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Antimicrobial activity of silver-carbon nanoparticles on the bacterial flora of bull semen: a pilot study

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Short title: Antimicrobial effect of silver-carbon nanoparticles on bacteriospermia in bovine

Abstract

The spermicidal effects of silver nanoparticles (AgNPs) hinder its application in the field of artificial insemination. In this study, silver-carbon NPs (Ag@C NPs) was synthesized and applied as an alternative antibiotic agent for bull semen extender. Ag@C NPs were characterized using X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), atomic absorption flame spectroscopy, transmission electron microscope (TEM), and high-resolution TEM (HR-TEM). Data analysis revealed the successful synthesis of Ag@C NPs with a particle size of 1-5 nm (average particle size of 2.5 nm) embedded into carbon. The antimicrobial activity of Ag@C NPs was tested against bacteriospermia of fresh semen collected from five fertile bulls (three ejaculates/bull). *Escherichia coli (E. Coli)*, *Staphylococcus aureus (S. aureus),* and *Pseudomonas aeruginosa* (*P. aeruginosa*) were isolated from fresh semen samples and identified by culture, staining, and conventional biochemical tests. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Ag@C NPs against bacteriospermia was determined at 5 and 37 ºC. Ag@C NPs showed efficient antimicrobial activity (MIC: 3.125 - 12.5 µg/mL) against the tested strains and strong bactericidal effect on *S. aureus,* and *P. aeruginosa* (MBC: 3.125 µg/mL), with no detrimental effect ($P > 0.05$) on the percentage of sperm motility (70.71±4.82; 74.65±4.46), plasma membrane integrity (68.39±4.31; 72.38±4.91), acrosome integrity (88.40±13.21; 86.77±14.23), and normal sperm morphology (86.85±7.43; 87.82±8.15) at concentrations of 15 and 30 µg/mL, respectively, after a cold storage of 48 h. However, Ag@C NPs showed a detrimental effect on sperm parameters in a dose dependent manner at concentrations ≥60 µg/mL. Ag@C NPs showed no adverse effect on the sperm´s ultrastructure with limited sperm

internalization at MIC. In conclusion, Ag@C NPs could be used as an alternative antibiotic agent for bull semen extender without a significant cytotoxic effect on the sperm during cold storage. However, further investigations for their effects on embryo production and female genitalia are still required.

Keywords: Nanoparticles; Sperm motility; Acrosome integrity; Nanomedicine; Spermicidal effect; Bull.

1. Introduction

Bacterial contaminations of bull semen may lead to breeding failure by its negative effect on the sperm parameters [1] and/or infection of the inseminated cows (i.e. endometritis) [2]. The microbial contamination of semen could reduce sperm quality in terms of motility, morphology and viability, and cause premature acrosome reaction [3]. Moreover, it could decrease mitochondrial activity and increase DNA fragmentation [4]. Notably, semen collection and sperm handling for artificial insemination (AI) are usually carried under non-septic conditions [5]. To avoid the adverse effects of bacterial contamination on semen quality, low concentrations of broad-spectrum antibiotics are added to the semen extenders to be used for AI [6]. The concern about the spermicidal effects of antimicrobials [7], and the emergence and spread of resistant bacteria forces the search for new alternative strategies to the use of antibiotics, for instance cationic antimicrobial peptides for boar semen preservation [8], physical removal of the bacteria during semen processing using a modified single layer centrifugation (with a tube insert [9, 10], human semen preparation by density gradient centrifugation using silane-coated silica particles [11] and the potential application of antimicrobial nanoparticles [12].

Silver-based materials have been recorded as an efficient antimicrobial agent in various fields such as health care, medicine, and food science [13, 14]. Silver nanoparticles (AgNPs) have emerged as an alternative approach to antibiotics but further studies to determine their effectiveness and safety are required [15, 16]. AgNPs are antimicrobial agents with a strong toxic effect on microorganisms [17]. Therefore, AgNPs are effective against opportunistic pathogens with great capacity to acquire antimicrobial resistance, as *Escherichia Coli (E. Coli)*, *Staphylococcus aureus (S.*

aureus), and Pseudomonas aeruginosa (P. aeruginosa) [18, 19], or even enhance the antibacterial activities of different antibiotics [20, 21]. Thus, the application of AgNPs could be one of the most promising strategies against antibiotic-resistant bacteria and thereby replacing antibiotics addition to semen extenders [22]. More recently, the AgNPs was considered as a safe and efficient antimicrobial agents for semen extenders in pigs [23, 24]. However, their toxic effects on the sperm should be taken into consideration before their widespread use in reproduction technologies [25, 26]. Furthermore, the current methods for the synthesis of AgNPs include wet chemical routes or thermal decomposition [27]. Residual chemical impurities might affect the biological activity of the prepared materials [28].

Hence, we investigated the possible use of silver-carbon nanoparticles (Ag@C NPs) as an alternative antimicrobial agent for bull semen extender and assess the biocompatibility/toxicity of those NPs for bull sperm. Ag@C NPs were synthesized via the pyrolysis of silver nitrate in the presence of melamine as a source of carbon and the present study was designed to: (i) isolate bacteria from fresh bull semen samples, (ii) assess the antimicrobial effect of Ag@C NPs by determining its minimum inhibitory concentrations (MICs) and minimum bactericidal concentration (MBC) against the isolated bacteria, and (iii) evaluate the effects of such NPs (at MIC) on sperm quality parameters and sperm ultrastructural morphology of chilled bull semen after 48 h of cold storage (5 ºC).

