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Assessment of reproductive and developmental effects of graphene oxide on Japanese medaka (*Oryzias latipes*).

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Abstract

Due to its unique properties, graphene oxide (GO) has potential for biomedical and electronic applications, however environmental contamination including aquatic ecosystem is inevitable. Moreover, potential risks of GO in aquatic life are inadequately explored. Present study was designed to evaluate GO as an endocrine disrupting chemical (EDC) using the model Japanese medaka (*Oryzias latipes*). GO was injected intraperitoneally (25–200 µg/g) once to breeding pairs and continued pair breeding an additional 21 days. Eggs laid were analyzed for fecundity and the fertilized eggs were evaluated for developmental abnormalities including hatching. Histopathological evaluation of gonads, liver, and kidneys was made 21 days post-injection. LD₅₀ was found to be sex-dependent. Fecundity tended to reduce in a dose-dependent manner during early post-injection days; however, the overall evaluation showed no significant difference. The hatchability of embryos was reduced significantly in the 200 µg/g group; edema (yolk and cardiovascular) and embryo-mortality remained unaltered. Histopathological assessment identified black particles, probably agglomerated GO, in the gonads of GO-treated fish. However, folliculogenesis in stromal compartments of ovary and the composition of germinal elements in testis remained almost unaltered. Moreover, granulosa and Leydig cells morphology did not indicate any significant EDC-related effects. Although liver and kidney histopathology did not show GO as an EDC, some GO-treated fish accumulated proteinaceous fluid in hepatic vessels and induced hyperplasia in interstitial lymphoid cells (HIL) located in kidneys. GO agglomerated in medaka gonads after 21-days post-injection. However, gonad histopathology including granulosa and Leydig cells alterations were associated with GO toxicity rather than EDC effects.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Figure 1. Experimental workflow and results of graphene oxide (GO) exposure in zebrafish.

The workflow illustrates the experimental design and outcomes. Male and female zebrafish were exposed to GO (24-200 µg/L) for 24 hours. Male fish were analyzed for testis and Leydig cells, while female fish were analyzed for ovaries and granulosa cells. The process continues through spermatogenesis, sperm, egg, and oogenesis, leading to external fertilization. A graph shows the percentage of embryos surviving to hatching over 100 hours for different GO concentrations. The results indicate no significant differences in gonad histomorphological changes, embryonic development, or hatching for the treated groups compared to the control.

EDC; Graphene oxide; Japanese medaka; gonads; liver; kidney

In recent years, graphene, a sp²-bonded carbon nanosheet, has been a promising biomaterial for diagnostic and therapeutic applications. The graphene family of nanomaterials including few-layer-graphene, ultrathin graphite, GO, reduced graphene oxide (rGO), graphene nanoplatelets, graphene nanosheets and others, varied in layer numbers, layer dimensions, surface chemistry, density or purity (Novoselov et al., 2012; Bianco, 2013; James and Tour, 2013). Because of their high water solubility (6.6 µg/mL in de-ionized water; Konios et al., 2014; Johnson et al., 2015), and use as precursors of chemically generating large-scale graphene structures, GO nanosheets have the potential to become a biomaterial for application both in vivo and in vitro models. As a graphene derivative, GO has a large two-dimensional plane, which provide ultrahigh, specific surface area to load drugs through surface adsorption, π - π interaction, hydrogen bonding, and others. GO is biocompatible and nontoxic which makes it a promising candidate for construction of drug carriers. However, several published reports indicate that GO is able to produce toxic effects in various animal models (reviewed by Ema et al., 2016) including fish (reviewed by Dasmahapatra et al., 2019). Moreover, even though GO could yield stable suspensions in water initially after being exfoliated into monolayer sheets, it may aggregate immediately in biomedica (blood or tissue fluids), especially after loading with anticancer drugs. Hence there is a need to investigate the toxic effects of GO considering all possible ways including various exposure routes, and to ensure that the methods used for its application have no toxic impact on animals (Kurantowicz et al., 2015).

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modifying the original toxicity of contaminants. Since fish occur in virtually all aquatic environments and play a major role in aquatic food webs, they are useful models for monitoring pollutants in aquatic environment. Small laboratory fish models, including zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), are widely used as experimental models for monitoring pollutants in the environment.

The toxicity of GO in fish, especially in zebrafish (*Danio rerio*) was found to be concentration-dependent (Bageppagari et al., 2019; Dasmahapatra et al., 2019; Yang et al., 2019), and did not affect embryonic development at lower concentrations. However, higher concentration showed significant impact on embryo mortality, hatching, and disorder in cardiovascularity (Bageppagari et al., 2019). Exposure of zebrafish embryos to GO for five consecutive days did not induce acute developmental toxicity, however, hatching and locomotor behavior were altered (Yang et al., 2019). Moreover, genes related to nervous and immune systems were upregulated (Yang et al., 2019). Proteomic analysis in zebrafish larvae identified down-regulation of several oxidative stress-related proteins by GO (Zou et al., 2018; Dasmahapatra et al., 2019). In tilapia fish, oral exposure of GO did not alter behavior, body weight, and the intestinal bacterial population after 30 days of treatment. However, the expression of several oxidative stress-related enzymes was down regulated significantly in liver, but remained unaltered in spleen, gill, intestine, and muscle (Ma et al. 2016). In *Geophagus iporangensis*, GO exposure decreased metabolic rate compared to control fish (Medeiros et al., 2019). Several oxidative stress-related enzymes were increased in the climbing perch (*Anabas testudineus*) after 24h of single intraperitoneal (ip) injection of GO (Paital et al., 2019).

Although in fish models, evaluation of GO was mainly focused on toxicological endpoints mediated probably by oxidative stress-related enzymes and pathways, the role played by GO as an EDC needs to be investigated. A recent report has indicated the potential of GO as a thyroid endocrine disruptor in tadpoles of *Xenopus laevis* (Li et al., 2019). Due to limited data, the effects of different nanoparticles on endocrine system need to be interpreted carefully (Iavicoli et al., 2013). Previously, our laboratory evaluated the toxic potential of GO administered orally to Sprague-Dawley rats using liver and kidney tissues. We observed that oral administration of higher doses of GO (20–40 mg/kg, five consecutive days) induced cellular damages in liver and kidneys as well as enhanced enzyme activities related to oxidative stress (Patlolla et al., 2016; 2017).

The aim of the present study was to assess the toxic potential of GO using Japanese medaka fish as an aquatic animal model. In addition, we also evaluated on the endocrine disrupting effects of GO targeting testis and ovary. Leydig cells in the testis produced male hormones (testosterone) while the perfollicular cells, especially theca and granulosa cells, in the ovary of medaka are responsible for female hormones (estrogen). This fish species is a recognized model to evaluate EDCs in the environment and several Test Guidelines (TG) are included in publications made by Organization for Economic Cooperation and Development (OECD, 2018). We used sexually mature, reproductively active male and female intact Japanese medaka fish maintained in glass aquaria under standard laboratory conditions (25±1°C; 16L: 8D light cycle). We focused on fecundity and histopathology of gonads (testis and ovary) as toxicological and reproductive (endocrine) endpoints 21 days after single ip injection of GO

(25–200 µg/g). In fish, fecundity can be characterized by a wide spectrum of disorders including endogenous hormone levels, activities of gonads, as well as the behavior of the fish during mating and external fertilization. However, fecundity in medaka is highly variable and reproducibility is questionable (Hutchinson et al., 2006) even though high concentrations of EDCs such as 17 alpha-ethinylestradiol (EE2) or 17 beta-trenbolone (TB) significantly reduced fecundity (Park et al., 2009). Moreover, in addition to histopathology of gonads, and because of the significant role played by liver and kidneys in fish reproduction (OECD, 2010, 2018), we conducted histopathological evaluation of these two organs focusing on EDC effects. To our knowledge, the toxicity and EDC effects of GO in reproductively active Japanese medaka adults remained unexplored.

2. Materials and Methods:

2.1. Synthesis of Graphene Oxide (GO):

The GO we used in the experiments was synthesized either in the laboratory, or obtained from a commercial source (Sigma-Aldrich, St. Louis, MO). The synthesis of GO in the laboratory from natural graphite powder was made by following modified Hummer's Method (Hummers and Offeman, 1958; Viraka Nellore et al., 2015). For details of synthesis, please see the supplementary data (Supplementary Figure (SF) 1A–B, SF2A–C; Supplementary Table 1).

Before ip injection, GO 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 and zeta potential of the synthesized GO after sonication were determined by Zeta Sizer Nano ZS (Malvern Instrument, MA, USA) (Supplementary Table 1).

2.2. Culture and maintenance of Japanese medaka fish.

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

Adult Japanese medaka fish (orange red variety), obtained from the University of Mississippi, Oxford, Mississippi, USA, by a protocol transfer agreement, and a breeding colony was set up at the Jackson State University, Jackson, MS, USA. For details of fish culture, please see the supplementary data file. Briefly, adult fish (6 females and 4 males) used as breeders were maintained in 25 L balanced salt solution (BSS, pH7.4) in glass tanks under standard laboratory conditions. The collection of eggs was generally made within 1–3 h after the light was turned on.

For GO experiments, sexually mature and reproductively active fish (one male and one female) were maintained for one week in 500 mL BSS in 1L glass jars. One week prior to GO (25, 50, 100, and 200 µg/g) and vehicle (water) injections, the eggs were collected from each individual pair, and the fertilized eggs cultured in embryo-rearing medium (ERM, pH 7.4). The embryos were allowed to hatch spontaneously until 14 days post fertilization (dpf) in 20 mL glass vials containing 10 mL ERM. 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 (1 µL/10 mg body weight). Some fish (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 and the collected data were pooled with the data generated from the fish injected with GO synthesized in the laboratory. After injection of either GO or vehicle, both male and female fish were returned to the respective tanks for continuation of breeding (same partners). The fish mortality data (died within 21 days post-injection period) 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 a significant difference ($p < 0.05$) only with controls (Figure SF3A). Although the r^2 is low, LD₅₀ was calculated by probit analysis (Finney 1971) which showed males are more GO-sensitive than females (Figure SF3B).

