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Research Article

Gentamicin preserves motility and viability of Zebrafish (*Danio rerio*) sperm inoculated with bacteria during short-term storage

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Abstract

Sperm short-term storage at low temperatures provides an advantage to control artificial reproduction in fish. However, spermatozoa lose their potential for motility and fertilization during the storage period that may be associated with microbial growth. In the present study, we used Zebrafish (*Danio rerio*) sperm to investigate the effects of bacterial contamination on spermatozoa motility and viability during short-term storage. Spermatozoa retained better motility characteristics during the storage period when sperm was diluted with an extender compared to the undiluted sperm. Inoculation of sperm with bacteria decreased spermatozoa motility in a concentration-dependent manner. Through the storage period, spermatozoa motility was not affected in sperm inoculated with 10^3 CFU/mL *Vibrio harveyi* or *Pseudomonas aeruginosa*. A significant decrease in spermatozoa motility was observed in the sperm inoculated with 10^8 CFU/mL *V. harveyi* and with 10^5 CFU/mL *P. aeruginosa* at 3 h post-storage. Next, we determined the effective concentrations of gentamicin to preserve spermatozoa against bacteria during short-term storage. The spermatozoa motility was similar to that of non-infected sperm when 0.08–0.15 and 0.02–0.04 mg/mL gentamicin was added to the sperm inoculated with 10^8 CFU/mL *V. harveyi* or *P. aeruginosa*, respectively. At 24 h post-storage, spermatozoa viability was significantly decreased in the sperm inoculated with 10^8 CFU/mL *V. harveyi* or *P. aeruginosa*, while it showed an increasing trend of spermatozoa viability in bacterially infected sperm with gentamicin. The results showed the detrimental effects of bacteria on spermatozoa motility during short-term storage, and determined effective dose of gentamicin to preserve spermatozoa against *V. harveyi* or *P. aeruginosa*. This study provides valuable information to develop artificial reproduction in Zebrafish research centers.

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Introduction

Zebrafish (*Danio rerio*) is one of the most prominent biological models that spawn in freshwater. Their small size, age of maturity, high fecundity, embryonic transparency, rapid development and genetic similarity to humans provide scientists with valuable advantages to conduct wide ranges of research in biological and biomedical sciences (Perry *et al.*, 2010; Piferrer and Ribas, 2020). Zebrafish laboratories around the world establish a novel protocol or develop and standardize the existed protocols for husbandry (Lawrence, 2007; Aleström *et al.*, 2020; Kotil *et al.*, 2024). In this context, methods for artificial reproduction and storage of sperm have been suggested (Yang *et al.*, 2007; Jing *et al.*, 2009; Wilson-Leedy *et al.*, 2009; Matthews *et al.*, 2018; Cheng *et al.*, 2021).

Short-term storage is a method of preserving sperm at low temperatures from hours to days that has been widely used for artificial reproduction of economically important fish species in aquaculture (Bobe and Labbé, 2009; Contreras *et al.*, 2020; Shazada *et al.*, 2024). It is also a biotechnology tool that enables scientists to manipulate spermatozoa for generating gynogenetic, androgenetic, hybrid, or polyploidy populations of fish (Piferrer *et al.*, 2009; Arai and Fujimoto, 2019) including Zebrafish (Corley-Smith *et al.*, 1996; Walker *et al.*, 2009; Setti *et al.*, 2023). Studies have shown better maintenance of spermatozoa motility, viability, and fertilizing ability when sperm short-term storage was performed using an extender (Christensen and Tiersch, 1996; Cejko *et al.*, 2018; Cheng *et al.*, 2022, 2021). However, during the period of storage, spermatozoa motility, viability and fertilizing ability decrease due to damages to morphology associated with induction of oxidative stress, energy depletion, damage to genome (DNA fragmentation), and stimulation of epigenetic modifications (Billard *et al.*, 1995; Hatef *et al.*, 2011; Dietrich *et al.*, 2021; Zhang *et al.*, 2022; Ubah *et al.*, 2023). So far, short-term storage protocol has been optimized for various fish species with consideration to the ionic composition, osmolality and pH of the extender, dilution ratio of sperm with extender, environmental temperature during storage, oxygen availability and supplementation of antioxidants to protect sperm from oxidative stress (Bobe and Labbé, 2009; Contreras *et al.*, 2020; Zhang *et al.*, 2022; Shazada *et al.*, 2024). However, little attention has been paid to the microbial contamination of sperm during storage.

Microbial growth has been observed during the period of sperm storage in fish along with decreases in spermatozoa motility (Jenkins and Tiersch, 1997; Viveiros *et al.*, 2010). The microbial contamination may originate from sperm itself or from the environment. Jenkins and Tiersch (1997) identified gram-negative (*Pseudomonas*) and gram-positive (*Bacillus* and *Streptococcus*) bacteria in short-term stored sperm of channel catfish in sterile and non-sterile extender, respectively. In sturgeons, both gram-negative (*Aeromonas*, *Psuedomonas* and *Vibrio*) and gram-positive (*Bacillus*, *Micrococcus* and *Staphylococcus*) bacteria have been identified from sperm that are fish pathogens including *A. hydrophila*, *A.*

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caviae, *M. luteus*, *P. putida* and *P. flourescens* (Wayman, 2003). It has been also observed that artificial contamination of sperm by some pathogens including *Mycobacterium* and *Edwardsiella* persists after cryopreservation (Norris *et al.,* 2018). Since the 1980s, antibiotics have been used to preserve spermatozoa during short-term storage. It has reported that gentamycin (Segovia *et al.,* 2000; Wayman, 2003; Viveiros *et al.,* 2010; Zidni *et al.,* 2023), ampicillin (Segovia *et al.,* 2000), oxytetracycline (Wayman, 2003), neomycin (Zidni *et al.,* 2023) or streptomycin plus penicillin (Stoss and Refstie, 1983; Saad *et al.,* 1988; Niksirat *et al.,* 2011; Cejko *et al.,* 2022) prolong storage time and retain spermatozoa motility, viability or fertilizing ability. However, the effects of bacteria on spermatozoa functions during short-term storage have been rarely studied. In this context, effective concentrations of bacteria and antibiotics that, respectively, decrease and protect spermatozoa functions remain largely unknown.