2. Materials and methods

2.1. Animal ethics and study location

Animal experiments described in this study were conducted in accordance with the ethical animal guidelines and regulations set by the Animal Care Committee of Assiut University, Egypt. The protocol was approved by the Committee Ethics of Animal Experiments (Permit number: 17300319).

Holstein Friesian bulls (4-8 years old; 350-450 kg body weight) were healthy, sexually mature and showed good libido. Animals were housed individually under semi-open sheds at Veterinary Teaching Hospital, Assiut University, Assiut, Egypt. They were kept on a balanced breeding bull diet which formulated according to NRC [29] of beef cattle and consisting of 3 kg of commercial concentrate mixture (containing 15% crude protein and 70% TDN), 3 kg Berseem hay and 3 kg of wheat straw. Drinking water was available at libitum. The experiment was conducted during the period from October, 2019 to May, 2020.

2.2. Synthesis and characterization of silver-carbon nanoparticles (Ag@C NPs)

Silver nitrate ($AgNO_3$) and melamine were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). $AgNO_3$ (2 g) and melamine (1.5 g) were dissolved in 10 mL ethanol and stirred for 10 min. The solvent was evaporated overnight at 80 °C. The product was calcined at 550 °C under nitrogen atmosphere with flow rate of 5 mL/min and washed with distilled water (5 x 100 mL) to remove unreacted species.

The crystallinity and phase purity of Ag@C NPs was determined using X-ray diffraction (XRD) (Phillips X'Pert, Cu, K_α radiation). The elemental compositions and their local structure were evaluated using X-ray photoelectron spectroscopy (XPS, Thermo

Fischer, K-alpha) instrument with monochromated, micro-focused Al Kα radiation (1486.6 eV). The particle size and morphology were estimated by transmission electron microscopy (TEM) and high-resolution TEM (HR-TME) images using TEM-2100 (JEOL, Japan, operated at accelerating voltage 200 kV). Atomic absorption flame spectrophotometer (AAF, Buck scientific 210 VGC) was used for measuring the percentage of silver in Ag@C NPs after the digestion in nitric acid.

2.3. Semen collection

This study was performed on ejaculates from five fertile bulls used for natural breeding. Three ejaculates were collected from each bull (n = 15) with a sterilized artificial vagina twice a week [30]. The bulls were allowed for at least one false mounting before collection. Immediately after semen collection, each ejaculate was assessed for volume, motility and concentration [31].

2.4. Microbiological evaluation of fresh semen samples

Immediately after collection, aliquots of 0.1 mL of fresh semen samples were aseptically inoculated into nutrient broth separately and incubated at 37 ºC overnight [32]. Then, samples were sub-cultured in several differential and selective agar culture media to isolate different bacterial species. MacConkey agar, EMB agar, Lactobacillus agar media base, eosin methylene blue agar (modified) levine, Mannitol salt agar, Sabaraud Dextrose and blood agar base (Oxoid 1td., Hampshire, UK), supplemented with 5% sterile defibrinated sheep blood and incubated in aerobic conditions at 37 ºC for 48 h were used. For detection of anaerobes, blood agar plates were incubated under anaerobic condition (the atmosphere was

150 enriched with 8% carbon dioxide) as previously described by Eggert-Kruse et al. [33]. One
151 representative colony of the most abundant morphologically distinct colonies were selected,
152 sub-cultured and grown in the same condition for further identification. Gram staining and
153 bacterial morphology [34] followed by conventional biochemical tests such as indole
154 production, methyl red and Voges-Proskauer tests, citrate utilization, oxidase, catalase,
155 coagulase and triple sugar iron tests [35] were performed. Isolates were inoculated in Brain
156 Heart Infusion (BHI) (BBL 11407, USA) broth and incubated at 37 °C for 24 h. The turbidity
157 of each aliquot was adjusted to 0.5 McFarland standards for all strains (equivalent to 1.5 ×
158 10^8 cfu/mL).
159
160 *2.5. Determining the minimum inhibitory concentration (MIC) and Minimum*
161 *Bactericidal Concentration (MBC) of silver-carbon nanoparticles*
162 The stock solution of Ag@C NPs was prepared as suggested by Mulfinger et al. [36].
163 In brief, we added 1 mg of the NPs powder into 10 mL of normal saline and sonicated to
164 obtain a homogeneous suspension of 100 µg/mL of Ag@C NPs (initial stock solution).
165 The antibacterial activity of Ag@C NPs was determined using the serial dilution
166 method in BHI broth. From the stock solution, we added 1 mL of Ag@C NPs suspension in
167 test tubes and diluted it further at two-fold serial dilutions to obtain concentrations ranged
168 from 0.049-100 µg/mL. Then, 5 µL of bacterial culture (1.5 x 10^8 cfu/mL) was added to all
169 the tubes containing the antimicrobial agent, Ag@C NPs (treated groups; BHI broth
170 inoculated with bacteria and Ag@C NPs) and to the tubes from the positive group (bacterial
171 growth control, BHI broth mixed with bacterial inoculum). Moreover, negative tubes (sterile
172 non inoculated BHI broth) were included. Then, the test tubes were shaken properly and

incubated at 5 and 37ºC for 24 h. Finally, the MIC of the Ag@C NPs was defined as the lowest concentration that inhibited the visible growth of a studied strain of bacteria [37]. The MIC was determined by the visual turbidity in the tubes after incubation. Once Ag@C NPs MICs were obtained, 50 µL from all tubes of no visible bacterial growth, starting on the tube which reached the MIC value, were subcultured onto BHI agar plates and then incubated at 37 ºC for 24 h and the total number of colonies on the culture plates was counted. The MBCs of the Ag@C NPs againts the tested isolates were determined according to Pérez-Díaz et al. [38] as the lowest concentration of Ag@C NPs that kills 99.9% of the initial bacterial load. The experiment was repeated five times.

