The *in vitro* effects of nanosilver colloid on kinematic parameters of ram spermatozoa

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Summary

This study investigated the concentration/time dependent effects of nanosilver colloid on the motion parameters of the ejaculated ram spermatozoa *in vitro*. After incubation of sperm samples for 30, 60, 120 and 180 min in the presence of nanosilver colloid (0, 0.001, 0.01, 0.1, 1 and 10 ppm), the motion parameters were evaluated by computer assisted sperm analysis. There was a significant ($P<0.05$) decrease in most motion of sperm parameters (the fast, slow and non-progressive motility, VCL, VSL, STR, MAD, ALH, WOB, VAP and LIN) in treated groups compared to their corresponding controls, especially after 120 min of incubation. On the other hand, there was a significant ($P<0.05$) increase in sperm immotility in comparison with the control group in all times at 1 ppm nanosilver colloid. At a concentration of 10 ppm, spermatozoa were completely inactivated. It is concluded that nanosilver colloid depresses sperm functions, especially motility parameters, which can be a causative agent for sperm infertility induced by nanosilver cytotoxicity.

Key words: Nanosilver colloid, Ram, Ejaculated sperm, Motion parameters

Introduction

Silver is one of the heavy metals, along with lead, mercury, cadmium, and gold, which, unlike other heavy metals, has a long history (over 6000 years) as a medicinal drug. Silver is non-toxic, and it can be an antibiotic under the suitable dosage (Alt *et al*., 2004). Silver vessels were used in ancient times to preserve water and wine and silver powder was believed by Hippocrates, the father of modern medicine, to have beneficial healing and antidisease properties and is listed as a treatment for ulcers (Chen and Schluesener, 2008). Silver compounds were a major weapon against wound infection in World War I until the advent of antibiotics (Chen and Schluesener, 2008). Metallic silver is subjected to new engineering technologies with resultant extraordinarily novel morphologies and characteristics. Silver is engineered into ultrafine particles whose size is measured in nanometers (nm). When these particles have at least one dimension which is less than 100 nm, they are named nanoparticles (Oberdörster *et al*., 2005; Warheit *et al*., 2007). Upon reaching nanoscale, like other nanomaterial and primarily by virtue of extremely small size, silver particles exhibit remarkably unusual physicochemical properties and biological activities. Great research efforts have been committed to this respect and yielded exciting and encouraging results (Elechiguerra *et al*., 2005; Evanoff Jr and Chumanov, 2005; Makarava *et al*., 2005; Lee and El-Sayed, 2006). As a consequence, applications of engineered silver nanoparticle (nanosilver), especially in the healthcare sector, have been and are still being heatedly explored.

Silver has long been known to have strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (Rai *et al*., 2009; Sotiriou and Pratsinis, 2010). Silver nanoparticles, which

have a high specific surface area and high fraction of surface atoms, will have high antimicrobial activity compared to bulk silver metal. Nanosilver colloid, a well-dispersed and stabilized silver nanoparticle solution will be more adhesive on bacteria and fungus, and so have enhanced antibacterial activity. Colloidal silver near a virus, fungus, bacterium or any other single cell pathogen disables its oxygen metabolism enzyme (Panacek *et al*., 2006; Rhee *et al*., 2008).

Colloidal silver has been used to treat different infections or parasitic diseases and many studies have been done to evaluate the toxicity of nanosilver on cells. While there is speculation that nanoparticles might pose potential reproductive harm, there has been little substantive evidence to support or refute these concerns, especially in farm animals. The objective of this study is to evaluate what the effects of concentration-time dependent nanosilver on the kinematic parameters of ram sperm *in vitro* are.

Materials and Methods

The study was performed on Bakhtiari sheep (n = 8) on a farm of the Agricultural Research Center (Shooli, Shahrekord, Iran) during October and November 2009. The rams were kept as a single flock in a semi-extensive production system. Bakhtiari rams had a mean weight of 95 ± 5 kg and body condition scores (BCS) of 3.0 to 4.0 (scale 0 to 5).

