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## Data Article

# Experimental datasets on the characterization of graphene oxide and its reproductive and developmental effects on Japanese medaka (*Oryzias latipes*) fish



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## ABSTRACT

The datasets of this article present the experimental parameters resulting from the synthesis and characterization of graphene oxide (GO) using scanning and transmission electron microscopy (SEM, TEM) and spectrophotometric (FTIR, AFM, EDX) methods, and the assessment of its toxicological and endocrine-disrupting effects on the Japanese medaka fish by acute toxicity testing, and histopathological evaluations. These datasets support the article "Reproductive and Developmental Effects of Graphene Oxide on Japanese Medaka (*Oryzias latipes*)". GO synthesis was performed following the modified Hummer's method. Its particle diameter and zeta potential were determined using Zeta Sizer Nano ZS analyzer, and characterized by SEM and TEM. After 5 min sonication in water, GO (25–200 µg/g) was injected intraperitoneally to the reproductively active male and female fish maintained as a breeding pair (one male, one female) in 500 mL balanced salt solution (BSS) in glass jars under standard laboratory conditions (25±1 °C; 16L:8D light cycle). The control fish were injected with water. The maximum volume of the injected material is 1 µL/10 mg body weight. To avoid move-

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ment, during injection the fish were briefly anesthetized in MS 222 (100 mg/L) and after injection transferred to BSS for recovery. LD<sub>50</sub> values of GO related to fish mortality were determined from the linear regression analysis using a software program. Reproductive activities (fecundity) were determined by daily collection of eggs 7 days before and 21 days after injection from a breeding pair and expressed as percent eggs laid every day post-injection relative to the average (mean of 7 days) eggs laid prior to injection. Developmental abnormalities of the embryos were assessed by culturing the collected fertilized eggs in ERM for a maximum period of 14 day-post fertilization (dpf). The fish that survived after 21 days post-injection were sacrificed and the entire fish excluding post-anal tail were cut into three small pieces and fixed in 4% paraformaldehyde containing 0.05% Tween 20. Histopathological evaluations of gonads (ovary and testis), liver, and kidneys were made in 5 µm thick sections stained mainly on hematoxylin and eosin (HE) following the guidelines published by OECD. The Photomicrographs of the sections were made using Olympus B-max 40 microscope attached to a camera with Q-capture Pro 7 software or in Nikon Eclipse 50i microscope attached to Nikon DS-Fi1 camera. Four types of follicles in the stromal compartments of the ovary, perinucleolar (PNO), cortical alveolar (CAO), early vitellogenic (EVO) and late vitellogenic (LVO) were considered as differentiating, and the post ovulatory and atretic follicles were considered as degenerating follicles, and counted in an entire section made through four different regions (anterior, upper middle, lower middle, and anal) of the ovary. The follicular data were expressed as percent follicles (individual follicles or differentiating or degenerating) or as the ratio of differentiating and degenerating follicles found in that particular region of the ovary. The data were analyzed either by one- or two-way ANOVA followed by post-hoc Tukey's multiple comparison test and expressed as means ± SEM.

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## Specifications Table

<b>Subject</b>	Pharmacology, Toxicology and Pharmaceutical Sciences
<b>Specific subject area</b>	Toxicology
<b>Type of data</b>	Table Image Graph Figure
<b>How data were acquired</b>	Zeta Sizer Nano ZS (Malvern Instrument, MA, USA), JEM-2100F Transmission electron microscope (TEM) (JOEL 2010, USA), Hitachi 5500 ultra-high-resolution scanning electron microscope (SEM) (TESCAN LYRA3), Energy-dispersive X-ray spectroscopy (EDX) (Thermo Noran System 7), Fourier Transform infrared spectroscopy (FTIR) (Thermo Nicolet Nexus 870 analysis), Atomic Force Microscope (AFM) (Veeco Dimension™3100), Ultra sonicator, LPX 750 (Cole Parmer) Microtome (Olympus cut 4055) Olympus B-max 40 microscope attached to a camera with Q-capture software (Media Cybernetics) Nikon Eclipse 50i microscope attached to Nikon DS-Fi1 Camera (Nikon Inc)
<b>Data format</b>	Raw and analyzed
<b>Parameters for data collection</b>	GO characterization by SEM, TEM, spectroscopy; statistical analysis on fish mortality (LD <sub>50</sub> ), embryo developmental abnormalities in vitro, and distribution of stromal follicles in the ovary; histopathological analysis of ovary, testis, liver and kidneys by standard histological techniques followed by visual inspection under microscope.
<b>Description of data collection</b>	GO was synthesized in the laboratory [1] using a standard protocol of Hummers and Offeman [2] or obtained commercially, characterized by electron microscopy (SEM and TEM) and spectroscopy procedures. GO was injected ip to fish once. The mortality was evaluated over 21-days post-injection, and the developmental abnormalities of the fertilized eggs were assessed in vitro for 14 dpf. Standard procedures of staining and photomicrography were used for histological data collection in ovary, testis, liver, and kidney. Stromal follicles in the ovary were characterized and counted [3].
<b>Data source location</b>	RCMI Center for Environmental Health, Jackson State University, Jackson, MS 39217, USA
<b>Data accessibility</b>	With the article
<b>Related research article</b>	Authors' names: Asok K. Dasmahapatra, Doris K. Powe, Thabitha P.S. Dasari, Paul B. Tchounwou [4] Title: Assessment of reproductive and developmental effects of graphene oxide on Japanese medaka ( <i>Oryzias latipes</i> ). Journal: Chemosphere doi:10.1016/j.chemosphere.2020.127221.

## Value of the Data

- The experimental fish, Japanese medaka (*Oryzias latipes*), an environmentally relevant model used for EDC studies, injected with graphene oxide (GO), intraperitoneally, which is more accurate method regarding the amount of precious nanomaterial introduced into the body of the experimental animal, that avoids excessive loss of nanomaterials agglomerated in the environment in waterborne exposure.
- The data will be useful to the U.S. Government agencies such as Environmental Protection Agency (EPA), Food and Drug administration (FDA), and many investigators including environmental biologist, nanotoxicologists, drug-designers, cancer researchers, endocrinologist, fish biologists, and biomedical scientist involved in drug development and nanomedicine.
- The toxicity data generated from this research will be useful for determining the LD50 of GO in Japanese medaka after intraperitoneal injection, as well as the no observed effect level (NOEL), or the lowest observed effect concentration (LOEC).

**Table 1A**

Effects of GO on the survivability of Japanese medaka adults after GO injection.