2.6. Biocompatibility/toxicity of silver-carbon nanoparticles at minimum inhibitory concentration (MIC)

After an initial semen evaluation, semen was diluted with prewarmed (37 ºC) TRIS-egg yolk (EY)-based extender (20% EY; v:v) [39], to get a final concentration of approximately 30 × 10^6 sperm/mL and split into six aliquots: control and treated aliquots exposed to Ag@C NPs at a dose-dependent concentrations (15, 30, 60, 120, and 240 µg/mL). Finally, the semen was chilled at 5 ºC for up to 48 h [40] and evaluated for motility, membrane integrity, morphology, and acrosome integrity at 0, 24 and 48 h, as described below.

2.7. Sperm evaluation

2.7.1. Sperm progressive motility

The percentage of progressive sperm motility in each semen sample (10 µL) was assessed using a phase-contrast microscope (Olympus, Tokyo, Japan) supplied with a warm stage adjusted to 37 °C [41].

2.7.2. Plasma membrane integrity

The plasma membrane was assessed using the Vital Test (Halotech DNA SL, Madrid, Spain) based on the red/green emission of two fluorescent dyes: acridine orange (AO) and propidium iodide (PI), respectively, as described by Dorado et al. [42] and sperm with intact plasma membrane were recorded (PMI, %).

2.7.3. Sperm acrosome

To evaluate the sperm acrosomes, the PI/peanut agglutinin–fluorescein isothiocyanate (FITC-PNA) double stain (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), was used as described by Dorado et al. [42]. The acrosome morphology of sperm was observed using an epifluorescence microscope (Olympus BX40, Tokyo, Japan). Ethanol-permeabilized bull sperm could be classified into two groups: (1) acrosome-intact sperm – AIS: the acrosomal region of the sperm head displayed bright green fluorescence; (PI+/FITC-PNA+), and (2) acrosome-reacted sperm – ARS (green fluorescent at the equatorial segment or no anterior acrosomalstaining; PI+/FICT-PNA-). All sperm showed red fluorescence due to counterstaining with PI.

2.7.4. Sperm morphology

Sperm morphology was examined by light microscopy evaluation (Olympus BH-2, Olympus Optical Co., Ltd., Tokyo, Japan) on smears stained with Diff-Quick (MedionDiagnostics AG, Düdingen, Switzerland) staining [43]. At least 200 sperm per slide were counted to determine the percentage of sperm with normal forms (NF, %).

2.7.5. Transmission electron microscopy (TEM) analysis

For TEM analysis, semen aliquots of each treatment (with and without exposure to Ag@C NPs and after 48 h of cold storage) were fixed by immersing in 4F1G in phosphate buffer solution (pH of 7.2) at 4 °C for 3 h. The samples were post-fixed in 2% OsO_4 in the same buffer at 4 °C for 2 h and washed. Specimens were dehydrated at 4 °C through a graded series of acetone. Then, samples were infiltrated and embedded in a liquid resin. After embedding, the resin blocks were cut into thin sections of 90 nm in thickness using LKB 2088 Ultracut ultramicrotome (Bromma, Sweden), followed by placing the sections on grid cobber and staining with uranyl acetate for 5 min, then lead citrate for 2 min before observation in the TEM (JEOL – JSM-1400 PLUS) [44].

2.8. Statistical analysis

Data were analyzed using SPSS statistics 21 for windows (IBM SPSS, Amonk, NY, USA). Sperm quality parameters before and after exposure to the NPs were analyzed by the General Linear Model (GLM) procedure. The difference among means was tested by Duncan's multiple range tests. Significant differences were considered when $P < 0.05$.

3. Results

3.1. Characterization of silver-carbon nanoparticles (Ag@C NPs)

The thermal decomposition of $AgNO_3$ in the presence of melamine leads to the formation of silver-carbon NPs (Ag@C NPs). X-ray diffraction (XRD) pattern of the synthesized Ag@C NPs is matched with the simulated XRD pattern of Ag powder (JCPDS 04-0783, Fig. 1). This observation indicated the successful synthesis of a pure phase of Ag. The XRD pattern showed three peaks at approximately 38.0°, 44.2°, and 64.3°, corresponding to the Miller indexes (111, 200, and 220, respectively). The XRD pattern confirmed the successful reduction of Ag^+ to Ag. The XRD pattern showed also a peak at 8.0° corresponding to the graphitic structure of C. The mechanism for the synthesis of Ag crystal via thermal decomposition of $AgNO_3$ is one step [45]. $AgNO_3$ is converted to Ag crystal and NO_2 (g) + $0.5O_2$ (g). Thus, the pyrolysis of $AgNO_3$-melamine leads to Ag@C NPs.

XPS was recorded to characterize the chemical composition inside Ag@C NPs (Fig. 2). The XPS survey showed peaks related to C, N, and Ag (Fig. 2a). There is the only main peak in the N 1s spectrum (Fig. 2b). This indicates the presence of N in one chemical environment at a binding energy of 398.9 eV, which belongs to the C–N=C triazine bonds predominantly present in carbonized melamine. The peak analysis of C 1s at binding energy 287.6 eV belongs to the C–C bond of the adventitious sp^2 C (Fig. 2c). Ag 3d spectrum depicted in is comprised of two central peaks at 367.8 eV of Ag 3d5/2 and 373.8 eV of Ag 3d3/2 (Fig. 2d). The peak positions and a binding energy difference (6 eV) of the two peaks confirmed the metallic state of Ag.

