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Effect of iron oxide and silver nanoparticles on boar semen CASA motility and kinetics

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ABSTRACT: The objective of the study was to investigate the potential toxic effect of iron oxide (Fe_3O_4) and silver (Ag/Fe) spherical nanoparticles (NPs) as alternative antimicrobial compounds on boar semen. The NPs' minimum inhibitory concentration was determined applying the *in vitro* antimicrobial activity evaluation test and included in the experiment. Totally, 9 ejaculates (3 boars; 3 ejaculates/boar) were extended in BTS without antibiotics at 30×10^6 spermatozoa/mL and divided in 3 aliquots corresponding to the following groups: 1) Control group (C): extended semen without treatment; 2) Iron oxide group (Fe): extended semen with Fe_3O_4 NPs of diameter 40 nm (0.192 mg/mL semen); and 3) Silver group (Ag): extended semen with Ag/Fe NPs of diameter 30 nm, consisted of Ag and a 5% of zero-valent Fe (0.128 mg/mL semen). Semen samples of all groups were incubated at 17° C for 30 min following NPs' removal through a magnetic field. All post treated samples were stored at 17° C for 48 h. Total motility (TM) and kinetics (progressive motility PM; rapid/medium/slow movement spermatozoa; static spermatozoa; VCL; VSL; VAP; LIN; STR; WOB; ALH; BCF; hyperactive spermatozoa) were evaluated by CASA system at 0, 24 and 48 h post treatment. Data were analyzed with a repeated measures mixed model. Group Fe did not differ from group C at any time point. TM and PM were lower at 24 h of storage in group Ag compared to groups C and Fe (all $P<0.001$). By 48 h of storage spermatozoa of group Ag were totally immotile and thus excluded from analysis. The comparison within groups and between storage time points showed that the values of TM, PM, VCL, VAP, ALH and BCF decreased after 24 h of storage in group Ag (all $P<0.05$), but not in groups C and Fe, while no significant differences were observed for the remaining parameters between successive time points within any group ($P>0.05$). In conclusion, Ag/Fe NPs demonstrated a harmful effect on boar spermatozoa, while the used concentration of the examined Fe_3O_4 NPs did not affect boar sperm CASA motility parameters enhancing further research about their application on semen handling.

Keywords: boar; semen; CASA; motility; nanoparticles

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INTRODUCTION

Semen collection in farm animals, including boars, is a procedure that takes place under no sterile conditions. Consequently, ejaculate contamination with bacteria is inevitable and a variety of bacteria from different genera has been identified after microbiological culture of semen samples (Akhter et al., 2008; Althouse et al., 2000). Semen extenders support the longevity of spermatozoa, but at the same time provide a particularly ideal environment for bacteria proliferation (Karageorgiou et al., 2016). Currently, antibiotics are the main constituent of their composition to avoid growth of bacteria and enhance reproductive performance (Salamon and Maxwell, 2000). A variety of antibiotic compounds have been used so far, to control microbial contamination in extenders. However, during the last decades, the antibiotics have been overused to control infections of animals and human beings and as a result, the antibiotic-resistant bacterial strains have been an emergency and a serious threat for the public health and animals' diseases management worldwide (Gyles, 2011). Also, regarding animals' reproduction, more recent studies have demonstrated that some antimicrobials may have a deleterious or toxic effect on bull (Azawi and Ismaeel, 2012) and equine (Varner et al., 1998) spermatozoa. Under these circumstances, an increasing scientific interest about novel compounds with antimicrobial properties has been demonstrated (Hajipour et al., 2012).

Nanotechnology as a promising and contemporaneous scientific field introduces nanomaterials, which are objects made of different shapes and sizes in nanometer scale. Due to their tiny size and their high surface to volume ratio, they provide specific physical, chemicals and biological activities (Hajipour et al., 2012). Specifically, nanoparticles (NPs) fabricated from metals, like silver (Rai et al., 2009), or metal oxides, like iron oxide (Azam et al., 2012), have received great attention by the scientific community. It is well known from the ancient years that the silver can demonstrate strong antibacterial action and nowadays it has been used as alternative agents to conventional antibiotics (Chaloupka et al., 2010). Also, iron oxide NPs have been widely used, for instance as magnetic resonance imaging contrast agents due to their magnetic properties (Babes et al., 1999), while its antimicrobial properties are well documented in the literature (Azam et al., 2012), too.