In the present study, we first investigated the effects of *Psuedomonas aeruginosa* and *Vibrio harveyi* on spermatozoa motility and viability in Zebrafish during short-term storage. *Psuedomonas* and *Vibrio* are pathogenic and isolated from the intestines of live Zebrafish, tissues of dead fish, or from their environments (Kent *et al.,* 2020). Next, we used gentamicin to determine the optimum concentrations that preserve spermatozoa against bacterial contamination during short-term storage. To our knowledge, this is the first study that shows detrimental and protective effects of bacterial contamination and gentamicin on spermatozoa motility and viability in a concentration-dependent and time-dependent manner. Our results provide novel information to develop artificial reproduction in Zebrafish.

Materials and methods

Chemicals

Nutrient Agar (NA), Nutrient Broth (NB), chloroform, NaCl, KCl, $CaCl_2$, $NaHCO_3$, $BaCl_2$ H_2SO_4 were purchased from Merck, Germany. Gentamicin was purchased from the Exir Pharmaceutical Co, Iran.

Animals: Ethics, husbandry and sperm collection

This study was performed at the Reproductive Biology Laboratory and Environmental Biotechnology Laboratory, School of Biology, University of Tehran, Iran. Manipulations with animals were performed according to a protocol approved by the Central Ethics Committee for Management, Realization, and Control of the Experiments on Animals issued by the University of Tehran, Iran (protocol code 79/337352, Feb. 5, 2020). SMHA has a certificate of professional competence for designing experiments and experimental projects.

Wild-type Zebrafish of 1-month-old were purchased from a pet market in Tehran, Iran. Zebrafish males (n=90) and females (n=90) were divided into 3 aquaria (each 30 L) at a ratio 1:1, maintained at 27 ^{o}C under 12D:12L photoperiod, and fed (total 3% body weight) with commercial food (BioMar, Aarhus, Denmark) in the morning and afternoon until maturity. Every 48 h, 80% of the water in each aquarium was renewed.

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To collect testicular sperm, each individual was first anesthetized with 2-phenoxyethanol (0.3 mL/L) to minimize suffering. Following cessation of opercular movement, the external body was wiped dry with a paper towel to avoid contamination from water, mucous, or urine. The unconscious Zebrafish were euthanized by pithing and spinal cord dislocation using a needle and scalpel (Canada Council on Animal Care, 2005), and dissected to remove the testes in a 1.5 mL sterile tube with or without an extender. The testes were cut to release the sperm without squeezing (Nayak *et al.*, 2023).

Origin and preparation of bacteria

The strains of *P. aeruginosa* and *V. harveyi* identified as PTCC 1074 and 1755, respectively, were obtained from the University of Tehran Microbiological Collection. The strains were cultured in the sterilized NA at 37°C for 24 h in an incubator (Memmert, Germany) to obtain sufficient colonies. They were then cultured in the sterilized NB at 32 ^{o}C for 24 h in a shaking incubator (Vision, Iran). The culture suspension (800 µL) was centrifuged at 37°C (6000 × rpm for 3 min), and isolated bacteria were gently re-suspended in 800 µL of NB containing 200 µL of pure glycerol, and stored at –20°C. Bacterial concentration was assessed using the McFarland standard (Lonsway, 2023) and adjusted to 1×10^{8} colony-forming units per mL (CFU/mL). The McFarland standard is prepared by mixing 0.5 mL of 1.175% $BaCl_2$•$2H_2O$ with 99.5 mL of 1% sulfuric acid H_2SO_4. The NA and NB media were sterilized at 121°C for 20 min.

Preparation and inoculation of sperm with bacteria

The released sperm from each male was first evaluated for sperm motility (see the next section for the method). Sperm samples with motility >90% were used for the experiments, and immediately diluted with an extender (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, and 2.38 mM $NaHCO_3$, pH 8.1±0.2) in a 1.5 mL Eppendorf tube, and kept in the refrigerator (4 ^{o}C) during the period of experiments. The osmolality of the extender with the same composition has been measured 343 mOsmol/kg (Cheng *et al.*, 2021). The spermatozoa concentration was adjusted to 2.5×10^{8} spermatozoa/mL in a total volume of 0.5 mL to assume that a similar number of spermatozoa are inoculated with bacteria. The spermatozoa concentration was counted using a hemocytometer according to Alavi *et al.* (2012). Briefly, sperm was diluted in the physiological saline 0.9% NaCl, 10 µL of the suspension was placed on the hemocytometer. A number of spermatozoa was counted allowing 10 min for spermatozoa sedimentation and calculated as the number of cells per mL. The bacterial was added into the extender at final infective concentrations of *P. aeruginosa* and *V. harveyi* were 1×10^{8}, 1×10^{5}, 1×10^{3} CFU/mL. The negative control tube was a sperm suspension in the extender without bacteria. The sperm of six males were used for each treatment.