Semen processing and preparation of sperm suspension

Samples of semen (n = 8) were collected using an artificial vagina from eight Bakhtiari rams. Generally, one ejaculate per ram was collected on a daily basis. All ejaculates were evaluated using a computer assisted sperm analysis (CASA) within 30-45 min after collection (keeping in 38°C). Ejaculates were only used if the volume was ≥0.5 ml and with sperm concentration ≥2 × 10^9 spermatozoa/ml and ≥60% motile cells. The presence of round cells (spermatogonia, spermatocytes, spermatides and leucocytes) were minimal (less than 1 × 10^6/ml) in all samples. Sperm concentration was adjusted to approximately 5 × 10^8 cells/ml by adding BO medium. This medium, which was first introduced by Brackett and Oliphant (1975), consisted of 112.0 mM-NaCl, 4.02 mM-Kcl, 2.25 mM-$CaCl_2$, 0.83 mM-NaH_2PO_4, 0.52 mM-$MgCl_2$, 37.0 mM-$NaHCO_3$, 13.9 mM-glucose, 1.25 mM-sodium pyruvate, and 31 mg/ml potassium penicillin G. Aliquots of sperm suspension in BO medium, 200 µl each, containing 2 × 10^7 cells, were incubated in the presence of 0.001, 0.01, 0.1, 1 and 10 ppm of nanosilver colloid (Nanocid, Iran) with an equal volume of BO medium (control) for 180 min at 38°C in 5% CO_2.

Measurement of motion parameters

Sperm motility parameters were measured by CASA (Hooshmand Fanavar, Iran), with the following settings: image collection speed, 20 frames per sec; analysis time per frame, less than 15 sec; sperm velocity that can be analysed, 0-180 µm/s; number of vision fields that were selected, 6/samples; magnifying power of microscope (object lens), ×4; Measurements were performed in Makler chambers 20 µm depth. Sperm motility parameters were analysed at four time intervals (30, 60, 120, 180 min) following incubation with different concentrations of nanosilver colloid. The sperm motility was assessed as rapid (class A), slow or sluggish (class B) and non-progressive motility (class C), or immotility (class D), all in percentages. The studied motion parameters can be defined as follows: straight line velocity (VSL), which represents the average velocity measured in a straight line from the beginning to the end of one track in micrometers per second; The curvilinear velocity (VCL), which is the average velocity measured over the actual point to-point track followed by the cell in micrometers per second; the average path velocity (VAP), which corresponds to the average velocity of the smoothed cell's pathway in micrometers per sec; the amplitude of lateral head displacement in micrometers (ALH); the beat cross frequency (BCF) is the frequency at which the sperm cell's head crosses the sperm cell's average pathway in Hertz; the linearity (LIN) which estimates linearity of a curvilinear path in percentage; the straightness (STR) estimates the proximity

of the cell's pathway to a straight line with 100% corresponding to the optimal straightness in percentage; the wobble (WOB), which is the measure of oscillation of the actual path about the average path. The mean angular displacement (MAD) which is the time average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory in degree (Verstegen *et al*., 2002).

Statistical analysis

All data are presented as means ± SEM. The statistical analysis was carried out using SPSS 14.0 software (SPSS Inc., New York, USA). The control and treatment groups were compared at each time interval using one way repeated measurement ANOVA followed by the Duncan's multiple range test. Differences were considered significant at a $P<0.05$.

Results

The results of nanosilver colloid effects on the motion parameters of the ejaculated sperm are shown in Tables 1 and 2.

After 30 min of incubation, the parameters of rapid progressive motility (class A), VCL, VSL, VAP, MAD, ALH and BCF did not show any significant variations at the five concentrations of nanosilver colloid (0.001, 0.01, 0.1, 1 and 10 ppm), while there was only significant ($P<0.05$) decrease in the non-progressive motility (class C), LIN, WOB and STR in the treated groups at a concentration of 1 ppm nanosilver colloid compared to control.

