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Effects of Whitlockite Nanoparticles on Germ Cell Development and Spermatogenesis in Japanese Medaka

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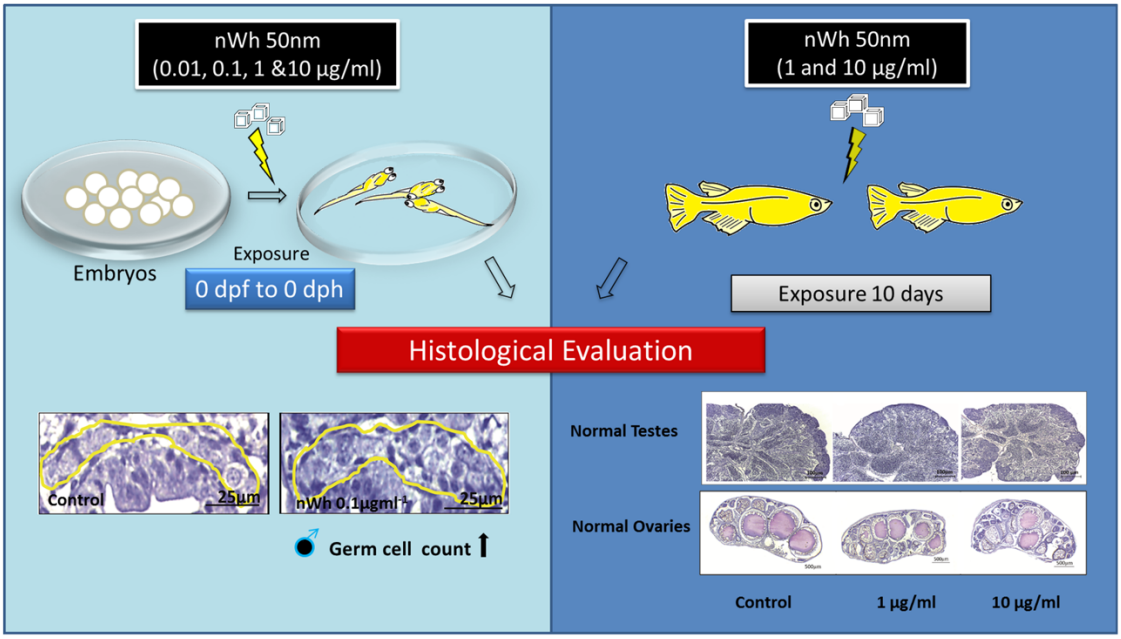
Graphical Abstract



Abstract

Whitlockite, the second most abundant bone mineral, was found promising in regenerative engineering of damaged bone tissue because of its high bone remodeling potential. However, no studies have investigated its effects on other organ systems and the environment. It is essential to study this aspect as whitlockite is known to dissolute at non-acidic pH. Hence, the effects of whitlockite nanoparticles on embryonic development, germ cell differentiation, and gametogenesis were explored here in an aquatic species, the Japanese medaka. Exposure to nano-whitlockite of 59.67±10.21 nm size showed no impact on medaka embryo development, survivability, and hatchability. However, it caused an increase in germ cell count in a significant number of XY males exposed to 0.1 µg L^{-1} nano-whitlockite compared to untreated controls and larvae exposed to 0.01, 1, and 10 µg L^{-1}. Interestingly, exposed XX females showed no such effect. Furthermore, exposure to nano-whitlockite at stages after attaining sexual maturity did not affect the reproductive systems of medaka males and females. Importantly, this study revealed that germ cell development during the embryonic period was sensitive to nano-whitlockite exposure, warranting detailed analyses and more studies in this regard using these particles.

Keywords Whitlockite nanoparticles; Japanese medaka; Germ cell proliferation

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Purpose and Rationale

Reports have shown that the nanoform of whitlockite (nWh) is an excellent bone remodeling agent and it supports bone regeneration through continuous release of phosphate and magnesium ions. However, it remains unclear whether nWh could harm reproduction and development by releasing these ions. As reproduction and development are highly susceptible to ionic imbalances, this study aimed to investigate the impact of nWh on embryogenesis, germ cell (GC) development, and gametogenesis. Therefore, the focus of this study was on finding the developmentally critical window, during which nWh influences the organisms adversely.