The egg collection was continued in control and GO-injected fish during 21 days post-injections days (21 days after injection), and the fertilized eggs were allowed for spontaneous hatching in ERM. The hatched embryos (larvae; stage 40; Iwamatsu, 2004) were used for morphological evaluation (complete or incomplete hatching, edema: both cardiac and yolk sac edema were considered as edema). The daily fecundity either as total eggs (both fertilized and unfertilized eggs) or as fertilized eggs during post-injection periods of each breeding pair was calculated by using the following formula:

$$\text{Daily fecundity} = \left(\frac{\text{number of eggs produced by a breeding pair}}{\text{average number of total eggs produced by the same breeding pair during pre-injection periods}} \right) * 100. \quad (i)$$

[The number of eggs indicates either total eggs (fertilized+ unfertilized) or only fertilized eggs].

2.3. Histopathological evaluation:

After 21 days post-injection, surviving fish were anesthetized in MS222 (Readmann et al., 2017), weighed to the nearest mg, and quickly cut into three small pieces excluding post anal tail and fixed in 4% paraformaldehyde (PFA) containing 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). For detail of histopathological evaluation, please see supplementary data file. Briefly, all tissues were paraffinized by following standard procedure and the serial sections of 5-µm thickness were cut in a manual rotary microtome (Olympus cut 4055). The sections were stained in hematoxylin/eosin (HE), following standard protocol. Some of the sections (testis) were stained in Masson's trichrome stain (Sigma-Aldrich, St. Louis, MO). The photomicrographs of the sections were taken either in an Olympus B-max 40 microscope attached to a camera with Q-capture Pro 7 software (Media Cybernetics, Inc, Rockville, MD) or in Nikon Eclipse 50i microscope attached to Nikon DS-Fi1 camera (Nikon Inc, Melville, NY). Those sections of the testis, ovary, liver or kidney were used for histopathological evaluation following OECD guidelines (OECD, 2010). Six types of follicles distributed in four different regions of the ovary (anterior, upper middle, lower middle and anal) in stromal compartments were considered for counting; 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. The specific features of these follicles

(differentiating and degenerating) are listed in Supplementary Table 2. The following formula are used for calculation (%) of the distribution of different types of differentiating and degenerating follicles in a particular section (5 µm thick) cut through in any of the four different regions (anterior, upper middle, lower middle, and anal) of the ovary.

$$\text{Any differentiating stromal follicles in \%} = \left[\frac{\text{number of that particular follicle}}{\text{sum of differentiating follicles}} \right] * 100; \text{ where any follicle indicates either PNO, CAO, LVO or EVO.} \quad (\text{ii})$$

$$\text{Degenerating follicles in \%} = \left[\frac{(\text{sum of atretic} + \text{post ovulatory follicles})}{(\text{sum of differentiating} + \text{degenerating follicles})} \right] * 100 \quad (\text{iii})$$

The ratio of degenerating and differentiating (degenerating: differentiating) follicles found in that particular section of the ovary were also determined.

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 µg/g groups (5 pairs for 25 µg/g, 4 pairs for 50 µg/g, 7 pairs for 100 µg/g, and 3 pairs for 200 µg/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, was made on HE stained slides. In addition, for careful observations of Sertoli cells and Leydig cells, Masson-Trichrome (MT)-stain (Sigma-Aldrich, St. Louis, MO) was used. For liver, the observations were mainly focused on hepatocytes distributed in the central vein regions, while for the kidneys, they were focused on the glomerulus, renal tubules, and interstitial tissues associated to glomerulus regions.

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 ± SD or SEM (GraphPad prism version 7.04 software; GraphPad Prism, San Diego, CA); $p < 0.05$ was considered as significant.

3. Results

3.1. Characterization of the GO synthesized in the laboratory:

We synthesized GO using the standard protocol (Hummers and Offeman, 1958). After purification, synthesized GO was extensively characterized using several microscopic and spectroscopic procedures. (For details, please see the Supplementary Figures, SF1A–B, SF2A–C).

3.2. Evaluation of the reproductive activity:

The physical conditions we provided during the experimental period allowed both control and GO-treated fish to maintain normal growth (Figure SF4), successful breeding, and laying eggs (both fertilized and unfertilized) into the environment (Figures 1A–B). The body weight of the fish remained unaltered except for the females of 200 µg/g group where it was increased significantly ($p < 0.05$) compared to the fish in pre- and post-injected vehicle-treated (control) fish (Figure SF4).

Reproductive activities of the experimental fish were evaluated as % eggs laid into the external environment by the female (total eggs) or the laid eggs (%) fertilized by the males (fertilized eggs) of each breeding pair (external fertilization) per day before and after GO or vehicle (water) administration (Figures 1A–B). Further, the fertilized eggs were evaluated for mortality, hatchability (complete and incomplete), and the hatched larvae were evaluated for edema (either yolk or cardiac or both) (Figures SF5A–F). Our data indicate that the breeding pairs surviving during the entire period of experiment (7-days before injection and 21-days after injection), were able to breed successfully and the calculated fecundity (%) during post-injection periods were initially low and then gradually returned to the status (both as total and fertilized) observed in pre-injection days. The data further indicate that in controls, and fish that received lower doses of GO (25 and 50 µg/g), steady-state increase (both total and fertilized eggs) was observed after 1 day post-injection. While higher doses (100 and 200 µg/g) required at least one week or longer to achieve the steady-state fecundity (Figures 1A–B). Further analysis of the data showed that the fecundity observed as total eggs in vehicle-injected control fish (n=21), on day 1 post-injection, showed a significant difference with the control fish on post-injection days 3 and 4 and continuously from 11–21 days ($p<0.05$). No significant difference was observed in total fecundity in control fish in 2–21 post-injection days (Figure 1A). Moreover, the total fecundity in day 1 post-injected fish received 25 µg/g GO (n=5) established significant difference with the same group only on day 18 post-injection ($p<0.05$). However, those fish that received 100 µg/g GO were significantly different with days 2, 3, and 4 fish only on post-injection day 18 ($p<0.05$). The total fecundity of the fish that received 50 µg/g (n=4) or 200 µg/g (n=3) GO, maintained equal status in post-injection days 1–21 (not significantly different in any days).

The fecundity data (%) when expressed as fertilized eggs remained almost identical with the data expressed as total eggs (Figure 1B). In controls (n=21), fecundity as fertilized eggs on day 1 showed significant difference with the fecundity (fertilized) of the control fish 11–21 days post-injection ($p<0.5$). Like total fecundity, in controls, the fecundity as fertilized eggs remained unaltered between post-injection days 2–21. In GO-treated fish, the fecundity as fertilized eggs of 25 µg/g group (n=5) on day 1 post-injection showed significant difference ($p<0.05$) with post-injection days 17, 18, and 21. In 100 µg/g group (n=7) post-injection days 2 and 3 were able to show significant difference ($p<0.05$) only with days 17 and 18. Moreover, as in total eggs, the fecundity as fertilized eggs maintained equal status in 1–21 days post-injection periods in fish that received either 50 (n=4) or 200 µg/g GO (n=3) (Figure 1B). We further observed that the laid eggs fertilized by the males in vehicle-treated controls or the fish received GO (50 µg/g and 200 µg/g) were found to be significantly less ($p<0.05$) than the eggs fertilized by the male fish before ip injection. However, other GO-treated groups (25- and 100 µg/g) did not show significant difference ($p<0.05$) either with the pre-injected or post-injected control fish (Figure SF5A).

Reproductive activities were further verified on the hatchability of the fertilized eggs laid by the female fish before and after GO injection (Figure SF5B). During this period, 6–13% of the embryos died (Figure SF5F) though the data were unable to show significant difference with pre- and post-injected controls as well as in different GO-treated groups (25–200 µg/g). Further, our evaluation as dead, hatched, unhatched, incompletely hatched larvae indicates that 24–61% of the fertilized embryos were successfully hatched (Figure SF5B) and 16–

55% of the fertilized eggs collected before (7 days) and after injection (21 day) periods were unable to hatch within 8–14 dpf time limits (remained within the chorion, showing heartbeats) (Figure SF5C). Moreover, the data of 200 µg/g group (n=3) show a significant difference with pre-and post-injected control fish (Figure SF5B) and only with pre-injected control fish with regard to unhatched conditions (Figure SF5C). Three to 8% of the larvae show edema (cardiac or yolk) which are also not significantly different between pre-injected or post-injected controls or GO-treated (25–200 µg/g) parents (Figure SF5D). Data on incomplete hatching (1–8%) were significantly different only in 25 µg/g group with the vehicle-injected control fish (Figure SF5E).

3.3. Histopathological evaluation of gonads, liver, and kidney.

3.3.1. Ovary: During development, medaka have a pair of ovaries, separated as right and left ovaries. As development proceeds, formation of an ovarian cavity on the dorsal side and the fusion of the paired ovaries into a single organ occurred (Nakamura et al., 2018). The mature ovary of medaka is covered with the ovarian epithelium on both dorsal and ventral sides, consisting of two major compartments. The ovarian cavity on the dorsal side to which the mature eggs are ovulated (Nakamura, 2018), and the stromal compartment on the ventral side, in which folliculogenesis, oocyte maturation, and production of steroid hormones by perifollicular cells (PFC; surface epithelial cells, theca and granulosa cells) occurred (Nakamura et al., 2011). The germinal epithelium (Figures 2A1, 2B1, 2C1, SF6A–B), which is equivalent to the surface epithelium in mammals, has basement membrane on the ventral side, which lies between stroma and ovarian cavity (Figures 2A1, 2B1, 2C1, SF6A–B). Germinal epithelium formed germinal cradles (Nakamura et al, 2011) containing oogonia, prefollicular and prethecal cells, epithelial cells, and occasionally small chromatin nucleolar oocytes (Norberg et al., 1999; Parenti and Grier, 2003; OECD 2010). In stromal compartments, PNOs, CVOs, EVOs, and LVOs are distributed within a variably apparent extravascular space (Figures 2A1, 2B1, 2C1, SF6A–B) (OECD, 2010). Moreover, degenerating follicles (atretic and postovulatory follicles) also exist in the stromal compartments of the ovary (Figures 2A, 2B, 2C, SF6A–B). The ovarian lumen, which is also a part of ovarian cavity, covered by the luminal epithelium, where the mature eggs are ovulated from the stromal compartment through the germinal epithelium (Nakamura et al., 2011).