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Spermatozoa motility and viability assessments

To activate spermatozoa motility, a drop of sperm sample (1 µL in case of sperm diluted with extender) was directly mixed with 19 µL distilled water (activation medium) on a glass slide under light microscopy at 10× objective magnification (Nikon Labophot-2) at room temperature (RT). BSA at a final concentration of 0.1% (*w*/*v*) was added into the activation medium to prevent sperm from sticking to the glass slides. No coverslip was used, and all observation was performed at RT. Spermatozoa motility was recorded using a (HDMI+USB camera (KaiLiwei, China), percentage of motile spermatozoa (%) and total duration of spermatozoa motility (s) were assessed visually at 10-15 s post-activation. Spermatozoa with forward movements were considered motile, and the total duration of spermatozoa motility refer to the period between sperm dilution with distilled water until forward movement was stopped for >99% of spermatozoa. For each sperm record, the percentage of motile spermatozoa was measured from the recorded videos by visual detection of a number of motile spermatozoa numbers / total number of spermatozoa × 100. For each sperm sample, two videos of motility were recorded, and the mean value was used in statistical analyses (Alavi *et al.*, 2012).

Sperm viability was assessed by evaluating the membrane integrity using the fluorochromes SYBR-14 dye and Propidium iodide (PI) (Live- Dead® Sperm Viability Kit, ThermoFisher Scientific) (n = 3) (Zhang *et al.*, 2023). Sperm suspension was centrifuged at 20 °C (250 *g* for 5 min), re-suspended in 0.5 mL extender, and incubated with 1 µL SYBR- 14 dye (final concentration of 100 nM) at 4 °C for 30 min in darkness. Next, 1 µL L PI (final concentration of 10 µM) was added and sperm suspension was incubated for 5 min before cytometric analyses using a BD FACSCalibur™ flow cytometer (Biosciences, USA).

Statistical analyses

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). As needed, data were $\log_{10}$-transformed to meet assumptions of normality. One-way ANOVA followed by Tukey's *post-hoc* test was performed to investigate storage time effects on spermatozoa motility of stored sperm with or without the extender. To investigate the effects of bacteria on spermatozoa motility during storage period, two-way ANOVA was performed to study effects of bacterial concentration, storage time and their interactions (bacterial concentration × storage time). Similarly, two-way ANOVA was used to investigate effects of gentamicin on spermatozoa motility during the period of storage where the main factors were gentamicin concentration, storage time and their interactions. When the interaction effects were significant, the models were revised into one-way ANOVA followed by Tukey's *post-hoc* test for each main factor i.e., bacteria/gentamicin and storage time. For spermatozoa viability, one-way ANOVA followed by Tukey's *post-hoc* test was performed to investigate storage time and treatment effects. All statistical analyses were performed with GraphPad Prism 9.1.0 (GraphPad Software,

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San Diego, CA, USA). Alpha was set at 0.05 to indicatea significant difference. Data are mean ± standard error of the mean (SE).

Results

Sperm suspension in the extender retains motility

In both undiluted sperm and sperm diluted with the extender, spermatozoa were in the quiescent state and their motility was activated when transferred to distilled water. In undiluted sperm, spermatozoa motility was not activated at 3 h post-storage and the percentage of motile spermatozoa was decreased at 1 h post-storage (p<0.05) (Fig. 1A). However, spermatozoa retained motility when sperm was diluted with extender with significant decrease observed at 9 h post-storage (p<0.05) (Fig. 1A). In fresh sperm, duration of spermatozoa motility was about 1 and 12 min for undiluted sperm and sperm suspended in the extender, respectively (p<0.05) (Fig. 1B). Duration of spermatozoa motility was decreased at 1 and 24 h post-storage in undiluted sperm and sperm suspended in extender, respectively (p<0.05) (Fig. 1B).

