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Size-Dependent Inhibition of Sperm Motility by Copper Particles as a Path toward Male Contraception

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Effective inhibition of sperm motility using a spermicide can be a promising approach in developing non-invasive male contraceptive agents. Copper is known to have contraceptive properties and has been used clinically for decades as intrauterine contraceptive devices (IUDs) for contraception in females. Beyond that, the spermicidal use of copper is not explored much further, even though its use can also subdue the harmful effects caused by the hormonal female contraceptive agents on the environment. Herein, the size, concentration, and time-dependent in vitro inhibition of bovine spermatozoa by copper microparticles are studied. The effectivity in inhibiting sperm motility is correlated with the amount of Cu^{2+} ions released by the particles during incubation. The copper particles cause direct suppression of sperm motility and viability upon incubation and thereby show potential as sperm-inhibiting, hormone-free candidate for male contraception. In addition, biocompatibility tests using a cervical cell line help optimizing the size and concentration of the copper particles for the best spermicidal action while avoiding toxicity to the surrounding tissue.

1. Introduction

Regulation of fertility and reproduction is one of the main targets in the worldwide human health sector, intending to grant equal access to effective contraception means for males[1] and females. Currently, female-only contraception methods dominate the market: intrauterine devices (IUDs), hormonal treatment through oral, injectables, or implants, as well as female sterilization are most widespread.[2] Some of these methods are invasive and associated with health problems, for instance, hormonal birth control in women is reported to have mild-to-severe side effects.[3] In the male contraceptive sector, there has not been much advancement besides barrier methods and vasectomy.[1,4] Despite a growing demand in the market, only around 30% of couples use a form of male contraception.[1] Lately, efforts to develop hormonal contraception for men have increased, but many are associated with side effects and social stigma, pending their approval. Hormonal approaches are divided into administration of pure androgens, combinations of testosterone with progestogens, and combinations with gonadotropin-releasing hormone antagonists,[5] all showing potential as male contraceptives, but longevity and reversibility of inhibition still have to be improved.[6]

Besides the medical drawbacks of hormonal contraception, the drastic effects on the environment have to be considered: The release of hormones into water bodies and finally exposure to aquatic life have shown to alter aquatic ecology.[7–10] Both estrogens and progestins have been considered as potent endocrine disrupters for both humans and aquatic life even at very low concentrations (as low as ngL^{-1}).[10–12] At lower concentrations, hormonal residues cause ineffective reproduction or feminization of male fish, and chronic exposure can lead to extinction of fish population.[9] Further, these hormones or their metabolites are very resistant to biodegradation and inefficient in most removal strategies.[13] Thus, the focus of contraceptive research toward a more sustainable approach is a viable strategy to restrain the overuse of hormonal substances.

The efficacy of contraceptive methods is also an important parameter to consider. These efficacy rates can be measured in terms of the pearl index which calculates unintended pregnancies in 100 women in a year of exposure. Male condoms have a pearl index of 2.5–5.9, which is higher compared with methods such as IUDs for females (0.16–1.26).[14] Therefore, prospective male contraceptives will only be impactful if they provide improved efficacy.

The development of a male contraceptive that completely ceases the hyperactivation or motility of the sperm cells and

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avoids the crossing of "blood–testis" barrier has been envisioned as a promising approach in the advancement of future male contraception.[4,15] Drugs or polymers targeting sperm motility or hyperactivation can be used as injectables,[16,17] where they stay within the seminal fluids and are ejaculated along with the semen. They show immediate response, so that they can be administered just before the intercourse as components of gels or condoms.[4] Here, we demonstrate the suitability of copper particles as such a motion-inhibiting agent, bypassing the environmentally critical use of hormones.

On the one side, copper is a nutritional trace element required for hemoglobin formation,[18] maintaining health of embryos and also involved in the ovulation process.[19] On the other side, copper in excess concentration (>62.5 μM concentration of Cu^{2+} ions[20]) can cause infertility or inhibition of reproductive processes.[21] As an inhibitor, copper affects spermatozoa motility,[22] by suppressing metabolic processes such as glucose consumption and oxidative processes of sperm cells.[20,23] In addition, the Cu^{2+} ions released from Cu form copper chelation complexes with mucoids which can curb spermatogenesis.[20,21] In addition, when present in the genital tract, it can induce inflammatory responses which are toxic for spermatozoa and embryos.[24] The main spermicidal action of copper can be attributed to the metabolic and kinetic inhibition of spermatozoa, as found in several other heavy metals as well.

The earliest reference of inhibition by cupric ions on mammalian spermatozoa was carried out by de Quatrefages in 1850.[25] Later, effects of copper in reproductive processes have been investigated.[26,27]

These spermicidal effects of copper in reproductive processes allowed the clinical development of Cu IUDs in the early 1920s, with Ernst Gräfenberg as one of the pioneers whose work is believed to have led to the first copper compounds in these devices (introduced through a silver wire).[28] Following that, Zipper et al. demonstrated the use of copper spirals in plastic IUDs.[29,30] These Cu wires locally excrete low concentrations of copper ions in the uterus and inhibit sperm survival after entering the female reproductive tract.[31] It is now proven to be an effective, nonhormonal, reversible female contraception method and is being widely used since the 1970s.[32,33] Safety of such devices is evaluated via apoptosis levels in surrounding tissues. These showed no increase in endometrial tissue after exposure to copper IUDs and is thus considered safe.[34] Despite this, the implantation not only involves a small surgery which leads to postsurgical pain and bleeding, but also causes disruptions in the menstrual cycle.[21]

Due to its compelling effectiveness and the absence of hormonal residues and metabolites in wastewater, the use of Cu in male contraception has also gained experimental implications.[35] The group of Kapur and Laumas developed the intravasal Cu (IVDs) which are inserted in the vas deferens and can be used as an effective and reversible method for male contraception.[36,37] Through many other research programs, the implantation of copper devices in the male reproductive system also included the lumen of the ductus deferens, epididymis, scrotum, etc.[23] The biggest concern is the direct implantation of these Cu IVDs in the scrotum and vas deferens-created toxicological effect on the tissues.[38] The development of copper as an effective and noninvasive male contraceptive is yet to be accomplished.