Groups	Controls Mean±SD; n=7	25 µg/g Mean±SD; n=2	50 µg/g Mean±SD; n=2	100 µg/g Mean±SD n=2	200 µg/g Mean±SD; n=4
Males	85.00±22.54	75.00±35.35	50.00±35.35	87.50±17.68	30.00±31.89*
Females	96.43±9.45	87.50±17.67	100.00±0.00	87.50 ±17.68	75.00±20.41
Breeding pairs	85.00±22.55	62.50±53.03	50.00±35.35	87.50±17.67	17.50±11.90*

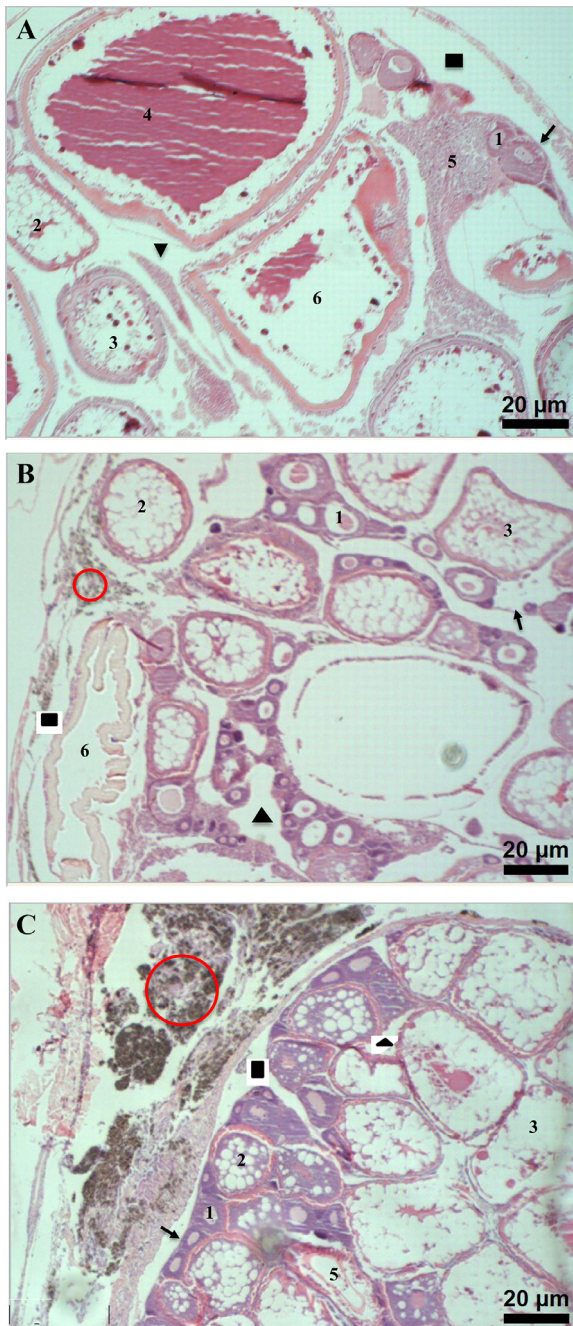
- The information on EDC activities of GO generated from this research will also be useful for OECD for setting guidelines for regulating the disposal of GO-containing compounds in the environment, as well as to determine its toxic potency to fish relative to other nanomaterials.
- Although limited in scope, the data generated from this research provide new insights into the toxicological and EDC effects of GO in medaka fish, and hence, contribute to the scientific knowledge of the potential environmental impacts of nanomaterials.

## 1. Data Description

The GO synthesized in the laboratory were characterized by using microscopic and spectroscopic procedures available at the Electron microscopy Core laboratory, Research Centers in Minority Institutions (RCMI), Center for Environmental Health, Jackson State University, Jackson, MS, USA (<https://www.jsums.edu/rcmi/electron-microscopy/>) and also at the Department of Chemistry, Physics, and Atmospheric Sciences, Jackson State University, Jackson, MS, USA ([www.jsums.edu/chemistry](http://www.jsums.edu/chemistry); [www.jsums.edu/physics](http://www.jsums.edu/physics))<sup>1</sup>. The scanning electron microscopic (SEM) images indicate the presence of single or few layers graphene sheets with a morphology resembling a thin curtain. The observed surface area of synthesized GO sheets in SEM images varied between one and few µm<sup>2</sup> and some wrinkles which were indicator of the presence of oxygen atoms as well as folding on edges were observed at several places. The transmission electron microscopy (TEM) images showed dark areas which indicate thick stacking nanostructure of several GO layers with the presence of oxygen functional groups. Areas of higher transparency in TEM images indicate much thinner films of a few layer of GO, resulted from stacking nanostructure exfoliation. FTIR analysis identified several functional groups such as OH (broad range from 3450 to 3250 cm<sup>-1</sup>), C=C, C-O attached to the GO sheets. Atomic force microscopy (AFM) analysis indicate that synthesized GO consists of few stacked layers. Typical measurement at two positions of the AFM analyzed data indicate the number of layers varied between 23 and 35 and the height of those layers ranged from 28 to 43 nm. Finally, the energy dispersive X ray spectroscopy (EDX) analysis of the synthesized GO indicate the ratio of carbon: oxygen is above 8. All these analysis are in agreement with the properties observed in GO by other investigators. Therefore, the GO we synthesized in the laboratory was used for the evaluation of reproductive and developmental effects on Japanese medaka fish (Tables 1-5; Figures 1- 4; ST1A-ST5).

The mortality of the fish was recorded 0–21 days post injections of both control (water) and GO-injected (25–200 µg/g, single ip injection; day of injection was considered as 0 day) fish. Each experiment was repeated at least twice and the percentages of survivability of the individual males, females, and as breeding pairs are presented in Table 1A as mean ±SD. The asterisk (\*) indicates significant difference ( $p < 0.05$ ) with corresponding control fish. In Table 1B the GO doses are log transformed (the corresponding log doses [#] are in parenthesis) and the median lethal dose (LD<sub>50</sub>) for mortality is analyzed by using a linear regression analysis program (GraphPad Prism, San Diego, CA). The percentage of mortality was converted to probability units (probit) [5]. Please see the supplementary Tables [ST1A and ST1B]

Fecundity (%) was calculated based on the total eggs laid by the female (total), and the eggs fertilized by the males (Fertilized) of each breeding pair relative to average total (fertilized+unfertilized) eggs (7 days) produced by the respective breeding pairs before injection. For total eggs, the control fish (vehicle-injected; n=21) on day 1 post-injection showed a significant



**Fig. 1.** Representative histopathological structure of the ovary of GO-injected fish (**Fig. 1A= Control; Fig. 1B, 1D, 1E, 1F, and 1G= 50 µg/g; 1C= 200 µg/g**) with different types of follicles, which are labelled by numerical numbers. 1= perinucleolar oocytes (PNO), 2= cortical alveolar oocytes (CAO), 3= early vitellogenic oocytes (EVO), 4= late vitellogenic oocytes (LVO), 5= post ovulatory, and 6= atretic follicles, are presented. Arrows (↖), squares (■), and triangles (▲) in black color indicate the position of the germinal epithelium, lumen, and extravascular spaces, respectively. The fish were sacrificed 21day after post-injection and histopathological evaluation was made on 5 µm thick sections stained in HE. The empty and filled arrows in black indicate the chorion and granulosa cells (GC), respectively. The oval shapes in red represent the accumulation of unidentified black particles (probably agglomerated GO or its metabolites) on the surface epithelial cells (granulosa cells) and initiate follicular atresia (**Fig. 1F**). However, the lack of agglomerated GO on the surface epithelium (granulosa) showed no evidence of atresia (**Fig. 1E**). When GO agglomerated inside the ovary and associated with the follicles, the shape and color (black) of the follicles gradually altered (**Fig. 3G**). (For interpretation of