In order to evaluate the effects of GO in the folliculogenesis of medaka ovary, we observed ip-injected GO accumulated and agglomerated in ovarian compartments in a dose-dependent manner (more in higher doses) and damaged near-by follicles probably by necrosis (Figures 2B2, 2B4, 2C1–3, SF6A–E). However, the histological structure of the rest of the ovary, where GO agglomerated were lacking, remained unaffected and probably continued normal folliculogenesis (Figures 2C1 and SF5C). In some areas of the ovary, interstitial fibrosis might occur that increased the formation of perivascular smooth muscles (Figures 2C1–2). Distributions of PNOs, CAOs, EVOs, and LVOs in the stromal compartments of the ovary of control fish (n=7) indicate that approximately 65% of the differentiating follicles are PNOs, 20% are CAOs, and 10–15% are vitellogenic (EVO and LVO) oocytes. Except PNO population in 50 µg/g group, individual counts of differentiating follicles are unable to show significant differences between control and GO-treated (25, 100, and 200 µg/g) fish after 21-

days post injection (Figure 2D). In 50 µg/g group, the PNO population in the stromal compartments of the ovary showed significant reduction ($p<0.05$) when compared with control or 100 µg/g groups, however, remained at the same level as in 25 and 200 µg/g groups (Figure 2D). Our data further indicate that in controls, approximately 90% of the stromal follicles are the differentiating follicles and 10% of the follicles are in degenerating stages (Figure 2E). GO at 25, 100 and 200 µg/g are unable to show significant differences with controls either as degenerating follicles (Figure 2E) or as a ratio of the degenerating and differentiating follicles (Figure 2F). In 50 µg/g group, the distribution of degenerating follicles increased significantly than in control and 100 µg/g groups, and remained at the same level as in 25 and 200 µg/g groups. Consequently, the ratios of degenerating and differentiating follicles (Figure 2F) are found to be significantly different (increase) in 50 µg/g group than control and 100 µg/g groups ($p<0.05$) and at the same level as in other two groups (25 and 200 µg/g) (Figure 2F).

The follicles are covered by chorion (zona radiata or vitelline envelope); in HE stain, the chorion appears to be a pale to dark eosinophilic outer membrane that surround the ooplasm of the oocytes (Figures 2A2–3, 2B2–3, 2C2–3, SF6D–E). Although a chorion-like structure exists in PNOs, in light microscopic observations, they become clearly visible in CAOs and gradually thicken as the oocytes mature to vitellogenic stages (Figures 2A2–3, 2B2–3, 2C2–3, SF6D–E). Next to chorion, the granulosa cell layer surrounds the chorion (Figures 2A2–3, 2B2–3, 2C2–3, SF6D–E) followed by theca cells; the surface epithelial cells remain external to theca layer. Among these cell layers, theca and granulosa cells secrete steroid hormones including estrogen. In medaka, the GCs in PFC of oocytes arranged as single cell layer with cuboidal to columnar appearance (Figures 2A2, 2B2, SF6D) even though remained thicker (GCs) at the vegetal hemisphere than at the animal hemisphere (Iwamatsu et al., 1988). The oocytes under spontaneous atrophy showed multiple layers in PFC (Figure 2A3). In atretic follicles, they become stratified (Figures 2B3, 2C3, and SF6E). In post-ovulatory follicles, the granulosa cells multiply and become hypertrophic (Figures 2A4, 2B4, and 2C4), if the fish are exposed to EDCs. Our observations indicate that follicular atresia can occur either in chorion or in granulosa cells, or both. It becomes spontaneous to the follicles probably when they are detracted from maturational processes. We observed a considerable number of atretic follicles in both control and GO-treated (25–200 µg/g) fish. Agglomerated GO can induce atresia in differentiating follicles of the ovary when it is in direct contact either with chorion or granulosa cells, or both (Figures 2B3, 2C2–3, SF6E). However, possibilities of spontaneous atresia cannot be ruled out in these regions. In postovulatory follicles, the granulosa cells become hypertrophic and multiplied (Figures 2A4, 2B4, 2C4); however, when they are associated with GO, it is very difficult to determine their identity (Figure SF6C). Moreover, like differentiating follicles, they appear to be normal in stromal compartments where GO agglomerates are lacking (Figures 2B, SF6C).

3.3.2. Testis: The testis of reproductively active medaka is a semi-translucent, elongated structure consisting seminiferous cysts (Satoh, 1974). Histologically, the testes of adult medaka comprise a lobular restricted type structure (Grier 1976; Parenti and Grier 2004) in which spermatogonia are found at the distal ends (periphery) of the lobules (Figures 3A1, 3B1, 3C1, SF7A–C), end blindly at the periphery of the organ (Miller et al., 2012). The

central region of the testis consists of efferent ducts (ED) and the lumens, which are flexible in diameter, filled with dark basophilic spermatozoa (Figures 3A1, 3B1–3, 3C1–3, SF7A–B). The flexibility of the lumen can increase or decrease the inner area, which is probably dependent on the invasion or evasion of the ductal walls. Sertoli cells (Figures 3A1–2, 3B1–2, 3C1–2, SF7A–C), compared to germinal cells, present in low numbers, usually as single cell, located adjacent to lobular septa. For histological assessment, the testis of medaka can be divided into two major compartments; the lobular and the interstitial. The lobular compartment consists of germinal components (spermatogonium, spermatocytes, spermatids), sperms (Figures 3A1, 3B1, 3C1, SF7A–C) and Sertoli cells (Figures 3A1, 3B1–2, 3C1–2 and SF7A–C). The interstitial compartment consists of Leydig cells (Figures 3A1, 3A3, 3B1, 3B3, 3C1, 3C3 and SF7A–C), peritubular myloid cells, connective tissue, efferent duct epithelial cells, endothelium of blood vessels, and circulating blood cells. The Leydig cells remain in small clusters in the stroma surrounding the seminiferous cysts and at the smaller terminal divisions of the ED (Gresik et al., 1973; Satoh, 1974). They have dense, dark round or oval nuclei with faintly vacuolated cytoplasm. Compared to germinal cells, interstitial cells are usually present in low numbers, either as single cell or as small aggregates within the interlobular interstitium, and have no close association with other cell types of the interstitium except with other Leydig cells (Gresik et al., 1973). In the lobular compartments, the germ cells have the ability to divide whereas the Sertoli cells, which are also known as the nurse cells, form spermatocyst with spermatogenic (Spermatogonia, spermatocytes, or spermatids) cells, provide nourishment to the testicular components, and remain undivided when spermatogenesis begins. The interstitial components also remain undivided during spermatogenesis. The Leydig cells secrete steroid hormones, which are necessary for gonadal maturation and function.

Our observations indicate that the ip-injected GO agglomerated on the peritoneal membrane or near the testis, and is likely to be associated with the periphery (Figure 3C1), gradually cross the basement membrane and incorporates into the testis in a dose-dependent manner (Figure SF7C). Germinal components (spermatocytes and spermatids) of the lobular compartments of medaka testis indicate that in both control and GO-treated fish (25–200 µg/g), within a particular cyst (lobule), all germinal elements were at the same stage of development. However, the stages of development of germinal elements differ from cyst to cyst (Figures 3A1, 3B1, 3C1, SF7A–C). Moreover, with current light microscopic studies, we did not observe any remarkable morphological variations in germinal cells (spermatogonium, spermatocytes, spermatids) or sperms and Sertoli cells (Figures 3A1, 3B1, 3C1, SF7A–B). However, apoptotic cells (identified by larger size and deep stained basic pycnotic nuclei) were observed in the ED system of the testis of both control and GO-treated (25–200 µg/g) fish (Figures 3A1, 3B1, 3C1, SF7A–B). The Sertoli cells with deep black nuclei and long cytoplasmic processes (Figures 3A1–2, 3B1–2, 3C1–2 SF 7A–C) also remained unaltered after GO treatment (25–200 µg/g) with respect to their shapes and sizes. We also observed that close association or agglomeration of GO inside the testis enhanced the spermatogonial lobules (cysts) in the periphery (Figure 3C1), or moved them toward ED of the testis (Figure SF7C). However, distribution of Leydig cells within the interstitial compartments are identical in control and GO-treated fish (25–200 µg/G); either present as

single or aggregates with no significant major alteration in histological structures or shapes (Figures 3A1, 3A3, 3B1, 3B3, 3C1, 3C3, and SF7A–C).

3.3.3. Liver: The hepatocytes of medaka are polyhedral in shape, with clear edges, broad cytoplasm with different degrees of hepatocellular intracytoplasmic vacuolation (Figures 4A1 and 4A2). Hepatocytes were radiating from the central vein creating a tubulo-sinusoidal arrangement in which hepatocytes arranged as cellular duplex surrounding a sinusoidal capillary (Hinton et al., 1984). Compared to males (Figure 4A1), cytoplasm of hepatocytes in female fish is basophilic in nature (Figure 4A2). Blood and blood cells are frequently seen in hepatic blood vessels. The muralium duplex suggested a possible mechanism for facilitating the nutrition of hepatocytes and releasing their products conveniently into the bloodstream (de Oliveira et al., 2016). The white vacuoles observed in HE stain indicate the existence of glycogen in hepatocytes. Administration of GO (25–200 µg/g) to male and female fish was unable to document GO agglomerates in the hepatocytes of liver, or induction of any noticeable changes in the histopathology of medaka liver after 21-day post injection. However, some GO-treated fish showed the accumulation of proteinaceous fluid in hepatic blood vessels (Figures SF8A1–2, and SF8B2). Hepatocyte basophilia, which is related to the synthesis of vitellogenin (the egg yolk precursor protein) or other proteins in the hepatocytes, compared to controls (male Figure 4A1, female Figure 4A2) also remained unaltered in GO-treated male (Figures 4B1, 4C1, SF8A1 and SF8B1) or female fish (Figures 4B2, 4C2, SF8A2 and SF8B2). Only one male fish injected with 100 µg/g GO (Sigma-Aldrich, St. Louis, MO) showed lesion in several sites of liver which can be compared with spongiosis hepatis (cystic degeneration) (Figure SF8C), a nonneoplastic lesion observed in Japanese medaka (Boorman et al., 1997; Ding et al., 2010). The spongiosis hepatis formation replaced large areas of liver parenchyma (Figure SF8C) that may affect the normal functions of liver. However, other control or GO-treated male and female fish (25–200µg/g) used in these experiments did not show similar cystic degeneration in the liver.