Table 1: Motility data at different time intervals by different concentrations of nanosilver colloid

Treatment	Parameter					
	Class A (%)	Class B (%)	Class C (%)	Class D (%)	VCL (µm/s)	VSL (µm/s)
After 30 min						
Control	40.2±3.9	20.7±1.8^{a}	6.1±1.0^{a}	32.7±5.7^{a}	67.5±7.2	47.9±4.5
0.001 ppm	44.7±4.9	15.6±2.7^{b}	6.1±1.1^{a}	33.3±7.9^{a}	66.4±7.3	48.2±5.0
0.01 ppm	40.7±4.5	18.0±2.3^{ab}	6.3±1.1^{a}	34.9±6.2^{a}	61.4±6.3	43.3±4.8
0.1 ppm	38.8±7.1	17.8±2.2^{ab}	6.3±1.1^{a}	36.9±8.8^{a}	58.2±9.1	39.5±7.1
1 ppm	23.9±14.2	7.8±3.5^{c}	3.4±1.4^{b}	64.8±18.7^{b}	36.4±19.4	24.5±14.2
10 ppm	0	0	0	100	0	0
After 60 min						
Control	31.9±5.1	18.7±2.0^{a}	7.3±0.3^{a}	42.0±4.6^{a}	51.3±5.0	34.7±4.5
0.001 ppm	33.9±7.1	15.9±2.1^{a}	4.3±0.6^{b}	45.7±9.2^{a}	52.1±8.8	39.0±8.1
0.01 ppm	41.1±7.4	14.4±1.1^{a}	5.3±0.5^{ab}	39.1±7.9^{a}	56.9±7.8	41.9±6.9
0.1 ppm	36.1±10.5	15.9±1.4^{a}	6.8±0.5^{ab}	41.0±11.4^{a}	56.0±12.4	37.5±9.6
1 ppm	18.4±11.3	6.4±4.1^{b}	2.8±1.7^{b}	72.1±17.1^{b}	27.7±16.9	19.7±12.1
10 ppm	0	0	0	100	0	0
After 120 min						
Control	37.5±5.9^{a}	17.9±2.3^{a}	6.6±0.5	37.8±5.9^{a}	62.2±7.8^{a}	45.4±7.6^{a}
0.001 ppm	31.0±8.1^{ab}	17.5±2.6^{a}	7.0±1.2	44.3±9.6^{a}	50.4±9.4^{ab}	33.4±8.2^{ab}
0.01 ppm	32.2±8.3^{ab}	16.6±1.8^{a}	6.5±0.6	44.4±10.7^{a}	49.7±8.9^{ab}	31.7±6.0^{ab}
0.1 ppm	21.2±3.7^{bc}	19.4±2.9^{a}	7.2±1.0	52.0±6.1^{a}	41.5±4.7^{b}	23.6±3.3^{bc}
1 ppm	10.1±6.5^{c}	8.0±4.9^{b}	3.3±2.1	78.4±13.3^{b}	16.8±10.6^{c}	8.8±5.7^{c}
10 ppm	0	0	0	100	0	0
After 180 min						
Control	23.7±5.4^{a}	19.1±1.8^{a}	5.6±1.1^{a}	51.4±5.8^{a}	42.7±6.2^{a}	26.9±3.5^{a}
0.001 ppm	24.1±6.5^{a}	13.8±2.1^{ab}	5.0±1.5^{a}	56.9±5.9^{a}	43.4±8.6^{a}	30.3±9.5^{a}
0.01 ppm	31.9±5.9^{a}	16.8±2.2^{a}	5.7±0.8^{a}	45.4±8.5^{a}	50.6±8.1^{a}	34.3±5.3^{a}
0.1 ppm	25.2±4.8^{a}	17.4±2.1^{a}	5.1±1.3^{a}	52.2±6.7^{a}	41.8±4.8^{a}	27.2±4.3^{a}
1 ppm	7.1±4.3^{b}	8.4±5.1^{b}	2.9±1.8^{b}	81.4±11.3^{b}	13.8±8.4^{b}	7.0±4.3^{b}
10 ppm	0	0	0	100	0	0