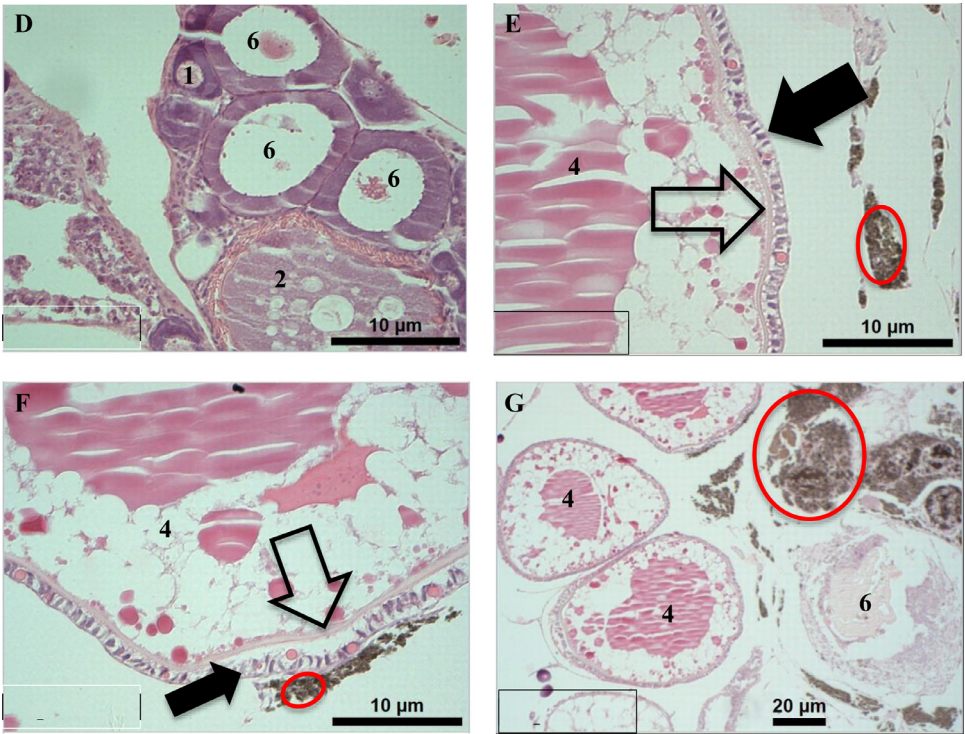


Fig. 1b. (Continued)

Table 1B

Conversion of percent mortality data to probability units (probits) for the determination of lethal doses (LD<sub>50</sub>).

Groups	Controls (0) n=7	25 µg/g (1.398)# n=2	50 µg/g (1.699)# n=2	100 µg/g (2.000)# n=2	200 µg/g (2.301)# n=4
Males	3.06	4.33	5.00	3.87	5.52
Females	3.25	3.92	2.67	3.87	4.33
Breeding pairs	3.96	4.69	5.00	3.87	5.95

Table 2

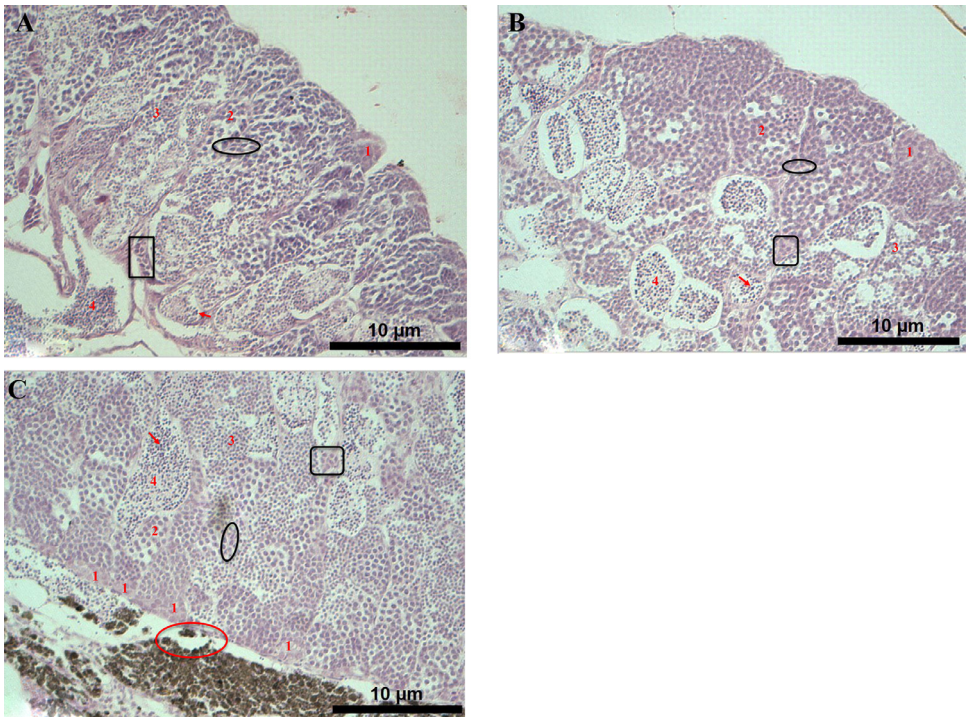
Effects of GO on the body weight of Japanese medaka.

Sex	Pre-injection	Post-injection Controls	25 µg/g	50 µg/g	100 µg/g	200 µg/g
Males	304.2±11.04 (n=46)	316.4±14.14 (n=22)	245.9±13.15 (n=7)	346.8±29.39 (n=4)	312±17.44 (n=7)	311.8±50.66 (n=5)
Females	348.3±11.83 (n=58)	351.5±12.51 (n=20)	319.8±22.84 (n=8)	433.8±33.0 (n=4)	327.5±14.96 (n=8)	491.2±40.81 (n=13) <sup>a, b</sup>

The body weight (mg) of the fish was recorded before and after 21 days post injections of both vehicle (water) and GO (25–200 µg/g, single ip injection). The data were expressed as means ±SEM;

<sup>a</sup> = *p*<0.05, compared to the pre-injected controls;