The unique properties of NPs have made them popular in different scientific fields, including medicine applications and biomedical research (Salata, 2004). Regarding semen technology, many published studies documented the antioxidant effect of nanoparticles on ram (Falchi et al., 2018), bull (Raana et al., 2015), rat (Afifi et al., 2015) and rooster (Safa et al., 2016) semen. Also, NPs have been used for sperm selection process in IVF protocols or for sperm purification and sorting for artificial insemination (Feugang et al., 2014). However, the last decades due to the extended use of NPs, there is an increasing concern about their possible side effects or toxicity in cellular level for humans' and animals' beings or even the environment. Recent studies have examined the effect of NPs on reproductive system and have focused mainly on spermatozoa indicating controversial results about their toxicity action on male gametes (Barkalina et al., 2014; Lafuente et al., 2016; Wiwanitkit et al., 2009).

The aim of the present study was to investigate the effect of iron oxide and silver spherical NPs on CASA motility and kinetic parameters of extended boar semen with the perspective of their possible use as alternative antimicrobial agents for semen handling.

MATERIALS AND METHODS

Reagents and media

All the reagents and chemicals used in the present study were of high analytical quality and were purchased from Sigma Aldrich, Seelze, Germany, unless otherwise stated. Semen samples were extended with Beltsville Thawing Solution (BTS: 205 mM glucose, 20.4 mM sodium citrate, 10.0 mM KCl, 15.0 mM $NaHCO_3$, 3.6 mM EDTA; pH 7.4; 290-300 mosmol/kg) without antibiotics.

Synthesis and dispersions of Fe_3O_4 and Ag/Fe NPs

Iron oxide (Fe_3O_4) nanoparticles and a silver-based (Ag/Fe) nanocomposite were used in this study. Fe_3O_4 nanoparticles were synthesized by the oxidative hydrolysis of Fe_2+ in aqueous media through the intermediate formation of green rust. Particularly, 5.56 g of $FeSO_4 \cdot 7H_2O$ were dissolved in 80 mL of 0.01 M H_2SO_4 solution and rapidly mixed with a second solution prepared by dissolving 1.84 g NaOH, 1.70 g $NaNO_3$ and 25 mL ethanol into 40 mL distilled water. Formed green rust precipitate was aged at 90° C for 24 h to receive spherical Fe_3O_4 nanoparticles with diameter around 40 nm. The produced solid was washed several times with distilled water until a conductivity below 1 mS/cm was obtained. On the oth-

er hand, the Ag/Fe nanocomposite was prepared by solar-powered physical vapor deposition (SPVD) in a Heliotron 2 kW glass vacuum chamber at the facilities of PROMES (France). A pellet of pressed Fe and Ag powder (5% wt. Fe) was evaporated under the focus of concentrated solar beam at a pressure of 70 torr controlled by a continuous flow of Ar. Particles were collected on a nanoporous ceramic filter located in the direction of pumping flow. The obtained Ag/Fe nanopowder maintained a composition around 5 % in zero-valent Fe with a particles' unit diameter of around 30 nm.

Taking into account the minimum inhibitory concentration (MIC) of the examined NPs after in vitro antimicrobial activity assessment, they were dissolved in distilled water and the following stock solutions were prepared: 1) solution of Fe_3O_4 NPs: 19.2 mg/ml distilled water; 2) solution of Ag/Fe NPs: 12.8 mg/ml distilled water. A fresh solution of each NP was prepared every week. Prior to their use, the NPs solutions were sonicated for 20 min to improve their dispersion stability.