The morphology and particle size of the prepared materials were evaluated using a TEM image (Fig. 3). The TEM image of Ag@C NPs illustrated a dark particle of Ag

embedded into a gray color sheet of C. The analysis of the TEM image reveals a particle size of 1-5 nm with an average particle size of 2.5 nm (Fig. 3a). The high-resolution TEM image showed lattice fringes of 0.24 nm corresponding to the plane (111) according to the hkl file extracted from the cif file of Ag (Fig. 3b). Atomic absorbance flame analysis (AAF) indicates that the Ag content is 320 ppm.

3.2. Microbiological evaluation of fresh semen samples

A total of three bacterial species were isolated in this study: *S. aureus*, *E. coli* and *P. aeruginosa*, *Anaerobes*, *Lactobacillus spp.* and, yeasts and molds were not found in any of the examined samples. The microbiological analysis of the ejaculates from the five bulls revealed that all semen samples were contaminated with bacterial pathogens belonged to different species. In detail, *S. aureus* and *E. Coli* were isolated from all semen samples, while, *P. aeruginosa* was identified in the semen samples of three out of five bulls.

3.3. Determining the minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of silver-carbon nanoparticles (Ag@C NPs)

The MIC and MBC values of the Ag@C NPs against all the tested bacterial species are showed in Table 1. After aerobic incubation of Ag@C NPs and bacteria at 37 ºC for 24 h, no turbidity was observed in the test tubes containing Ag@C NPs at concentrations ranged from 100 to 3.125 µg/mL referring to the inhibition of bacterial growth. While in the lower concentrations (1.56 to 0.1953 µg/mL), the turbidity was obvious indicating the bacterial growth. The MIC value of Ag@C NPs was of 3.125

286 µg/mL against *S. aureus* and *P. aeruginosa* and 12.5 µg/mL against *E. coli* species. The
287 same MICs of the Ag@C NPs were obtained when the tubes were incubated at 5 ºC
288 (Table 1). For control tubes, the turbidity (bacterial growth) was noticed in positive
289 control tubes, whereas the absence of turbidity (no bacterial growth) was observed in the
290 negative control ones. For MBC values, the bactericidal activity of Ag@C NPs was
291 obvious at 3.125 µg/mL against *S. aureus* and *P. aeruginosa*. However, Ag@C NPs had
292 no bactericidal effect against *E. coli* at 100, 50, 25 and 12.5 µg/mL.
293
294 *3.4. Effect of silver-carbon nanopoarticles (Ag@C NPs) on sperm quality and*
295 *ultrastructural morphology of sperm.*
296 Ag@C NPs (at 15-30 µg/mL) did not affect ($P > 0.05$) the assessed sperm
297 parameters (motility, membrane integrity, morphology, and acrosome integrity) at
298 different time points (0, 24 and 48 h) after cold storage (Table 2). While, Ag@C NPs
299 used at higher concentrations (≥60 µg/mL) were detrimental for all sperm parameters
300 evaluated in a dose-dependent manner (Table 2). The most common sperm abnormality
301 observed was bent tail.
302 TEM investigation of sperm exposed to the Ag@C NPs for 48 h showed that
303 Ag@C NPs have no obvious adverse effect on the ultrastructure of bovine sperm in
304 comparison to control. The plasma membrane was normal and intact at different parts of
305 the sperm. Additionally, the sperm head showed an intact acrosomal membrane which
306 completely surround the acrosomal ground substance. The morphology of nucleus and
307 mitochondria was normal and did not show any detrimental changes. Thus, a low
308 proportion of internalization has been shown (Figs.4 and 5).

4. Discussion

The present study was conducted to determine the main bacterial species present in fresh bulls semen. *S. aureus* and *E. coli* were isolated from all of the examined samples. These results are expected if we considered the ubiquitous nature of such microorganisms, widely present in the surrounding environment [46]. Similar results were reported in previous studies on the semen of several species including bull [47], buffalo [48], ram [49], and boar [50]. The higher occurrence of *S. aureus* and *E. coli* in the current study has significant importance due to the implication of such pathogens in breeding failure in cattle [51-53].

We could also detect *P. aeruginosa* in 60% (data not shown) of the tested ejaculates. This finding is in accordance with previous studies performed in semen and prepuce samples of bull and buffalo [48, 54]. Indeed, *P. aeruginosa* might adversely affect sperm viability and decrease the conception rate [55]. On the other hand, we could not detect other kinds of microorganisms in the fresh semen such as anaerobes, *Lactobacillus spp*, yeasts, and molds. Anaerobic bacteria are not commonly present in semen samples, because they are oversensitive and may be damaged during transportation or cultivation of the samples when exposed to oxygen [56].

Different concentrations of Ag@C NPs were used herein to clarify their possible antimicrobial effects and determinate the MICs and MBCs values against *S. aureus*, *E. coli* and *P. aeruginosa* isolates obtained from fresh bull semen. The MIC of the newly synthesized Ag@C NPs was low for the tested strains (3.125-12.5 µg/mL), thereby indicating that the bacterial flora found in fresh semen samples was highly suceptible to

Ag@C NPs after incubation for 24 h at 37 ºC. Moreover, it is noteworthy that Ag@C NPs could exhibit a strong effect against the examined isolates at cold temperature (5 ºC). Higher MIC value was reported by Borah et al. [57] for *S. aureus* (60 µg/mL) when Ag-citrate NPs with size of 10 nm were used. Additionally, higher MIC values (90 and 100 µg/mL) were recorded for *E. coli MTCC 739* and *S. aureus NCIM 5021*, respectively [58]. While, MIC values of AgNPs synthesized by UV photoreduction for *P. aeruginosa* were extremely low (1 and 2 µg/mL) [59].