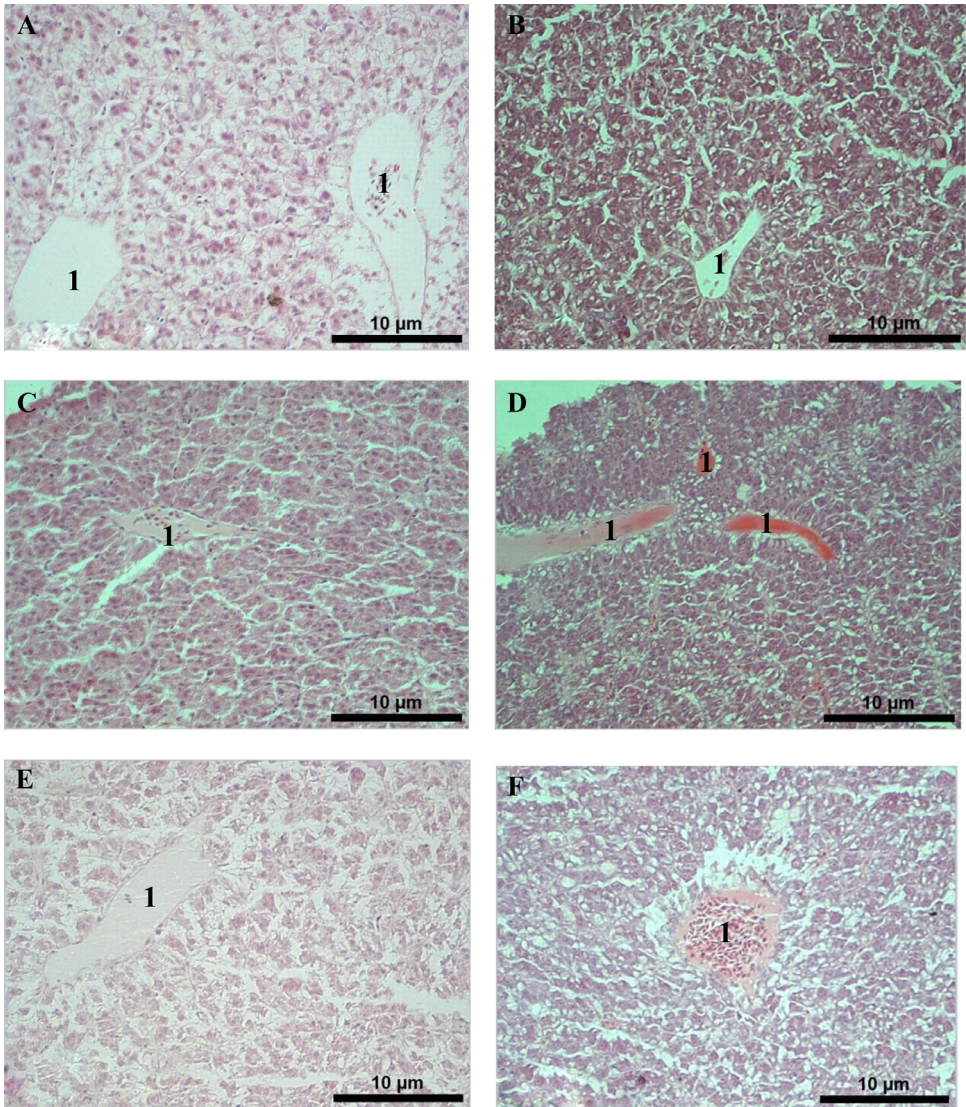
<sup>b</sup> = *p*<0.05, compared to post-injected controls. n= number of observations. [Please see the supplementary Tables ST2]



**Fig. 2.** Representative figure showing the effects of GO (2A= control [vehicle no GO]; 2B= GO 50 µg/g; 2C= GO 200 µg/g) on the testicular histopathology of Japanese medaka after 21 days post injection of GO. Paraffin cut sections were stained in HE. The oval shapes and the rectangular shapes in black indicate the Sertoli cells and Leydig cells, respectively. The germinal components of the testis are marked by numerical numbers (1) spermatogonia, (2) spermatocytes, (3) spermatids, (4) sperms. The arrow head in red indicate apoptotic cells (probably the sperm heads) in ED system of testis. The oval shape in red (Fig. 2C) indicates that GO was agglomerated on the germinal epithelium and induced spermatogonial cysts (1) in the testis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

difference ( $p < 0.05$ ) with control fish on days 3 and 4, and then days 11–21, continuously; no significant differences were observed in the fecundity of the control fish from days 2–21 post-injection days. The fish that received 25 µg/g GO ( $n = 5$ ) showed a significant difference ( $p < 0.05$ ) with day 1 fish only on day 18. Those fish that received 100 µg/g ( $n = 7$ ) showed a significant difference on day 18 with days 2, 3, 4, and 5. Those fish that received 50- and 200 µg/g GO ( $n = 4$  and 3, respectively) showed no significant differences with any day. For fertilized eggs, control fish fecundity (fertilized eggs) in post-injection day 1 showed a significant difference ( $p < 0.05$ ) with the fecundity of control fish from post-injection days 11–21; however, no significant difference was observed in fecundity (fertilized) of the control fish from days 2–21 post-injection days. In GO-treated fish, fecundity of 25 µg/g group on day 1 showed a significant difference ( $p < 0.05$ ) with post-injection days 17, 18 and 21. In 100 µg/g group post-injection days 2 and 3 showed a significant difference with days 17 and 18. The fish that received 50 and 200 µg/g did not show any significant difference with any of these days. [Please see the supplementary Tables ST3A and ST3B].