Introduction

Whitlockite (WH), the most stable form of calcium phosphate at acidic pH conditions, is the second most abundant mineral in bone [1, 2]. WH was reported to be the predominant component of bone during the mineralization process [3], making it an excellent bone remodeling agent. The bone regeneration potential of nano WH was proven by several studies in vitro and in vivo [4-8]. nWh was found to stimulate osteogenesis through the continuous release of phosphorus and magnesium ions [8]. However, most properties of nWh, including its toxicity potential, remain underexplored because of its narrow thermodynamic stability and high similarity with hydroxyapatite (HAP) at physiological conditions. The impact of nWh on reproduction and development must certainly be analyzed as these processes are vulnerable to perturbations in intracellular ion concentrations. It is of paramount importance that germ cells (GCs), which carry the genetic information needed to generate a new organism, remain unscathed throughout its journey from one generation to the next. Damages to reproductive organs and GCs can result in reproductive failure and the extinction of a species. Graphene quantum dots (GQDs) with excellent biocompatibility affected GC development in medaka embryos adversely [9], warranting the need to screen each nanoparticle (NPs) developed for human use. Therefore, we examined the reproductive toxicity potential of nWh using Japanese medaka (*Oryzias latipes*), an aquatic model organism approved by the Organization for Economic and Cooperative Development (OECD) for toxicity screening. Several studies have demonstrated that medaka and zebrafish were suitable for such purposes as exogenous substances, including NPs caused adverse effects on embryonic development by entering through pore canals on egg chorions [10-12]. Further, both medaka and zebrafish have external fertilization and development, which can be tracked using an optical microscope because of their transparent egg chorions. Nevertheless, medaka has several advantages over zebrafish, making it a better model for studies involving reproductive toxicity as the former is a gonochorist-like mammal with sexually differentiated gonads at the time of hatching. Furthermore, medaka has XX/XY chromosomal mode of sex determination, despite which gonadal sex differentiation in this species remains manipulatable using exogenous factors. Sexual differentiation in medaka during early developmental stages is well-characterized, morphologically distinguishable, and several biomarkers are available to identify sexual genotype and phenotype [13]. These aspects make medaka an attractive model organism compared to zebrafish for studies analyzing the impact of chemical agents on reproductive organs.

Materials and Methods

2.1 Materials

nWh particles were synthesized using calcium hydroxide, magnesium hydroxide, and phosphoric acid (Sigma Aldrich, USA). Bouin's reagent was prepared using picric acid, acetic acid (Merck Specialities Pvt Ltd, Mumbai), and formaldehyde (Sigma, St. Louis, U.S.A.). Davidson's reagent was prepared by formaldehyde solution (Sigma, St. Louis, U.S.A.), acetic acid, glycerol (Merck Specialities Pvt Ltd, Mumbai), ethanol (Hayman Group Ltd, Witham, U.K.), and MilliQ ultrapure water (Millipore, Billerica, MA). Xylene and Paraffin wax used for histology were purchased from Merck Specialities Pvt Ltd, Mumbai.

2.2 Animal model

Japanese medaka was maintained under laboratory conditions, temperature 26 - 28°C, light/dark cycles 14/10 h, and humidity 50–60%. Adults were fed 4 times a day, while larvae were fed with commercially available feed (Artemia, Ocean Star International, INC.USA). The study was

conducted after obtaining Institutional Animal Ethics Committee approval (Ref. No.: IAEC/2019/2/4), strictly adhering to their animal experimentation and maintenance guidelines.

Methodology

2.3a Synthesis

nWh particles were synthesized following previously published protocols [1]. Briefly, calcium hydroxide (0.37M) and magnesium hydroxide (0.13M) were mixed in distilled water at 80ºC. During vigorous stirring, phosphoric acid (0.5M) was added at a rate of 12.5 ml/min. The precipitate was centrifuged and washed with distilled water and freeze-dried to obtain nWh.

2.3b Characterization of nWh

The size and morphology of nWh were determined using high-resolution transmission electron microscopy (HR-TEM, (Tecnai G2 TF20 S-TWIN)) at 200 kV. Elemental composition of nWh was carried out using an energy dispersive X-ray spectroscope (EDS) connected to a field-emission scanning electron microscope (FE-SEM; JEOL JSM-7610 F Plus). The functional groups were identified by Fourier transform infrared (FT-IR) spectroscopy. The crystalline structure of nWh was determined by X-ray diffraction (XRD), and characteristic peaks of the particles were confirmed using WH JCPDS (70-2064) database.