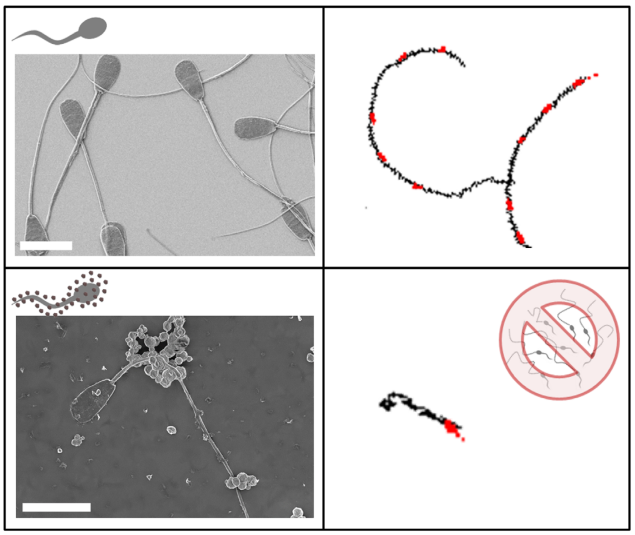

Figure 1. Exemplary scanning electron microscopic (SEM) images of bovine spermatozoa in the absence and presence of copper microparticles (Cu_2 particles) with the following example track are shown in the upper and lower panel, respectively. The tracks represent the motion of sperm cells in sperm medium (control) and in the presence of 0.1 g L^{-1} Cu_2 particles in sperm medium, incubated for 15–30 min. The tracks are tracked for 220 frames (5.5 s). Scale bar 10 μm.

Micro- or nanoscience has evolved as a remarkable tool in biomedical applications.[39,40] The use of micro- or nanoparticles is more advantageous than their larger conjugates due to their high surface-to-volume ratio and their additional intrinsic properties.[41] The photothermic or magnetothermic effect of metal or metal–oxide nanoparticles has been explored as a reversible, nonsurgical method for male contraception.[42,43] However, all these methods involve intravenous administration and generate testicular hyperthermia, which might damage the testicles and permanently suppress the spermatogenesis process.[44,45]

Besides a variety of catalytic applications,[46] copper micro- or nanoparticles are used as biocidal agents for the reduction and elimination of microbes.[47]

In this manuscript, the contraceptive effect of copper microparticles on bovine spermatozoa is explored. Copper particles of three different sizes are synthesized according to a recently developed strategy[46] and their effect on the overall sperm motility, velocity, and viability is investigated (see **Figure 1**). To demonstrate a dependence on particle concentration, the release of Cu^{2+} ions is estimated by spectrophotometric and inductively coupled plasma–optical emission spectrometry (ICP–OES) methods.

2. Results and Discussion

2.1. Characterization of Copper Particles

To study the sperm inhibition effect by differently sized particles, we synthesized copper particles of three different size ranges via an assisted polyol reaction approach: smaller particles of 0.2 μm

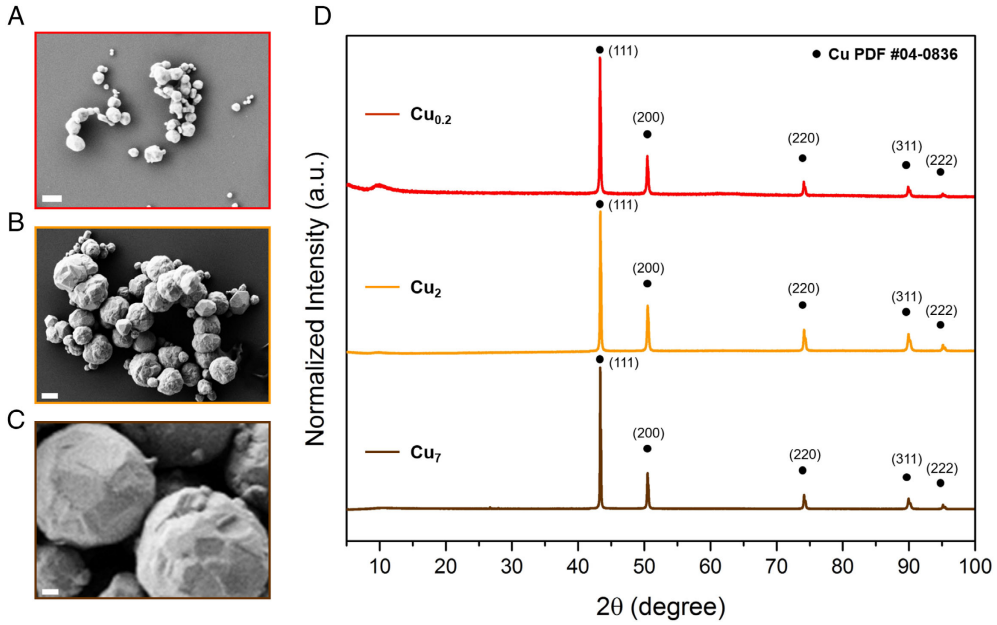

Figure 2. Characterization of the copper particles: SEM images of A) $Cu_{0.2}$, B) Cu_2, and C) Cu_7 particles. Scale bar: 1 µm. D) X-ray diffraction (XRD) reflexes of $Cu_{0.2}$, Cu_2, and Cu_7 particles, compared with the standard FCC phase of Cu.

($Cu_{0.2}$), medium-sized particles of 2 µm (Cu_2), and larger particles of 7 µm (Cu_7) (**Figure 2**A–C).