Recently, it has been reported that carbon-based nanoparticles showed strong antimicrobial activity [60]. However, the antimicrobial activity of the C nanostructures is not fully understood till the moment [61]. It has been proposed that the direct physical contact between the bacterial cells and C nanomaterials lead to cell death [62], in addition to the bacterial membrane damage due to the nanoparticle-induced oxidative stress [60, 63]. Furthermore, ultrastructural analysis of bacterial cells indicated that the antibacterial mechanism of Ag@C was the physical interaction with cell membrane, the large formation of cell-nanocomposite aggregates, and faster destructibility of cell membrane, thereby resulting in cells death [64]. Interestingly, our work showed the bactericidal activity of the Ag@C NPs against *S. aureus* and *P. aeruginosa* at 3.125 µg/mL (MBCs); however, Ag@C NPs had no bactericidal activity against *E. coli* after co-incubation for 24 h. Hence, the obtained result for MBC revealed that *E. coli* was less sensitive to Ag@C NPs, as previously reported by Lara et al. [65] who found that the positive charges of AgNPs could be trapped and blocked by lipopolysaccharide. The resistance of some pathogens to AgNPs was previously reported such as *S. aureus* [66] and some

strains of *E. coli* [67] through different mechanisms including negative regulation of porins, chromosomal resistance genes or plasmids with resistance genes [68].

The MBCs determined in the current study showed the strong bactericidal activity of Ag@C NPs against both *S. aureus* and *P. aeruginosa*, but not for *E. coli*. The MBCs in the present study were lower than that previously obtained by Qais et al. [69] and Parvekar et al. [70], who reported that 32 µg/mL and 0.625 mg/mL were the MBC of AgNPs against *S. aureus*, respectively. The differences between the present study and the previous ones might be due to the different sources and size of the AgNPs, presence of C, and Ag^{+} ions content. Notably, the environmental conditions could interfere with the antimicrobial activity of the nanoparticles. When ZnO NPs were encouraged by temperature, the antimicrobial effectiveness of the NPs was increased by induction of ROS generation. In this context, the current study evaluated the antibacterial effect of AgNPs at 5 ºC to mimic the effect of temperature during semen chilling [71]. However, the micro milieu of the semen extender (i.e. pH, dissolved oxygen and nutrients) could also interfere with the antibacterial activity of the presented NPs [72]. Thus further studies are needed to confirm the antimicrobial efficiency of Ag@C NPs in the semen extender, artificially inoculated with the isolated bacteria, after 48 h of cold storage.

Our results showed that Ag@C NPs at concentrations of ≤ 30 µg/mL did not affect the different sperm parameters after 48 h of cold storage. Similar biocompatibility findings were previously recorded in humans [73] and pigs [74], using low concentrations of AgNPs. In the same way, Seo et al. [75] reported that 30 µg/mL of synthesized Ag-C nanostructures showed potential antibacterial activity with minor adverse effect toward human and animal cells. On the other hand, the used Ag@C NPs

was spermicidal at higher concentrations (≥60 µg/mL). In harmony, AgNPs showed detrimental changes in sperm characteristics in a dose dependent manner [25, 73].

Of note, the high rate of AgNPs internalization into sperm altered the sperm physiology (decreased viability; and increased mitochondrial and morphological abnormalities) by inducing reactive oxygen species (ROS) generation and lead to poor fertilization and embryonic development in mice [26]. In the present study, TEM showed attachment of Ag@C NPs to the sperm plasma membrane, but provided no evidence for the penetration ability of such NPs into the bull sperm at MIC. However, the percentage of reacted acrosome was increased by increasing the Ag@C NPs concentrations. In the same context, Taylor et al. [76] suggested the impairment of sperm functions by interacting of gold NPs with the sperm membrane in a dose-dependent manner. Further research is required to determine the impact of Ag@C NPs attachment with the sperm membrane on fertilization rates and embryonic development. Notably, our Ag@C NPs had no negative impact on sperm ultrastructure. The presence of C and the lower content of Ag in the newly synthetized Ag@C NPs could be the main reasons for lowering its spermicidal effect.

5. Conclusions

Overall, the current research detected different bacteria in fresh bull semen. The minimum inhibitory concentration (MIC) of the novel synthesized type of AgNPs that contain carbon (Ag@C NPs) was low enough to have no adverse effect on the measured sperm parameters. Moreover, Ag@C NPs had no detrimental effect on the ultrastructure of bull sperm and limited affinity for sperm internalization. As far as we know, this is the

first study to apply the carbon-based nanoparticles as an alternative antibiotic for the semen. However, further investigation for its effect on the embryo production and female genitalia still required.

Conflict of interest

The authors declare that they have no competing interests.

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References

[1] González-Marín C, Roy R, López-Fernández C, Diez B, Carabaño MJ, Fernández JL, et al. Bacteria in bovine semen can increase sperm DNA fragmentation rates: a kinetic experimental approach. Anim Reprod Sci 2011;123:139-48.

[2] Givens MD, Marley MSD. Pathogens that cause infertility of bulls or transmission via semen. Theriogenology 2008;70:504-7.

[3] Villegas J, Schulz M, Soto L, Sanchez R. Bacteria induce expression of apoptosis in human spermatozoa. Apoptosis 2005;10:105-10.

[4] Althouse GC, Pierdon MS, Lu KG. Thermotemporal dynamics of contaminant bacteria and antimicrobials in extended porcine semen. Theriogenology 2008;70:1317-23.