Animals, semen samples collection and dilution

Semen samples were collected from 3 sexually mature (2-2.5 years old) and healthy boars (crossbred) of a 700-capacity pig farm in Northern Greece. Totally, nine ejaculates (3 boars; 3 ejaculates/boar) were collected weekly by an experienced farm employee using the gloved hand technique and were transported in an isothermal glass vessel (37° C) to the farm laboratory. The gelatinous portion was discarded with a cotton gauze and the semen sample was estimated for volume, concentration, and motility. Sperm concentration was assessed by a photometer (SDM1, Minitube®, Germany). Sperm motility was subjectively evaluated by a phasecontrast microscope with a heated stage at 37° C. Only ejaculates with volume >200 ml, concentration >200x10^6 spermatozoa/ml, total sperm number/ejaculate > 40×10^9, and gross motility >70% were further processed.

Semen samples with acceptable quality were extended in BTS without antibiotics (30×10^6 spermatozoa/ml). The extended semen samples were reexamined microscopically and those with gross motility >70% were packaged in vials of insemination and were transported (17° C) in less than 60 min in a portable semen storage unit (Minitube®, Germany) to the Unit of Biotechnology of Reproduction (UBR), Clinic of Farm Animals, Faculty of Veterinary Medicine, Aristotle University of Thessaloniki.

Semen processing with NPs

Upon arrival in the UBR, each semen sample was divided in 3 aliquots and the following three experimental groups were prepared: 1) control group (C): extended semen without any treatment; 2) iron oxide group (Fe): extended semen with Fe_3O_4 NPs (0.192 mg Fe_3O_4/ml semen); 3) silver group (Ag): extended semen with Ag/Fe NPs (0.128 mg AgFe/ml semen).

Pretrial: Determination of coincubation time of semen with NPs

In a pretrial (data not shown), the beneficial/detrimental coincubation period of semen samples with NPs was evaluated. The C and NPs groups were incubated at 17° C (the appropriate storage temperature of extended boar semen) for 30, 45 and 60 min following the NPs' removal through a magnetic field (as it is described in the main trial) and total and progressive motility were assessed by CASA. The incubation period of 45 and 60 min were excluded because the values of the examined CASA parameters were significantly decreased in NPs groups, compared to the control group, whilst interestingly boar spermatozoa in Ag group were completely immotile after 60 min of coincubation. Consequently, the coincubation period of 30 min was selected for further research because no differences observed for the evaluated CASA parameters between the control and the NPs groups.

Main trial: Investigation of effect of NPs on boar semen quality

The three experimental groups were stored at 17° C for 30 min after Fe and Ag/Fe NPs supplementation to groups Fe and Ag, respectively, following NPs' removal through a magnetic field. For NPs' removal, tubes were placed in a plastic rack equipped with magnets, remained in vertical position for at least 5 min and the post-treated semen was transferred to a new tube, while the NPs were discarded. This procedure was repeated three times to remove completely the NPs. Finally, the control and the NPs post treated samples were stored at 17° C for 48 h. Total motility and kinetics (progressive motility; rapid/medium/slow movement spermatozoa; static spermatozoa; VCL; VSL; VAP; LIN; STR; WOB; ALH; BCF; hyperactive spermatozoa) were evaluated by CASA at 0, 24 and 48 h post treatment.

Sperm motility and kinetics evaluation by CASA

Sperm motility was evaluated by a Computer Assisted Semen Analysis (CASA) system (Sperm Class Analyser®, Microptic S.L., Automatic Diagnostic Systems, Spain) and a microscope (AXIO Scope A1, Zeiss, Germany) equipped with a heating stage and a camera (Basler scA780 54fc, Germany). The analysis performed by Sperm Class Analyser® software (SCA® v.6.3.; Microptic S.L., Automatic Diagnostic Systems, Spain) with the following configurations: 4-6 fields were recorded (×100) for each semen sample, >500 spermatozoa, 25 frames/sec, region of particle control 10-18 microns, progressive movement of >45% of the parameter STR, circumferential movement <50% LIN, depth of field 10 μm, and temperature of the microscope plate 37° C. The debris incorrectly identified as spermatozoa were manually removed before final analysis.