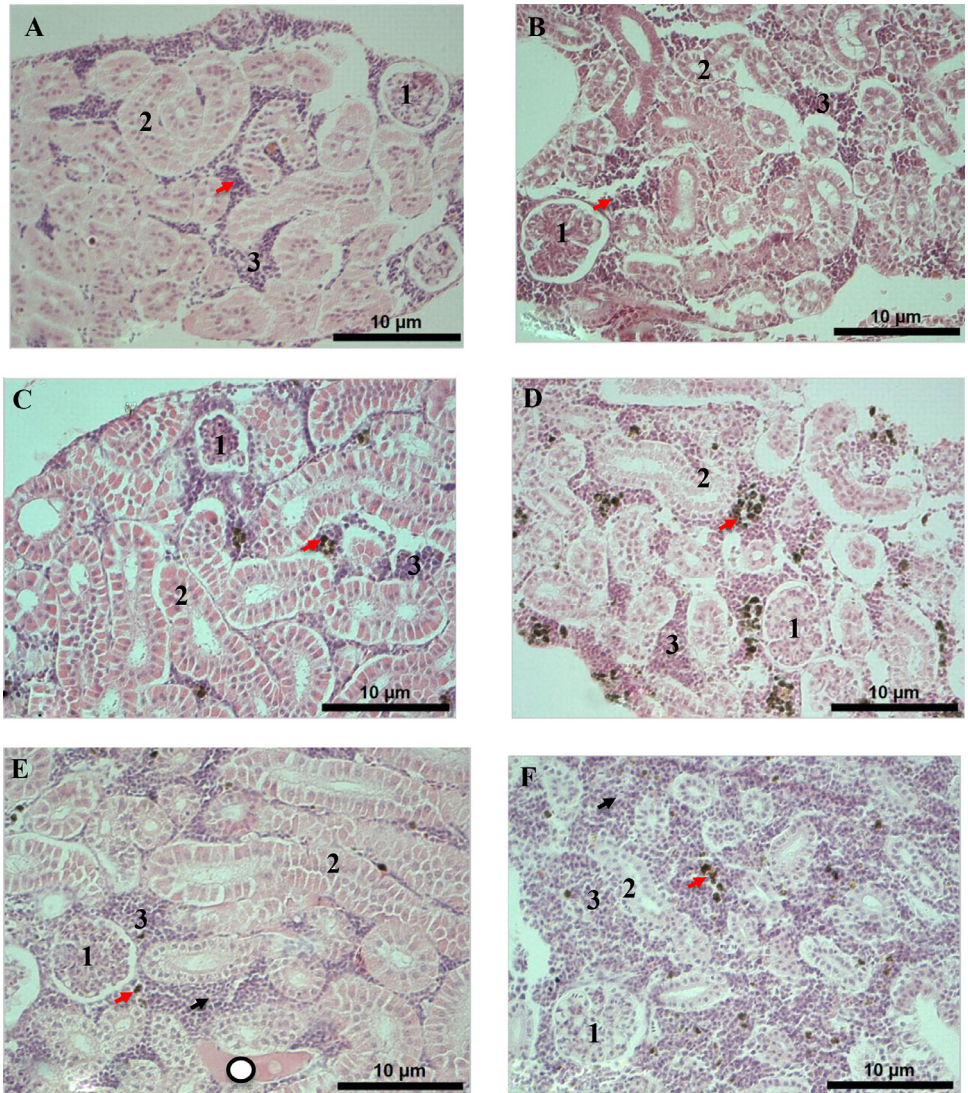
The asterisk (\*) on the data are significantly different ( $p < 0.05$ ) from the pre-injected fish and the pound symbol (#) indicates that the data are significantly different ( $p < 0.05$ ) from post-injected control fish. The fertility was evaluated during 21 days post-injection period as [the number of eggs fertilized by male fish/ average number of total eggs (fertilized+ unfertilized) laid by the female fish during preinjection period] X100. The hatchability of the embryos was



**Fig. 3.** Effects of GO on the liver Histopathology of Japanese Medaka. Representative sections presented above are from livers of medaka treated with vehicle (control, no GO, Fig. 3A, male fish, Fig. 3B, Female fish), 50 µg/g (Fig. 3C, male fish; Fig. 3D, female fish), 200 µg/g (Fig. 3E, male fish; Fig. 3F, female fish) sacrificed after 21day post-injection and histopathological evaluation were made on 5 µm thick sections stained in HE. 1= central vein. Fig. 3C and 3E (males) and 3D and 3F (females) showed accumulation of proteinaceous fluid in the hepatic vessels.

evaluated as number of embryos hatched during pre-injection (7 days) and post-injection (21 days) periods with normal swimming activity. Unhatched embryos; those were unable to come out from the chorion, either as a whole or a part of the body, on or before 14 dpf. The survivability of the unhatched embryos was evaluated by checking their heartbeats. Edema of the hatched embryos was evaluated on or before 14 dpf when the embryos were hatched completely and unable to swim due to the edema in yolk or heart. The embryos were considered incompletely hatched when the head remained inside the chorion and the tail or any other body parts re-





**Fig. 4.** Effects of GO on the kidney histopathology of Japanese Medaka. Representative sections of kidneys of medaka fish treated with vehicle (**Control, no GO, Fig. 4A, male fish, Fig. 4B, female fish**), 50 µg/g (**Fig. 4C, male fish; Fig. 4D, female fish**), 200 µg/g (**Fig. 4E, male fish; Fig. 4F, female fish**) are presented. GO was administered ip to both male and female medaka and histopathological observations on kidneys are made 21 day post-injection (HE staining). The components of kidneys are marked in numerical numbers. 1= glomerulus; 2= renal tubules; 3= lymphomyeloid interstitial tissues. Arrows in red indicate melanomacrophage and melanomacrophage centers (MMCs) in the lymphomyeloid interstitial tissue. Accumulation of proteinaceous fluid observed in the renal blood vessels of male and female fish injected with GO (50 and 200 µg/g) compared to controls were observed after 21 days post-injection. Interstitial cell hyperplasia (HIL) in the kidney of a male (**Fig. 4E**) and female fish injected with 200 µg/g GO was observed. HIL marked by black arrow. Accumulation of proteinaceous fluid in the in the lymphomyeloid tissue (black circle) was also observed in male fish injected with 200 µg/g GO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 3**  
Effects of GO on the Fecundity of Japanese medaka.