3.3.4. Kidney: The kidneys of medaka are mesonephric, both the cranial (head) and trunk portions composed of glomeruli, renal tubules, and interstitial lymphomyeloid tissues (Figures 5A1, male; 5A2, female). Structurally, the glomerulus is divided into vascular and epithelial regions. The vascular region is the core structure of glomerulus and consists of capillary network and mesangium. The vascular region is surrounded by a flat epithelium of Bowman's capsule. There are at least two types of tubular segments identified by PAS staining. The proximal convoluted segments with narrow lumen are lined with tall columnar epithelial cells with PAS positive brush border on apical surfaces. The distal convoluted segments with round and wide lumen, lined by low columnar epithelial cells, and the apical surface is PAS negative (Mochizuki et al., 2005). Interstitial lymphomyeloid cells have large nuclei, stained positive with hematoxylin, and filled the gaps of renal tubules. Brown and blackish pigments, probably the melanomacrophages and melanomacrophage centers (MMC) are occasionally seen in interstitial lymphoid tissues (Mochizuki et al., 2005; Steinel and Bolnick, 2017; Stosik et al., 2019). Compared to controls, administration of GO (25–200 µg/g) was unable to induce any significant alteration in the histopathology of glomerulus, and renal tubules either in males (Figures 5B1, 5C1, SF9A1–2) or females (Figures 5B2, 5C2, SF9B1–2) as observed by low microscopy. Moreover, we have observed

hyperplasia in interstitial lymphoid tissues (HIL) mostly at the cranial region of kidneys of some male and female fish injected with GO (Figures SF9C1–2). The kidneys showing HIL became basophilic due to increase in interstitial cell populations. Moreover, the lumen of the tubules in HIL region became compact and in some cases, the cilia of the epithelial cells probably detached from the cell surfaces and clumped at the center that reduced the tubular passages, even though they (cilia) remained undetectable in HE staining (SF9C1–2). In some fish, the renal tubules were tightly packed, seldom showing any interstitial lymphomyeloid cells in between them. However, the reproductive activities of these fish having HIL were identical with other GO-injected fish (25–200 µg/g).

4. Discussion:

As a sequel of our previous investigations on evaluation of toxic effects of GO in mammalian model (Patlolla et al., 2016; 2017), we continued our studies in a fish model and evaluated the toxicity and the EDC effects of GO. We used Japanese medaka fish, a recognized model for characterization of EDCs (Hinton et al., 2005; Urushitani et al., 2007; Scholz and Mayer 2008; Dang, 2016; OECD 2018), for the research. Several physiological and molecular markers in medaka including hepatic vitellogenin, testis-ova, altered morphology of anal and dorsal fins, genetic sex identification by polymerase chain reaction (PCR), are useful parameters which can be considered for identification and characterization of EDCs disposed in the aquatic environment (Urushitani et al., 2007; OECD 2018; Dang and Kienzler, 2019).

In our previous studies on rats, the GO we used was obtained from commercial source, and we administered multiple doses of GO orally and repeatedly to the experimental animals (Patlolla et al., 2016; 2017). However, in the present experiment, we synthesized GO in our laboratory by modified Hummer's method (Viraka Nellore et al., 2015), injected ip to the sexually mature, reproductively active, intact adult male and female fish at the doses of 25–200 µg/g of body weight (some fish were also injected with GO obtained from commercial source). The experimental fish as a pair (one male and one female) bred successfully for 7 days (one week) before injection and continued breeding for another 21 days (three weeks) after injection. The collected eggs were used for the estimation of fecundity (Figures 1A–B) and the developmental disorders (Supplementary Figures SF5A–F). After 21-days post-injection, the survived fish were sacrificed and the gonads (testis and ovary), livers, and kidneys were used for histopathological analysis.

The physicochemical properties of the synthesized GO as verified by SEM, TEM, AFM FTIR spectra and zeta potentials (SF1A–B, SF2A–C; Supplementary Table 1) showed excellent similarities with the results reported previously by us (Patlolla et al., 2016; 2017) as well as by other investigators (Chen et al., 2016; Paital et al., 2019). We observed after sonication GO was agglomerated in the water over time, however, because of the negative value of zeta potential (–30.5 mV) the solution was considered stable (Patlolla et al., 2017). Although the conventional method of exposure to nanomaterials in fish is immersion, direct administration of GO or EDCs by ip-injection(s) have also been reported (Zhang et al., 2002; Kiparissis et al., 2003; Dang 2016; Dorelle et al., 2017; Movahedinia et al., 2018; Dang and Kienzler, 2019; Paital et al 2019). To avoid agglomeration of GO in the

environment, we preferred to use ip injection as an alternative method, because the route of administration of nanomaterials has significant impact on the retention and biological function of the compounds (Ema et al., 2016). The ip-route of GO was inadequately investigated in fish but seems to be safe, convenient, rapid, and less stressful than intravenous (IV) injections used in mammals (Kurantowicz et al., 2015), which is not very convenient in a small laboratory fish like medaka.

In our research, the fish injected with GO were able to maintain normal breeding and produced both fertilized and unfertilized eggs (Figures 1A–B) with dose-dependent increase of body weight in female and remained unaltered in males (Figure SF4). Moreover, with these limited data, we have documented that the males are more sensitive to GO than females (calculated LD₅₀ for males is 175.39 and for females are 2,901.20 µg/g) (Figures SF3A–B). Our observations on body weights (Figure SF4) are similar with the observations made by Ma et al (2016) who have exposed adult tilapia fish to GO (total 4 mg GO fed for 30 days) and observed non-significant increase in body weight of the experimental fish ($p=0.668$, $n=6$) without affecting the growth and behavior of the fish. Further, the accumulated GO has the potential to agglomerate in the body fluids that can affect the incorporation of GO into specific organs. However, we have observed noticeable accumulation of unidentified black particles (probably GO or its metabolites) in the proximity of the injection site, especially between the connective tissues of the abdominal skin, muscles, peritoneal membrane, and in gonads (ovary and testis) after 21 days post-injection (Figures 2B2, 2C1–3, SF6A–C and SF7). In Wistar rats, ip-injection of GO nanoparticles retained in the body, mostly as agglomerates, and the largest agglomerate appeared to be 10 mm in diameter, which are mostly seen at the proximity of the injection site (Kurantowicz et al., 2015). We therefore think that the animal model (Japanese medaka) we used, and the mode of administration (ip injection) we followed, provide a new insight into the toxicological and endocrine disrupting potential of GO.

Our data on fecundity apparently show a dose-dependent reduction by GO at least in the early days of post-injection (Figures 1A–B). However, the statistical analysis of the data (fecundity) was unable to show significant difference between the water-injected controls and various GO-treated groups at all-time points; either as total eggs (fertilized and unfertilized) or as fertilized eggs (25–200 µg/g, single ip injection). Considering the impact of GO on gonadal activity, we hypothesize that the egg laying activity of the fish is a functional endpoint of the ovarian function/activity of the female fish (Figure 1A) and the fertilized eggs (Figure 1B) found in post mating period represent the testicular activity/function of the male fish. Although our data are unable to establish a significant difference, available reports indicate that in other models such as *Caenorhabditia elegans* chronic exposure to GO was able to reduce fecundity, and size of the animals; however, acute exposure failed to do that (Rive et al., 2019). In *Spodoptera frugiperda*, the diet containing 1mg/g GO during larval rearing, reduced fecundity and fertility of the insects (Martins et al., 2019). In house cricket (*Acheta domesticus*) multigenerational exposure to GO reduced fecundity and induced cellular damage (Dziewiecka et al., 2018). Moreover, our data on Japanese medaka indicate that fecundity increased gradually with time even though it did not reach up to the same level observed during pre-injection periods (Figures 1A–B). We expect that despite the deposition of GO agglomerates in the different organs of medaka

especially in gonads (Figures 2C1–3, 3C1, SF6B–E, SF7B–C), there is a possibility of depuration of GO over time as observed in other EDC studies (Miller et al., 2012). The fertilizing activity of the male medaka reduced significantly during 14 days exposure to EE2, a xenoestrogen and a known EDC disposed in the environment, and the fish were unable to recover completely to the pre-exposure period during depuration probably due to the impairment of the male reproductive capacity by the xenoestrogen (Miller et al., 2012). Although our data on fecundity were unable to document GO as an indicator of EDC, we hypothesize that the injected GO may have the ability to impair gonadal functions and will be considered as a potential EDC disposed in the environment.