2.3c In vivo exposure to nWh

Embryos: Fertilized eggs of Qurt strain of medaka were exposed to nWh at 0.01, 0.1, 1.0, and 10 µg/ml till the hatching day. Survival and hatchability were recorded from day of fertilization to the day of hatching in three replicated experiments. The exposed and untreated embryos were screened for developmental defects under a stereomicroscope (Leica MSV269, Taiwan).

Adults: Sexually mature males and females were anesthetized using 0.02% of MS-222 (Sigma Aldrich, China) to record body weights. The fish were exposed to 1 and 10 µg/l of nWh in aquarium water for 10 days. The rearing medium was changed every day, and particles were added freshly. The fish were anesthetized and weighed upon the termination of exposure.

2.3d Histology

Hatched larvae were fixed in freshly prepared Bouin's reagent overnight at 4ºC. The fixed larvae were then dehydrated using increasing concentrations of absolute ethanol and stored in 100% ethanol at −20ºC until histological evaluation. The liver, kidney, gut, and gonads of adult fish were dissected and fixed (testes in Bouin's reagent and ovaries in Davidson reagent) overnight at 4ºC and dehydrated in increasing concentrations of absolute ethanol. The samples were then stored in 100% ethanol at -20ºC until histological evaluation. Liver and gonads were weighed prior to fixation.

Fixed tissues were processed for histology following standard protocols and embedded in paraffin. Embryos and testes were sectioned at 5 µm and ovaries at 8 µm thickness using a manual microtome (Thermo scientific MICROM HM 340E, Germany) attached with a section transfer system (MICROM 61534, Germany). Sections were stained with hematoxylin-eosin using standard protocols. The GCs were counted manually under the microscope (LEICA ICC50 HD, Germany).

2.3e Statistical Analysis

Data were analyzed statistically by one-way ANOVA and significance was determined by Bonferroni's Multiple Comparison test using Graph pad prism.

Results and Discussion

3.1 Characterization of nWh

HR-TEM revealed the rhombohedral structure and uniform particle size (59.67±10.21 nm) of nWh (Figure 1A). EDS data confirmed the presence of Ca, Mg, P and O in nWh (Figure 1B, Table 1).

Table 1. Atomic percentage of Ca, P, Mg and O in whitlockite nanoparticles

Element	Weight %	Atomic %
O	47.35	66.36
Mg	3.05	2.82
P	18.74	13.57
Ca	30.86	17.26