Days (post-injection)	Controls (n=21)		25 µg/g (n=5)		50 µg/g (n=4)		100 µg/g (n=7)		200 µg/g (n=3)	
	(total eggs)	(fertilized eggs)	(total eggs)	(fertilized eggs)	(total eggs)	fertilized eggs	total eggs	fertilized eggs	total eggs	fertilized eggs
1	26.70 ±8.02	21.40 ±7.63	29.30 ±13.72	10.76 ±7.38	69.97 ±31.01	63.39 ±34.54	23.22 ±9.77	23.20 ±9.78	54.78 ±41.76	57.48 ±57.48
2	62.74 ±9.99	53.72 ±10.62	62.07 ±18.93	37.42 ±15.78	29.43 ±15.57	22.85 ±14.35	8.62 ±7.09	8.63 ±7.09	7.20 ±4.72	0
3	69.69 ±9.95	54.40 ±10.24	56.26 ±16.92	39.87 ±18.49	67.26 ±21.64	60.27 ±22.89	15.76 ±10.40	15.82 ±10.45	1.83 ±1.84	0
4	69.14 ±8.24	61.24 ±9.28	77.66 ±8.13	69.15 ±12.46	80.68 ±11.07	80.68 ±13.26	17.86 ±16.25	17.98 ±16.37	7.35 ±7.35	0
5	64.64 ±8.30	58.07 ±9.02	92.21 ±9.22	92.57 ±9.47	72.06 ±15.16	65.31 ±18.49	18.43 ±16.16	18.55 ±16.27	28.11 ±14.10	3.38 ±3.38
6	63.89 ±8.53	60.16 ±9.21	74.173 ±6.21	74.27 ±5.54	73.04 ±11.34	72.38 ±12.74	23.58 ±10.58	23.65 ±10.63	22.44 ±17.23	10.14 ±10.14
7	61.98 ±8.04	56.99 ±8.73	76.80 ±4.86	69.89 ±1.65	55.03 ±18.83	45.03 ±14.74	37.87 ±17.61	37.99 ±17.69	94.74 ±45.29	89.29 ±44.88
8	64.66 ±8.24	56.59 ±7.87	62.60 ±13.00	60.64 ±15.23	69.68 ±14.77	52.01 ±16.03	46.49 ±18.50	46.47 ±18.53	61.73 ±6.86	56.07 ±5.85
9	53.53 ±8.26	54.67 ±8.26	81.28 ±9.05	78.15 ±6.51	48.29 ±19.01	40.51 ±21.76	56.29 ±21.64	56.25 ±21.63	62.70 ±5.11	47.90 ±10.20
10	58.18 ±6.79	55.63 ±7.45	90.38 ±7.54	89.98 ±8.97	39.76 ±17.67	38.98 ±18.96	71.75 ±19.44	69.02 ±22.11	73.13 ±32.80	61.10 ±25.57
11	67.94 ±6.89	66.48 ±7.32	88.65 ±4.84	86.95 ±4.65	101.78 ±32.45	107.70 ±39.98	58.16 ±16.42	58.14 ±16.46	80.39 ±7.14	69.17 ±10.58
12	73.64 ±5.96	71.39 ±6.78	90.21 ±12.05	86.29 ±15.26	40.01 ±14.25	42.90 ±15.42	61.31 ±13.55	61.23 ±13.51	35.16 ±20.93	37.84 ±27.49
13	78.99 ±5.31	74.10 ±6.19	86.16 ±6.74	85.60 ±7.77	19.47 ±10.23	20.62 ±12.67	63.06 ±10.68	63.08 ±10.74	48.72 ±27.87	44.92 ±29.85
14	74.38 ±7.43	76.76 ±7.07	93.20 ±3.65	93.69 ±3.79	41.00 ±11.79	21.14 ±15.28	66.72 ±15.32	67.26 ±15.45	51.40 ±20.20	47.58 ±18.46
15	73.62 ±6.15	71.71 ±6.93	93.78 ±4.51	83.55 ±2.30	36.43 ±15.63	33.78 ±16.90	65.77 ±13.00	65.68 ±12.95	59.29 ±10.15	50.06 ±6.22
16	84.82 ±7.69	84.01 ±7.15	88.41 ±6.90	93.54 ±6.57	39.00 ±12.79	33.12 ±17.44	69.87 ±18.82	71.09 ±18.95	34.07 ±2.27	17.89 ±9.34
17	73.52 ±9.21	80.86 ±8.95	83.96 ±14.06	109.04 ±11.49	42.22 ±23.86	46.33 ±24.78	60.24 ±12.35	89.94 ±22.97	38.83 ±21.07	41.03 ±22.77
18	83.73 ±8.97	79.53 ±9.22	113.24 ±12.27	109.04 ±11.49	45.86 ±22.67	47.71 ±25.28	89.99 ±22.97	89.94 ±22.97	58.03 ±25.84	50.29 ±25.21
19	82.26 ±6.33	74.17 ±8.11	92.84 ±10.95	91.80 ±12.59	59.42 ±24.45	61.58 ±28.41	75.98 ±12.96	75.92 ±12.93	46.03 ±19.52	40.88 ±24.07
20	81.93 ±8.15	81.30 ±8.12	89.97 ±10.21	90.59 ±10.30	62.92 ±26.27	63.78 ±29.82	72.88 ±15.46	76.48 ±15.95	32.92 ±25.31	20.37 ±20.37
21	68.26 ±9.24	68.73 ±9.32	102.82 ±6.99	103.30 ±6.89	45.99 ±24.20	47.46 ±24.73	73.42 ±16.24	69.78 ±15.57	30.24 ±26.33	24.07 ±24.07

mained outside the chorion. The dead embryos were evaluated as embryos remained inside or outside the chorion with no heartbeats. (Please see the supplementary Tables ST4A, ST4B, ST4C, ST4D, and ST4E).

**2. Experimental design, materials and methods**

*2.1. Synthesis of Graphene Oxide (GO)*

The GO used in the experiments was either synthesized in the laboratory, or obtained from commercial source (Sigma-Aldrich, St. Louis, MO). The synthesis of GO in the laboratory was

**Table 4**

Effects of GO on fertility and embryonic development of Japanese medaka.

Parameters investigated	pre-injection	post-injections				
		Controls	graphene oxide ( $\mu\text{g/g}$ )		100	200
			25	50		
Fertility	96.7 $\pm$ 0.65 (n=45)	86.84 $\pm$ 2.69* (n=26)	93.12 $\pm$ 2.21 (n=5)	82.13 $\pm$ 7.97* (n=4)	90.58 $\pm$ 1.82 (n=7)	75.79 $\pm$ 8.69* (n=3)
Hatchability	60.79 $\pm$ 2.63 (n=40)	56.54 $\pm$ 3.82 (n=22)	60.09 $\pm$ 8.73 (n=5)	54.88 $\pm$ 11.41 (n=4)	54.06 $\pm$ 9.86 (n=6)	24.04 $\pm$ 7.78*# (n=3)
Unhatched	13.45 $\pm$ 1.88 (n=36)	23.95 $\pm$ 4.31 (n=19)	18.99 $\pm$ 10.06 (n=5)	19.66 $\pm$ 9.80 (n=3)	32.39 $\pm$ 8.56 (n=7)	62.52 $\pm$ 17.97 (n=2)
Edema	7.53 $\pm$ 0.83 (n=36)	5.23 $\pm$ 0.80 (n=19)	5.70 $\pm$ 2.32 (n=5)	4.60 $\pm$ 1.11 (n=3)	6.80 $\pm$ 2.07 (n=7)	2.97 $\pm$ 2.97 (n=2)
Incomplete hatching	3.27 $\pm$ 0.71 (n=36)	1.68 $\pm$ 0.52 (n=19)	7.27 $\pm$ 1.49# (n=5)	1.62 $\pm$ 0.59 (n=3)	2.03 $\pm$ 0.90 (n=7)	1.10 $\pm$ 0.11 (n=2)
Dead embryos	13.12 $\pm$ 2.21 (n=36)	11.61 $\pm$ 2.62 (n=19)	9.20 $\pm$ 3.17 (n=5)	6.68 $\pm$ 0.84 (n=3)	7.72 $\pm$ 1.95 (n=7)	11.46 $\pm$ 5.37 (n=2)

**Table 5**

Follicular distribution of differentiating and degenerating follicles in the stromal compartments of the ovary of Japanese medaka fish.

Follicular types		Control	graphene oxide ( $\mu\text{g/g}$ )		100	200
		(n=28)	25 (n=20)	50 (n=16)	(n=28)	(n=12)
Differentiating	perinucleolar	65.41 $\pm$ 2.02	59.11 $\pm$ 2.60	51.55 $\pm$ 2.25 <sup>a</sup>	65.09 $\pm$ 2.57 <sup>a</sup>	62.22 $\pm$ 2.81
	Cortical	21.06 $\pm$ 1.22	26.53 $\pm$ 1.92	28.92 $\pm$ 1.87	24.68 $\pm$ 2.04	26.18 $\pm$ 2.19
	alveolar	6.68 $\pm$ 0.95	8.15 $\pm$ 1.21	8.13 $\pm$ 1.56	5.49 $\pm$ 0.65	5.75 $\pm$ 1.05
	vitellogenic	6.55 $\pm$ 0.85	6.16 $\pm$ 1.12	11.39 $\pm$ 1.70	5.72 $\pm$ 0.81	5.84 $\pm$ 1.45
Degenerating	late vitellogenic	9.98 $\pm$ 1.03	15.16 $\pm$ 2.48	21.99 $\pm$ 2.33 <sup>a</sup>	11.04 $\pm$ 1.39 <sup>a</sup>	17.92 $\pm$ 3.44
	Ratio (degenerating: differentiating)	0.11 $\pm$ 0.01	0.22 $\pm$ 0.05	0.30 $\pm$ 0.04 <sup>a</sup>	0.13 $\pm$ 0.02 <sup>a</sup>	0.25 $\pm$ 0.06

The data are expressed as means  $\pm$ SEM of the % distribution of the differentiating and degenerating follicles found in the stromal compartments of the ovary. n= number of observations.

