiment was repeated three times.

Evaluation of Sperm Movement by CASA

The kinetic characteristics of PM10-treated and untreated selected motile sperm 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 for CASA were those previously established by Castellini and Lattaioli [17] and the acquisition rate was set at 100 Hz. For each sample, two drops and six microscopic fields were analysed.

Sperm motion parameters were recorded, including **VCL**: Curvilinear velocity; **LIN**: Linearity; **ALH**: Amplitude of lateral head displacement; **VSL**: Straight Line Velocity; **WOB**: VAP/VCL; **VAP**: Smoothed Path Velocity; **STR**: Straightness (ratio of VSL/VAP); **BCF**: Beat Cross Frequency. In particular, we analyzed:

- Motility (%), the number of motile spermatozoa within the analysis field was divided by the sum of the motile plus immotile spermatozoa within the field. Sperm motility was categorized into 3 grades: progressive motility (rapid and slow), non progressive motility, and immotile sperm;
- Curvilinear velocity (VCL, μm/s) 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;
- Linearity (LIN, %), the percentage of VSL/VCL ratio (VSL, straight-line velocity, defined as the distance between the start and end points of the track divided by the time taken for spermatozoa to cover the track);
- Amplitude of lateral head displacement (ALH, μm) the average deviation from the smoothed path based on the difference in linearity between the smoothed and the real path.

We particularly considered good parameters VSL and LIN as indicators of sperm progression, whereas VCL and ALH were noted as measures of sperm vigour. LIN was also used to describe sperm swimming patterns.

AnnexinV/Propidium Iodide Assay

The detection of apoptosis (phosphatidylserine externalization) and necrosis (disrupted membranes) in treated and untreated semen samples was performed with a Vybrant Apoptosis Assay kit (Invitrogen Ldt, UK) made up of Annexin V-FITC and Propidium iodide as reported in Moretti *et al*. [16]. Observations were made and photographs were taken with a Leitz Aristoplan (Leica, Wetzlar, Germany) light microscope equipped with a fluorescence apparatus. A total of 100 spermatozoa from each sample were scored. By staining cells with FITC-Annexin V (AnV, green fluorescence), and simultaneously with the non vital dye Propidium iodide (PI, red fluorescence), it is possible to recognize intact cells (AnV negative, PI negative), early apoptotic cells (AnV positive, PI negative), damaged sperm with phosphatidylserine externalization (AnV positive, PI positive) and damaged necrotic sperm (AnV negative, PI positive).

Statistical Analysis

Statistical analysis was performed using the version 8 SAS system (Sas Institute Inc. Cary, NC 27513, USA). The Kolmogorov-Smirnov test was used to verify normal or non-normal distribution of values. Differences in the characteristics of basal samples and treated samples and their significance were evaluated using the Kruskal-Wallis test since the variables were not normally distributed. When a statistically significant difference was found among the groups, the Mann Whitney U-test was then used between pairs of groups. In this case, Bonferroni's correction was applied to reduce falsely significant results and $P \leq 0.05$ was considered significant.

RESULTS

Swim up selected samples exhibited a progressive motility ranging from 90% to 95%.

The incubation of sperm with 10 μg/ml PM10 for 4 hours did not affect sperm motility. On the contrary, a decrease in progressive motility was evident at 50 μg/ml and 75 μg/ml PM10; particularly, at 50 μg/ml PM10 we observed that the 35% of sperm exhibited non progressive motility, while at 75 μg/ml PM10, 50% of sperm were immotile (Fig. **1**). It is noteworthy that at 50 μg/ml PM10, non-progressive motility was characterized by spermatozoa displaying circular trajectories.

Among the parameters of sperm motility assessed with the CASA system reported in Table **1**, curvilinear velocity (VCL), linearity (LIN) and the amplitude of lateral head displacement (ALH) were considered as more representative for this study.

Using the Kruskall-Wallis test, the VCL variable was not statistically significant among the analysed PM10 concentrations; for the other variables, when a statistical difference was detected, the Mann Whitney U-Post Hoc test was applied to compare the pairs. The LIN variable was not affected by a 10 μg/ml PM10 treatment (no significant difference between group I and group IV). The LIN variable was significantly lower in sperm treated with 50 μg/ml and 75 μg/ml PM10 (significant differences between group I and groups II/III, between group IV and groups II/III and between groups II and III). The ALH variable was significantly higher in group II *vs*. controls (Table **1**). The other CASA kinetics parameters reported in Table **1**, such as VSL, WOB, VAP, STR and BCF, showed significant differences between groups II/III compared to group IV, at times reached significant differences between groups I and II, but did not highlight any significant difference in values between groups I and IV.

All the treated and untreated swim up specimens were then processed by Annexin V/PI.

This assay showed that the percentages of necrosis and apoptosis were similar to controls in the samples incubated with 10 μg/ml PM10. In samples incubated with 50 μg/ml PM10, only a slight increase in sperm with PS externalization (apoptosis) was noted, whereas a substantial increase in necrosis, up to 30% of sperm, was evident in samples treated with 75 μg/ml PM10 (Fig. **2**).