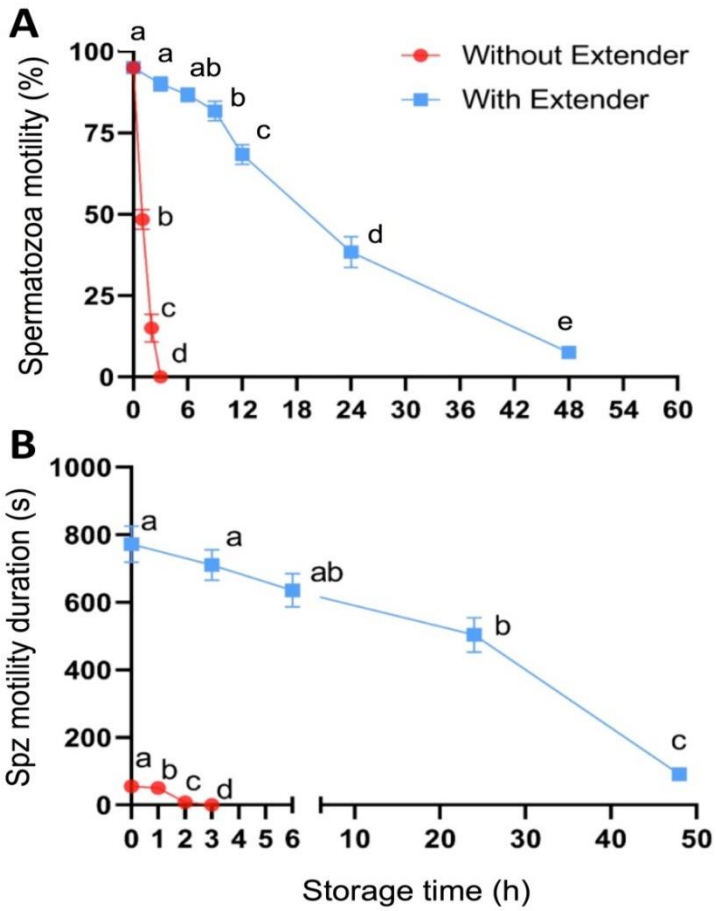

Figure 1: Short-term storage of Zebrafish (*Danio rerio*) sperm. Percentage of motile spermatozoa (A) and duration of spermatozoa motility (B) were assessed during the period of storage, and compared between intact and diluted sperm in an extender (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, and 2.38 mM $NaHCO_3$, pH 8.1 ± 0.2). Data are expressed as mean ± SE (n=6). For each treatment, different small letters show significant differences during the period of storage. ANOVA with Tukey's *post hoc* test, p<0.05.

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Bacterial infection decreases spermatozoa motility

From two-way ANOVA, effects of bacterial concentration × storage time interactions on spermatozoa motility were significant ($F_{df,error}$ =10.7$_{18,140}$ for *V. harveyi* and $F_{df,\ error}$ =13.7$_{18,140}$ for *P. aeruginosa*; p=0.001). The models were revised into separate one-way ANOVA to investigate the effects of bacterial concentrations and storage time.

When effects of bacterial concentrations were studied at the same storage time, spermatozoa motility did not differ between non-infected sperm and inoculated sperm with 10^3 CFU/mL *V. harveyi* or *P. aeruginosa* (p>0.05). Spermatozoa motility was significantly decreased in sperm inoculated with 10^8 CFU/mL *V. harveyi* and with 10^5 CFU/mL *P. aeruginosa* at 3 h post-storage. At 6-24 h post-storage, a decrease in spermatozoa motility was significant at 10^5 CFU/mL *V. harveyi* and *P. aeruginosa*. Increasing bacterial concentration to 10^8 CFU/mL decreased spermatozoa motility compared to non-infected sperm or sperm inoculated with 10^3 or 10^5 CFU/mL *V. harveyi* or *P. aeruginosa* at each storage time (p<0.05).When effects of storage time were studied at the same bacterial concentration (Fig. 2), spermatozoa motility was decreased at 9 h post-storage in non-infected sperm and sperm inoculated with 10^3 CFU/mL *V. harveyi* or *P. aeruginosa* (p<0.05). At 10^5 CFU/mL, spermatozoa motility was decreased at 6 and 3 h post-storage when sperm was inoculated with *V. harveyi* or *P. aeruginosa*, respectively (p<0.05). At 10^8 CFU/mL, spermatozoa motility was decreased at 3 h post-storage when sperm was inoculated with either *V. harveyi* or *P. aeruginosa* (p<0.05).

Gentamicin preserves spermatozoa motility against bacteria

In this experiment, gentamicin was added into the extender where sperm was inoculated with 10^8 CFU/mL *V. harveyi* or *P. aeruginosa*. Two-way ANOVA showed significant effects of gentamicin concentration × storage time interactions on spermatozoa motility ($F_{df,error}$ =32.5$_{30,210}$ for *V. harveyi* and $F_{df,\ error}$ =10.2$_{30,210}$ for *P. aeruginosa*; p=0.001). The models were revised into separate one-way ANOVA to investigate the effects of bacterial concentrations and storage time.

When effects of gentamicin concentrations were studied at the same storage time, spermatozoa motility was similar to that of non-infected sperm at each time post-storage when 0.08–0.15 and 0.02–0.04 mg/mL gentamicin was added into sperm inoculated with *V. harveyi* and *P. aeruginosa*, respectively (p>0.05). Spermatozoa motility in the presence of 0.3 mg/mL gentamicin was similar to that of sperm inoculated with 10^8 CFU/mL *V. harveyi* (p>0.05). In the presence of 0.6 mg/mL gentamicin, spermatozoa motility in sperm inoculated with 10^8 CFU/mL *V. harveyi* was not activated at 3 h post-storage. Spermatozoa motility in the presence of 0.005 and 0.01 mg/mL gentamicin was higher than that of sperm inoculated with 10^8 CFU/mL *P. aeruginosa* at 3–12 h post-storage (p<0.05), however, it was lower than that of non-infected sperm and sperm with 0.02 and 0.04 mg/mL gentamicin (p<0.05).