$^{a, b, c}$ Mean ± SEM with the different indices in each column (within the same times) are significantly different ($P<0.05$). Class A: sperm with rapid progressive motility, Class B: sperm with slow progressive motility, Class C: sperm with non-progressive motility, and Class D: immotile sperm. VCL: Curvilinear velocity, and VSL: straight line velocity

Table 2: Motility data at different time intervals by different concentrations of nanosilver colloid

Nanosilver	Parameters						
	VAP (μm/s)	MAD (°)	ALH (μm)	BCF (Hz)	LIN (%)	WOB (%)	STR (%)
After 30 min							
Control	53.4±5.1	14.6±3.0	2.5±0.2	0.7±0.1	52.2±1.8^{a}	65.7±1.4 ^{a}	67.3±3.0^{a}
0.001 ppm	53.2±5.6	15.9±2.6	2.4±0.2	0.8±0.1	54.0±3.2^{a}	66.0±3.4^{a}	68.5±4.1^{a}
0.01 ppm	48.2±5.1	15.4±2.2	2.3±0.2	0.7±0.1	52.3±3.3^{a}	64.2±3.1 ^{a}	67.4±3.7^{a}
0.1 ppm	44.8±7.5	14.6±3.3	2.4±0.3	0.6±0.2	49.8±4.8^{a}	63.8±3.7^{a}	64.8±4.8^{a}
1 ppm	27.8±15.6	9.4±5.3	1.4±0.7	0.4±0.3	28.1±13.7^{b}	35.9±16.1^{b}	37.1±16.9^{b}
10 ppm	0	0	0	0	0	0	0
After 60 min							
Control	39.5±4.7	10.9±1.4	2.1±0.2^{a}	0.5±0.1	45.6±3.5^{a}	59.5±3.3^{a}	60.7±2.8^{a}
0.001 ppm	43.0±8.3	9.1±1.8	1.9±0.2^{a}	0.6±0.1	48.8±6.3^{a}	61.5±5.6^{a}	62.4±5.9^{a}
0.01 ppm	46.5±7.2	12.0±1.9	2.1±0.2^{a}	0.6±0.1	52.1±5.6^{a}	64.5±4.8^{a}	64.9±5.2^{a}
0.1 ppm	42.8±10.5	13.2±3.6	2.3±0.4^{a}	0.6±0.2	46.8±6.7^{a}	60.4±5.8^{a}	62.3±6.5^{a}
1 ppm	22.0±13.5	6.3±3.9	1.0±0.7^{b}	0.3±0.2	21.3±13.1^{b}	26.2±16.1^{b}	27.1±16.6^{b}
10 ppm	0	0	0	0	0	0	0
After 120 min							
Control	50.5±7.6^{a}	11.4±2.1^{a}	2.2±0.1^{a}	0.6±0.1^{a}	49.6±4.1^{a}	63.4±3.1^{a}	64.2±3.9^{a}
0.001 ppm	38.3±8.5^{ab}	10.9±3.1^{a}	2.1±0.2^{a}	0.5±0.1^{ab}	44.2±5.7^{a}	58.8±4.7^{a}	60.5±5.0^{a}
0.01 ppm	36.7±6.7^{ab}	12.4±4.1^{a}	2.2±0.3^{a}	0.5±0.1^{ab}	46.1±4.3^{a}	60.4±3.7^{a}	62.6±5.0^{a}
0.1 ppm	28.3±3.5^{b}	10.1±2.5^{a}	2.0±0.2^{a}	0.3±0.1^{bc}	36.5±3.0^{a}	52.2±2.5^{a}	54.2±3.5^{a}
1 ppm	10.9±6.9^{c}	4.7±3.0^{b}	0.8±0.5^{b}	0.1±0.1^{c}	15.3±9.4^{b}	21.8±13.4^{b}	23.1±14.2^{b}
10 ppm	0	0	0	0	0	0	0
After 180 min							
Control	31.4±4.1^{a}	8.8±2.7^{a}	1.9±0.3^{a}	0.4±0.1	40.8±2.0^{a}	56.2±1.6^{a}	56.1±2.6^{a}
0.001 ppm	34.3±9.3^{a}	6.8±1.1^{ab}	1.7±0.2^{a}	0.4±0.2	39.6±5.1^{a}	53.9±4.5^{a}	53.7±4.1^{a}
0.01 ppm	39.1±6.0^{a}	10.4±2.9^{a}	2.1±0.4^{a}	0.5±0.1	45.8±3.6^{a}	60.4±3.4^{a}	59.7±4.1^{a}
0.1 ppm	31.5±4.5^{a}	8.2±1.6^{a}	1.9±0.2^{a}	0.4±0.1	41.3±3.8^{a}	55.8±3.9^{a}	55.3±3.4^{a}
1 ppm	8.8±5.4^{b}	3.1±1.9^{b}	0.8±0.5^{b}	0.1±0.1	14.9±9.1^{b}	21.2±13.0^{b}	22.1±13.5^{b}
10 ppm	0	0	0	0	0	0	0