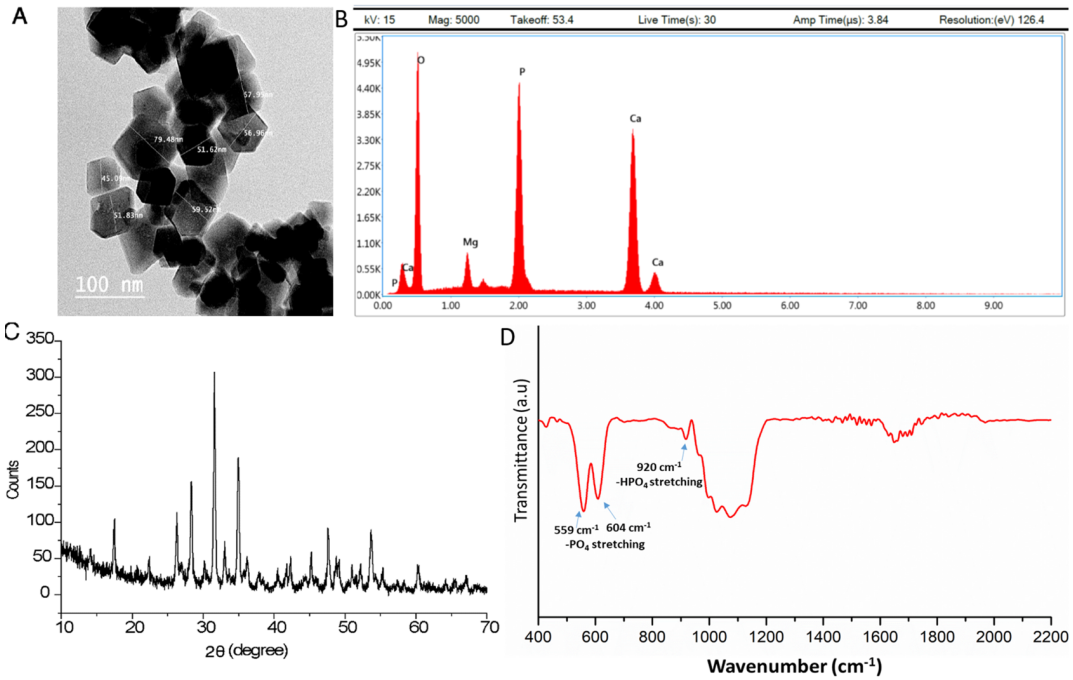

Figure 1. *Physico-chemical characterization of nWh.* ***(A)*** *TEM image showing rhombohedral structure and size around 59.67±10.21 nm.* ***(B)*** *EDS data showing percentages of Ca, Mg, P and O.* ***(C)*** *XRD analysis with characteristic peaks of WH.* ***(D)*** *FTIR spectrum showing relevant peaks for functional groups, PO_4 and HPO_4.*

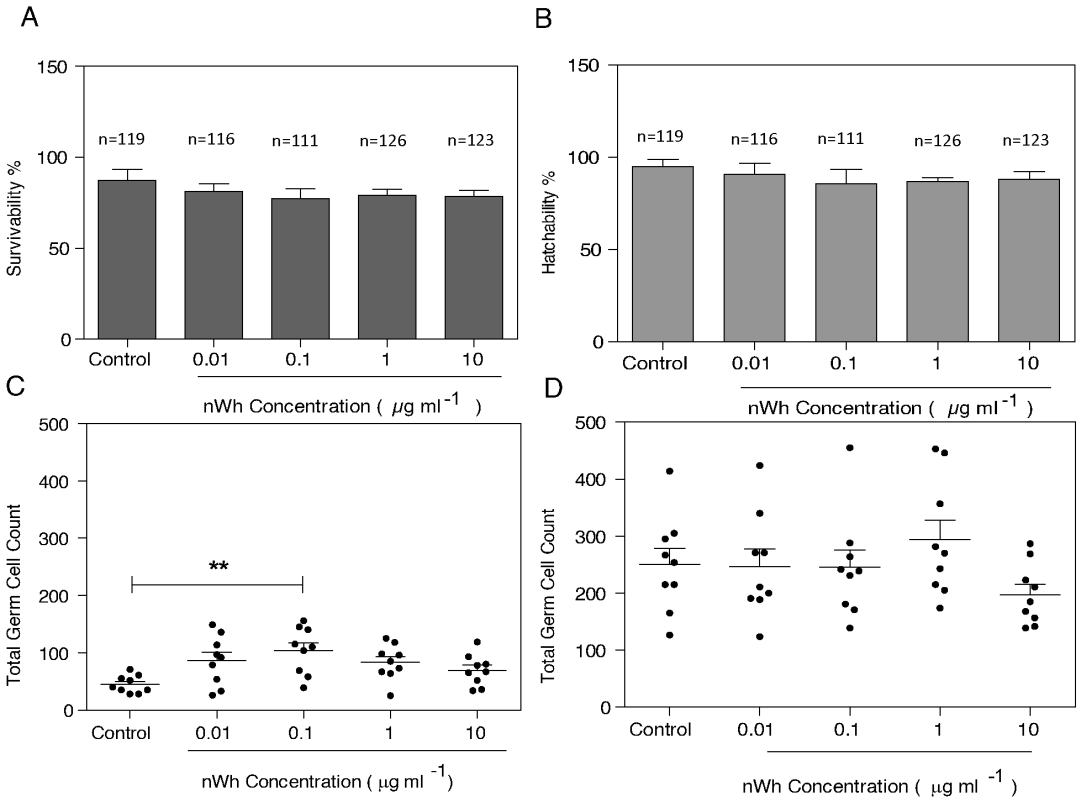

Figure 2. *Effects of nWh on medaka embryos.* ***(A)*** *Survivability of embryos.* ***(B)*** *Hatchability of embryos. Numbers within parentheses above the bars in (A) and (B) indicate the total number of embryos used in 3 triplicate experiments. None of the concentrations tested showed any significant difference from the Control group.* ***(C)*** *Impact of nWh on the total number of GCs in male embryos.* ***(D)*** *Impact of nWh on the total number of GCs in female embryos. Each dot in (C) and (D) represents an individual data point (N = 9). ** indicates P≤0.005.*

The calculated ratio of (Ca+Mg)/P for the prepared nWh were 1.48, as compared to the theoretical value of 1.42. The stoichiometric ratio of Ca:Mg:P was found to be 1.27:0.2:1, compared to the theoretical ratio of 1.28:0.14:1 [1]. The values obtained were close to the theoretical values. XRD of nWh NPs was compared with previously known XRD patterns of WH (JCPDS 70-2064) to prove its crystallinity (Figure 1C), and FT-IR spectroscopy revealed the functional groups of nWh (Figure 1D). These results were consistent with the previous data on nWh, confirming that the NPs synthesized were the same [14].