An additive-facilitated polyol method is used to synthesize the $Cu_{0.2}$ and Cu_7 particles where simultaneous competing nucleation leads to the formation of copper microparticles in different size ranges.[46] Cu_2 particles are synthesized by a simple hydrothermal method using ascorbic acid as a stabilizer and reducing agent.

The X-Ray diffraction pattern (Figure 2D) of these particles exhibits similar reflexes attributed to metallic copper. These reflexes comply very well with the standard face-centered-cubic (FCC) phase of copper (JCPDS No. 040 836) and the sharp reflexes depict the crystalline nature of samples.

Although these copper particles have positive surface charges (see Table S1, Supporting Information), we did not observe any significant charge-based interaction with the sperm cells like positively charged Fe_2O_3 particles[48] or IRONSperm.[49]

2.2. Amount of Copper Ions Released in Aqueous Media

As mentioned earlier, Cu^{2+} ions cause alterations in metabolic properties and are responsible for the immobilization of sperm cells.[50,51] At higher concentrations, copper ions decrease the mitochondrial activity of the sperm cells,[20] which can further affect the sperm's acrosome reaction and penetration ability, ultimately impairing the interaction between female and male gametes and thus the fertilizing capacity.[52]

The toxic action of these cupric ions on the sperm cells is extensively influenced by the concentration of ions in the sample solution.[20,51] Therefore, the amount of Cu^{2+} ions released from differently sized colloidal microparticles is investigated and correlated with the size-dependent effects on sperm cells.

To quantify and compare the amount of Cu^{2+} ions liberated by these differently sized particles, spectrophotometry and ICP–OES as two different approaches were used (detailed in the Experimental Section). From both methods, the percentage of Cu^{2+} ions released from a known amount of copper particles was calculated and compared for deionized (DI) water and sperm medium (SP-TALP).

In the spectrophotometric method, a complexation reaction between a cationic polymer polyethyleneimine (PEI) and Cu^{2+} ions was utilized.[53,54] The resulting Cu–PEI moiety showed an absorbance peak at 275 nm and a significantly lower absorbance at 630 nm in aqueous medium. The absorbance spectra were plotted with a known concentration of Cu^{2+} ions (500 µM) in water (**Figure 3**A) and SP-TALP (Figure 3B). The absorbance at 275 nm for these spectra was plotted against Cu^{2+} ion concentrations (µM) and a calibration curve was obtained in both, water and SP-TALP (see inset of Figure 3A,B).

For both media, the absorption intensity showed a linear positive correlation with Cu^{2+} ion concentration. However, in the case of SP-TALP, the transitions from lower to higher wavelength and the attained absorbance maxima were much greater than that observed in the case of DI-water. As the SP-TALP is composed of different bases, phosphates, hydrocarbons, proteins, and minerals, there is a possibility of complexation of these medium constituents with the cationic polymer PEI, leading to absorption discrepancies.

A quantitative measurement of the copper-ion concentration for differently sized particles was obtained using ICP–OES analysis.

As the starting concentrations (in $g\,L^{-1}$) of the particles in both methods were different, we estimated the percentage of Cu ions released from a given amount of particles to make a uniform comparison (see Section S7, Supporting Information).