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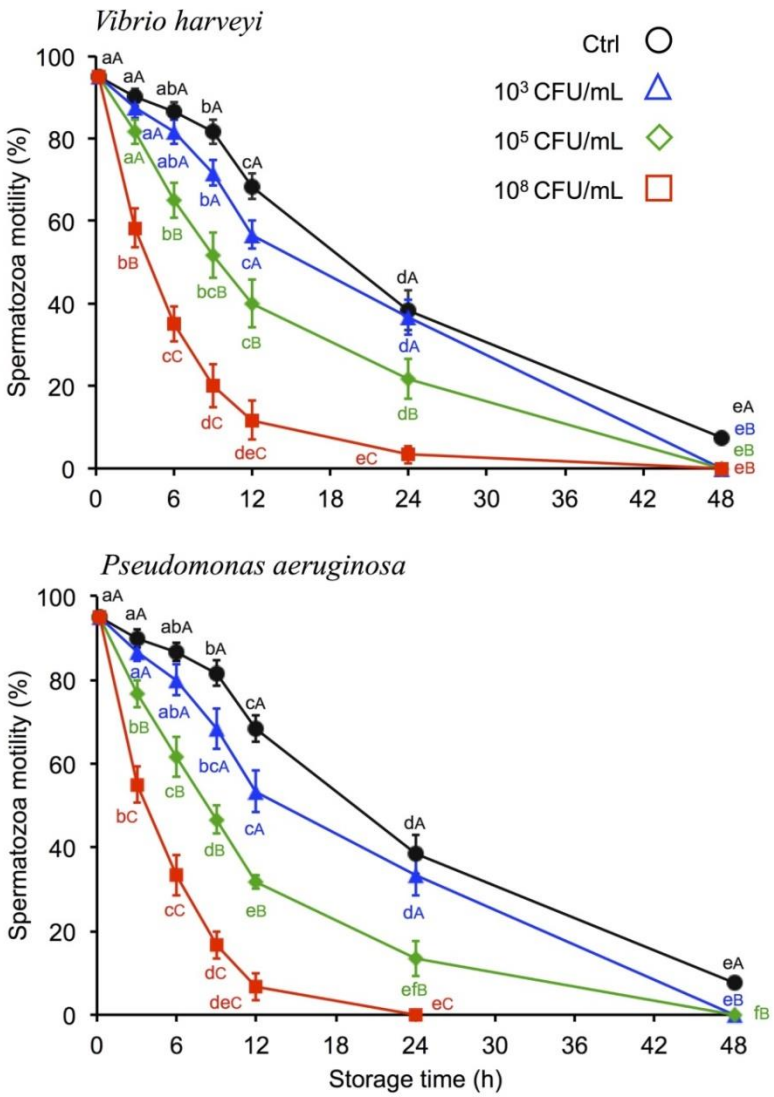

Figure 2: Percentage of motile spermatozoa following short-term storage of Zebrafish (*Danio rerio*) sperm inoculated with *Pseudomonas aeruginosa* and *Vibrio harveyi*. Diluted sperm (2.5×10^8 spermatozoa/mL) in an extender (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, and 2.38 mM $NaHCO_3$, pH 8.1 ± 0.02) was infected with different concentrations of bacteria. At each storage time, different capital letters show the effects of bacterial concentrations. For each treatment, different small letters show the effects of storage time. Data are expressed as mean ± SE (n = 6). ANOVA with Tukey's *post hoc* test, p<0.05.

When effects of storage time were studied at the same gentamicin concentration (Fig. 3), in sperm inoculated with 10^8 CFU/mL *V. harveyi* without gentamicin, spermatozoa motility was decreased at 3 h post-storage, while it was decreased at 12, 6 and 3 h post-storage in infected sperm containing 0.15, 0.08 and 0.3–0.6 mg/mL gentamicin, respectively (p<0.05).

At 10^8 CFU/mL *P. aeruginosa* without gentamicin, spermatozoa motility was decreased at 3 h post-storage, while it was decreased at 9, 6, and 3 h post-storage in infected sperm containing 0.04, 0.02, and 0.005–0.01 mg/mL gentamicin, respectively (p<0.05).

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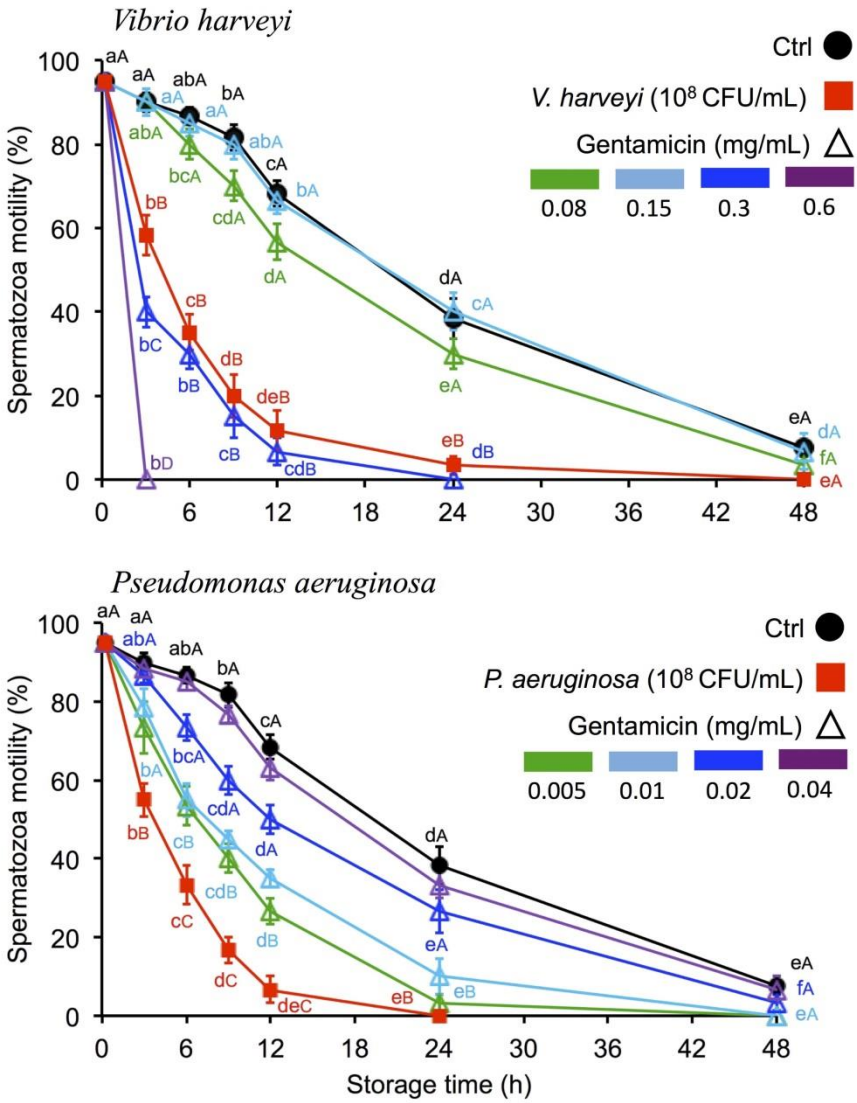