$^{a, b, c}$ Mean ± SEM with the different indices in each column (within the same times) are significantly different (P<0.05). VAP: Average path velocity, MAD: Mean angular displacement, ALH: Amplitude of lateral head displacement, BCF: Beat cross frequency, LIN: Linearity, WOB: Wobble, and STR: Straight line rate

The slow progressive motility (class B) also significantly (P<0.05) decreased at concentrations of 0.001 and 1 ppm.

After 60 min of incubation, the parameters of rapid progressive motility, VCL, VSL, VAP, MAD and BCF did not show any significant variations at the four concentrations of nanosilver colloid (0.001, 0.01, 0.1, and 1 ppm), while there was only a significant (P<0.05) decrease in the slow progressive motility, LIN, ALH, WOB and STR in the treated groups at a concentration of 1 ppm nanosilver colloid compared to control. The non-progressive motility also significantly decreased at concentrations of 0.001 and 1 ppm.

After 120 min of incubation, the parameters of rapid progressive motility, VCL, VSL, VAP and BCF significantly decreased at 0.1 and 1 ppm nanosilver colloid, while slow progressive motility, MAD, ALH, LIN, WOB and STR only significantly decreased at 1 ppm nanosilver colloid in the treated groups compared to control. The non-progressive motility did not significantly change.

After 180 min of incubation, the parameters of rapid progressive motility, slow progressive motility, non-progressive motility, VCL, VSL, VAP, MAD, ALH, LIN, WOB and STR only significantly (P<0.05) decreased at 1 ppm nanosilver colloid, while BCF did not change compared to control.

In all times, the parameter of immotility (class D) significantly increased at a concentration of 1 ppm nanosilver colloid. At a concentration of 10 ppm nanosilver colloid, sperm of all samples were immotile after 30 min.