Reproductive activities were further verified on the hatchability of the fertilized eggs laid by the female fish before and after GO injection (Figure SF5B). We hypothesize that the injected materials (water or graphene oxide) are adsorbed into the ovary/testis and consequently into the gametes (eggs/sperms) that can affect either the fertilizing ability (remained unfertilized) of the gametes (sperms and ovum), or the morphogenesis of the embryos during post-fertilization periods, possibly due to transgenerational inheritance (Pooma et al., 2014). If the gametes are unable to be released into the environment for external fertilization, they will be degenerated within the gonads (follicular atresia or degeneration of germinal components). Although we are unable draw a definite conclusion on the effects of GO on embryonic development of medaka exposed through parenteral administration, available reports indicate that in house cricket exposed to GO throughout life cycle show a significantly decreased their reproductive capabilities. Moreover, there are possibilities to produce multigenerational harmful effects on this species (house cricket) by GO (Dziewiecka et al., 2018). In zebrafish embryos or larvae, exposed to GO either by microinjection or waterborne, remained unresponsive or induced several morphological disorders including yolk sac and pericardial edema, abnormal tail flexures, malfunction of heart and cardiovascular system, hatching delay, enhanced mortality and others in a concentration dependent manner (Dasmahapatra et al. 2019). In mammals, oral administration of GO to lactating mothers induced growth retardation of offspring; moreover, intravenous injection of rGO during late gestation period caused maternal death and abortion in mice (Fu et al., 2015; Xu et al., 2015; Ema et al., 2016). However, no adverse effects of S-GO (55 nm) or L-GO (238 nm) were observed on male reproduction in mice injected either intravenously or intraperitoneally (Liang et al., 2015). We injected GO once to both male and female fish, which make difference with others. Our observations in the reproductive activities of medaka and on developing embryos and larvae are found to be very minimal compared to the observations made by others in other animal models like insects (Dziewiecka et al., 2018), zebrafish (Dasmahapatra et al., 2019) or in mammals (Fu et al., 2015; Liang et al 2015; Ema et al., 2016).

Our studies further extend to evaluate the histopathological changes in gonads (ovary and testis), liver and kidney of the experimental fish. Histopathologic assessment of the gonads is a valuable endpoint in EDC studies (Hutchinson et al., 2006). Generally, estrogenic (EE2) or androgenic (TB) chemicals have the potential to induce sex skewing and producing intersex phenotypes targeting estrogen, androgen or steroidogenic pathways (Dang 2016; Dang and Kienzler, 2019). In medaka spontaneous induction of intersex gonad is a very common phenomenon depending on culture conditions, breeding colonies used, and other

factors (Grim et al., 2007; Miller et al., 2012; Cheung et al., 2014); however, despite severe intersex gonads, medaka males are still capable of successful reproduction (Flynn et al., 2017) and probably show no impact on fecundity. EDC can also cause malformation of accessory structures such as oviducts in females or efferent ducts in the testis of males that can adversely affect reproduction (Flynn et al., 2017).

In the present experiment, histopathological evaluation of the ovary or testis of the experimental fish after 21 days post-injections of different doses of GO did not show any testicular tissues in ovary or ovarian components in testis (sex reversal) of the experimental fish. Therefore, we conclude from our data that the medaka colony we maintained in the laboratory, or the experimental protocol we followed to evaluate GO as an EDC, are unable to induce sex reversal in medaka, either spontaneously (Figure 2A1 and 3A1) or after GO injections (25–200 µg/g) (Figures 2B1, 2C1, 3B1, 3C1, SF6A–B and SF7A–B). The deposition of unidentified black particles (probably the agglomerated GO), 21-days post-injections on gonads, ovarian follicles, or in the adjacent periphery of the germinal epithelium of ovary and testis, indicate that the injected GO agglomerates into the gonadal tissues of medaka in a dose-dependent manner (Figures 2C1, 3C1, SF6B–E, SF7B–C). Our data further indicate that despite the presence of GO inside the gonads, the gametogenesis process is continuing in ovary (Figure SF6C) and testis (Figure SF7C). Moreover, the existence of both differentiating and degenerating follicles (Figures 2B1, 2C1, SF6A–B) in the stromal compartments of the ovary, and substantive number of Sertoli and Leydig cells in the testis (Figures 3A–C, SF7A–C), indicate that any histological damages induced by GO are limited to the regions where GO agglomerates are accumulated within the organs. Alternatively, the organs are able to recover from GO-induced damages, if any, within 21-days post injection periods. Our observations on fecundity (Figures 1A–B) indicate that the fish received higher doses of GO (100–200 µg/g) are able to produce fewer eggs (both as total and fertilized) in the early part of post-injections (first 7 days or more) than the eggs produced in the late part of the experiment (14–21 days). Moreover, our data on follicular counts (Figures 2D and 2E), except 50 µg/g groups, are also unable to establish significant differences between vehicle-treated controls and GO-treated (25, 100, and 200 µg/g) fish. Furthermore, we observed that the ovaries themselves, tended to eliminate agglomerated GO from the ovarian compartments probably by an invagination of dorsal ovarian epithelium (Nakamura, 2018), originated from the intact ovarian structures (Figure SF6C). Therefore, we predict that the stem cells in the germinal epitheliums of ovary and testis of medaka are still active in the fish treated with GO, and the presence of GO inside the gonads are unable to prevent their functions, completely. Reports document that structural alteration of the seminiferous tubules and interstitium observed in male Wistar rats exposed to a high dose of GO (10 mg/kg BW, every alternative day for 15 days) (Nirmal et al., 2017). However, in ICR-strain male mice nanoscale GO showed no toxicity with regard to histopathological changes in testis and epididymis (Liang et al., 2015). No histopathological changes were observed in the testis of BALB/c mice after single intravenous injection of GO (Qu et al., 2013). Moreover, single-walled carbon nanotubes (SWCNT-COOH) and rGO did not affect human sperm viability in vitro even though SWCNT-COOH generated significant super oxide species (Asghar et al., 2016). However, the metabolites of TB (androgenic in nature) reduced the primary ovarian follicles (similar to PNOs in our studies) and increased

vitellogenic follicles (similar to EVOs and LVOs in our studies) in medaka ovary (Forsgren et al., 2014). Despite the observations made by other investigators, with our present experimental data, we have reason to believe that the destructive effects of GO (probably by necrosis), if any, was either restricted only in the GO accumulated sites of the organ, or the fish were able to recover the damages induced by GO agglomerates within 21 days of depuration.

In teleost, PFC of the ovarian follicles (theca and GC) are the hormone producing cells (estrogen and progesterone), and the Leydig cells in the testes secrete male hormones (testosterone). Exposure to an EDC (TB) has been associated with an increase in the height and number of GCs, which give PFC a pseudostratified columnar appearance (OECD 2010). Moreover, hyperplasia in Leydig cells and hypertrophy of Sertoli cells are also significant indicators of EDC effects in testis of fish (OECD 2010). GO has been documented as an inducer of size-dependent toxicity on Sertoli cells (TM4) and Leydig cells (TM3) in vitro (Gurunathan et al., 2019), and atrophy of seminiferous tubules and damaged interstitium by nano GO in Wistar rats in vivo (Nirmal et al, 2017). Although we did not measure the endogenous hormone levels (estrogen or testosterone), from the cellular morphology of GC layers (Figures 2B2–3, 2C2–3, SF6A–B) and Leydig cells (Figures 3B1, 3B3, 3C1, 3C3 SF7A–C), by light microscopy studies, it is very difficult to predict the hormonal status of the fish after GO-injections. The morphology of GC in the follicles adjacent to GO agglomeration sites were altered (multiple layer) expecting a hormonal alteration, however, the data on follicular counts (Figure 2E), except 50 µg/g, did not support the hypothesis. We predict that steroid hormones produced by GC or Leydig cells of Japanese medaka remained unaltered or at the same level after GC injections as in controls. Alternatively, any alterations in GC or Leydig cell morphology, induced by GO in the earlier phase of treatment, could recover during the late part of the treatment either by depuration or by other means.

The toxic effects of GO on liver and kidney of fish were reported by many investigators (Chen et al., 2016; Souza et al; 2017; Dasmahapatra et al., 2019 (review); Paital et al., 2019). To our knowledge, it is unknown to us, whether GO is able to induce any EDC effects in liver and kidneys of medaka. In the present study, we did not observe any histopathological variation in the liver (Boorman et al., 1997; OECD 2010) or kidney (OECD 2010) of male and female medaka after 21 days post-injection of GO. The hepatocytes in male fish due to the activation of vitellogenin gene by estrogenic EDCs, can turn the cytoplasm of the hepatocytes basophilic. Consequently, EDCs are able to modulate the basophilicity of the hepatocytes in female fish, possibly by enhancing the expression of vitellogenin or other genes. Moreover, alteration in cytoplasmic vacuolization, and accumulation of proteinaceous fluid in the hepatic blood vessels were significant indicators of EDC activities, especially in fish (OECD, 2010). In the present experiment, we did not measure the vitellogenin or other protein contents of medaka in the liver, or serum. However, our histopathological observations are able to document a marked difference in basophilia of the hepatocytes between male (Figure 4A1) and female fish (Figure 4A2), and unable to establish remarkable difference between control and GO-treated male (Figures 4B1, 4C1, SF8A1, SF8B1) or female (Figures 4B2, 4C2, SF8A2 and SF8B2) fish. The accumulation of proteinaceous fluid in some male and female fish (Figures SF8A1–2, and SF8B2) are probably the blockage of the hepatic vessels by GO.

EDCs, which are estrogenic in nature, are able to induce renal impairments in kidneys of fish including swelling of tubular epithelial cells and necrosis, dilution of Bowman's capsule, interstitial fibrosis, casts, and hyaline droplets in tubules or glomerulus (OECD, 2010). However, our light microscopy studies are unable to document any of these changes in the glomerulus, tubules, either in vehicle-injected control male (Figure 5A1) or female (Figure 5A2) or in GO-treated male (Figures 5B1, 5C1, SF9A1–B1), and female (Figures 5B2, 5C2, SF9A2–B2) fish. Although we are unable to claim the incorporation/agglomeration of GO nanoparticles in the kidneys, we observed substantive number of black particles in the head kidneys of both control and GO-treated fish, probably MMC, within the interstitial lymphomyleyoid cells found in medaka kidneys (Mochizuki et al., 2005). MMCs are aggregates of pigmented phagocytes found primarily in poikilotherm lymphoid tissues including liver, spleen and kidneys. We did not see similar MMC-like components in liver of both male and female medaka; however, we observed substantive number of MMC-like structure in spleen of GO-injected male and female medaka (data not shown). MMCs play significant role in mediating immunologic disorders, and we are confused with the GO (which were also black in color), we did not pay any attention to MMCs we observed in kidneys. Moreover, the presence of HIL in kidneys suggests a probable immune-related mechanism associated with GO. Immunomodulatory effects of GO was studied in both mammalian models (Ema et al., 2016) as well as in adult zebrafish (Dasmahapatra et al., 2019, review). Expect HIL, the histopathological studies in kidneys of both male and female fish do not indicate any functional overload of vitellogenin, or spiggin (an EDC-sensitive renal glue protein found in male three-spined stickleback, *Gasterosteus aculeatus*, during breeding season, Hahlbeck et al., 2004, Jolly et al., 2009), or any other proteins (seen in livers of some GO-injected fish) induced by GO.