Figure 3: Gentamicin protects spermatozoa motility in Zebrafish (*Danio rerio*) during short-term storage. Diluted sperm (2.5×10^8 spermatozoa/mL) in an extender (180 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2$, and 2.38 mM $NaHCO_3$, pH 8.1 ± 0.02) was infected with different concentrations of bacteria, and different concentrations of gentamicin were added. At each storage time, different capital letters show the effects of gentamicin concentrations. For each treatment, different small letters show the effects of storage time. Data are expressed as mean ± SE (n = 6). ANOVA with Tukey's *post hoc* test, $p<0.05$.

Gentamicin retains viability of spermatozoa against bacteria

In this experiment, spermatozoa viability was compared between non-infected sperm and sperm infected by 10^8 CFU/mL *V. harveyi* and *P. aeruginosa* with or without gentamicin. Within the period of 24 h storage, spermatozoa viability did not differ in sperm inoculated with *P. aeruginosa* in the presence or absence of 0.04 mg/mL gentamicin compared to non-infected sperm ($p>0.05$). For all treatments of *P. aeruginosa*, spermatozoa viability was decreased at 24 h post-storage ($p<0.05$). In contrast, spermatozoa viability was affected by *V. harveyi* with a decrease observed at 24 h post-storage ($p<0.05$), but it remained unchanged in infected sperm containing

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0.15 mg/mL gentamicin (p>0.05). Spermatozoa viability was non-significantly increased when 0.15 mg/mL gentamicin was added to sperm inoculated with 10^8 CFU/mL *V. harveyi* (Fig. 4).

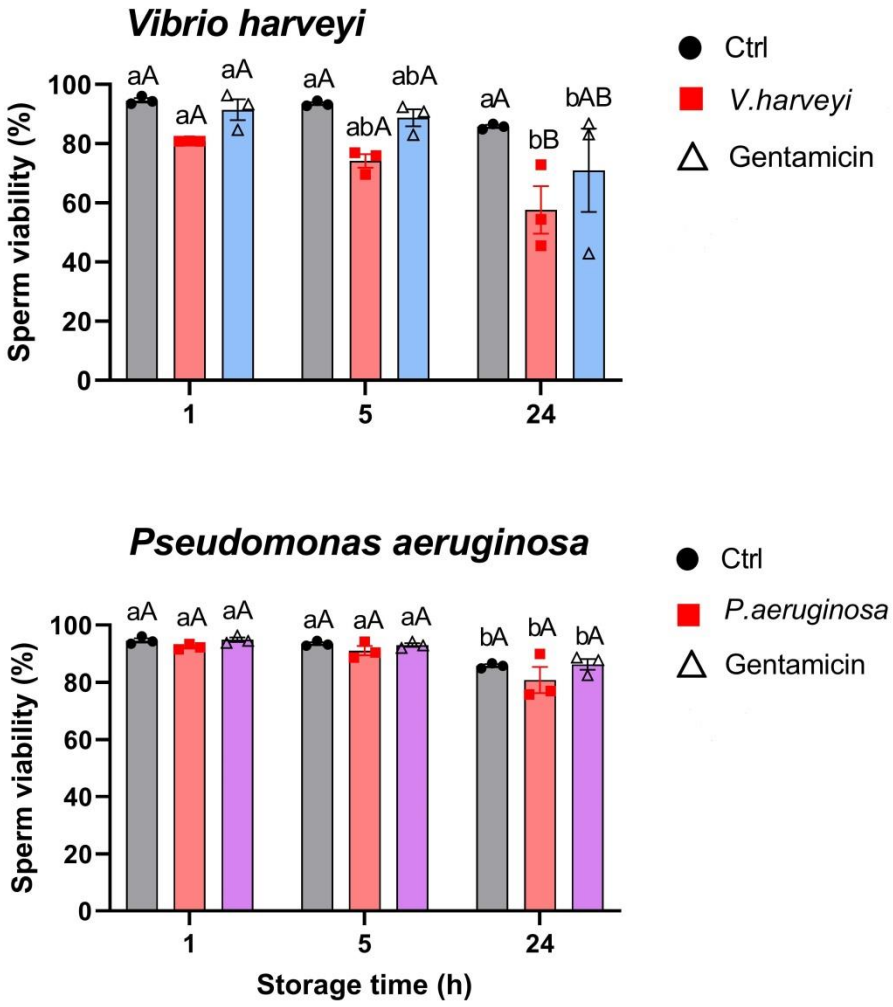

Figure 4: Gentamicin protects spermatozoa viability in Zebrafish (*Danio rerio*) during short-term storage. Spermatozoa viability in short-term stored sperm of Zebrafish inoculated with 1 × 10^8 (CFU/mL) *Pseudomonas aeruginosa* and *Vibrio harveyi* was assessed in the presence of 0.04 and 0.15 mg/mL gentamicin, respectively. For each treatment, different small letters show significant effects of storage time. At each storage time, different capital letters show significant differences among treatments. Data are expressed as mean ± SE (n = 3). ANOVA with Tukey's *post hoc* test, p<0.05.