For each group, a 10 μl semen sample was placed on the preheated (37° C) Makler chamber (Makler® counting chamber, 10 μm deep, Sefi Medical Instruments, Israel) and the following CASA motility and kinetic parameters were evaluated: 1) total motility; %, 2) progressive motility; %, 3) rapid, medium, slow and static movement spermatozoa (static<10<slow<25<medium<45<rapid μm/sec); %, 4) VCL-curvilinear velocity; μm/sec, 5) .VSL-straight line velocity; μm/sec, 6) VAP-average path velocity; μm/sec, 7) ALH-amplitude of lateral head displacement; μm, 8) BCF-beat/cross-frequency; Hz, 9) LIN-linearity (VSL/VCLx100), 10) STR-straightness (VSL/VAPx100), 11) WOB-wobble (VAP/VCLx100) and 12) hyperactive spermatozoa (VSL >97 μm/sec, ALH >3.5μm, LIN <0.32); %.

Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems version 9.3 (SAS Institute Inc., 1996, Cary, N.C., U.S.A.). Normality of the data was tested using the Shapiro-Wilk Test (PROC UNIVARIATE). Parameters that did not follow a normal distribution were normalized by logarithmic transformation. For reasons of clarity the means and SEM of the not transformed data are presented. Statistical analysis was conducted with a repeated measures mixed model (PROC MIXED). The model included group, time and their interaction as fixed effects and boar as a random effect. Semen sample within boar was defined as the subject of the repeated observations. Pairwise comparisons were performed with the PDIFF command incorporating the Tukey adjustment. Statistically significant difference was defined as $P<0.05$.

RESULTS

Casa motility and kinetics

For all the CASA evaluated parameters, there was no difference between groups at 0 h post treatment (Fig. 1; Table 1). Group Fe did not differ from group C at any time point for all the evaluated CASA parameters (Fig. 1; Table 1). Values of total and progressive motility were lower (all $P<0.001$) at 24 h of storage in group Ag compared to groups C and Fe (Fig. 1). In all nine replicates, semen samples of group Ag were immotile after 48 h of storage (17° C) and thus excluded from analysis. Static spermatozoa decreased after 24 h of storage post treatment in group Ag and after 48 h in groups C and Fe ($P<0.0001$). The comparison within groups and between storage time points showed that the values of total motility, progressive movement spermatozoa, VCL, ALH (Fig. 1) as well as VAP and BCF (Table 1) were decreased after 24 h of storage post treatment in group Ag (all $P<0.05$), but not in groups C and Fe. Also, in group C, the values of LIN were lower ($P<0.0001$) after 24 h and 48 h of storage post treatment, while no differences were observed in the other two groups (Table 1). The remaining parameters did not differ significantly ($P>0.05$) between successive time points within any group (Table 1).

Table 1. Sperm kinetic parameters (mean ± SEM) of semen samples evaluated by CASA at 0, 24, 48 h of storage (17° C) post coincubation (30 min) with nanoparticles.