Conclusion:

Taken together, our data indicate that single ip injection of GO (25–200 µg/g) to reproductively active intact adult medaka male and female show significant accumulation of GO agglomerates in the gonads after 21 days post-injections. However, as observed by light microscopy, administration of GO, ip (25–200 µg/g), is unable to disrupt significant reproductive activities of medaka as evidenced by everyday fecundity and from the histopathological evaluation of gonads (ovary and testis), liver and kidneys. Although we did not study the oxidative-stress mediated mechanisms, the damaging effects of GO we observed in gonads and livers are probably mediated through necrosis. HILs observed in kidneys indicate the immunological response of the organ to GO. To evaluate the EDC activities of GO, more studies at the cellular levels including biochemical, immunological and molecular biological approaches are necessary. Moreover, transgenerational studies will be an important endpoint for the evaluation of EDC effects of GO in aquatic models such as fish.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References:

- Asghar W, Shafiee H, Velasco V, Sah VR, Guo S, El Assal R, Inci F, Rajagopalan A, Jahangir M, Anchan RM, Mutter GL, Ozkan M, Ozkan CS, Demirci U. Toxicological study of single-walled carbon nanotubes and reduced graphene oxide in human sperm. *Sci Rep*. 6:30270 Doi: 10.1038/srep30270 (2016). [PubMed: 27538480]
- Bangeppagari M, Park SH, Kundapur RR, Lee SJ. Graphene oxide induces cardiovascular defects in developing zebrafish (*Danio rerio*) embryo model: in vivo toxicity assessment. *Sci Total Environ*. 673: 810–820 (2019). [PubMed: 31005017]
- Bianco A Graphene: safe or toxic? The two faces of the medal. *Angew. Chem. Int. Ed* 52:4986–4997(2013).
- Boorman GA, Botts S, Bunton TE, Fournie JW, Harshbarger JC, Hawkins WE, Hinton DE, Jokinen MP, Okihiro MS, Wolfe MJ. Diagnostic criteria for degenerative, inflammatory, proliferative nonneoplastic and neoplastic liver lesions in medaka (*Oryzias latipes*): consensus of a National Toxicology Program pathology working group. *Toxicol. Pathol* 25:202–210 (1997). [PubMed: 9125779]
- Chen M, Yin J, Liang Y, Yuan S, Wang F, Song M, Wang H. Oxidative stress and immunotoxicity induced by graphene oxide in zebrafish. *Aqua. Toxicol* 174: 54–60(2016).
- Cheung CH, Chiu JM, Wu RS. Hypoxia turns genotypic female medaka fish into phenotypic males. *Ecotoxicology*, 23:1260–1269(2014). [PubMed: 25011919]
- Dang Z Interpretation of fish biomarker data for identification, classification, risk assessment and testing of endocrine disrupting chemicals. *Environ Int*. 92–93:422–441(2016).
- Dang Z, Kienzler A. Changes in fish sex ratio as a basis for regulating endocrine disruptors. *Environ Int*. 130:104928 (2019). [PubMed: 31277008]
- Dasmahapatra AK, Dasari TPS, Tchounwou PB. Graphene-based nanomaterial toxicity in fish. *Rev. Environ. Contam. Toxicol* 247: 1–58(2019). [PubMed: 30413975]
- De Oliveira LB, Leonardo AS, de Lima EM Macro- and microstructural descriptions of the zebrafish (*Danio rerio*) liver. *Folia Morphol*. 75: 382–387(2016).
- Ding L, Kuhne WW, Hinton DE, Song J, Dynan WS. Quantifiable biomarkers of normal aging in the Japanese medaka fish (*Oryzias latipes*). *PLoS One* 10 11; 5 (10):e13287.
- Dorelle LS, Da Can RH, Vazquez GR, Hocht C, Shimizu A, Genovese G, Lo Nostro FL. The SSRI fluoxetine exhibits mild effects on the reproductive axis in the cichlid fish *Cichlasoma dimerus* (teleostei, cichliformes). *Chemosphere*, 171:370–378(2017) [PubMed: 28030789]
- Dziewiecka M, Witas P, Karpeta-Kaczmarek J, Kwasniewska J, Flasz B, Balin K, Augustyniak M. Reduced fecundity and cellular change in *Acheta domesticus* after multigenerational exposure to graphene oxide nanoparticles in food. *Sci Total Environ*. 635: 947–955 (2018). [PubMed: 29710616]
- Ema M, Hougaard KS, Kishimoto A, Honda K. Reproductive and developmental toxicity of carbon-based nanomaterials: a literature review. *Nanotoxicology* 10:391–412(2016). [PubMed: 26375634]
- Finney DJ *Probit Analysis*, 3rd Ed. Cambridge University Press, Cambridge, UK (1971)
- Flynn K, Lothenbach D, Whiteman F, Hammermeister D, Touart LW, Swintek J, Tatarazako N, Onishi Y, Iguchi T, Johnson R. Summary of the development the US environmental protection agency's medaka extended one generation reproduction test (MEOGRT) using data from 9 multigenerational medaka tests. *Environ. Toxicol Chem* 36:3387–3403(2017). [PubMed: 28857258]
- Forsgren K, Qb S, lavado R, Cwiertny D, Schlenk D. Trenbolone acetate metabolites promote ovarian growth and development in adult Japanese medaka (*Oryzias latipes*). *Gen Comp Endocrinol*. 201:1–7(2014). [PubMed: 24681151]

- Freixa A, Acuna V, Sanchis J, Farre M, Barcelo D, Sabater S. Ecotoxicological effects of carbonbased nanomaterials in aquatic organisms. *Sci Total Environ.* 619–620:328–337(2018).
- Fu C, Liu T, Li L, Liu H, Liang Q, Meng X. Effects of graphene oxide on the development of offspring mice in lactation period. *Biomaterials*, 40:23–31(2015). [PubMed: 25498802]
- Grier HJ Sperm development in the teleost *Oryzias latipes*. *Cell Tissue Res.* 168:419–431(1976). [PubMed: 1277277]
- Grim KC, Wolfe M, Hawkins W, Johnson R, Wolf J. Intersex in Japanese medaka (*Oryzias latipes*) used as negative controls in toxicologic bioassays: a review of 54 cases from 41 studies. *Environ. Toxicol. Chem* 26:1636–1643(2007). [PubMed: 17702336]
- Gresik EW, Quirk JG, Hamilton JB. A fine structural and histochemical study of the Leydig cell in the testis of teleost, *Oryzias latipes* (Cyprinodontiformes). *Gen Comp Endocrinol.* 20:86–98(1973). [PubMed: 4689594]
- Gurunathan S, Kang M-H, Jeyaraj M, Kim J-H. Differential cytotoxicity of different sizes of graphene oxide nanoparticles in Leydig (TM3) and Sertoli (TM4) cells. *Nanomaterials*, 2019,9, 139; doi:10.3390/nano9020139.
- Hahlbeck E, Katsiadaki I, Mayer I, Adolfsson-Erici M, James J, Bengtsson B-E,. The juvenile three-spined stickleback (*Gasterosteus aculeatus* L) as a model organism for endocrine disruption II- kidney hypertrophy, vitellogenin and spiggin induction. *Aquat Toxicol.* 70:311–326(2004). [PubMed: 15588642]
- Hinton DE, Kullman SW, Hardman RC, Volz DC, Chen PJ, Carney M, Bencic DC. Resolving mechanism of toxicity while pursuing ecotoxicological relevance. *Mar Pollu Bull.* 51:635–648(2005).
- Hinton DE, Walker ER, Pinkstaff CA, Zuchelkowski EM. Morphological survey of teleost organs important in carcinogenesis with attention to fixation. *Natl Cancer Inst. Monogr* 65:291–320(1984). [PubMed: 6087142]
- Hummers WS, Offeman RE. Preparation of graphitic oxide. *J. Am.Chem. Soc* 80:13–39 (1958).
- Hutchinson TH, Ankley GT, Segner H, Tyler CR. Screening and testing for endocrine disruption in fish- biomarkers as “signposts,” not “traffic lights,” in risk assessment. *Environ.Health Perspec* 114:106–114(2006).
- Iavicoli I, Fontana L, Leso V, Bergamaschi A. The effects of nanomaterials as endocrine disruptors. *Int J Mol Sci.* 14:16723–16801(2013).
- Iwamatsu T Stages of normal development in the medaka *Oryzias latipes*. *Mech Dev* 121:605–618(2004). [PubMed: 15210170]
- Iwamatsu T, Ohita T, Oshima E, Sakai N. Oogenesis in the medaka *Oryzias latipes*-stages of oocyte development. *Zool Sci.* 5:353–373(1988).
- James DK, Tour JM. Graphene: powder, flakes, ribbons, and sheets. *Acc Chem Res.* 46:2307–2318(2013). [PubMed: 23276286]
- Johnson DW, Dobson BP, Coleman KS. A manufacturing perspective on graphene dispersions. *Curr. Opin. Colloid Interface Sci* 20:367–382(2015).
- Jolly C, Katsiadaki I, Morris S, Le Belle, N., Dufour S, Mayer I, Pottinger TG, Scott AP. Detection of the antiandrogenic effect of endocrine disrupting environmental contaminants using in vivo and in vitro assays in the three-spined stickleback. *Aquat Toxicol.* 92:228–239(2009). [PubMed: 19307032]
- Konios D, Stylianakis MM, Stratakis E, Kymakis E. Dispersion behavior of graphene oxide and reduced graphene oxide. *J Colloid Interface Sci.* 430:108–112(2014). [PubMed: 24998061]
- Kurantowicz N, Strojny B, Sawosz E, Jaworski S, Kutwin M, Grodzik M, Wierzbicki M, Lipinska L, Mitura K, Chwalibog A. Biodistribution of a high dose of diamond, graphite, and graphene oxide nanoparticles after multiple intraperitoneal injections in rats. *Nanoscale Res. Lett* (2015) 10:398 DOI 10.1186/s11671-015-1107-9. [PubMed: 26459428]
- Li M, Zhu J, Wang M, Fang H, Zhu G, Wang Q. Exposure to graphene oxide at environmental concentrations induced thyroid endocrine disruption and lipid metabolic disturbance in *xenopus laevis*. *Chemosphere* 236:124834(2019). Doi: 10.1016/j.chemosphere. 2019. 124834. Epub 2019 Sep 13. [PubMed: 31549672]