Discussion

In the present study, we found that nanosilver colloid could show adverse

effects on the most kinematic parameters of sperm in a time-concentration dependent manner. At the concentration of 10 ppm nanosilver colloid, spermatozoa were completely inactivated after 30 min. This data is evidence of nanosilver toxicity on spermatozoa. The adverse effects of nanosilver have also been confirmed by other studies. For a limited number of cell lines tested (Hussain *et al*., 2006) (C18-4 germ cell line, BRL 3A liver cell line and PC-12 neuroendocrine cell line), exposure to silver nanoparticles significantly decreased the function of mitochondria. This is a shared characteristic of all cellular responses (Braydich-Stolle *et al*., 2005; Hussain *et al*., 2005; Hussain *et al*., 2006). It has been well established that dysfunction of mitochondria is an early and key step towards apoptosis. Thus mitochondria seem to be sensitive targets of cytotoxicity of silver nanoparticles. However, the mechanism of silver nanoparticles action on mitochondrion is yet to be elucidated. In the study with BRL 3A liver cell line, depletion of glutathione level and increased reactive oxygen species (ROS) were found in association with mitochondrial perturbation, suggesting that oxidative stress might mediate the cytotoxicity of silver nanoparticles (Arabi, 2006; Chen and Schluesener, 2008). Spermatozoa are protected by various antioxidants and antioxidant enzymes in the seminal plasma and in the spermatozoon itself. It has been reported that there is negative coloration between lipid peroxidation by ROS and sperm motion parameters such as STR, BCF, ALH, VCL, VSL, LIN and VAP which agreed with our study (Imai and Nakagawa, 2003; Peris *et al*., 2007).

Recently, it has been found that Ag+ perturb mitochondria through interactions with thiol groups of the mitochondrial inner membrane (Almofti *et al*., 2003). On the other hand, anti-microbial activity of silver nanoparticles suggest that silver nanoparticles also exert their anti-microbial effects through interaction with the proteins thiol groups (Elechiguerra *et al*., 2005; Morones *et al*., 2005). Proteins and enzymes with thiol groups within mammalian cells like glutathione, thioredoxin, superoxide dismutase (SOD) and thioredoxin peroxidase, are key components of the cell's antioxidant defense mechanism, which is responsible for neutralizing the oxidative stress of ROS largely generated by mitochondrial energy metabolism (Chen and Schluesener, 2008). Silver nanoparticles may deplete the antioxidant defense mechanism, leading to ROS accumulation. The accumulation of ROS can initiate an inflammatory response and perturbation, and destruction of the mitochondria take place (Costa *et al*., 2010). The mitochondria and oxidative phosphorylation are the main source of energy in the sperm and it has been confirmed that impaired mitochondria changed most of the motion parameters of the sperm (Hung *et al*., 2008). Therefore the reduction of VCL, VSL, VAP, MAD, ALH, LIN, WOB and STR in our data, which are dependent on mitochondrial activity is likely, due to the harmful effect of silver nanoparticles on sperm mitochondria. Besides mitochondria destruction, damage to cell membranes appears to be another part of nanosilver's mechanism of cytotoxicity that precedes mitochondrial perturbation. It is known that thiol-group containing proteins are abundant in the cell membrane. Detrimental protein-nanosilver interactions are highly likely and lipoperoxidation may play some role (AshaRani *et al*., 2008; Chen and Schluesener, 2008).

Braydich-Stolle *et al*. (2005) utilized a cell line with spermatogonial stem cell characteristics to test the *in vitro* toxicity of several types of nanoparticles. The results showed that of all the tested materials, silver nanoparticles were the most toxic with manifestations like drastic reduction of mitochondrial function, increased membrane leakage, necrosis and induction of apoptosis. The findings are of significant practical implications because silver nanoparticles are now able to access human sperm *via* a variety of commercialized products like contraceptive devices and maternal hygiene items, which may result in fertility problems. In addition, the application of silver nanoparticles as a powerful disinfection agent in the animal farm has recently been recommended and some studies have shown that nanoparticles deposit in the testes and therefore cross the blood testes barrier if accidentally used

orally (McAuliffe and Perry, 2007). This study warns about the adverse affects of silver nanoparticles concentrations of more than 1 ppm on tools such as semen collection and artificial insemination tools.

It is concluded that nanosilver colloid depresses sperm functions, especially motion parameters, which could be evidence of sperm infertility induced by nanosilver cytotoxicity.

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