Variable	Group C			Group Fe			Group Ag			P value		
	0h	24h	48h	0h	24h	48h	0h	24h	48h	Group	Time	G*T
Rapid (%)	59.9±5.4$^{\#}$	43.3±5.4$^{\#}$	36.6±5.4$^{\#}$	65.6±5.4$^{\#}$	42.5±5.4$^{\#}$	44.5±5.4$^{\#}$	64.8±5.4$^{\#}$	21.7±6.6*	-	0.015	<.0001	0.2163
Medium (%)	24±2.4$^{\#}$	23.5±2.4$^{\#}$	24.1±2.4$^{\#}$	22.6±2.4$^{\#}$	24±2.4$^{\#}$	18.3±2.4$^{\#}$	22.9±2.4$^{\#}$	14±2.9$^{\#}$	-	0.0595	0.2051	0.212
Slow (%)	12.3±2.4$^{\#}$	21.7±2.4$^{\#}$	23.5±2.4$^{\#}$	9.5±2.4$^{\#}$	24.2±2.4*	21.5±2.4*	10.1±2.4$^{\#}$	20.5±3$^{\#}$	-	0.935	<.0001	0.7937
Static (%)	3.7±4.4$^{\#}$	11.5±4.4$^{\#*}$	15.8±4.4*	2.2±4.4$^{\#}$	9.3±4.4$^{\#*}$	15.6±4.4*	2.2±4.4$^{\#}$	43.8±4.4*	-	0.0228	<.0001	0.2673
VSL (μm/sec)	26.9±3$^{\#}$	32.5±3$^{\#}$	30.4±3$^{\#}$	27±3$^{\#}$	29±3$^{\#}$	34±3$^{\#}$	28.4±3$^{\#}$	23.4±3.6$^{\#}$	-	0.3819	0.2163	0.5629
VAP (μm/sec)	45±3.7$^{\#}$	32.3±3.7$^{\#}$	29.6±3.7$^{\#}$	47.2±3.7$^{\#}$	34.7±3.7$^{\#}$	33±3.7$^{\#}$	47.2±3.7$^{\#}$	24.2±4.6*	-	0.157	<.0001	0.7847
BCF (Hz)	5.6±0.24$^{\#}$	5.2±0.24$^{\#}$	5.2±0.24$^{\#}$	5.6±0.24$^{\#}$	5.2±0.24$^{\#}$	5.3±0.24$^{\#}$	5.6±0.24$^{\#}$	4.2±0.29*	-	0.0068	0.0046	0.2569
LIN (%)	45.6±3.2$^{\#}$	55.4±3.2*	58.6±3.2*	42.5±3.2$^{\#}$	52.7±3.2$^{\#}$	54.6±3.2$^{\#}$	44.7±3.2$^{\#}$	52.1±3.2$^{\#}$	-	0.5195	<.0001	0.9787
STR (%)	54.1±3.2$^{\#}$	57.2±3.2$^{\#}$	62.9±3.2$^{\#}$	51±3.2$^{\#}$	57±3.2$^{\#}$	54.5±3.2$^{\#}$	53.3±3.2$^{\#}$	59.1±3.2$^{\#}$	-	0.4228	0.0064	0.7545
WOB (%)	78.2±2.2$^{\#}$	79.1±2.2$^{\#}$	78.7±2.2$^{\#}$	76.9±2.2$^{\#}$	79.1±2.2$^{\#}$	76.6±2.2$^{\#}$	77.8±2.2$^{\#}$	75±2.6$^{\#}$	-	0.509	0.0665	0.8752
Hyper. (%)	5.1±2.7$^{\#}$	3.5±2.7$^{\#}$	2.5±2.7$^{\#}$	6.5±2.7$^{\#}$	5.1±2.7$^{\#}$	9.5±2.7$^{\#}$	5.1±2.7$^{\#}$	0.2±3.3$^{\#}$	-	0.1656	0.0476	0.6926

Group C: extended semen without any treatment, **Group Fe:** extended semen with Fe_3O_4 NPs (0.192 mg Fe_3O_4/ml semen) **and Group Ag:** extended semen with Ag/Fe NPs (0.128 mg Ag Fe/ml semen).
Rapid: rapid movement spermatozoa (%), **Medium:** medium movement spermatozoa (%), **Slow:** slow movement spermatozoa (%), **Static:** static spermatozoa (%), **VSL:** straight line velocity (μm/sec), **VAP:** average path velocity (μm/sec), **BCF:** beat/cross-frequency (Hz), **LIN:** linearity (VSL/VCL x 100), **STR:** straightness (VSL/VAP x 100), **WOB:** wobble (VAP/VCL x 100), **Hyper.:** hyperactive spermatozoa (%).
G*T: Group*Time.
Different symbols (#, *) denote significant differences between evaluation times within a group.