[5] Hosie J, Rowe SM, Morton JM, Tranter WP, Cavalieri J. Use of a sanitary sheath at artificial insemination by nonprofessional technicians does not markedly improve pregnancy rates to artificial insemination in pasture-based dairy cows. J Dairy Sci 2019;102:5588-98.

[6] Morrell J. Artificial Insemination: Current and future trends. In: Malafi M, editors. Artificial insemination in farm animals. London: IntechOpen; 2011, p. 1–17.

[7] Aurich C, Spergser J. Influence of bacteria and gentamicin on cold-stored stallion spermatozoa. Theriogenology 2007;67:912-8.

[8] Schulze M, Junkes C, Mueller P, Speck S, Ruediger K, Dathe M, et al. Effects of cationic antimicrobial peptides on liquid-preserved boar spermatozoa. PloS one 2014;9:e100490.

[9] Loskutoff NM, Huyser C, Singh R, Walker DL, Thornhill AR, Morris L, et al. Use of a novel washing method combining multiple density gradients and trypsin for removing human immunodeficiency virus-1 and hepatitis C virus from semen. Fertil Steril 2005;84:1001-10.

[10] Fourie J, Loskutoff N, Huyser C. Elimination of bacteria from human semen during sperm preparation using density gradient centrifugation with a novel tube insert. Andrologia 2012;44:513-7.

[11] Nicholson CM, Abramsson L, Holm SE, Bjurulf E. Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different g forces. Hum Reprod 2000;15:662-6.

[12] Tsakmakidis IA, Samaras T, Anastasiadou S, Basioura A, Ntemka A, Michos I, et al. Iron oxide nanoparticles as an alternative to antibiotics additive on extended boar semen. Nanomaterials (Basel) 2020;10:1568.

[13] Wakshlak RB-K, Pedahzur R, Avnir D. Antibacterial activity of silver-killed bacteria: the "zombies" effect. Sci Rep 2015;5:9555.

[14] Abdelhamid HN, Wu H-F. Facile synthesis of nano silver ferrite (AgFeO2) modified with chitosan applied for biothiol separation. Mat Sci Eng C 2014;45:438-45.

[15] Abalkhail T, Alharbi S, Salmen S, Wainwright M. Bactericidal activity of biosynthesized silver nanoparticles against human pathogenic bacteria. Biotechnol Biotec Eq 2016;31:1-7.

[16] Abdelhamid HN, Wu H-F. Proteomics analysis of the mode of antibacterial action of nanoparticles and their interactions with proteins. TrAC-Trend Anal Chem 2015;65:30-46.

[17] Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv 2009;27:76-83.

[18] Kim JS, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomed- Nanotechnol 2007;3:95-101.

[19] Liao S, Zhang Y, Pan X, Zhu F, Jiang C, Liu Q, et al. Antibacterial activity and mechanism of silver nanoparticles against multidrug-resistant *Pseudomonas aeruginosa*. Int J Nanomed 2019;14:1469-87.

[20] Shahverdi AR, Fakhimi A, Shahverdi HR, Minaian S. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against *Staphylococcus aureus* and *Escherichia coli*. Nanomed-Nanotechnol 2007;3:168-71.

[21] Abdelhamid HN, Talib A, Wu H-F. Facile synthesis of water soluble silver ferrite (AgFeO2) nanoparticles and their biological application as antibacterial agents. RSC Adv 2015;5:34594-602.

[22] Hill EK, Li J. Current and future prospects for nanotechnology in animal production. J Anim Sci Biotechnol 2017;8:26.

[23] Feugang JM, Rhoads CE, Mustapha PA, Tardif S, Parrish JJ, Willard ST, et al. Treatment of boar sperm with nanoparticles for improved fertility. Theriogenology 2019;137:75-81.

[24] Pérez-Duran F, Acosta-Torres LS, Serrano-Díaz PN, Toscano-Torres IA, Olivo-Zepeda IB, García-Caxin E, et al. Toxicity and antimicrobial effect of silver nanoparticles in swine sperms. Syst Biol Reprod Med 2020;66:281-9.

[25] Wang E, Huang Y, Du Q, Sun Y. Silver nanoparticle induced toxicity to human sperm by increasing ROS (reactive oxygen species) production and DNA damage. Environ Toxicol Pharmacol 2017;52:193-9.

[26] Yoisungnern T, Choi Y-J, Han JW, Kang M-H, Das J, Gurunathan S, et al. Internalization of silver nanoparticles into mouse spermatozoa results in poor fertilization and compromised embryo development. Sci Rep. 2015;5:11170.

[27] Yuan B, Guo J, Bai S. *In-situ* thermally induced reduction of silver nitrate by polyvinyl alcohol to prepare three-dimensional porous Ag substrate with excellent

Con formato: Español (España)

adsorption and surface-enhanced raman scattering properties. J Mater Chem C 2020;8:6478-87.

[28] Prajapati P, Agrawal YK. Analysis and impurity identification in pharmaceuticals. Rev Anal Chem 2014;33:123-33.

[29] National Academies of Sciences, Engineering, and Medicine. Nutrient Requirements of Beef Cattle. 8th ed. Washington: The National Academies Press. 2016.

[30] Barszcz K, Wiesetek D, Wasowicz M, Kupczyńska M. Bull semen collection and analysis for artificial insemination. J Agric Sci 2011;4:1-10.

[31] Murphy EM, Kelly AK, O'Meara C, Eivers B, Lonergan P, Fair S. Influence of bull age, ejaculate number, and season of collection on semen production and sperm motility parameters in Holstein Friesian bulls in a commercial artificial insemination centre. J Anim Sci 2018;96:2408-18.