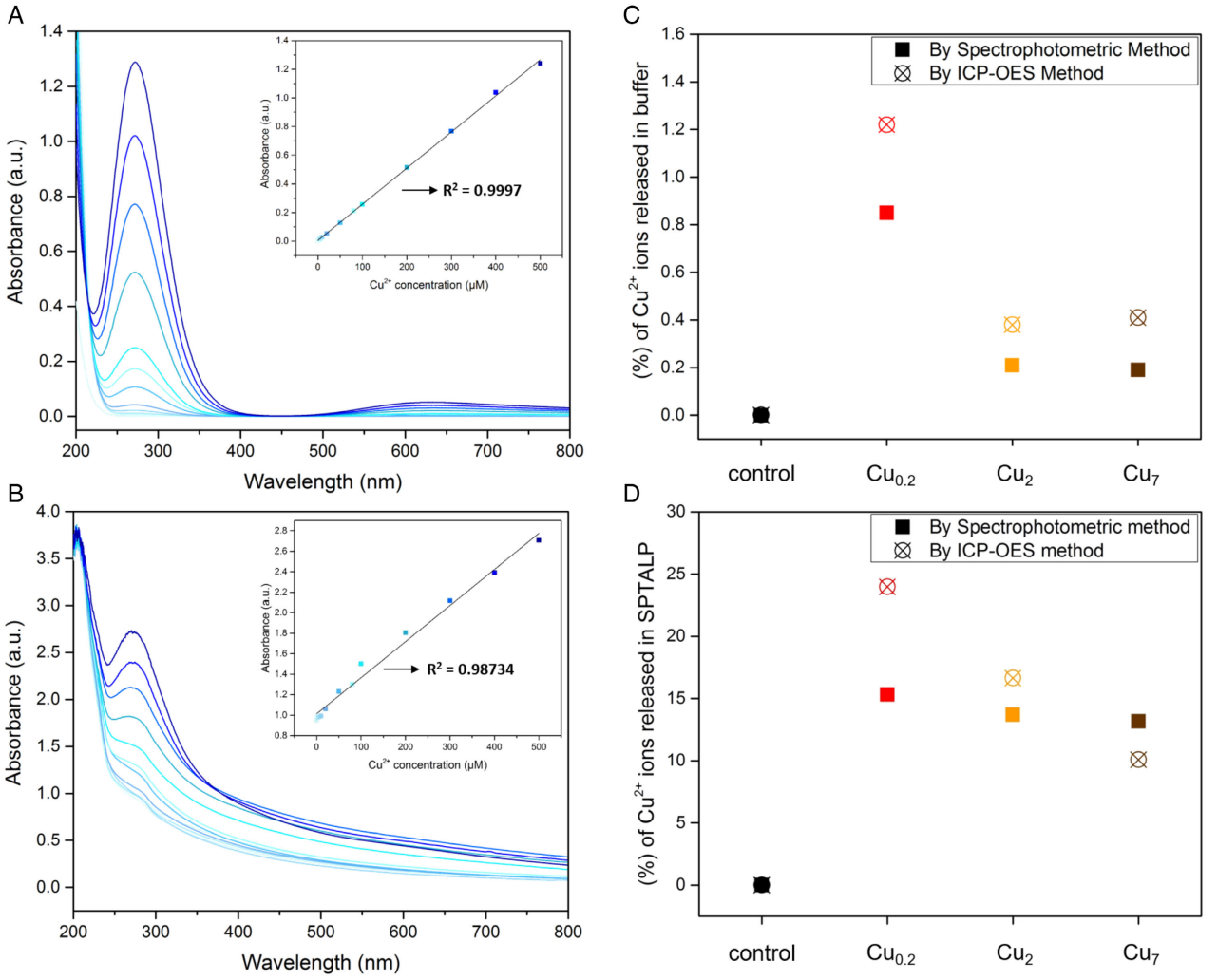

Figure 3. Detection of Cu^{2+} ions: Absorbance spectra of PEI in the presence of different concentration Cu^{2+} ions (500 μM) for spectrophotometric determination in A) DI–water and B) SP-TALP. The inset displays the Cu^{2+}–PEI complex absorbance versus Cu^{2+} ion concentration (500 μM). The percentage of cupric ions released from differently sized copper samples by spectrophotometry and ICP–OES is compared in C) DI-Water and B) SP-TALP.

Figure 3C,D shows the percentage of Cu^{2+} ions released in aqueous and SP-TALP, respectively, for different size ranges of copper particles. The trend in the amount of Cu^{2+} ions released by both methods was found to be similar. However, it is interesting to note that the amount of Cu^{2+} ions released in water is much lower compared with SP-TALP, for instance, in case of $Cu_{0.2}$ particles, by ICP–OES, a maximum of 1.22% was determined in water as compared with 23.95% release in SP-TALP. This is probably due to the presence of proteins and bases in SP-TALP which favors dissolution by complexation of metal ions. As expected, a clear decrease in the amount of Cu^{2+} ions released with a surge in particle size is observed. This reduction is not so prominent in case of SP-TALP, when quantifying the Cu^{2+} ions using spectrophotometry. However, a significant decrease of Cu^{2+} ions percentage is observed using the ICP–OES. This discrepancy can be explained by the possible complexation of PEI and SP-TALP and the resulting error associated with the spectrophotometric analysis in SP-TALP. The ICP–OES gives more accurate and reliable results.

2.3. Evaluation of Sperm Motility in the Presence of Cu Particles

The effect of differently sized copper particles in the inhibition of sperm cells is studied by evaluating the motility and the velocity of the sperm cells under varying particle concentrations and incubation time. Particle concentrations of 0.1, 0.5, and 1 g L^{-1} were used and these tests were conducted over three time intervals of 15 min each (see "sperm motility assay" in Section 4). As time progressed, the number of motile sperm cells decreased, so to maintain homogeneity in the measurement results, a control sample (without the Cu particles) was also measured at every interval. This incubation period was estimated over time intervals and not exact time (in minutes) because, as there is a control

sample alongside the Cu-incubated sample, studying the motion of one sample in microscope changes the incubation time for the other, so it is preferable to indicate a time interval within which these studies were conducted.

Figure 4A–C shows the average overall sperm motility in percentage of sperm cells after incubating with 0.2, 2, and 7 μm particles, respectively. Similarly, in Figure 4D–F, the average curvilinear velocity (VCL) is plotted for the same sizes.

The mean percentage of motile spermatozoa is compared by altering the incubation conditions. The motility of sperm cells in the presence of $Cu_{0.2}$ particles within the first interval (0–15 min) drops to around 11% (89% of the sperm population was immotile) from its control value with particle concentration as low as $0.1\,g\,L^{-1}$. The motility further drops to less than 5% from its control value in the following interval (within 15–30 min). The percentage of motile sperm finally drops to zero at the higher interval (30–45 min). For higher concentrations, no motile sperms were found and to indicate the zero-percent motility, a column bar in the negative direction is plotted (see Figure 4A).

With the increasing Cu particle size (see Figure 4B), the effect on sperm motility changes. Analogous to the $Cu_{0.2}$ particles, the motility of the sperm cells decreases with rise in the concentration of the Cu_2 particles but the rate of decrease is much slower compared with the smaller Cu particles. The cells are comparatively more motile at similar concentration ranges. The motility drops to 53% at $0.1\,g\,L^{-1}$ concentration of Cu, to 32% at $0.5\,g\,L^{-1}$, and to 26% at $1\,g\,L^{-1}$ concentration compared with the control during the first interval. In the second interval, the motility gets further reduced to 12% at $0.1\,g\,L^{-1}$, to 7% at $0.5\,g\,L^{-1}$, and completely diminishes at $1\,g\,L^{-1}$ concentration of Cu. In the third interval, the sperm sample with the lowest concentration of Cu show some motility but it is not even 1% compared to its control.

With Cu_7 particles, a not-so prominent decrease in the sperm motility was noted. At the highest Cu concentration ($1\,g\,L^{-1}$) and longest incubation time (third interval), still 38% sperm cells were found to be motile compared with the control (see Figure 4C). As there was not such a drastic decrease in sperm motility even at the highest Cu concentration, the experiments with lower concentrations were not performed.