DISCUSSION

The present study sheds more light on the impact of PM10 exposure on human sperm using an *in vitro* model. To our knowledge, this is the first study that has evaluated the effect of PM10 on a swim up selected fraction of human spermatozoa.

One of the major problems in evaluating the direct damage of chemical compounds in human samples is the well known broad variability of semen parameters, such as concentration, morphology and motility, detectable in infertile and fertile subjects, as well as in different ejaculates from the same individual. For this reason, our experiments were performed in a swim up selected fraction of sperm which was as homogeneous as possible.

Particulate matter sampling in Milan revealed a mean annual PM10 concentration of 56 $\mu g/m^3$, with maximum daily peaks of over 200 $\mu g/m^3$ in winter. These values exceed the annual mean limit of 40 $\mu g/m^3$ and daily mean limit of 50 $\mu g/m^3$ imposed by EU air quality directive 99/30/CE [14].

Although the PM10 concentrations used in this study are higher than that measured in the air, they are not potentially harmful to human sperm *in vitro*.

Treatment with 10 μg/ml PM10 did not affect motility and vitality. However, we cannot exclude that this low concentration might compromise the integrity of sperm DNA.

Sperm motility was negatively affected only at PM10 concentrations higher than 10 μg/ml. In particular, at 50 μg/ml PM10 sperm displaying a circular trajectory, also demonstrated by the ALH value, were observed. As the PM10 concentration increased, circular motility, progressive

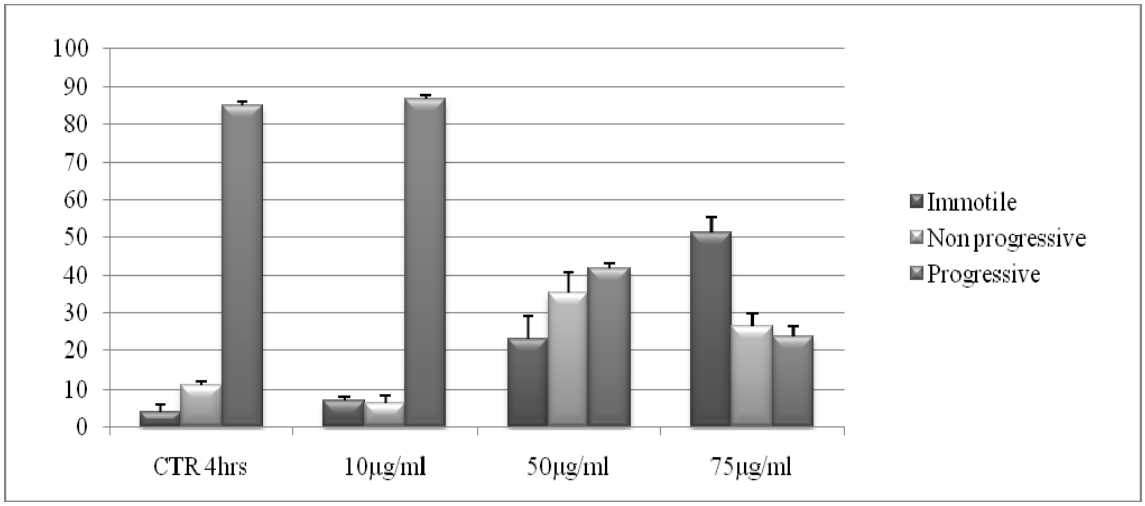

Fig. (1). Mean and standard deviations in the motility percentage of sperm treated with different PM10 concentrations after 4 hours of incubation. CTR= controls, under the same conditions without PM10.

Table 1. Medians and Ranges of Kinetic Parameters Evaluated with the CASA System

Variable	Group 1 PM10 10 µg/ml	Group II PM10 50 µg/ml	Group III PM10 75 µg/ml	Group IV Controls	Kruskal-Wallis Test (p-Value)	Mann Witney U Post hoc test
VCL	121.19 33.71-205.85	116.84 12.65-209.75	121.27 13.85-199.4	120.04 33.71-205.85	0.2737	
VSL	44.74 4.54-115.64	35.09 0.84-94.65	30.13 0.84-75.18	40.35 2.34-109.35	<.0001	IxII**;IxIII**;IIxIII**; IIxIV**;IIIxIV**
VAP	74.07 13.21-117.67	64.73 6.25-108.51	63.24 6.25-101.83	71.59 6.25-112.94	<.0001	IxII**;IxIII**;IIxIV**; IIIxIV**
LIN	0.35 0.03-0.97	0.33 0.01-0.94	0.2 9 0.01-0.87	0.38 0.01-0.91	<.0001	IxII*;IxIII**;IIxIII*; IIxIV**;IIIxIV**
STR	0.67 0.05-0.98	0.62 0.02-0.98	0.51 0.02-0.95	0.68 0.02-0.97	<.0001	IxII**;IxIII**;IIxIV**; IIIxIV**
WOB	0.62 0.27-1	0.57 0.33-0.98	0.54 0.27-0.95	0.59 0.3-0.94	<.0001	IxIII**;IIxIV**;IIIxIV**
ALH	2.93 0.82-6.05	3.09 0.57-5.67	3.1 0.59-5.33	2.79 0.68-6.41	0.0211	IIxIV*
BCF	15 0-29.63	15 0-28	12.5 0-27.14	16 3-31	<.0001	IxIII**;IIxIII**;IIxIV**; IIIxIV**