3.2 Effects on embryonic development

Male and female larvae were separated based on the presence and absence of leucophores on the head region, respectively [15]. No developmental anomalies were noticed in nWh-treated embryos when compared with the untreated controls. Survivability of nWh-treated embryos was comparable to that of the untreated, proving excellent biocompatibility of the particles (Figure 2 A, B).

3.3 Effects on gonadal development

GC count is an essential morphological characteristic that distinguishes the initiation of gonadal sex differentiation in medaka [16, 17]. Therefore, the impact of nWh on GC proliferation and development was investigated. GC count in nWh-treated XY larvae was significantly higher than that of untreated control XY larvae. This phenomenon was exhibited by 66.67% of larvae exposed to 0.1 µg/ml of nWh, while only a small percentage of larvae in other dosage groups showed such a response (Figure 2C, 3A-B).

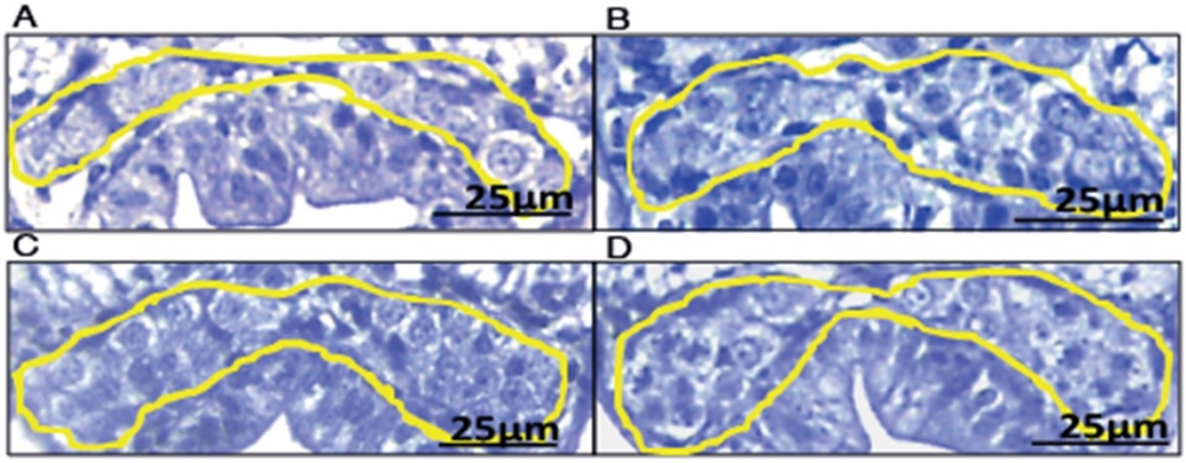

Figure 3. *Gonadal histology showing effects of nWh on GCs in male and female embryos (N = 9).* ***(A)*** *Germ cells in the gonadal region of control male embryos.* ***(B)*** *Germ cells in the gonadal region of male embryos exposed to 0.1 µg/ml nWh.* ***(C)*** *Germ cells in the gonadal region of control female embryos.* ***(D)*** *Germ cells in the gonadal region of female embryos to 0.1 µg/ml nWh. The gonadal region is marked with yellow dotted lines. Scale bar, 25 µm.*

GC count of nWh-treated XX larvae remained unaltered (Figure 2D, 3C-D). This kind of non-monotonic response was observed in the case of larvae exposed to GQDs [9]. It has already been demonstrated that physico-chemical properties of engineered nanoparticles (ENPs) could vary at different concentrations [18], and thus, ENPs could exhibit remarkable properties at lower doses, suggesting nonmonotonic dose response observed in this study to be not an unusual norm. For example, several ENPs were found to aggregate at higher doses, making it impossible for the particles to cross the cell membrane, while the same ENPs at lower doses were found to make an entry into the cells [19, 20].

