IN VITRO EFFECT OF SILVER ENGINEERED NANOPARTICLES ON HUMAN SPERMATOZOA

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Abstract. Silver (Ag) NPs are among the most commercialized NPs due to their antimicrobial potential. They are highly attractive for possible applications in manufacture of medical device. However there is a serious lack of information concerning their impact on the human health and the environment. Moreover studies on the effects of NPs on ejaculated sperm are rather limited. For these reasons our study explored the in vitro effects of Ag NPs on human ejaculated spermatozoa.
Ag NPs have been produced, characterized, and furnished by Colorobbia Industry, Sovigliana (Vinci, Florence, Italy). Aliquots of total semen were incubated at 37°C for 60 minutes (min) and 120 min at the concentration of 125 µM, 250 µM, and 500 µM of engineered Ag NPs. The control was represented by specimens of semen samples treated with the same procedure without NPs. After the incubations, sperm motility was evaluated following WHO guidelines and sperm viability was evaluated by Eosin Y test. At the end of incubation with Ag NPs the samples were processed by a Field Emission Gun-based Scanning Transmition Electron Microscope/ Energy Dispertion Spectrometry (STEM/EDS).
We observed that sperm motility percentage was significantly reduced in semen samples treated with 125 µM, 250 µM and 500 µM of Ag NPs after 60 min and 120 min of incubation respect to controls ($P<0.001$; $P<0.01$, 125 µM at 60 min). Sperm viability percentage significantly decreased in a progressive manner after 125 µM ($P<0.05$), 250 µM ($P<0.05$) and 500 µM ($P<0.001$) Ag NPs incubation at 60 min and 120 min. We did not find any significant difference between the values assessed after 60 min of NPs incubation and those estimated after 120 min of incubation. In the control samples, the sperm motility and the sperm viability percentages significantly decreased after 120 min of incubation ($P<0.001$) respect to the basal values. Ag NPs were undetectable in all treated samples by STEM/EDS.
These in vitro results show a decline in sperm motility and viability in even at the lowest concentration used and the cytotoxic effect occurs in a dose dependent manner. It is noteworthy that in each experiment, for each concentration of NPs used, the percentage of sperm viability was always higher than the percentage of sperm motility; it means that spermatozoa were viable but immotile. Moreover Ag NPs was undetectable in all the treated samples by STEM/EDS analysis. We may hypothesize that Ag NPs, under aqueous conditions, release Ag+ that could damage sperm membrane and/or penetrate inside the cells and interfere with disulphide bonds of proteins of the periassonemal structures of the sperm tail.
Keywords: silver nanoparticles, human sperm, sperm motility, sperm viability.

INTRODUCTION

The fast growing of nanotechnology led to an increased of the industrial production of nanoparticles (NPs). They have dimensions below 100 nm and they seem to have different physical and chemical properties from their bulk materials. Their use have been proposed for industrial and biomedical applications. NPs are attractive for medical purposes for their important and unique features, such as their surface to mass ratio that is much larger than that of other particles, their quantum properties and their ability to adsorb and carry other compounds [1].
However, despite the wide application of nanomaterials, there is a serious lack of information concerning the impact of NPs on human health and on the environment.
Our interest was focussed on the reproductive field where studies on the effects of NPs on ejaculated sperm are rather limited.
Wiwanitkit et al. [2] showed that sperm motility was affected when human spermatozoa were incubated with gold NPs, which were also able to penetrate in the sperm head and tail.
On the other hand, Makhluf et al. [3] demonstrated that the motility and the ability to undergo the acrosome reaction of bovine spermatozoa were not affected by the presence of the magnetite NPs. The same group proved that magnetite NPs conjugated to anti-protein kinase C (PKC) antibody can be utilised for PKC localization and inhibition of its function in sperm cells [4].
Although in vivo testing will continue to provide the most relevant information on human hazards, there is an economic and ethical impetus to minimize the burden of animal testing for rapid and validated alternative models of in vitro cytotoxicity [5].

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For these reasons our study explored the in vitro effects of silver (Ag) NPs on human ejaculated spermatozoa.

MATERIALS AND METHODS

Ag NPs characterization

The Ag NPs suspension was prepared by a polyol-mediated synthesis with bottom up approach using an organometallic precursor of Ag. This procedure enables to obtain metal NPs dispersion in an organic medium or in water by means a rapid nucleation of metallic salts. The procedure involves an initial phase in which reagents were added in high-temperature environment of reaction containing a solvent, such as glycolic solvent medium, with a high capacity for complexing. The next step concerns the crystal growth stage performed by mixing the reagents including tensioactives such as polyvinilpyrrolidone (PVP), at room temperature. The monitoring of the kinetic of reaction and the choice of solvents and complexing agents can control the size and shape of synthesized NPs. The obtained nano-suspensions were stable for long periods of time (more than one year) without showing evident phenomena of aggregation of NPs and changes in chemical characteristics.

The morphology and the hydrodynamic dimension of NPs have been determined with Field Emission Gun-based Scanning Transmition Electron Microscope/ Energy Dispertion Spectrometry (FEG-STEM/EDS, model Supra 40 by Zeiss Oberkoken, Germany) available at Colorobbia Industry (Montelupo Fiorentino, Florence) and with Dynamic Light Scattering, Nano-S model by Malvern.

The size of used Ag NPs was 65nm. The concentration of Ag NPs stock solution was 1% in distilled H_2O. PVP (<1%) was the stabilizing agent.

Human semen samples

Semen samples were obtained from 10 healthy donors recruited at the Interdepartmental Centre for Research and Therapy of Male Infertility, University of Siena. All participants in this study signed a written informed consent. Semen samples were collected by masturbation after 3-5 days of sexual abstinence and examined after liquefaction for 30 min at 37°C. Volume, pH, concentration, motility, and morphology were evaluated according to WHO guidelines [6]. Sperm viability was evaluated by Eosin Y test.