<sup>a</sup> indicates that the data are significantly different ( $p < 0.05$ ) compared to corresponding controls. The following formulas were used during the calculation of stromal follicles:

(i) Any differencing stromal follicles in % = [number of that particular follicle/ sum of differentiating follicles] \* 100; where any follicle indicates either PNO, CAO, LVO or EVO.

(ii) Degenerating follicles in % = [(sum of atretic + post ovulatory follicles)/(sum of differentiating + degenerating follicles)] \* 100

made following modified Hummer's Method [1,2] from natural graphite powder. A mixture containing 0.501 g of graphite (Sigma-Aldrich, St. Louis, MO), 0.503 g of sodium nitrate and 23 ml of concentrated  $\text{H}_2\text{SO}_4$  (98%) (Sigma-Aldrich, St. Louis, MO) was added in a beaker and stirred for 20 min. After stirring, 3.007 g  $\text{KMnO}_4$  along with 3 mL  $\text{H}_2\text{SO}_4$  was added each time (approximately 30 min for 3 times and 60 min for 2 times) to the mixture (total 5 times) followed by the addition of 60 ml nanopure water. Finally 3 mL of  $\text{H}_2\text{O}_2$  (30%) (Sigma-Aldrich, St. Louis, MO) was added to the mixture until no gas was generated. Centrifugation (8500 rpm for 2 h) was carried out four times until the pH reached near neutral. Finally, GO was lyophilized to dryness and different physical properties were evaluated by standard techniques.

GO, used for ip injection, either synthesized in the laboratory or obtained from commercial source, was dissolved to desired concentration (1-2 mg/mL) in nanopure water and sonicated for 5 min (2s on-1s off pulse, 225 W) by a probe sonicator (ultrasonicator LPX 750, Cole Parmer,

Chicago, IL, USA). The particle diameter (zeta size  $154.8 \pm 40.32$  d.nm) and zeta potential (-40.7) of the synthesized GO after sonication were determined by Zeta Sizer Nano ZS (Malvern Instrument, MA, USA)

## 2.2. Culture and maintenance of Japanese medaka fish

Adult Japanese medaka fish (orange red variety), initially obtained from the University of Mississippi, University, Mississippi, USA, by a protocol transfer agreement, and a breeding colony was set up at the Jackson State University (JSU), Jackson, MS, USA in the Plant Science building. The adult fish (6 females and 4 males) were maintained in 40 L tanks in 25 L balanced salt solution (BSS, 17 mM NaCl, 0.4 mM KCl, 0.3 mM MgSO<sub>4</sub>, 0.3 mM CaCl<sub>2</sub>, pH was adjusted to 7.4 by adding required amount of NaHCO<sub>3</sub> at  $25 \pm 1$  ° C with 16L: 8D light cycle) and were used as breeders. The recirculated medium passed through disposable bio and carbon filters. The BSS was changed every two- three weeks, depending on pH (7.0–8.5) and ammonia concentration (1–3 ppm). The fish bred successfully in this environment and the eggs were generally collected within 1–3 h after the light was turned on. Sexually mature and reproductively active Japanese medaka fish (one male and one female) were maintained for one week 500 mL BSS at 25 °C, with 16 L: 8D light cycle, in 1 L glass jars. The medium was changed every day. After one week of acclimatization, when both male and female fish were breeding successfully (evaluated by counting the fertilized eggs collected), the fish were selected for experimental use. One week prior to GO (25, 50, 100, and 200 µg/g) and vehicle (water) injections, the eggs were collected from each individual pair (one male and one female), separated from the clutch, and counted. The fertilized eggs were allowed to hatch spontaneously until 14 days post fertilization (dpf) in 20 mL glass vials containing 10 mL embryo rearing medium (ERM, 17 mM NaCl, 0.4 mM KCl, 0.6 mM MgSO<sub>4</sub>, 0.36 mM CaCl<sub>2</sub>, pH 7.4). The ERM was replaced every day after 8 dpf. In our facility, the fertilized medaka eggs are initiated hatching on 8 dpf. All the fertilized eggs are unable to hatch at the same time; we therefore maintained the eggs until 14 dpf and those, which were unable to hatch after 14 dpf, even though heartbeats found to be normal, were considered as unhatched (Table 4, ST4C). During culture (0–14 dpf), the dead (Table 4, ST4F) and hatched embryos (Table 4, ST4B) were removed from the culture vials. After one successful week of egg collection, GO was injected ip to the fish (both males and females) and the controls (both males and females) were injected with nanopure water; the maximum volume of the injected material is 1 µL/10 mg body weight (volume was adjusted to nearest 10 mg). Other than synthesized GO, a few pairs (4 pairs for both 50 and 100 µg/g and 9 pairs for 200 µg/g doses) were injected with GO obtained from commercial source (Sigma-Aldrich, St Louis, MO) and the data collected were pooled with the data generated from the fish injected with GO, synthesized in the laboratory. To avoid movement during injection, the fish were anesthetized in MS 222 (Sigma-Aldrich, St. Louis, MO), 100 mg/L in BSS [6], and after injection, returned to regular BSS for quick recovery. When the fish recovered completely from anesthesia (judged from normal swimming behavior), both male and female fish are returned to the respective tanks for continuation of breeding (same partners). With these conditions, not all injected fish survived 21 days post-injections (Table 1A, ST1A). The mortality data were analyzed by two way ANOVA followed by post-hoc Tukey's multiple comparison test. The mortality of individual males, or as a breeding pair injected with 200 µg/g GO showed significant difference ( $p < 0.05$ ) only with controls (Table 1A). The mortality data were further analyzed to determine the median lethal dose (LD<sub>50</sub>) of GO required for 50% mortality of the fish (Table 1B, ST1B) by probit (probability) analysis [5]. The linear regression analysis was made by using GraphPad Prism, version 7.1, San Diego, CA. Although the data showed very low  $r^2$  values, with this limited data, the calculated LD<sub>50</sub> for males are 175.39 µg/g ( $r^2=0.19$ ;  $Sy.x=0.80$ ;  $Y=0.8106X+3.181$ ), females are 2901.2 µg/g ( $r^2=0.19$ ;  $Sy.x=0.78$ ;  $Y=0.8172X+2.204$ ), and as breeders are 110.75 µg/g ( $r^2=0.19$ ;  $Sy.x=0.96$ ;  $Y=0.88X+3.249$ ) [5].