Increased GC count in XY larvae exposed to nWh indicated that these cells could override the genetically programmed mitotic arrest that earmarks testicular development in normal XY larvae. Previous studies have revealed the role of Mg^{2+} in spermatogenesis [21, 22]. However, its involvement in the initiation of spermatogonia development has not been studied yet. Our previous study had investigated the release of Ca, $Mg^{2+,}$ and P ions from nWh for 48 h and found that 2.2±0.1 ppm of Mg^{2+} was released [14].

However, it needs to be determined whether it was Mg^{2+} ions, P/Ca ions, or a combination of these three ions that released the GCs in XY embryos from mitotic arrest. An increase in GC count and entry into meiosis during early developmental stages induced ovarian differentiation in genetically male embryos of medaka [23]. Such sex reversal could lead to skewing of sex ratios towards one phenotype and eventually extinction of the species. Previous reports have shown that developmental exposure to endocrine disruptors [23, 24] or pesticides [25, 26] can induce persistent effects that manifest in adulthood [27]. Hence, our data indicated that nWh could be potentially hazardous to GC development and, thereby, reproduction in XY medaka. The evidence obtained by this study on GC development emphasized the requirement for in-depth analysis of developmental effects of nWh in higher vertebrate models.

3.4 Effects on gametogenesis

No mortality and changes in body weights in response to nWh were observed compared with that of the untreated controls, suggesting nWh to be biocompatible with adult fish (Figure 4 A, D).

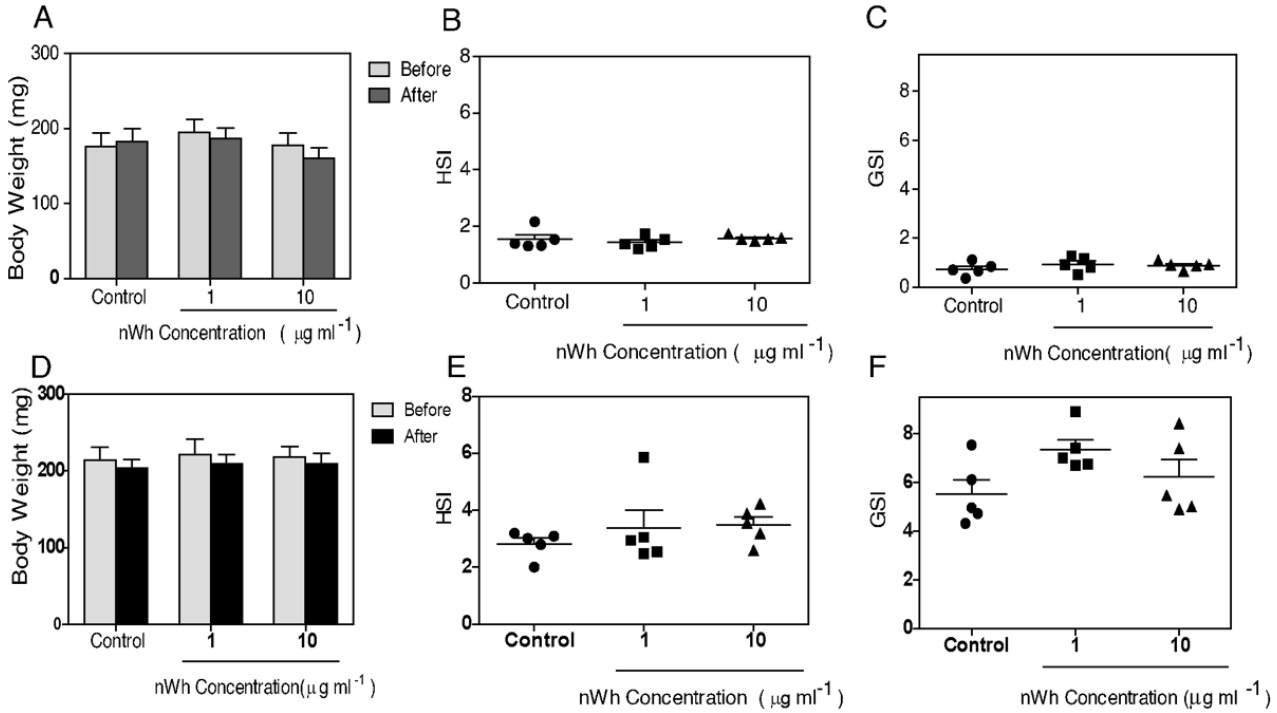

Figure 4. *Effects of nWh on sexually mature males and females of medaka.* ***(A)*** *Body weights of males before and after the exposure.* ***(B)*** *Hepato-somatic indices (HSI) of males.* ***(C)*** *Gonado-somatic indices (GSI) of males.* ***(D)*** *Body weights of females before and after exposure.* ***(E)*** *HSI of females.* ***(F)*** *GSI of females. Each dot in dot plots represents an individual data point (N = 5).*