- Liang S, Xu S, Zhang D, He J, Chu M. Reproductive toxicity of nanoscale graphene oxide in male mice. *Nanotoxicology*, 9:92–105(2015). [PubMed: 24621344]
- Ma K, Zhang S, Ye B, Ouyang J, Yue GH. New view of graphene oxide biosafety on water environment using an eatable fish as a model. *RSC Adv.* 6:29619–29623(2016).
- Martins CHZ, de Souza M, Fonseca LC, Martinex DST, Alves OL. Biological effects of oxidized carbon nanomaterials (1D versus 2D) on *Spodoptera frugiperda*: Material dimensionality influences on the insect development, performance and nutritional physiology. *Chemosphere*, 215:766–774(2019). [PubMed: 30352373]
- Medeiros AMZ, Coa F, Alves OL, Teodoro Martinez DS, Barbieri E. Metabolic effects in freshwater fish *Geophagus iporangensis* in response to single and combined exposure to graphene oxide and trace elements. *Chemosphere* 243:125316 Doi:10.1016/j.chemosphere.2019.125316.
- Miller HD, Clark BW, Hinton DE, Whitehead A, Martin S, Kwok KW, Kullman SW. Anchoring ethinylestradiol induced gene expression changes with testicular morphology and reproductive function in the medaka. *PLoS ONE* 7(12):e52479 Doi:10.1371/journal.pone.0052479. (2012). [PubMed: 23300682]
- Mochizuki E, Fukuta K, Tada T, Harada T, Watanabe N, Matsuo S, Hashimoto H, Ozato K, Wakamatsu Y. Fish mesonephric model of polycystic kidney disease in medaka (*Oryzias latipes*) pc mutant. *Kidnet Int.* 68:23–34(2005).
- Movahedinia A, Salamat N, Kheradmand P. Effects of environmental endocrine disrupting compound benzo(a)pyrene on thyroidal status of abu mullet (*Liza abu*) during short-term exposure. *Toxicol Reports*, 5:377–382(2018).
- Nakamura S, Kobayashi K, Nishimura T, Tanaka M. Ovarian germline stem cells in the teleost fish, medaka (*Oryzias latipes*). *Int J Biol Sci.* 7:403–409(2011). [PubMed: 21547057]
- Nakamura YT. All oocytes attached to the dorsal ovarian epithelium in the ovary of medaka, *Oryzias latipes*. *Zoolog Sci.* 35:306–313(2018). [PubMed: 30079832]
- Nirmal NK, Awasthi KK, John PJ. Effects of nano-graphene oxide on testis, epididymis and fertility of Wister rats. *Basic Clin.Pharmacol. Toxicol* 121:201–210(2017).
- Norberg B, Kjesbu OS, Taranger GL, Andersson E, Stefansson SO. The teleost germinal epithelium: a unifying concept. *Proceedings of the 6th international symposium on the reproductive physiology of fish*, pp 233–236 (1999).
- Novoselov KS, Fal'ko VI, Colombo L, Gellert PR, Schwab MG, Kim K. A roadmap for graphene. *Nature*, 490(7419)192–200. (2012). [PubMed: 23060189]
- OECD. "Guidance document on the diagnosis of endocrine-related histopathology in fish gonads", OECD Series on Testing and Assessment, No. 123, OECD, Paris, <https://www.oecd.org/chemicalsafety/testing/42140701.pdf> (2010)
- OECD. Revised guidance document 150 on standardized test guidelines for evaluating chemicals for endocrine disruption, OECD series on testing and assessment, OECD Publishing, Paris 10.1787/9789264304741-en (2018).
- Paital B, Guru D, Mahapatra P, Panda B, Parida N, Rath S, Kumar V, Saxena P, Srivastava A. Ecotoxicological impact assessment of graphene oxide on lipid peroxidation at mitochondrial level and redox modulation in fresh water fish *Anabas testudineus*. *Chemosphere*, 224:796–804(2019). [PubMed: 30851531]
- Parenti LR, Grier HJ. Patterns and processes in the evaluation of fishes. *Annual meeting of the society of integrative and comparative biology Toronto, Canada*, p.8 (2003).
- Parenti LR, Grier HJ. Evaluation and phylogeny of gonad morphology in boney fishes. *Integr Comp Biol.* 44:333–348 (2004). [PubMed: 21676719]
- Park JW, Tompsett AR, Zhang X, Newsted JL, Jones PD, Au DW, Kong R, Wu RS, Giesy JP, Hecker M. Advanced fluorescence in situ hybridization to localize and quantify gene expression in Japanese medaka exposed to endocrine disrupting compounds. *Environ. Toxicol. Chem* 28: 1951–1962(2009). [PubMed: 19469586]
- Patlolla AK, Randolph J, Kumari SA, Tchounwou PB. Toxicity evaluation of graphene oxide in Sprague-Dawley rats. *Int J Environ. Res. Public Health* 13:380 Doi: 10.3390/ijerph13040380 (2016). [PubMed: 27043588]

- Patlolla AK, Randolph J, Tchounwou PB. Biochemical and histopathological evaluation of graphene oxide in Sprague-Dawley rats. *Austin J Environ Toxicol.* 3 (1): pii1021 (2017).
- Pooma A, Colafarina S, Fontecchio G, Chichirico G. Transgenerational effects of NMs In: Capco D, Chen Y (eds) *Nanomaterials. Advances in Experimental Medicine and Biology* vol 811, Springer, Dordrecht DOI 10.1007/978-017-8739-0_12. (2014).
- Readman GD, Owen SF, Knowles TG, Murrell JC. Specis specific anesthetics for fish anesthesia and euthanasia. *Sci Rep.* 7:7102 (2017). doi:10.1038/s41598-01706917-2. [PubMed: 28769117]
- Rive C, Reina G, Wagle P, Treossi E, Palermo V, Bianco A, Delogu LG, Rieckher M, Schumacher B. Improved biocompatibility of Amino-functionalized graphene oxide in *Caenorhabditis elegans*. *Small* 2019, 15, 1902699 (2019),
- Satoh N An ultrastructural study of sex differentiation in the teleost *Oryzias latipes*. *J Embryol. Exp. Morph* 32:195–215(1974). [PubMed: 4452829]
- Scholz S, Mayer I. Molecular biomarkers of endocrine disruption in small model fish. *Mol Cell Endocrinol.* 295:57–70(2008).
- Souza JP, Barretta JF, Santos F, paino MM. Toxicological effects of graphene oxide on adult zebrafish (*Danio rerio*). *Aqua Toxicol* 186:11–18(2017).
- Steinel NC, Bolnick DI. Melanomacrophage centers as a histological indicator of immune functions in fish and other poikilotherms. *Front Immunol* 8:827 (2017). [org/10.3389/fimmu.2017.00827](https://doi.org/10.3389/fimmu.2017.00827). [PubMed: 28769932]
- Stosik MP, Tokarz-Deptula B, Deptula W. Melanomacrophages and melanomacrophage centers in Osteichthyes. *Cent Eur J Immunol.* 44:201–205(2019). [PubMed: 31530990]
- Urushitani H, Kato Y, Tooi O, Santo N, Kawashima Y, Ohta Y, Kisaka Y, Lange A, Tyler CR, Johnson RD, Iguchi T. Medaka (*Oryzias latipes*) for use in evaluating developmental effects of endocrine active chemicals with special reference to gonadal intersex (tes-tis ova). *Environ Sci.* 14:211–233(2007). [PubMed: 17975535]
- Viraka Nellore BP, Kanchanapally R, Pedrza F, Sinha SS, Pramanik A, Hamme AT, Arslan Z, Sardar D, Ray PC. Bio-conjugated CNT-bridged 3D porous graphene oxide membrane for highly efficient disinfection of pathogenic bacteria and removal of toxic metals from water. *ACS applied materials & interfaces*, 7:19210–19218(2015). [PubMed: 26273843]
- Xu S, Zhang Z, Chu M. long-term toxicity of reduced graphene oxide nanosheets: effects on female mouse reproductive ability and offspring development. *Biomaterials*, 54:188–200(2015). [PubMed: 25907052]
- Yang X, Yang Q, Zheng G, Han S, Zhao F, Hu Q, Fu Z. Developmental neurotoxicity and immunotoxicity induced by graphene oxide in zebrafish embryos. *Environ Toxicol.* 34: 415–423(2019). [PubMed: 30549182]
- Zhang L, Khan IA, Moral CM. Characterization of the estrogenic response to genistein in Japanese medaka (*Oryzias latipes*). *Comp. Biochem. Physiol, Part C:* 132:203–211(2002).
- Zou W, Zhou Q, Zhang X, Mu L, Hu X. Characterization of the effects of trace concentrations of graphene oxide on zebrafish larvae through proteomic and standard methods. *Ecotoxicol. Environ. Safety*, 162:192–200 (2018). [PubMed: 29990731]

High lights

- Single IP-injected graphene oxide (GO) tended to interrupt medaka breeding.
- Agglomerated GO nanoparticles were observed in medaka gonads 21days post-injections.
- Histopathological alterations of medaka gonads by GO are found to be minimum.
- Morphology of granulosa and Leydig cells in gonads remained unaltered by GO.
- No endocrine disrupting effects of GO were observed in liver and kidneys of medaka.