* p<0.05; ** p<0.01 after ***Bonferroni's correction***

VCL: Curvilinear velocity; **LIN**: Linearity; **ALH**: Amplitude of lateral head displacement; **VSL**: Straight Line Velocity; **WOB**: VAP/VCL; **VAP**: Smoothed Path Velocity; **STR**: Straightness (ratio of VSL/VAP); **BCF**: Beat Cross Frequency. The significant values reported in the last column were obtained by applying the Mann Whitney U-test among the pairs of groups.

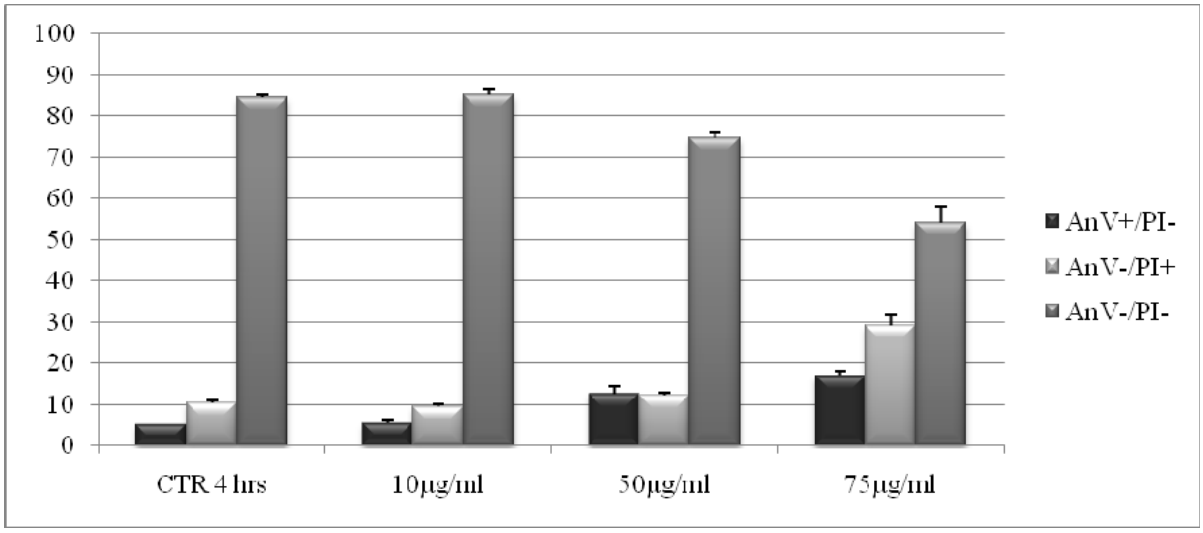

Fig. (2). Means and standard deviations of Annexin V (AnV)-FITC and Propidium iodide (PI) assays performed in swim up selected sperm (nuclei). Intact cells appeared unstained (AnV- PI-), apoptotic cells are green stained with FITC-Annexin (AnV+ PI-), necrotic cells are red stained (AnV- PI+). CTR= controls, under the same conditions without PM10.

motility and linearity of sperm trajectory decreased and concomitantly immotile sperm increased.

Screening with Annexin V/PI confirmed that 50% of sperm were still viable at the highest PM10 concentration used in the study.

A recent *in vivo* study by Hammoud *et al.* [11] carried out in the Salt Lake City area reported a negative correlation between PM 2.5 values and sperm motility. However, the effect on motility was minimal and was unlikely to affect the fertility of the studied population.

Different results were reported in another study by Hansen *et al.* [12], demonstrating that levels of particulate matter below the current National Ambient Air Quality Standards were not associated with significant decrements in sperm outcomes, however some results have suggested effects on sperm concentration and morphology.

The results obtained in the present study obviously cannot be extrapolated into an *in vivo* outcome, yet they supply an indication of the effects of PM10 on sperm cells. It should be considered that it is possible that PM10 does not

act directly on sperm cells due to the presence of the blood-testis barrier, a very selective structure that protects these cells from most toxic agents that are accidentally introduced into the organism. However it is known that PM10 may induce a dose dependent increase in interleukins, well known inflammatory mediators [14, 18-21]. This phenomenon may be an indirect cause of chronic inflammation which could contribute to compromise male reproductive function.

CONCLUSION

These preliminary results might indicate that PM10 are not potentially harmful to spermatozoa *in vitro*, but that they can be deleterious only over a very high level (50-75 μg/ml) that is difficult to reach *in vivo*, and that motility and viability are partially affected by these higher levels.

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