The effectiveness of $Cu_{0.2}$ over Cu_2 and Cu_7 particles can be correlated with the amount of Cu^{2+} ions liberated by the Cu particles in the SP-TALP. As the size of the particle decreases,

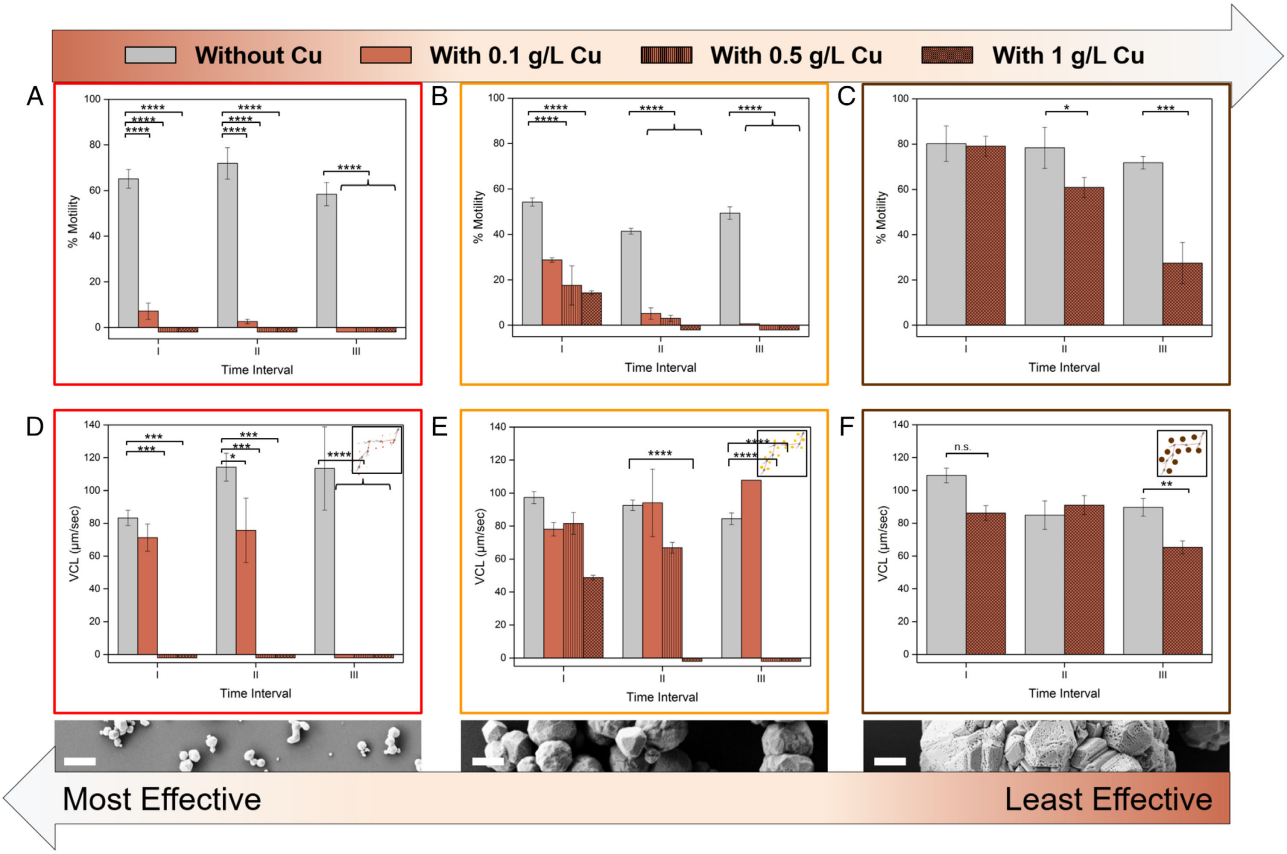

Figure 4. Average motility and VCL of sperm cells in comparison with a control postincubation over 3 time intervals with A & D) $Cu_{0.2}$, B & E) Cu_2, and C & F) Cu_7 particles. The SEM images of $Cu_{0.2}$, Cu_2, and Cu_7 particles are shown below for reference. The inset shows a schematic representation for estimation of motility (in A, B, and C) and VCL (in D, E, and F), respectively. First, second, and third time intervals correspond to the time range of 0–15, 15–30, and 30–45 min, respectively. Statistical treatment was performed with one-way ANOVA with Bonferroni method in origin; $n \geq 3$; Data presentation: Avg ± standard deviation (SD). The significance level was set at: **** ($P < 0.0001$); *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$); n.s (statistically nonsignificant). Scale bar 2 μm.

more Cu^{2+} ions are released into the medium (see Section 2.2) and it is more effective in inhibiting sperm motility.

The VCL of the sperm cells lie in the range from 60 to 120 μm s^{-1}. With the Cu particles of different size ranges and concentrations, the motile sperm maintained their velocity within this velocity range. There was a slight decrease in the velocity compared with the control, for instance, in the case of $Cu_{0.2}$ particles, (see Figure 4D), the velocity decreases by 15% in the first interval and by 34% in the second interval compared with the control. A similar decreased tendency was observed for Cu_2 particles (see Figure 4E) in the first interval and for Cu_7 particles in first and third (see Figure 4F). Although this decrease was not consistent enough to state that the presence of copper particles might influence the velocity of the motile sperms. The average path velocity (VAP) and straight line velocity (VSL) show a similar trend to the VCL and are plotted in Figure S2, Supporting Information.

2.4. Evaluation of Viability of Sperm Cells and HeLa Cells in the Presence of Cu Particles

In addition to the sperm motility studies, we also performed viability tests on sperm to evaluate the spermicidal effect. Sperm viability studies were performed with bovine spermatozoa under the same particle concentrations and sizes as in the motility assays but only at the longest time interval (third interval). As shown in **Figure 5**A, the sperm viability decreased with increasing particle concentration for all three sizes of particles. The effect was strongest in the smallest particle size 0.2 μm. A concentration of 0.5 g L^{-1} was sufficient to kill all sperm cells after 30–45 min of incubation. With 2 μm Cu particles, only a small portion of cells (about 4%) were alive after this incubation period with 0.5 g L^{-1} particles and the same was seen with the 7 μm particles. The particle size had a similar effect on the viability as it did on the motility, meaning that with increasing particle size, the toxicity effect on sperm cells was reduced. However, in this case, it can be noticed that the 2 μm particles had the most spermicidal effect at a low concentration of 0.1 g L^{-1} compared to other sizes of particles.