Discussion

Gamete handling needs to be improved to develop *in vitro* fertilization. In this context, sperm handling mainly includes proper methods to (a) collect good quality sperm, (b) maintain spermatozoa in the quiescent state until being used for fertilization, and (c) activate spermatozoa to achieve high fertilization (Beirão *et al.*, 2019). In the present study, Zebrafish sperm was inoculated with two species of bacteria, and spermatozoa motility and viability were assessed during a short-term storage period (Fig. 5). As shown in former studies (Jing *et al.*, 2009; Cheng *et al.*, 2021), spermatozoa showed better motility characteristics when sperm was stored in the extender. Infection of Zebrafish sperm

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with *P. aeruginosa* and *V. harveyi* significantly decreased spermatozoa motility in a bacterial concentration-dependent manner. When sperm was inoculated with 1×10^8 CFU/mL bacteria, gentamicin protected the adverse effects of *P. aeruginosa* and *V. harveyi* on spermatozoa motility at 0.02–0.04 mg/mL and 0.08–0.15 mg/mL, respectively. Assessment of spermatozoa viability showed that it was higher in the presence of gentamicin compared to bacterially infected sperm.

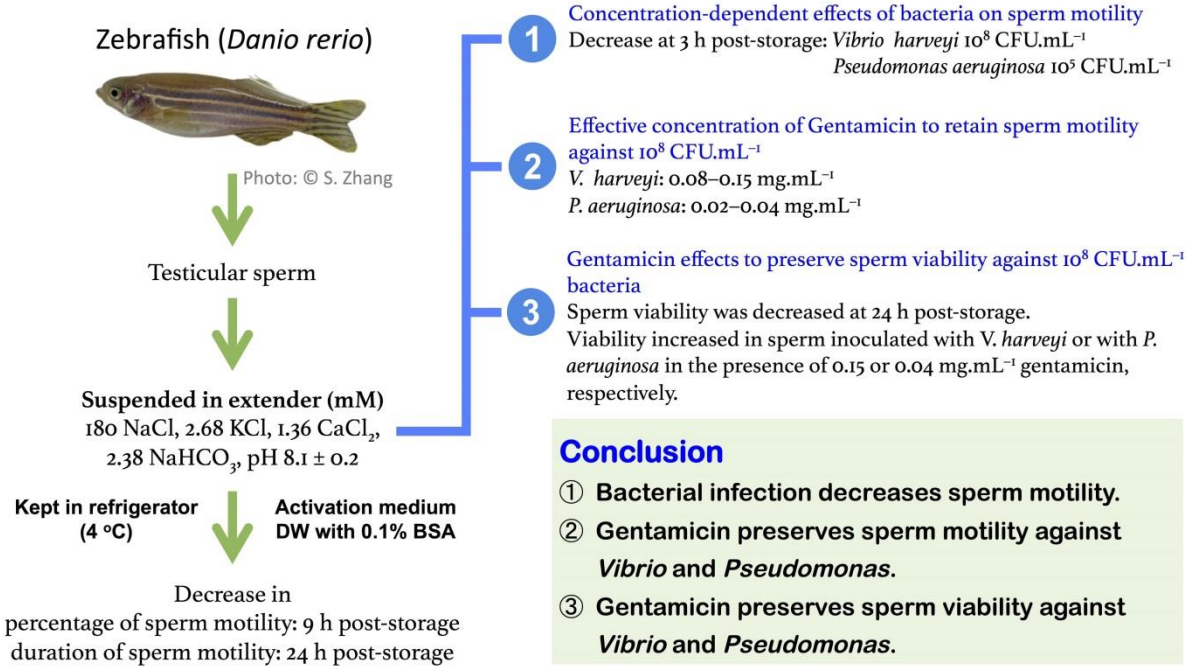

Figure 5: Graphical summary of the present study. Bacterial contamination decreases motility and viability of Zebrafish (*Danio rerio*) sperm during short-term storage. Gentamicin protects motility or viability of sperm inoculated with *Pseudomonas aeruginosa* and *Vibrio harveyi*.

Spermatozoa were in a quiescent state in the extender, and their motility was activated after mixing with distilled water (Jing *et al.*, 2009; Wilson-Leedy *et al.*, 2009; Cheng *et al.*, 2021). We did not measure the osmolality of sperm or the extender, however, the osmolality of seminal plasma in Zebrafish and similar extender has been reported to be 268 and 343 mOsmol/kg, respectively (Cheng *et al.*, 2021). Activation of spermatozoa motility after dilution with distilled water was due to the fact that a hypo-osmotic environment triggers spermatozoa motility initiation in Zebrafish (Takai and Morisawa, 1995), similar to most freshwater fish (Alavi *et al.*, 2019). Similar to previous studies on Zebrafish sperm (Jing *et al.*, 2009; Cheng *et al.*, 2021), this study showed higher spermatozoa motility characteristics when sperm was stored in the extender. Duration of spermatozoa motility that highly depends on spermatozoa ATP stores (Cosson, 2010) was significantly shorter in undiluted sperm than sperm diluted with extender (Fig. 1B). It has been shown that extender protects spermatozoa against damage to mitochondria, which provide energy for spermatozoa motility, and damage to the plasma membrane and axoneme that are

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involved in spermatozoa motility signaling (Saad *et al.,* 1988; Hatef *et al.,* 2011; Dietrich *et al.,* 2021).