Furthermore, no differences could be noticed in hepato-somatic and gonado-somatic indices, which indicated the presence of healthy liver and gonads, respectively, compared to the controls (Figure 4 B-C, E-F). Though Mg^{2+} showed beneficial effects on spermatogenesis through Ca homeostasis [21], no alterations were seen in the gonads of adult medaka exposed to nWh (Figure 5 A-F), suggesting the NPs to be biocompatible with the reproductive systems.

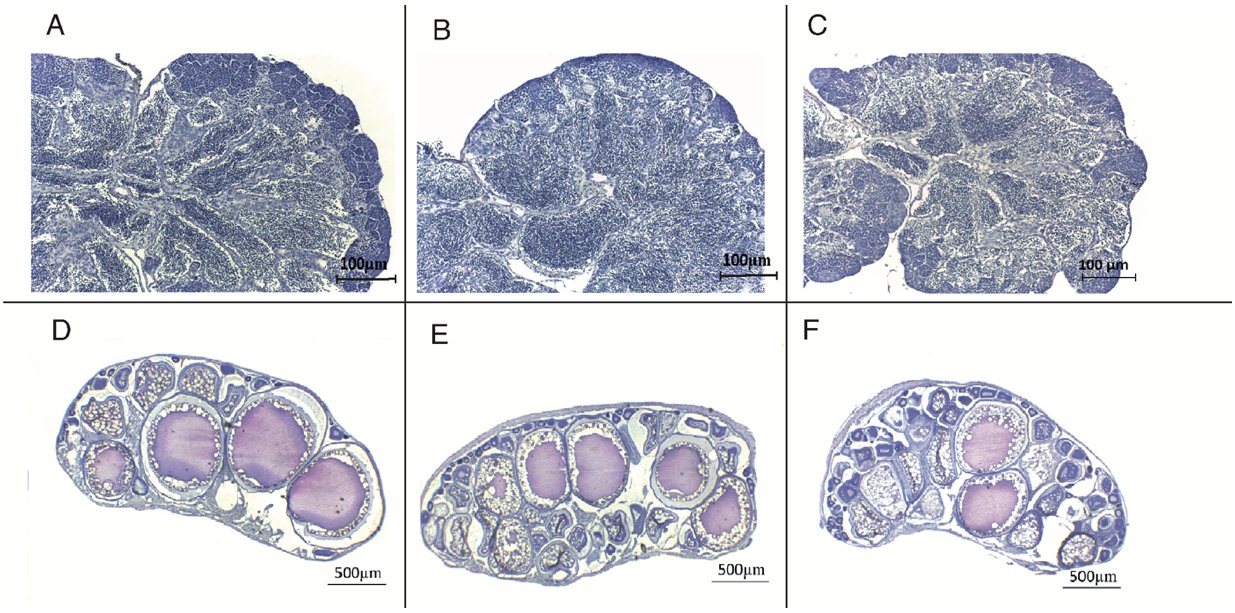

Figure 5. *Gonadal histology of adult males and females of medaka (N = 5).* ***(A - C)*** *Testicular histology of control males* ***(A),*** *males exposed to nWh at 1 µg/L* ***(B)*** *and 10 µg/L* ***(C)****.* ***(D - F)*** *Ovarian histology of control females* ***(D),*** *females exposed to nWh at 1 µg/L* ***(E)*** *and 10 µg/L* ***(F)****. Scale bar: (A – C), 100 µm; (D – F), 500 µm.*

Conclusion

Whitlockite of 59.67±10.21 nm size showed excellent biocompatibility with embryonic development; however, it increased germ cell count in genetically male embryos of medaka compared to untreated controls. The adult fish showed no adverse effects in response to nano-whitlockite exposure. This study revealed an important insight about nano-whitlockite that it could be toxic to germ cell development during embryogenesis, warranting more studies in this regard in other species.

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Conflict of interest

Authors declare no conflict of interest. For a signed statement, please contact the journal office at editor@precisionnanomedicine.com

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