Furthermore, viability tests were performed on a cervical carcinoma cell line (HeLa) to investigate the toxicity of these particles on somatic tissue. This was tested by exposing cervical carcinoma cells HeLa in 2D culture to the Cu particles for the same time periods and concentrations as reported earlier, before performing cytotoxicity stains. As shown in Figure 5B, the HeLa cells after 30–45 min of incubation with 0.1 g L^{-1} concentration of any of the three particle sizes showed no significant reduction in cell viability. With 0.5 g L^{-1} concentration, the smallest particles (0.2 μm) had a strong toxic effect, while the larger 2 and 7 μm Cu particles still maintained acceptable cell viability at this concentration (86% and 92%, respectively, compared with 99.7% viability in the control). At the highest concentration of 1 g L^{-1}, all particle sizes showed a strong cytotoxic effect on the HeLa cells, killing most of them within the third interval (30–45 min). Fluorescence microscopic images of viability of sperm cells and HeLa cells after incubation with copper particles of different sizes and in different concentrations are shown in Supporting Information (Figure S3 and S4) and illustrate this effect.

3. Conclusion

Copper particles of three different sizes were synthesized using an assisted polyol method.[46] When these particles were incubated with bovine spermatozoa, they showed inhibiting effects on sperm motility and viability, which were studied in vitro. A strong size-dependent inhibition of sperm cell motility was noted, with the smaller particles being more effective inhibitors compared with larger ones. Further, an increasing particle concentration led to stronger inhibition of sperm motility. The viability tests carried out at the longest time interval (30–45 min) also resulted in similar results as the motility studies. With an

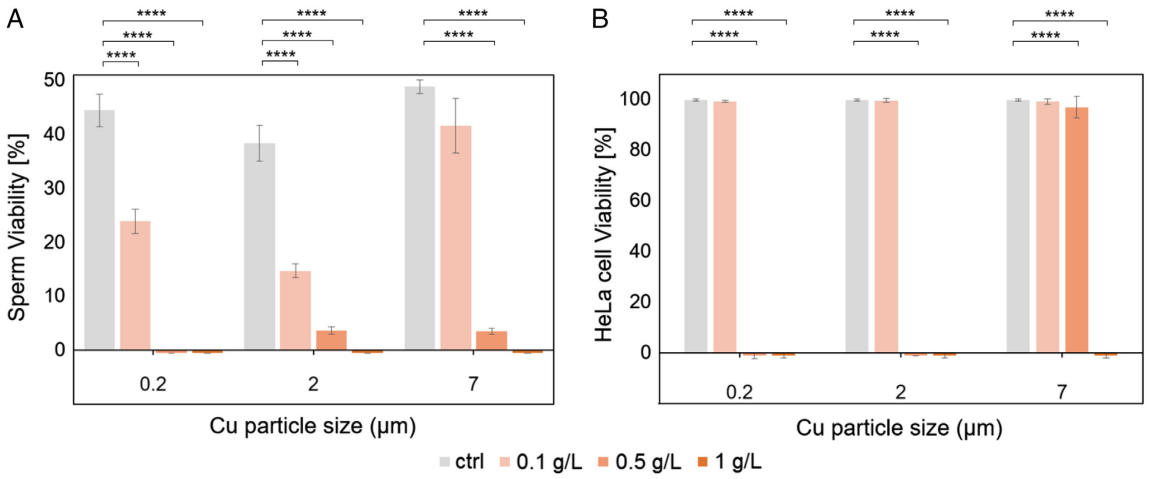

Figure 5. Viability of A) sperm cells and B) HeLa cells, after incubation at third time interval (30–45 min) with Cu particles of concentrations 0.1, 0.5, and 1 g L^{-1} and sizes $Cu_{0.2}$, Cu_2, and Cu_7 respectively. Statistical treatment was performed with one-way ANOVA with Bonferroni method in origin. Data presentation: Avg ± standard error of the mean (SEM); $n = 3$. The significance level was set at: **** ($P < 0.0001$); *** ($P < 0.001$); ** ($P < 0.01$); * ($P < 0.05$); n.s (statistically nonsignificant) SEM = Standard error of the mean.

increase in particle concentration and decreasing particle size, the viability of sperm cells is reduced.

As Cu^{2+} ions are known to be responsible for the inhibition of sperm cells,[22] the extent of release of Cu^{2+} ions is correlated with the particle size by spectrophotometry and ICP–OES in both, water and SP-TALP. This does not exclude additional effects caused by the particles, which, however, would be more difficult to quantify. In addition, SP-TALP favors an increased dissolution of metallic microparticles, releasing more Cu^{2+} ions, which is probably due to the presence of complexing proteins and bases in the medium. Therein, spectrophotometry was found to be less reliable for quantification of copper ions in complex media compared with ICP–OES.

Nano/microparticles are characterized by a high surface-to-volume ratio, which favors rapid dissolution of ions from the copper particles, which is one of the main advantages of its use compared with macroscopic copper structures. Besides this size-dependent effect, another large benefit compared with traditional hormonal contraceptives is the reduced burden on wastewater systems. It is well studied that the influx of hormones and their metabolites have drastic effects on aquatic systems[9,11] and eventually human lives.[13]

The cell toxicity of the copper microparticles on cervical carcinoma HeLa cells with maximum exposure time (30–45 min) was investigated in order to test the biocompatibility of these differently sized particles in different concentrations. It can be noted that a balance is required in targeting strongest spermicidal action of the microparticles while maintaining good tissue compatibility. From our results, we can conclude that either a low concentration of the smallest microparticles (0.2 µm, 0.1 g L^{-1}) could be used or the 2 µm particles at low or medium concentrations (0.1 to 0.5 g L^{-1}). This would achieve the desired effect of sperm inhibition (shown by significantly decreased sperm motility and viability), while maintaining healthy tissue compatibility. Further optimization of concentrations and particle sizing would be required to obtain the optimum combination of spermicidal effect and tissue compatibility. Nevertheless, our study provides good implications on the benefit of copper microparticle size tuning for optimum spermicidal effect of copper.