[32] Moses NA, Uchenna I, Michael OJIJoP, Sciences B. Semen Culture: A comparative analysis between solid media and liquid media supplementation. IOSR J Pharm Biol Sci 2013;5:67-72.

[33] Eggert-Kruse W, Rohr G, Ströck W, Pohl S, Schwalbach B, Runnebaum B. Anaerobes in ejaculates of subfertile men. Hum Reprod Update 1995;1:462-78.

[34] Wehr H. Michael, Frank Joseph F.. Standard methods for the examination of dairy products. 17th ed. Washington: American Public Health Association Inc. 2004.

[35] Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S, Hartigan PJ. Veterinary microbiology and microbial disease. 2nd ed. Iowa: Iowa State University Press. 2011.

511 [36] Mulfinger L, Solomon SD, Bahadory M, Jeyarajasingam AV, Rutkowsky SA, Boritz
512 C. Synthesis and study of silver nanoparticles. J Chem Educ 2007;84:322.
513 [37] Krishnan R, Arumugam V, Vasaviah SK. The MIC and MBC of silver nanoparticles
514 against *Enterococcus faecalis* - A Facultative Anaerobe. J Nanomed Nanotechnol
515 2015;6:1-4.
516 [38] Pérez-Díaz MA, Boegli L, James G, Velasquillo C, Sánchez-Sánchez R, Martínez-
517 Martínez RE, et al. Silver nanoparticles with antimicrobial activities against
518 *Streptococcus mutans* and their cytotoxic effect. Mat Sci Eng C Mater 2015;55:360-6.
519 [39] Singh A, Singh V, Narwade B, Mohanty T, Atreja S. Comparative quality
520 assessment of buffalo (*Bubalus bubalis*) semen chilled (5°C) in egg yolk- and soya milk–
521 based extenders. Reprod Domest Anim 2012;47:596-600.
522 [40] Crespilho AM, Nichi M, Guasti PN, Freitas-Dell'Aqua CP, Sá Filho MF, Maziero
523 RR, et al. Sperm fertility and viability following 48h of refrigeration: Evaluation of
524 different extenders for the preservation of bull semen in liquid state. Anim Reprod Sci
525 2014;146:126-33.
526 [41] Khalil WA, El-Harairy MA, Zeidan AEB, Hassan MAE, Mohey-Elsaeed O.
527 Evaluation of bull spermatozoa during and after cryopreservation: Structural and
528 ultrastructural insights. Int J Vet Sci Med 2017;6:S49-S56.
529 [42] Dorado J, Acha D, Ortiz I, Galvez MJ, Carrasco JJ, Gomez-Arrones V, et al. Effect
530 of extender and amino acid supplementation on sperm quality of cooled-preserved
531 Andalusian donkey (*Equus asinus*) spermatozoa. Anim Reprod Sci 2014;146:79-88.

[43] Hidalgo M, Rodriguez I, Dorado J. Influence of staining and sampling procedures on goat sperm morphometry using the Sperm Class Analyzer. Theriogenology 2006;66:996-1003.

[44] Tahmasebi E, Masoomi MY, Yamini Y, Morsali A. Application of mechanosynthesized azine-decorated Zinc (II) metal–organic frameworks for highly efficient removal and extraction of some heavy-metal ions from aqueous samples: A comparative study. Inorg Chem 2015;54:425-33.

[45] Wang R-C, Gao Y-S, Chen S-J. Simple synthesis and size-dependent surface-enhanced Raman scattering of Ag nanostructures on TiO2 by thermal decomposition of silver nitrate at low temperature. Nanotechnology 2009;20:375605.

[46] Kadariya A, Smith T, Thapaliya D. *Staphylococcus aureus* and Staphylococcal food-borne disease: An ongoing challenge in public health. BioMed Res Int 2014;2014:827965.

[47] Paray A, Bhakat M, Lone S, Mohanty T, Sinha R, Ur Rahman J, et al. Role of preputial washing in reducing microbial load and improving bovine semen quality. Asian Pac J Reprod 2018;7:97-102.

[48] Andrabi SMH, Khan LA, Shahab M. Isolation of bacteria in semen and evaluation of antibiotics in extender for cryopreservation of buffalo (*Bubalus bubalis*) bull spermatozoa. Andrologia 2016;48:1166-74.

[49] Yániz JL, Marco-Aguado MA, Mateos JA, Santolaria P. Bacterial contamination of ram semen, antibiotic sensitivities, and effects on sperm quality during storage at 15°C. Anim Reprod Sci 2010;122:142-9.

554 [50] Bennemann P, Machado S, Girardini L, Tonin A. Bacterial contaminants of semen
555 and antimicrobial susceptibility profile in boar studs of Southern Brazil. Rev MVZ
556 Cordoba. 2018;23:6637-48.
557 [51] Mitra J, Chowdhury S, Panda S, Chakraborty M, Singha A. Microbiological
558 evaluation of bovine frozen semen samples in West Bengal, India. Explor Anim Med Res
559 2016;6:185-91.
560 [52] Wolff H, Panhans A, Stolz W, Meurer M. Adherence of *Escherichia coli* to sperm: a
561 mannose mediated phenomenon leading to agglutination of sperm and *E. coli*. Fertil
562 Steril 1993;60:154-8.
563 [53] Földi J, Kulcsár M, Pécsi A, Huyghe B, de Sa C, Lohuis JACM, et al. Bacterial
564 complications of postpartum uterine involution in cattle. Anim Reprod Sci 2006;96:265-
565 81.
566 [54] Smole I, Thomann A, Frey J, Perreten V. Repression of common bull sperm flora
567 and *in vitro* impairment of sperm motility with Pseudomonas aeruginosa introduced by
568 contaminated lubricant. Reprod Domest Anim 2010;45:737-42.
569 [55] Corona A, Cossu I, Bertulu A, Cherchi R. Characterisation of bacteria in fresh
570 semen of stallions during the breeding season. Anim Reprod Sci 2006; 94:85-8.
571 [56] Moretti E, Capitani S, Figura N, Pammolli A, Federico MG, Giannerini V, et al. The
572 presence of bacteria species in semen and sperm quality. J Assist Reprod Genet
573 2009;26:47-56.
574 [57] Borah D, Deka P, Bhattacharjee P, Changmai A, Yadav RNS. *Ocimum sanctum*
575 mediated silver nano particles showed better antimicrobial activities compared to citrate