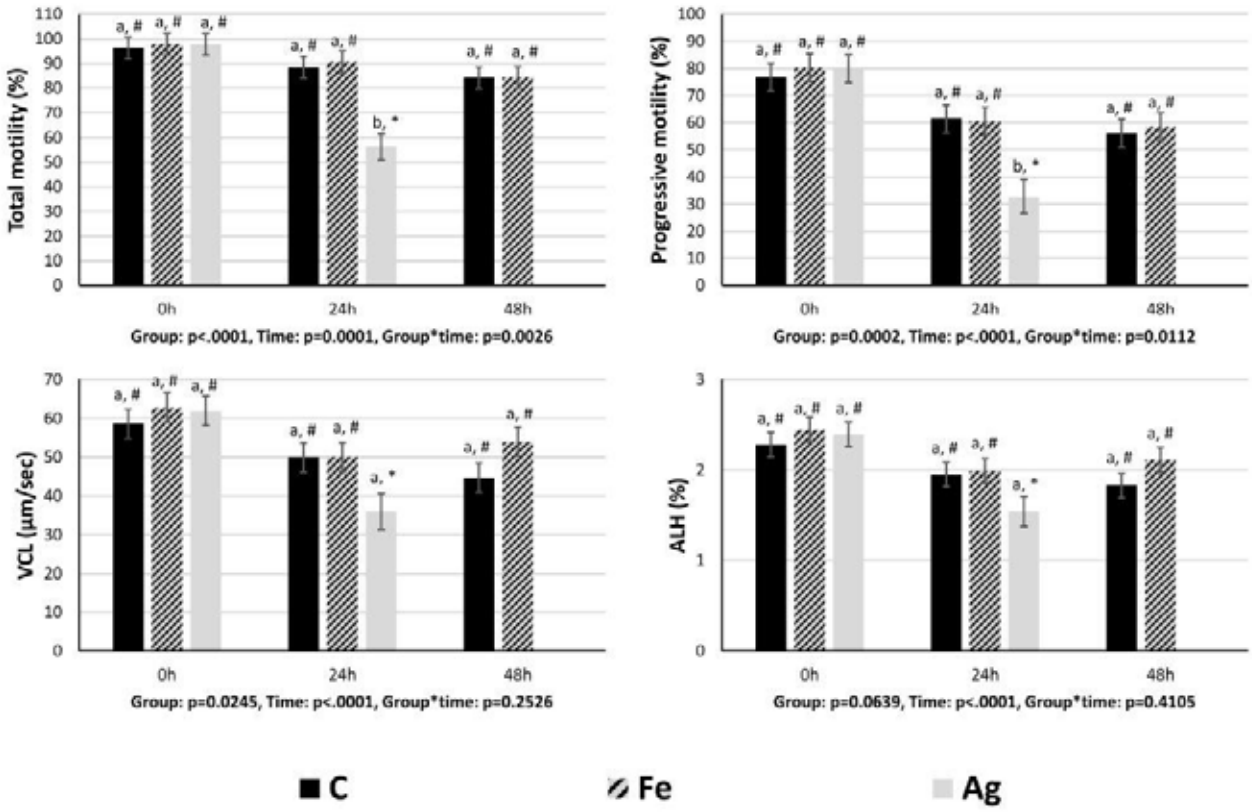

Figure 1. Effect of iron oxide (Fe; Fe_3O_4) and silver oxide (Ag; Ag/Fe) nanoparticles on Total and Progressive motility, VCL and ALH of boar spermatozoa at 0, 24, 48 h of storage (17° C) post co-incubation (30 min) with nanoparticles.
Group C: extended semen without treatment, Group Fe: extended semen with Fe_3O_4 NPs of diameter 40 nm (0.192 mg/mL semen), and Group Ag: extended semen with Ag/Fe NPs of diameter 30 nm, consisted of Ag and a 5% of zero-valent Fe (0.128 mg/mL semen). All the values are expressed as mean ± SEM. Different subscripts (a, b) denote significant differences between groups for each evaluation time point. Different symbols (#, *) denote significant differences between evaluation times within a group.

Characterization of nanoparticles

The morphological characteristics of the tested nanoparticles were studied by transmission electron microscopy (Fig. 2). Iron oxide nanoparticles appear to consist of well-defined spherical units with an average size around 40 nm. The silver-based nanomaterial shows a higher degree of aggregation in the building units and a wider size distribution with an average around 30 nm.