The inhibition of bovine spermatozoa by copper microparticles has been demonstrated and such particles prove to be very useful components for prospective use in lubricants or as spermicidal agents for male contraception. In future studies, besides the efficacy for human sperm, the compatibility of the here-presented materials needs to be evaluated in combination with lubricants and potentially with latex, before proceeding to clinical tests.

4. Experimental Section

Materials and Methods: TL-Sperm (Caisson Labs, USA), sodium pyruvate (100 mM, Gibco, Thermofisher, Germany), gentamicin sulfate (Cassion labs, USA), bovine serum albumin (Sigma Aldrich, Germany), Dulbecco Eagle's Minimal Essential Medium (DMEM) (Gibco, Thermofisher, Germany), propidium-iodide(PI) and SYBR-14 (sperm live/dead viability kit, Thermofisher, L7011), and cytotoxicity test (LIVE/DEAD Viability kit, Thermofisher, L3224) were purchased and used without any purification. Straws of cryo-preserved bovine semen (Masterrind GmbH, Meißen, Germany) was stored in liquid N_2 until use.

$CuSO_4$ (Reagent Plus, $\geq$ 99%), H_3BO_3 ($\geq$ 99.5%), H_3PO_4 (85%), and HNO_3 (65%) were obtained from Sigma Aldrich, Germany. CH_3COOH (100%) was obtained from VWR, Germany, and polyethylenimine (M.Wt - 10 000) from Polysciences, Germany.

We selected three differently sized copper particles in the range 0.1–0.3 µm labeled as $Cu_{0.2}$, 1–2 µm labeled as Cu_2, and 6–7 µm labeled as Cu_7 (see Figure 2). The preparation of $Cu_{0.2}$ and Cu_7 is shown in the study by Chattopadhyay et al.[46] Cu_2 particles were formed by solvothermal synthesis described in detail in Supporting Information. The particles were characterized using XRD (Bruker D2 phaser diffractometer) and Scanning electron microscope (Zeiss DSM 982 GEMINI electron microscope).

Preparation of Sperm Medium (SP-TALP): SP-TALP is a sperm-specific medium; it is a tyrode-modified albumin lactate pyruvate medium. In preparation of 10 mL SP-TALP, 9.5 mL of TL-Sperm was taken and it was supplemented with 500 µL of sodium pyruvate (100 mM), 50 µL of gentamicin sulfate and 60 mg of bovine serum albumin. The medium was kept at 37 °C prior to the motility assay.

Sperm–Particle Interaction: The synthesized Cu particles were added in SP-TALP in a concentration of 2 g L^{-1} (stock Cu particle solution). The samples were sonicated for 10 min to properly disperse the particles. They were kept in the incubator at 37 °C for at least 30 min before starting the experiments. The stock solution of Cu particles (2 g L^{-1}) was diluted with sperm cells to make 0.1, 0.5 and 1 g L^{-1} solutions. A control sample was also prepared without any Cu particles. The dilutions are shown in **Table 1**.

Sperm Preparation: A straw of bovine semen was taken out from cryogenic storage and quickly thawed in a water bath (at 37 °C) for 2 min. The straw was emptied in a 1.5 mL eppendorf tube and to it 500 mL of SP-TALP was added. The sperm sample was then washed by centrifuging at 100 g for 7 min. The supernatant was removed and the washing was continued two more times. Finally, the sperm pellet was resuspended in 500 mL of SP-TALP and kept in the incubator at 37 °C.

Sperm Motility and Velocity Tests: To understand the influence of the particles on sperm cells, the total amount of motile cells (motility) and velocity of the sperm cells were characterized using an inverted microscope (Axio observer, Carl Zeiss Microscopy GmbH) and recorded with an attached Zeiss camera (Axiocam 702 Mono). The movies were recorded at a frame rate of 40 fps for at least 200 frames with 10× magnification. After incubating the Cu particles with spermatozoa, videos were taken after every 15 min time range for three distinct intervals marked as first, second, and third. The time ranges for first, second, and third time intervals were 0–15, 15–30, and 30–45 min, respectively.

The videos were analyzed using ImageJ software with CASA plugin.[55] With the help of this plugin, we calculated the motility of the sperm cells which was given as the ratio of number of motile sperms to the total number of sperms.

$$\%\text{Motility} = \frac{\text{Number of motile sperms}\,(N)}{\text{Total number of sperms}\,(N_0)} \times 100\% \quad (1)$$

The velocity of the sperm cells was calculated in multiple modes.[56] Here we took into account three different velocities: VCL, VSL, and VAP obtained directly from the plugin.[55] To maintain an equivalency within the results, control samples were correspondingly measured in parallel to every particle inhibition case and from the same straw of semen.

Sperm Viability Tests: A sperm live/dead test was performed (Live/dead sperm viability kit L7011, Molecular Probes) to analyze the inhibitory effect

Table 1. Dilution table for preparation of required concentrations of Cu particles.