Although previous studies showed antibiotics preserve spermatozoa motility and fertilizing ability during short-term storage in various fish species (Saad *et al.,* 1988; Christensen and Tiersch, 1996; Segovia *et al.,* 2000; Niksirat *et al.,* 2011; Cejko *et al.,* 2022; Zidni *et al.,* 2023), however bacteriology of the stored sperm remained largely unknown. Some species of *Pseudomonas* and *Vibrio* which are pathogens to fish including Zebrafish (Austin and Austin, 2016; Kent *et al.,* 2020; Manchanayake *et al.,* 2023) have been isolated from the flora of the catfish testes (Jenkins and Tiersch, 1997) or from short-term stored sperm of sturgeons (Wayman, 2003). Also, former studies showed bacterial growth during sperm short-term storage (Stoss and Refstie, 1983; Jenkins and Tiersch, 1997; Viveiros *et al.,* 2010). The present study, for the first time to our best knowledge, determines and quantifies the detrimental effects of *P. aeruginosa* and *V. harveyi* on sperm storage. The range of bacterial concentrations examined in the present study (10^3, 10^5, and 10^8 CFU/mL) has been chosen with respect to the bacterial population measured in Piracanjuba, *Brycon orbignyanus* sperm: 1×10^4, 2.4×10^6 and 1.1×10^7 CFU/mL at 0, 2 and 8 d post-storage (Viveiros *et al.,* 2010). A similar bacterial population has been also reported in fresh and short-term stored sperm of mammals, for instance 10^3–10^5 CFU/mL in boar sperm (Althouse and Lu, 2005). These results show that inoculation of sperm with bacteria decreased spermatozoa motility in a concentration-dependent manner, with a significant decrease at 10^8 CFU/mL *V. harveyi* and at 10^5 CFU/mL *P. aeruginosa* at 3 h post-storage (Fig. 2). However, *V. harveyi* and *P. aeruginosa* at 10^3 CFU/mL were without significant effects on spermatozoa motility. Similar to mammals (Sepúlveda *et al.,* 2014), our results show that a bacterial load of 10^8 CFU/mL resulted in the highest reduction of spermatozoa motility.

For the first time, we evaluated spermatozoa membrane integrity in short-term stored sperm inoculated with bacteria, as bacterial contamination of sperm may lead to a decrease in membrane stability through direct damage to the plasma membrane (Diemer *et al.,* 2003). Results showed a decrease in spermatozoa viability when sperm was inoculated with 1×10^8 CFU/mL *V. harveyi* or *P. aeruginosa* for 24 h (Fig. 4), suggesting that detrimental effects might be attributed to membrane damage. Data also suggest higher impacts of *V. harveyi* on spermatozoa viability compared to *P. aeruginosa*. This might be due to the multi-functional effects of bacteria to decrease spermatozoa viability in association with oxidative stress or energy stores (Tvrdá *et al.,* 2022).

Finally, we used gentamicin, an antibiotic widely used in fish sperm storage, to investigate whether it protects spermatozoa against *V. harveyi* and *P. aeruginosa*. The Zebrafish sperm was inoculated with 1×10^8 CFU/mL in the presence of various gentamicin concentrations, and spermatozoa motility was assessed. Spermatozoa motility of diluted sperm with extender was activated in the presence of examined concentrations

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of gentamicin similar to control (Data are not shown). Similarly, Viveiros *et al.* (2010) reported that gentamicin at 1.0 mg/mL had without effects on spermatozoa motility in non-infected sperm of *B. orbignyanus*. Our results showed that spermatozoa motility was similar to that of non-infected sperm when 0.08–0.15 and 0.02–0.04 mg/mL gentamicin was added to sperm inoculated with 1×10^8 CFU/mL *V. harveyi* or *P. aeruginosa*, respectively (Fig. 3). The effective concentrations are consistent with those observed for sperm of Nile tilapia, *Oreochromis niloticus* (0.5 mg/mL) (Segovia *et al.,* 2000) and of *B. orbignyanus* (0.1–1 mg/mL) (Viveiros *et al.,* 2010). A recent study also showed that higher concentrations of gentamicin (100–800 mg/mL) were efficient in protecting sperm of spotted halibut, *Verasper variegatus* during storage (Zidni *et al.,* 2023). Although spermatozoa motility was retained in the presence of gentamicin, spermatozoa viability was not significantly different when 0.15 and 0.04 mg/mL gentamicin were added to sperm inoculated with 1×10^8 CFU/mL *V. harveyi* and *P. aeruginosa*, respectively. However, a non-significant increase was observed in sperm inoculated with *V. harveyi*. We observed that higher concentrations of gentamicin (0.3 mg/mL) were without effects on protecting spermatozoa motility against *V. harveyi*, and its lower concentrations (0.005-0.01 mg/mL) were not as effective as higher concentrations in protecting spermatozoa against *P. aeruginosa*.

Conclusions

The present study shows that inoculation of sperm with *V. harveyi* and *P. aeruginosa* decreases spermatozoa motility in a bacterial concentration-dependent manner, with a high decrease at 1×10^8 CFU/mL. It also determines the optimum concentrations of gentamicin to protect spermatozoa against *V. harveyi* and *P. aeruginosa*. It was observed that gentamicin at 0.08–0.15 and 0.02–0.04 mg/mL protect spermatozoa against detrimental effects of 1×10^8 CFU/mL *V. harveyi* or *P. aeruginosa*, respectively. This suggests that effective concentrations of gentamicin differ for *V. harveyi* and *P. aeruginosa*. Results of the present study provide valuable information to develop methods for short-term storage of Zebrafish sperm in artificial reproduction that may be used for generations of gynogenetic, androgenetic, hybrid, or polyploidy populations.

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Conflicts of Interest

The authors declare no conflicts of interest.

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