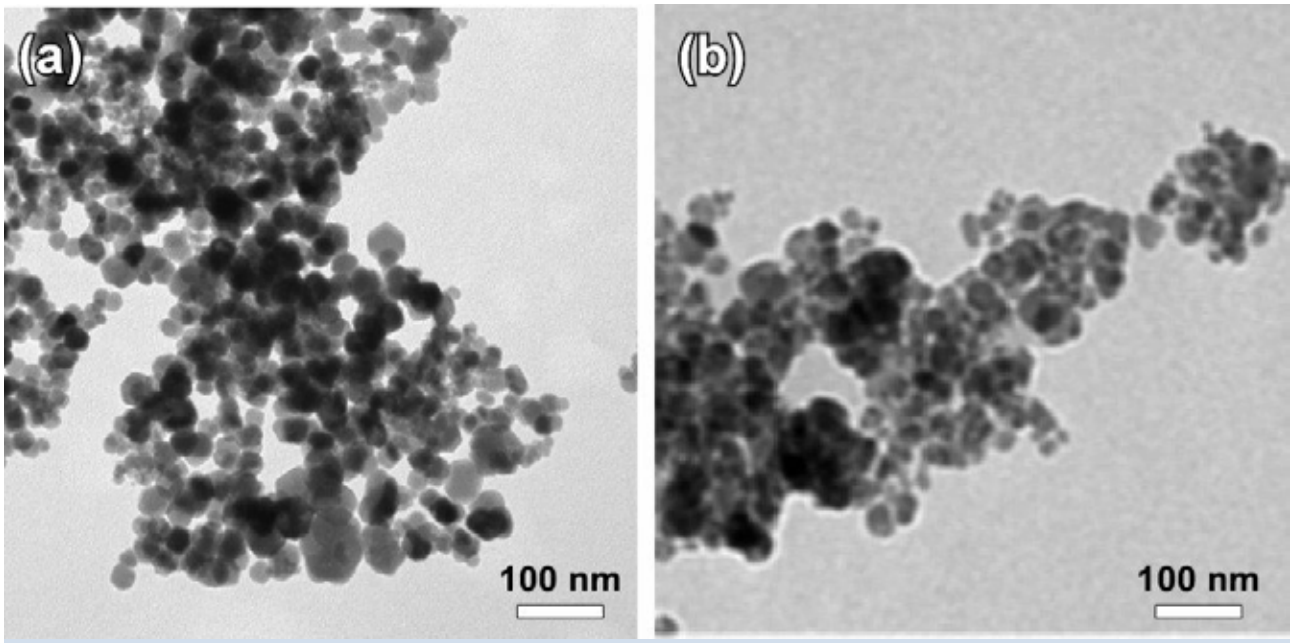

Figure 2. Transmission electron microscopy images of Fe_3O_4 (a) and Ag/Fe (b) nanoparticles.

DISCUSSION

In the present study, we investigated the possible toxic effect of the iron oxide (Fe_3O_4) and silver (Ag/Fe) spherical nanoparticles on boar sperm motility parameters as a first approach before performing further research of their potential use as alternative agents to conventional antibiotics for semen handling.

It is undoubtedly accepted that nanoparticles applications in different fields of modern life have been increasing. However, it is well known that there are potential side effects caused by NPs exposure and their extended use has raised a serious concern about their safety for human and animals' health (Johnston et al., 2010). In *in vivo* trials, it is documented that nanoparticles can penetrate the bloodtestis barrier (Lan and Yang, 2012) and under *in vitro* conditions they may cause necrosis, apoptosis and mitochondrial dysfunction (Braydich-Stolle et al., 2005). Although the toxicity of NPs on reproductive system is well documented in the literature (Ema et al., 2010), a lot of studies examined a variety of them, like iron oxide (Özgür et al., 2018), silver (Asare et al., 2012), gold (Moretti et al., 2013), zinc oxide (Barkhordari et al., 2013) under *in vitro* or *in vivo* conditions providing contradictory results and conclusions about their toxic effect on sperm (Braydich-Stolle et al., 2005) or their beneficial use for semen handling (Durfey et al., 2019; Özgür et al., 2018). Specifically, concerning the iron oxide NPs on sperm quality, according to Özgür et al., (2018), the Rainbow trout spermatozoa were exposed to magnetite (Fe_3O_4) NPs over 24 h and the values of VCL, VSL and VAP decreased, while the intraperitoneally administration of maghemite (γ-Fe_2O_3) NPs decreased the motility and kinetic parameters of mouse epididymal spermatozoa (Varzeghani et al., 2018). However, in our study the iron oxide (Fe_3O_4) NPs interacted with spermatozoa for 30 min and were carefully removed through a magnetic field. This restricted period of sperm co-incubation with NPs as well as the different kind of NPs and species may excuse the different results. Some reports also enhance the hypothesis that the time of interaction may be involved in NPs potential toxic activity. Specifically, the iron oxide (Fe_2O_3) NPs used for sperm purification of boar and bovine insemination doses did not provide any toxicity to purified spermatozoa, while the fertilizing capacity of nanopurified semen used for artificial insemination was not affected (Feugang et al., 2014; Odhiambo et al., 2014). Also, the Fe_3O_4