Concentration of particles [g L^{-1}]	Amount of stock Cu particle solution [µL]	Amount of Sperm [µL]	Amount of SP-TALP [µL]
0	0	50	50
0.1	5	50	45
0.5	25	50	25
1.0	50	50	0

of the copper particles on the viability of the sperm. The three sizes of particles $Cu_{0.2}$, Cu_2, and Cu_7 were used in the same three concentrations: 0.1, 0.5, and 1 g L^{-1} as in the sperm motility analysis. Stock solutions were prepared by diluting $Cu_{0.2}$, Cu_2, and Cu_7 particles in DMEM (Gibco Dulbecco's Modified Eagle Medium, Thermofisher) to achieve a concentration of 2 g L^{-1}. These were then diluted with thawed bovine sperm cells (initial concentration of bovine sperm cells in the straw was 3×10^7 sperm mL^{-1}) and DMEM medium to get the working concentrations mentioned in Table 1, preparing a total of nine samples. In addition, three control samples without Cu particles were prepared, one for each particle size. As third interval time resulted in the lowest motility, the incubation time at 37 °C of the mixture was set to 30–45 min. 10 min before completing this interval, 1 µm propidium-iodide(PI) and 1 mL diluted SYBR-14 (1:50) were added to each vial to stain dead (red) and live (green) cells, respectively. Several images for each sample were taken using Leica Thunder Fluorescence Microscope with a 10× objective, bright-field channel, and green fluorescent (for SYBR 14, 480 nm excitation, 530 nm emission) and red fluorescent (Propidium iodide, 510 excitation, 630 nm emission) channel. Viability was analyzed with ImageJ software by counting the red (dead) and green (live) cells.

Biocompatibility Study with Cervical Carcinoma Cell Line HeLa: To further investigate the biocompatibility of the Cu particles with somatic cells of the reproductive tract, a cytotoxicity test (LIVE/DEAD Viability kit, Thermofisher, L3224) was performed on HeLa cells. HeLa cells were seeded at 100 000 cells/petri dish in 2 mL DMEM medium and cultured for 20–48 h until an appropriate cell coverage was obtained. The Cu particles were added to the HeLa petri dishes using the same particle sizes, concentrations, and incubation times. After 20 min incubation, 0.1 µL mL^{-1} calcein and 1 µL mL^{-1} ethidium homodimer-1 were added to stain live and dead HeLa cells, respectively. The samples were incubated additional 10 min before starting imaging. The resulting stained cells were imaged in the Leica Thunder microscope in the bright-field, green, and red fluorescent channels (Calcein 494/517 nm; Ethidium homodimer 528/617 nm).

The spectrophotometric determination of cupric ions was commenced following the literature.[54]

$Cu_{0.2}$, Cu_2, and Cu_7 were each added to a vial, containing either DI–water or SP-TALP to make the final concentration to 5 g L^{-1}. The mixture was sonicated for 30 min and kept overnight to make the dissolved ions be in equilibrium with the medium. On the following day, the sample mixture was centrifuged and the supernatant was taken for further use. Next, the supernatant was diluted with PEI, buffer, water, and SP-TALP to make a final concentration of 0.1 g L^{-1}.

Stock solutions of 0.1 M copper (II) sulfate, 9.4 g L^{-1} polyethylenimine (PEI), and Britton–Robinson (BR) buffer of pH 6 were prepared. The buffer solution was prepared by mixing 0.04 M each of H_3PO_4, H_3BO_3, and CH_3COOH; the pH was then adjusted by 0.2 M NaOH solution. The analysis was done in DI–water and SP-TALP for each case separately.

For water, 30 µL of PEI was added from the stock to make the final concentration of the solution 0.094 g L^{-1}; then, 600 µL of BR buffer was added. Subsequently different volumes of 0.1 M $CuSO_4$ were added to make the Cu concentration 0–500 µM. The mixture was diluted with DI–water to make the final volume to 3 mL. For SP-TALP, the same procedure was followed, and just the volume of the measuring samples was adjusted with SP-TALP.

The UV absorption spectra were measured using Cary 50 Scan UV–vis spectrophotometer in the range 200–800 nm using a 1 cm path long quartz cuvette.

Detection of Cu^{2+} Ions by ICP–OES Method: ICP–OES (iCAP 7400 from Thermo Scientific, Waltham, USA) was used to determine the copper concentration. Initially, eight standards were prepared by diluting a solution containing 1000 mg L^{-1} copper in 2 M HNO_3 (Bernd Kraft, Duisburg, Germany). The respective copper concentrations of the standards were 500, 100, 50, 10, 5, 1, 0.5, and 0.1 mg L^{-1}. From the fivefold measurement of all standards, a calibration curve was constructed. The given detection limit was calculated by the software QtegraTM from Thermo Scientific with 0.02 mg L^{-1}. The concentration of every sample was determined fivefold.

For the experiment, 8 mg of Cu particles ($Cu_{0.2}$, Cu_2, and Cu_7) were taken in a vial and 7.68 mL of DI–water or SP-TALP was added to each of them. The mixture was sonicated for 30 min and kept overnight for maximum dissolution of Cu^{2+} in solution. After that, the sample was centrifuged and the supernatant was collected. Next, 1.32 mL of concentrated HNO_3 was added to the supernatant, to make the final concentration 890 mg L^{-1}. The solution was further filtered with 2 µm nylon syringe filter to obtain a clean solution without any turbidity. Similarly, two control samples of just DI–water and SP-TALP were also measured.

For both these methods, the detected amount of Cu^{2+} ions was evaluated by the percentage of Cu^{2+} ions released in the medium from the initial starting concentration, elaborately detailed in Supporting Information (Section S7).

All the data were used directly without any preprocessing.

Statistical Treatment: For Motility and Velocity of the Sperm Cells, the data were presented as the average with the standard deviation (avg ± SD). Each statistical data were obtained by analyzing at least 3–4 videos (sample size (n)) with 2–4 semen straws and with at least 100 datasets (number of sperm cells evaluated).

For Viability Tests, the data was presented as average ± SEM (standard errors of the mean). Images of at least 200 stained sperm cells per condition per replicate for three replicate samples were prepared ($n = 3$).

Statistical treatment on motility, VCL, and viability in Figure 4 and 5 was performed with a one-way ANOVA with Bonferroni method (in Origin). Significance was defined as $P \leq 0.05$. *R*-square values below 0.3 were not taken into account for the statistical significance analysis. Details of the statistical values can be found in the Supporting Information (Section S8).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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bovine spermatozoa, copper, male contraception, microparticles, sperm motility, sperm viability, spermicide

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