Experimental design

For each sample, aliquots of total semen were incubated at 37°C for 60 min and 120 min at the concentration of 125 µM, 250 µM and 500 µM of Ag NPs. The control was represented by specimens of semen samples treated with the same procedure without NPs. The experiments were repeated ten times. In each aliquots sperm motility and viability were evaluated. At the end of incubation the samples were processed for STEM/EDS.

FEG-STEM/EDS

Human ejaculated sperm samples were incubated with Ag NPs and then fixed in cold Karnovsky fixative and maintained at 4°C for 2 h. Fixed specimens were washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, postfixed in 1% buffered osmium tetroxide for 1h 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 nickel grids. The samples were then observed with the STEM/EDS available at Colorobbia Industry (Sovigliana Vinci, Florence).

Statistical analysis

The data are reported as median and interquartile difference (75° - 25°). In the control group, the comparisons between the values of basal semen and values after 60 min and 120 min incubation were analysed by Friedman test for repeated measures. When a statistically significant difference was found, the Dunn's multiple comparison test was used between pairs of groups. The comparisons between the values of sperm motility and viability percentage in control group and after Ag NPs treatment at 60 min and 120 min were analysed with the statistical methods previously described. A P value <0.05 (two-tailed) was considered statistically significant. All analyses were performed by GraphPad Software Inc. version 5.00 for Windows.

RESULTS

The analysed human semen samples were examined by optical microscopy and classified according to WHO guidelines: the concentration, measured as sperm/ml, ranged from $75x10^6$ to $196 x10^6$ (> 10 < 50 centile) the motility from 42% to 67% (> 5 < 75 centile), the sperm normal morphology from 28% to 32% (> 75 < 90 centile) and the viability from 65% to 88% (> 10 ≤ 90 centile).

Sperm aliquots of total semen and separated spermatozoa were incubated for 60 min and 120 min with 125 µM, 250 µM, and 500 µM of Ag NPs. Aliquots treated under the same conditions without NPs were used as controls. After incubation, the sperm motility and the sperm viability were evaluated in all samples and the obtained values are reported in Table I.

In the control samples, the sperm motility and the sperm viability percentages significantly decreased after 120 min of incubation (P<0.001) respect to the basal values.

The sperm motility percentage was significantly reduced in semen samples treated with 125 µM, 250 µM, and 500 µM of Ag NPs after 60 min and 120 min of incubation respect to controls (P<0.001; P<0.01, 125 µM at 60 min).

Sperm viability percentage significantly decreased in a progressive manner after 125 µM (P<0.05), 250 µM (P<0.05) and 500 µM (P<0.001) Ag NPs incubation at 60 and 120 min.

We did not find any significant difference between the values evaluated after 60 min of NPs incubation and those estimated after 120 min of incubation.

Table1.
The medians and interquartiles differences of sperm motility percentage and viability percentage measured at 60 min and 120 min after incubation with 125 µM, 250 µM and 500 µM Ag NPs. The experiments were repeated in 10 different semen samples.

NPs concentration	Sperm Motility %			Sperm Viability %		
	Basal	60'	120'	Basal	60'	120'
Control	*51 (13)*	*44.5 (10)*	*40 (5)* °°°	*70 (9)*	*67 (11)*	*62.5 (7)*°°°
Ag 125 µM		35.5 (12)**	32.5 (15)***		63 (10)*	60 (9)**
Ag 250 µM		35 (28)***	23.5 (16)***		61.5 (14)*	53.5 (21)**
Ag 500 µM		22.5 (17)***	11(14)***		41 (12)***	30.5 (13)***

* p<0.05; ** p<0.01; *** p<0.001 vs control

°°° p<0.001 vs basal value

Ag NPs were undetectable in all samples by STEM/EDS.

Discussion

Ag NPs are among the most commercialized NPs due to their antimicrobial potential. Therefore they are highly attractive for possible applications in manufacture of medical device [7; 8]. One of the most interesting potential usage is related to the ability of Ag NPs to bind to HIV-1, preventing the infection of host cells [9]. Thus it is important to test the safety, the bioavaibility, the biocompatibility of Ag NPs, and consequently their possible cytotoxicity. In order to acquire insights on the Ag NPs in vitro effects, we used highly differentiated cells as ejaculated human spermatozoa.

The period of incubation (60 min and 120 min) with Ag NPs did not significantly influence the sperm motility and viability. Regarding the concentrations (125 µM, 250 µM, 500 µM) we observed a decline in sperm motility and viability even at the lowest concentration used, and the cytotoxic effect occurs in a dose dependent manner.

It is noteworthy that in each experiment, for each concentration of Ag NPs used, the percentage of sperm viability was always higher than the percentage of sperm motility; it means the spermatozoa were viable but immotile. For this reason it is possible to hypothesize that Ag NPs could interfere with membrane receptors or cell signaling involved in motility maintenance. At this purpose Braydich-Stolle et al. [10] demonstrated that Ag NPs at a concentration ≥ 10µg/ml induced a significant decline in spermatogonia stem cells proliferation, disrupting GDNF/Fyn kinase signaling. Moreover our results show that Ag NPs was undetectable in all the treated samples by STEM/EDS analysis. We may hypothesize that Ag NPs, under aqueous conditions, release Ag+ [11] that could damage sperm membrane and/or penetrate inside the cells and interfere with disulphide bonds of proteins of the periassonemal structures of the sperm tail.

In conclusion, the toxicity of Ag NPs is evident in particular at very high concentrations (250 µM -500 µM) that are probably difficult to reach in vivo; in addition the possible genotoxicity of Ag NPs cannot be excluded and urge to be explored.

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