NPs coated with lectins and annexin V were used for boar semen selection without providing any toxic effect (Durfey et al., 2019, 2017). Although the experimental approach of the present study is different, our results are interestingly in accordance with the previous mentioned studies and demonstrated that the examined iron oxide spherical NPs interacting with spermatozoa for a short period of time had no negative effect on boar semen CASA motility parameters.

Silver NPs are very popular among others NPs and have been widespread explored for human biomedical applications, drug delivery systems, dental applications, wound healing, and catheters compartments thanks to their interesting chemical, physical and biological/antimicrobial action related to their tiny size (Burdușel et al., 2018) without providing adverse effects to healthy cells (Stensberg et al., 2011). However, their extended use warrants their potential toxicity assessment during the last decades. The available data reported that they can negatively affect the physiological function and structure of organs (Lin et al., 2017; Ribeiro et al., 2018), while there are controversial results about their toxicity on male reproductive system (Asare et al., 2012; Garcia et al., 2014; Terzuoli et al., 2012). Taking into account that human is constantly exposed to silver NPs and the social impact of human fertility capacity, the reported *in vivo* and *in vitro* studies focus mainly on human (Terzuoli et al., 2012) or on animals' experimental models examining either a time or dose effect of NPs as well as a possible interference of their size (Gromadzka-Ostrowska et al., 2012). The results of the present study demonstrated a harmful effect of the examined silver NPs on CASA motility parameters of boar semen. Although no detrimental effect on group Ag was observed at 0 h post NPs removal, the values of total motility, progressive movement spermatozoa, VCL, VAP, ALH, and BCF were lower at 24 h and interestingly, the spermatozoa of group Ag were immotile 48 h post NPs removal. In accordance with the results of our study, Terzuoli et al., (2012) observed that human sperm motility decreased after 60- and 120-min of co-incubation even at the lowest concentration of the used Ag NPs. On the contrary, Garcia et al., (2014) found that the intravenously administration of Ag NPs had no negative effect on CASA parameters of mice semen, even though the values of VSL, VCL, VAP, ALH, STR were lower 60 days post administration but these differences were not statistically significant. Also, Moretti et al., (2013) did not find any harmful effect of Ag NPs in low concentrations post co-incubation, but a dose depended NPs' effect was observed on sperm motility. Surprisingly, in a preliminary study (data not shown), we found that there was a decrease in sperm motility even after 45 min of co-incubation of boar sperm with Ag NPs, while according to Tiedemann et al., (2014) when boar semen was *in vitro* supplemented with Ag NPs, no negative effect on CASA motility parameters was observed even 120 min post coincubation. These contradictory findings could be attributed to the different synthesis, size, shape, or even the concentration of the administered NPs which could affect their properties, as well as their action on cells. It is well documented that there is a size depended and dose depended adverse effect of NPs on sperm quality and testis health (Gromadzka-Ostrowska et al., 2012; Terzuoli et al., 2012). Moreover, it has been observed that when NPs are coated with other molecules like annexin V, lectins (Durfey et al., 2017), BSA (Grade et al., 2012) their potential toxic effect could be alleviated as well as the cell culture media composition could reduce or enhance their cytotoxic action (Grade et al., 2012).

CONCLUSIONS

In conclusion, under the experimental conditions of the present study the Ag/Fe NPs exerted a harmful effect on boar spermatozoa motility and kinetic parameters evaluated by CASA, while the used concentration of Fe_3O_4 NPs did not affect boar sperm CASA parameters revealing a need for further research about their applications on semen processing. Also, it is necessary to investigate further their effect on boar spermatozoa by assessing more sperm quality and functional parameters, as well as their antimicrobial potential by microbiological cultures.

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CONFLICT OF INTEREST

No conflict of interest is declared by the authors.

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