The egg collections were continued in control and GO-injected fish during 21 days post-injections every day (7 days before injection and 21 days after injection; total 28 days), counted,

checked for fertility, separated from the clutch and the fertilized eggs are allowed for spontaneous hatching in a 20 mL scintillation vial containing 10 mL ERM. Some of the breeding pairs lost their partners (male or female) during the experimental period; however, the surviving females (no males) laid unfertilized eggs occasionally. These unfertilized eggs were counted after collection, however, excluded during data analysis (only surviving breeding pairs are considered in data analysis). The hatched embryos (larvae; stage 40 [7]) were used for morphological evaluation (complete or incomplete hatching, edema: both cardiac and yolk sac edema were considered as edema). Daily fecundity in post-injection periods of each breeding pair were evaluated (Table 3; ST3A, ST3B) as percent eggs (either as total considering both fertilized and unfertilized eggs or only fertilized eggs, excluding the unfertilized eggs from the count) considering the average number of eggs (average of 7 days) laid during pre-injection periods as 100, keeping the breeding pairs constant (same breeding pair). The following formula used during fecundity calculation.

- (i) Daily fecundity = (number of eggs produced by a breeding pair per day / average number of total eggs produced by the same breeding pair during pre-injection periods) \* 100, where the number of eggs represent either total eggs (both fertilized+ unfertilized) or only fertilized eggs.


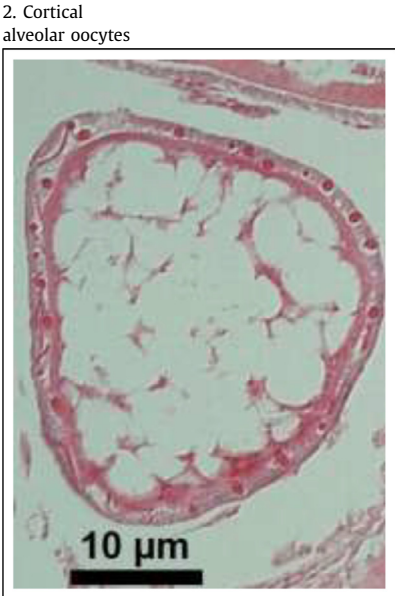
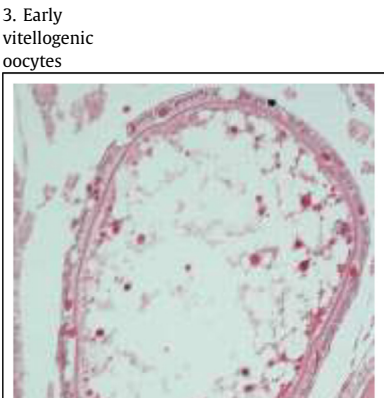
### 2.3. Histopathological evaluation of gonads, liver and kidney

After 21 days post-injection, surviving fish were anesthetized in MS222, weighed to the nearest mg, and fixed in 4% paraformaldehyde (PFA) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) excluding the post-anal tail. The bodies were quickly cut into three small pieces before fixation. All tissues dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin (60 °C). Serial sections of 5- $\mu$ m thickness of the paraffinized tissues were cut in a manual rotary microtome (Olympus cut 4055) and the sections were stained in hematoxylin/eosin (HE) following standard protocol. The photomicrograph of the sections were taken in an Olympus B-max 40 microscope attached to a camera with Q-capture Pro 7 software (Media Cybernetics, Inc, Rockville, MD). The whole body sections that contain testis or ovary and the liver and kidney were used for histological evaluation following the guidelines published by OECD [3]. The distribution of ovarian follicles varied throughout in a given section as well as in the different regions of the ovary. To make it consistent, we have counted six types of follicles distributed in the stromal compartments of the ovary; four of them as differentiating follicles (perinucleolar oocytes, PNO; cortical alveolar oocytes, CAO; early vitellogenic oocytes, EVO; and late vitellogenic oocytes, LVO) and two of them (atretic and postovulatory) as degenerating follicles (Table 6). We considered four different regions of the ovary (anterior, upper middle, lower middle and anal part) for oocyte count. We counted all these follicles (maturing and degenerating) in one whole section (5  $\mu$ m thick) and the PNO, CAO, EVO, and LVO were expressed as percent (%) of total differentiating follicles distributed in that particular section (stromal compartment) of the ovary. The degenerating follicles (sum of atretic and postovulatory follicles) were expressed as (%) of total follicles (differentiating and degenerating) found in the stromal compartment of that particular section. The ratio of degenerating and differentiating follicles found in that particular section of the ovary were also determined (Table 5, ST5). The specific features of these differentiating and degenerating follicles were listed in Table 6.

Although among 21 surviving breeding pairs as controls, we used only 7 pairs for histological analysis. However, all the surviving breeding pairs of 25–200  $\mu$ g/g groups (5 pairs for 25  $\mu$ g/g, 4 pairs for 50  $\mu$ g/g, 7 pairs for 100  $\mu$ g/g, and 3 pairs for 200  $\mu$ g/g) were used for histological analysis. For testis, the visual assessment of the gametogenic precursors (Spermatogonia, spermatocytes and spermatids), mature gametocytes (sperms), the Sertoli cells and Leydig cells, were made on HE stained slides. For the liver, observations were mainly focused on hepatocytes distributed in the central vein regions and for kidneys in glomerulus, renal tubules, and interstitial tissues associated to glomerulus regions.

**Table 6**

Specific features considered for histological examination of the different types of follicles distributed in the stromal compartments of the ovary of Japanese medaka [3]:

Stage	follicles	features
Differentiating	1. Perinucleolar oocyte	(i) Nucleus increases in size with multiple nucleoli, generally at the periphery of the nucleus (ii) Basophilic cytoplasm (iii) Late perinucleolar oocytes may have small clear or amphophilic vacuoles in the cytoplasm
		(i) Appearance of the cortical alveoli (yolk vesicles) within the ooplasm. (ii) Chorion becomes distinctly evident (iii) Perifollicular cells are visualized
		(i) Centralized appearance of spherical, eosinophilic, vitellogenic yolk granules/globules (ii) Thick chorion separates the ooplasm from the perifollicular cells
		

## 2.4. Statistical analysis

Data were analyzed by either one- or two-way ANOVA followed by post-hoc Tukey's multiple comparison test and expressed as mean  $\pm$ SD or SEM (GraphPad prism version 7.04 software; GraphPad Prism, San Diego, CA);  $p < 0.05$  was considered as significant.

## Ethics Statement

No human subjects were involved in this research.

The Institutional Animal Care and Use Committee (IACUC) of the Jackson State University, Jackson, MS, approved all experimental animal protocols.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have, or could be perceived to have, influenced the work reported in this article.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.dib.2020.106091](https://doi.org/10.1016/j.dib.2020.106091).

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