Con formato: Francés (Francia)

576 stabilized silver nano particles against multidrug resistant bacteria. J Pharm Res
577 2013;7:478-82.
578 [58] Agnihotri S, Mukherji S, Mukherji S. Size-controlled silver nanoparticles
579 synthesized over the range 5–100 nm using the same protocol and their antibacterial
580 efficacy. RSC Adv 2014;4:3974-83.
581 [59] Kora AJ, Arunachalam J. Assessment of antibacterial activity of silver nanoparticles
582 on *Pseudomonas aeruginosa* and its mechanism of action. World J Microb Biot
583 2011;27:1209-16.
584 [60] Maleki Dizaj S, Mennati A, Jafari S, Khezri K, Adibkia K. Antimicrobial activity of
585 carbon-based nanoparticles. Adv Pharm Bull 2015;5:19-23.
586 [61] Al-Jumaili A, Alancherry S, Bazaka K, Jacob MV. Review on the antimicrobial
587 properties of carbon nanostructures. Materials (Basel) 2017;10:1066.
588 [62] Kang S, Pinault M, Pfefferle LD, Elimelech M. Single-walled carbon nanotubes
589 exhibit strong antimicrobial activity. Langmuir 2007;23:8670-3.
590 [63] Gurunathan S, Han JW, Dayem AA, Eppakayala V, Kim J-H. Oxidative stress-
591 mediated antibacterial activity of graphene oxide and reduced graphene oxide in
592 *Pseudomonas aeruginosa*. Int J Nanomedicine 2012;7:5901-14.
593 [64] Dinh NX, Quy NV, Huy TQ, Le A-T. Decoration of silver nanoparticles on
594 multiwalled carbon nanotubes: Antibacterial mechanism and ultrastructural analysis. J
595 Nanomater 2015;2015:814379.
596 [65] Lara H, Ayala-Nunez V, Ixtepan Turrent L, Rodríguez-Padilla C. Bactericidal effect
597 of AgNPs against multidrug-resistant bacteria. World J Microb Biot 2009;26:615-21.

[66] Li XZ, Nikaido H, Williams KE. Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol 1997;179:6127-32.

[67] Radzig MA, Nadtochenko VA, Koksharova OA, Kiwi J, Lipasova VA, Khmel IA. Antibacterial effects of silver nanoparticles on gram-negative bacteria: influence on the growth and biofilms formation, mechanisms of action. Colloid Surface B 2013;102:300-6.

[68] Salas-Orozco M, Niño-Martínez N, Martínez-Castañón G-A, Méndez FT, Jasso MEC, Ruiz F. Mechanisms of resistance to silver nanoparticles in endodontic bacteria: A literature review. J Nanomater 2019;2019:7630316.

[69] Qais FA, Shafiq A, Khan HM, Husain FM, Khan RA, Alenazi B, et al. Antibacterial Effect of silver nanoparticles synthesized using *Murraya koenigii* (L.) against multidrug-resistant pathogens. Bioinorg Chem Appl 2019;2019:4649506.

[70] Parvekar P, Palaskar J, Metgud S. Antibacterial efficacy of silver nanoparticles against *Staphylococcus aureus*. Res Microbiol 2020;162:542-9.

[71] Saliani M, Jalal R, Kafshdare. Goharshadi E. Effects of pH and temperature on antibacterial activity of Zinc oxide nanofluid against *Escherichia coli* O157: H7 and *Staphylococcus aureus*. Jundishapur J Microbiol 2015;8:e17115.

[72] Varier KM, Gudeppu M, Chinnasamy A, Thangarajan S, Balasubramanian J, Li Y, et al. Nanoparticles: Antimicrobial applications and its prospects. In: Naushad M, Rajendran S, Gracia F, editors. Advanced nanostructured materials for environmental remediation. Cham: Springer International Publishing; 2019. p. 321-55.

Con formato: Español (España)

[73] Moretti E, Terzuoli G, Renieri T, Iacoponi F, Castellini C, Giordano C, et al. *In vitro* effect of gold and silver nanoparticles on human spermatozoa. Andrologia 2013;45:392-6.

[74] Tiedemann D, Taylor U, Rehbock C, Jakobi J, Klein S, Kues WA, et al. Reprotoxicity of gold, silver, and gold–silver alloy nanoparticles on mammalian gametes. Analyst 2014;139:931-42.

[75] Seo Y, Hwang J, Kim J, Jeong Y, Hwang MP, Choi J. Antibacterial activity and cytotoxicity of multi-walled carbon nanotubes decorated with silver nanoparticles. Int J Nanomedicine 2014;9:4621-9.

[76] Taylor U, Barchanski A, Petersen S, Kues WA, Baulain U, Gamrad L, et al. Gold nanoparticles interfere with sperm functionality by membrane adsorption without penetration. Nanotoxicology 2014;8 Suppl 1:118-27.