Figure 1: Effects of GO on the Fecundity of Japanese medaka.

Fecundity was calculated on the total eggs laid by the female (Fertilized+ unfertilized, **Figure 1A**), and the eggs fertilized by the males (Fertilized, **Figure 1B**) of each breeding

pair. Sexually mature, reproductively active, adult male and female medaka were maintained in standard laboratory conditions and eggs were collected (both fertilized and unfertilized) for 28 consecutive days (7-days before injection and 21-days after injection). GO (either synthesized in the laboratory or obtained from Sigma-Aldrich, St. Louis, MO) was sonicated in water and injected ip (single injection; 25–200 µg/g) to both male and female fish. Control fish were injected with vehicle (water). Data were analyzed by two-way ANOVA followed by a post-hoc Tukey's multiple comparison test and $p < 0.05$ was considered as significant. Data were expressed as mean $\pm$ SEM (n=3–21). The data presented in **Figure 1A** as total eggs (both fertilized and unfertilized), which indicate that the fecundity sharply decreased during 1st day post-injection and gradually increased. The control fish (vehicle-injected; n=21) on day 1 post-injection showed significant difference ($p < 0.05$) with control fish on days 3 and 4, and then days 11–21, continuously; no significant difference 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 significant difference ($p < 0.05$) with day 1 fish only on day 18. Those fish that received 100 µg/g (n= 7) showed 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 difference with any days. The data presented in **Figure 1B** are the fecundity based on fertilized eggs. In control fish (n=21), fecundity (fertilized eggs) in post-injection day 1 showed significant difference ($p < 0.05$) with the fecundity of control fish from post-injection days 11–21; however, no significant difference is 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 (n=5) on day 1 showed significant difference ($p < 0.05$) with post-injection days 17, 18 and 21. In 100 µg/g group (n=7) post-injection days 2 and 3 showed significant difference ($p < 0.05$) with days 17 and 18. The fish that received 50 and 200 µg/g (n= 4 and 3, respectively) did not show significant difference with any of these days.

Figure 2: Effects of GO on the Ovarian Histopathology of Japanese Medaka.

Control (vehicle-injected) and GO-injected (25–200 µg/g) medaka females were sacrificed after 21 days post-injection and histopathological evaluation were made on 5µm thick sections stained in HE (Figures 2A–C). Data (mean± SEM) presented as histograms (Figures 2D, 2E, and 2F) were analyzed by one-way ANOVA followed by post-hoc Tukey's multiple comparison test and $p < 0.05$ is considered as significant. Representative histopathological structures of the ovary of control fish (**Figure 2A1**) and GO-injected fish (**Figure 2B1, 25 µg/g and Figure 2C1, 100 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 follicles, and 6= atretic follicles. Arrows (□), squares (■), and triangles (▲) in black color indicate the position of the germinal epithelium, lumen, and extravascular spaces, respectively, in the ovary. **Figures 2A2 (control), 2B2 (25 µg/g) and 2C2 (100 µg/g)** represent the magnified view of chorion (empty arrows) and the perfollicular cells, especially the granulosa cells (solid arrows) in the surface of vitellogenic oocytes. **Figures 2A3 (control), 2B3 (25 µg/g), and 2C3 (100 µg/g)** represent the magnified view of chorion (empty arrows) and the perfollicular cells, especially the granulosa cells in the surface of atretic follicles (black arrows). **Figures 2A4 (control), 2B4 (25 µg/g), 2C4 (100 µg/g)** represent the magnified view of PFC, especially the granulosa cells (black arrows) in postovulatory follicles. **Figure 2D** represents the distribution (percentage) of differentiating follicles (PNOs, CAOs, EVOs and LVOs) in the stromal compartments of control (vehicle-injected), and GO-injected (25–200 µg/g) fish. **Figure 2E** represents the distribution (percentage) of degenerating follicles (atretic and post-ovulatory) in the stromal compartments of control, and GO-injected (25–200µg/g) fish. **Figure 2F** represents the follicular ratio (degenerating: differentiating) observed in the stromal compartments of the ovary of control and GO-injected (25–200 µg/g) fish. Asterisk (*) indicates the data are

significantly different ($p<0.05$) from the control fish and dollar symbol (\$) indicates the data are significantly different ($p<0.05$) from 50 µg/g GO-injected fish. The oval shapes in red (**Figures 2B2, 2B4, 2C, 2C2, and 2C3**) represent the accumulation of unidentified black particles (probably agglomerated GO) in the stromal compartments of the ovary. The rectangular shapes in red (**Figures 2C1 and 2C2**) indicate the development of perivascular smooth muscle-like structure in the ovary after GO (100µg/g) injection. We used 7 control fish, 5 fish from 25 µg/g group, 4 fish from 50 µg/g, 7 fish from 100 µg/g and 3 fish from 200 µg/g for follicular analysis. Moreover, the photographs of the ovary of GO-treated fish presented in the manuscripts are from 25 µg/g (Figures 2B1–2B4) and 100µg/g (Figures 2C1–2C4) groups. Other groups (50 and 200 µg/g) are included in supplementary data (SF2D for 50 µg/g and SF2E for 100 µg/g).

Figure 3: Effects of GO on the testicular histopathology of Japanese medaka. Representative photomicrographs of the histological sections of the testis of a control (**Figure 3A**) and GO-injected fish (**Figure 3B, 25 μg/g; Figure 3C, 100 μg/g**) after 21 days post injection are presented. The testicular components of both control and GO-treated fish (HE stain) are marked in numerical numbers; 1= spermatogonia; 2= spermatocytes; 3= spermatids; 4= sperms; The oval shapes and the rectangular shapes in red color indicate Sertoli cell and Leydig cells, respectively. The triangles in red indicate apoptotic cells (probably the sperm heads) in ED system of testis. The photomicrographs of the testis of both control and GO-treated fish (25 and 100μg/g) indicate that the cysts showed isogenic distribution of a germinal component (a particular cyst consists of identical components having variation with other cysts). Apoptotic germinal components (sperm) were mostly found in ED system (pointed by triangles in red). Agglomeration of GO in testicular components was found to be dose-dependent (pointed by empty arrow in red in Figure 3C1).

Figures 3A1 (control), 3B1 (25 µg/g), and 3C1 (100 µg/g) show the different germinal components of testis (spermatogonia, spermatocytes, spermatids), sperms, Sertoli cells, and Leydig cells. **Figures 3A2 (control), 3B2 (25 µg/g), and 3C2 (100 µg/g)** show the Sertoli cells in MT stains (the nuclei stained black and the cytoplasmic arms stained green). **Figures 3A3 (control), 3B3 (25 µg/g), and 3C3 (100 µg/g)** show Leydig cells in MT stain (the nuclei are black and collagens are green). Although the photographs of the GO-treated testes presented in the manuscripts are from 25 µg/g (Figures 3B1–3) and 100µg/g (Figures 3C1–3) groups, histological sections of the testis of other GO-treated groups (50- and 200 µg/g) are presented in supplementary data (Supplementary Figures SF7A **for 50 µg/g** and SF7B **for 200 µg/g**).

Figure 4: Effects of GO on the liver histopathology of Japanese medaka.

GO was injected IP to both male and female medaka, and histopathological observations on liver were made 21 day post-injection (HE staining). Representative photomicrographs of control male fish (**Figure 4A1**), control female fish (**Figure 4A2**), GO (25 μg/g) male (**Figure 4B1**), female (**Figure 4B2**); GO (100 μg/g) male (**Figure 4C1**) and female (**Figure 4C2**) fish. 1= sinusoidal cavity showed muralis duplex arrangement of hepatocytes in both control and GO-treated (25- and 100 μg/g) male and female fish. Vacuoles in the cytoplasm of hepatocytes seem to remain identical in both male and female fish (control or GO-treated) represent glycogen contents of the liver. The hepatocytes in female fish (Figures 4A2, 4B2, and 4C2) are more basophilic in nature than male fish (Figures 4A1, 4B1, and 4C1). GO injection was unable to establish any significant difference with regard to the histopathology

of the organ. The photographs presented in the manuscripts are from 25 µg/g (Figures 4B1–2) and 100µg/g (Figures 4C1–2) groups. Histological sections of the liver of other GO-treated groups (50 and 200 µg/g) are presented as Supplementary Figures: SF8A1, male, SF8A2 female, for 50 µg/g; and SF8B1, male, and SF8B2, female, for 200 µg/g.

Figure 5: Effects of GO on the kidney histopathology of Japanese medaka.

GO was injected ip to both male and female medaka and histopathological observations on kidneys were made 21 day post-injection (HE staining). Representative photomicrographs of control male fish (**Figure 5A1**), control female fish (**Figure 5A2**), GO (25 µg/g) male (**Figure 5B1**), female (**Figure 5B2**), GO (100 µg/g) male (**Figure 5C1**) and female (**Figure 5C2**) fish. The components of kidneys are marked in numerical numbers. 1= glomerulus; 2= renal tubules; 3= lymphomyleoid interstitial tissues. Arrows in red indicate MMCs in the lymphomyleoid interstitial tissue. No significant alteration in the histopathology of GO-injected fish with controls were observed after 21 days post-injection. The photographs presented in the manuscripts were from 25 µg/g (Figures 5B1–2) and 100µg/g (Figures 5C1–2) groups. Histological sections of the kidneys of other GO-treated groups (50- and 200

µg/g) are included as supplementary data (Figures SF9A1, male, and SF9A2 female for 50 µg/g; SF9B1, male, and SF9B2, female, for 200 µg/g).

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